

Biosecurity of infectious diseases in veterinary medicine

Edited by

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Biosecurity of infectious diseases in veterinary medicine

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Editorial: Biosecurity of infectious diseases in veterinary medicine

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biosecurity, infectious diseases, One Health, antimicrobial resistance (AMR), risk assessment

Editorial on the Research Topic

Biosecurity of infectious diseases in veterinary medicine

The global animal health landscape is constantly under threat from new and re-emerging infectious diseases. Apart from the negative impacts on animal welfare and the decrease in livestock productivity, they also pose serious risks to public health through the transmission of diseases from animals to humans. Effective biosecurity is crucial in preventing the introduction and spread of pathogens within and between animal populations. This Research Topic explores various aspects of biosecurity in veterinary medicine, focusing on new approaches, critical evaluations, and the complex interplay among human behavior, policies, and technologies. The contributing articles collectively aim to improve the management of infectious disease risks, thereby protecting animal health, food security, and public health under the “One Health” approach.

At the core of any successful biosecurity program there are strict cleaning and disinfection (C&D) protocols. While evaluating these procedures is essential, it can also be challenging. One of the studies included in this topic reviewed different methods for assessing C&D, including visual inspections, ATP bioluminescence, microbiological and molecular analyses, emphasizing the need for a comprehensive, individualized approach to ensure effective hygiene management (Makovska et al.). Additionally, research into new disinfection agents offers promising alternatives. For example, a study evaluating chlorous acid water as a disinfectant used at the pre-surgically stage in cattle found it to be as effective and comparable to standard approved methods, potentially decreasing preparation time in field settings (Ichii et al.). Another research explored the efficacy of UV254 irradiation for inactivating major swine viruses like African Swine Fever Virus (ASFV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), and Porcine Epidemic Diarrhea Virus (PEDV) in both water and air, providing useful information for decontamination of the environment on swine farms (Qiu et al.). These studies reflect the ongoing scientific work that has been done to improve and innovate the physical and chemical barriers against pathogen transmission.

Further emphasizing the element of human intervention, a survey of North American swine producers' biosecurity practices showed the dynamics affecting their adoption of biosecurity plans, varying according to farm size and level of perceived disease risk. This work highlights the need for updated assessments and the introduction of artificial intelligence systems, such as machine learning, for risk assessment evaluation, recognizing the role of demographics and risk perception in adoption (Chepkwony et al.).

Effective biosecurity involves more than just technical procedures, it is actually heavily influenced by human behavior, policy, and communication strategies, and because of these elements, it is essential that farmers are understood, and encouraged to engage. Another study from a research group of China provides valuable insights into how legislative regulation affects biosecurity investments by pig producers, showing that effective law enforcement and tailored regulations can significantly boost prevention efforts, especially among medium-scale farmers (Liu and Tao). In a different context, a pilot intervention in Tanzania demonstrated the effectiveness of a participatory approach in improving biosecurity practices on small and medium-scale pig farms. By collaboratively creating checklists and fostering cooperation between farmers and livestock field officers, significant improvements in overall farm production and biosecurity compliance were achieved, proving the effectiveness of grassroots, capacity-building strategies in areas with limited resources (Auplish et al.). Crucially, effective communication also emerges as a central theme. A study exploring stakeholder perspectives on communication methods for biosecurity advocates for collaborative, personalized, and sustainable approaches, emphasizing direct interaction, practical learning, and the integration of technological tools to promote behavioral change (Moya et al.). These articles as a whole illustrate that the effective implementation of biosecurity measures requires an integrated approach of the attitudes, knowledge, and cooperation of all stakeholders involved in the biosecurity assurance.

Disease-specific biosecurity intervention is essential for specific disease concerns. Infectious Bronchitis (IB) in poultry, for instance, is an infectious avian disease that can potentially lead to severe economic losses. An investigation of IB outbreaks on a broiler farm revealed that external and internal biosecurity deficits coupled with inappropriate vaccine selection facilitated the introduction and spread of wild viral strains, highlighting the extreme importance of good biosecurity practices (Maletić et al.). In dairy cattle, *Salmonella* Dublin poses an enzootic threat. A study from Denmark identified specific farm sections where lower biosecurity scores were associated with a higher risk of *S. Dublin* introduction and establishment, indicating that current biosecurity levels may be insufficient to counteract infection pressure from the surroundings (Pedersen et al.).

Furthermore, to address the limitations of existing biosecurity assessment tools, particularly for diverse farming structures, a new biosecurity risk assessment tool (BioscoreDairy) has been developed and optimized for pasture-based dairy farms in Ireland. This innovative tool combines a questionnaire on management practices with an audit of cattle movement records, providing enterprise-specific risk categorization and benchmarking capabilities (O Donovan et al.). Such tools are crucial to identify specific vulnerabilities and guiding targeted interventions.

Besides direct disease control, the threat of antimicrobial resistance (AMR) is an unresolved issue. A systematic review of *Streptococcus* infection in bovine mastitis in Ethiopia revealed an extremely alarming rate of *Streptococcus* spp., and high rates of resistance to widely prescribed antimicrobials like penicillin. This

makes evidence-based risk management and strict antimicrobial use standards imperative to avoid AMR (Fenta et al.). Moreover, the characterization of canine circovirus, an emerging pathogen, emphasizes the necessity of understanding its genetic variability, risk of cross-species transmission, and diagnostic challenges starting from a One Health approach in consideration of its significance in animal and public health (Ferreira da Silva et al.).

This Research Topic captures the dynamic and multidimensional nature of biosecurity in veterinary medicine. From C&D efficacy and new disinfectants to the general impact of laws and farmer engagement, and from unique disease control strategies to general risk assessment tools and emerging pathogen surveillance, each article brings its contribution with important issues and knowledge.

The joint findings underscore the fact that effective biosecurity is not an unchanging concept but an evolving discipline that requires continuous novelty in technology, dynamic policy tools, different communication strategies, and cooperative comprehension of animal and human behavior. In conclusion, what emerges from this collection is that for the future, interventions in biosecurity will require a more concerted and multi-disciplinary approach against infectious disease and for the safeguarding of welfare and health of animals and humans across the globe within the context of One Health approach.

Author contributions

JF: Conceptualization, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MGC: Writing – review & editing. GA: Writing – review & editing. GR: Writing – review & editing. MR: Writing – review & editing.

Conflict of interest

GR was employed by I-Vet srl.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Use of conjoint analysis to weight biosecurity practices on pasture-based dairy farms to develop a novel audit tool—BioscoreDairy

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Risk assessments are important tools to identify deficits in biosecurity management practices. A major strength of some existing tools is that they facilitate cross-country comparisons. However, a weakness is their failure to account for unique intra-national farming enterprise structures such as, for example, pasture-based dairying. Currently, there are no suitable biosecurity risk assessment tools applicable to pasture-based dairying as practiced in Ireland. In addition to a need for enterprise-specific biosecurity risk assessment tools, the weighting of risk scores generated by these tools needs to be context-specific to ensure validity in assessing biosecurity risks in the farming sector of interest. Furthermore, existing biosecurity audits rely exclusively on respondent recall to answer questions about management practices. To address each of these limitations of existing biosecurity risk assessment tools we developed and optimised a new biosecurity risk assessment tool (BioscoreDairy) designed to assess the biosecurity status of pasture-based dairy farms in Ireland. It consists of two parts, a biosecurity questionnaire and a cattle movement records audit. A questionnaire was developed on biosecurity management practices appropriate for a pasture-based dairy system. Multiple national expert groups were leveraged to provide weightings for the different management practices in the questionnaire using the best-worst scaling methodology of MaxDiff. The results of this process provided a numerical categorisation that could then be used to assign scores to the individual biosecurity management practices. These practices were grouped into three biosecurity areas; risk of disease entry, speed of disease spread and diagnosis of infection. Within each of these three areas, a traffic light system was used to compare a farm's biosecurity risks to other similar farms—least risk (green; within the top third of farms), concerning practice (amber; middle third) and worst practice or greatest risk (red; lowest third). In addition to these scores, the cattle introduction profile of a herd over the previous 3 years, based on nationally recorded data, was audited, compared amongst dairy farm enterprise subtypes, and included in the BioscoreDairy report. BioscoreDairy is therefore the first biosecurity risk assessment tool tailored to pasture-based dairy farm systems, both for individual farm reporting and for benchmarking against comparable farms.

KEYWORDS

biosecurity, risk assessment tool, questionnaire, best-worst scaling, scores, weightings, dairy, cattle

1 Introduction

Biosecurity is defined by the World Organisation of Animal Health as “a set of management and physical measures designed to reduce the introduction (bioexclusion), establishment and spread (biocontainment) of animal diseases, infections or infestations to, from or within an animal population” (1). To identify biosecurity deficits in farm practices and compare such practices with similar farms, various biosecurity audit tools have been developed. Such assessments are usually based on a questionnaire designed for use by farmers (self-assessment) or, more commonly, by professional service providers (external assessment). These questionnaires may be pathogen/disease-specific, e.g., John’s disease (2) or they may be pathogen/disease-agnostic, e.g., (3). In the intensive animal production sectors, there is evidence of the beneficial impact of implementation of biosecurity measures on antimicrobial use and on production indices (4).

As biosecurity measures are adopted to a lesser degree on cattle farms compared to other more intensive enterprise types (5), the need for comprehensive biosecurity auditing of cattle farms is possibly even greater. Additionally, some of the lack of adoption of biosecurity measures on cattle farms may be explained by the limited suite of robust biosecurity audit tools currently available to assess, and hence facilitate benchmarking of biosecurity performance. This is important given that previous research has demonstrated that benchmarking motivates farmers to improve management practices (6).

Audit tools that have been published to date are generally developed to address biosecurity concerns within a particular farming sector, e.g., pig farming, or within a specific country [e.g., (2)]. An exception to this is the Biocheck, a robust suite of questionnaires which have been developed in Belgium but are used internationally for multiple enterprises; pigs (7), poultry (8), dairy (9) and beef (10). A major advantage of these tools is that they facilitate cross-country comparisons and benchmarking. However, within each farming sector, there may be significant variation in how farms are managed and even the epidemiology of livestock infectious diseases between countries. This is arguably greatest in the dairy sector (compared to pig or poultry sectors), which displays variations of seasonal vs. non-seasonal calving, confinement vs. pasture-based systems, both within- and between countries. It is therefore possible that more generic tools, which aim to facilitate greater ‘external’ comparisons between countries, may be less applicable and/or valid internally. Accordingly, McCarthy et al. (11) assessed publicly available dairy cattle biosecurity questionnaires internationally and concluded that none adequately suited pasture-based dairy farming, such as that practiced in Ireland.

Audit tools should weight biosecurity deficits according to their perceived risk with respect to farm biosecurity. However, it is likely that the weighting and prioritisation of biosecurity practices for one country may differ to that of another. In addition, terminology and language

concerning different practices is likely to differ between countries, so some local ‘translation’ is likely to result in more accurate data collection regarding biosecurity practices. This process is particularly important if the tool is to be used by farmers rather than, for example, being collected by a veterinarian. Finally, a further potential weakness of existing tools is their limited ability to gather accurate data on cattle movements into the farm, when they rely on farm recall. Cattle introductions are the most important risk factor for introduction of infectious pathogens into a herd (12). As with all answers in a questionnaire, there is a risk of gathering inaccurate information due to recall, recency or other responder cognitive biases (13). While this may not be critical for some information (e.g., whether milk recordings are carried out or not), it is essential that cattle introductions are accurately documented when assigning a biosecurity risk status to a farm. There is therefore a need to collate accurate/objective data on cattle introductions with other farmer biosecurity behaviours to get a more complete perspective on the farm’s biosecurity status.

In Ireland the requirement for a robust, holistic biosecurity audit tool has become more important with the recent major demographic changes in the dairy industry. Irish dairy farming is based predominantly on small herds (mean 90 cows) which are seasonal calving, pasture-based, and family-run (14). Nationally, there is high regional density of dairy cattle, high inter-farm cattle movements, with some infectious endemic diseases under legislative control (e.g., bovine tuberculosis, bovine brucellosis, bovine viral diarrhoea), others under voluntary control (e.g., John’s disease, mastitis/SCC, infectious bovine rhinotracheitis) and many others with no recognised national control programme (e.g., leptospirosis, salmonellosis, cryptosporidiosis) (15). Since the European Union milk production quota was abolished in 2015, the Irish national dairy herd has expanded significantly (16). This expansion highlighted the need to address biosecurity risks from increased cattle movements. Hence the National Farmed Animal Biosecurity Strategy (NFABS 2021–2024) was introduced in 2021 (17). This strategy was designed to place increased emphasis on prevention of disease entry and spread within a herd.

To deliver the national biosecurity strategy a context-specific biosecurity audit tool tailored to pasture-based dairy production is needed. Therefore, the objective of this study was to develop a farmer-facing biosecurity scoring audit tool for use on pasture-based dairy farms, based on an expert-weighted risk assessment (RA) questionnaire and cattle movement data to capture and benchmark dairy farms.

2 Materials and methods

2.1 Risk assessment (RA) questionnaire development

A novel biosecurity RA questionnaire was developed using participatory design methodology. The aim was to produce a farmer-facing questionnaire that would assess farm biosecurity-related

Abbreviations: BWS, Best Worst Scaling; RA, Risk Assessment; DAFM, Department of Agriculture Food and Marine; AHI, Animal Health Ireland; TWG, Technical Working Group; NFABS, National Farmed Animal Biosecurity Strategy.

behaviours and performance generically (i.e., not disease-specific) across multiple infectious diseases and create national benchmarks. Unlike existing tools, the overall design was not predetermined by the bioexclusion and biocontainment dichotomy. The questionnaire for this new biosecurity tool was initially based on McCarthy et al. (11). Questions from this survey were cross checked with two existing publicly available biosecurity questionnaires: Biocheck (18), and the Irish John's Control Program (IJCP) Veterinary Risk Assessment and Management Practices (VRAMP) tool (19), to ensure no management practices were overlooked. The full list of questions were compiled and reviewed to find duplicate questions, which were excluded.

The consolidated list of questions were reviewed by the Animal Health Ireland (AHI) Biosecurity Technical Working Group (TWG).¹ The backgrounds of the membership of this group comprised veterinary practitioner, university veterinarian, pharmaceutical company veterinarian, AHI veterinarian, Department of Agriculture, Food and the Marine veterinarian, research veterinarian, dairy specialist agricultural adviser, sire performance centre manager and health psychologist. To aid with this technical review, an evaluation document with three key guidelines was circulated to members. This document specified that each reviewer should independently review the questions with a focus on three key criteria: (1) alternative information source—could this information (or a reasonable alternative) be provided automatically from the national Animal Introduction Movement (AIM) database maintained by the Department of Agriculture Food and Marine. AIM is the central data base of Ireland for cattle, pigs, sheep and goats (20) (yes/no), (2) redundancy—could this question be removed or amalgamated with another question in a way that would not lead to significant loss of vital information, and (3) practicality—is this a practice that could reasonably be modified on a commercial (dairy) farm? Following this review, the questionnaire was revised by two of the authors (JFM and CMA) based on the feedback gained from the technical review process.

Next, the biosecurity questionnaire was reviewed in full by a panel consisting of two of the authors (CMA and JFM), two dairy farm advisors, and a Department of Agriculture Food and the Marine (DAFM) veterinarian, to find consensus on the language used in the document with a particular emphasis on language that would be familiar to a dairy farmer, without compromising the specific information the question aimed to collect. Finally, the questionnaire was then sent to five dairy farmers (four male, one female; farm managers of dairy research herds) who were asked to complete it and to provide additional feedback on the technical content, the language and the format.

The farmers' comments were used to revise the questionnaire again by members of the project team (CMA, JFM, LOG, TG). The survey was then divided into 4 sections: risk of disease entry, speed of disease spread within herd, diagnosis of infection and baseline resilience/vaccination. Next, the questionnaire was reviewed by a chartered health psychologist from Animal Health Ireland (AB) to identify any language which may influence responses, and to identify any weaknesses within the questionnaire's clarity.

Irrespective of the enterprise a biosecurity questionnaire is designed for, the language used and the responders' perception of the meaning of questions may influence the answers provided. One way of reducing this bias is to conduct cognitive interviews (CI) with pilot respondents. Cognitive interviews are conducted in order to evaluate individuals' understanding of the survey through "think aloud" protocols and verbal probes (21). They are a qualitative development method used to aid the development of the survey by helping the design team to investigate the clarity of the survey and gain the responders perception of each question. They also highlight whether the survey achieves the overall objective (22). Thus, five cognitive interviews (CI) were carried out by the first author (SOD) to get feedback from dairy farmers. These interviews were focused on identifying questions that were unclear and resulted in confusion for the farmer. The CIs were carried out either in-person or online via video call. The farmers were provided with a copy of the revised questionnaire to which they had to respond orally, reading the question aloud and choosing their answer. Where hesitation or a reaction to the question and/or answer was observed, they were asked what caused such reaction or confusion. From this process any questions which caused confusion or misunderstanding were highlighted. So too were answer options which may have been omitted. Finally, all cognitive interviews were reviewed and from there the questionnaire was edited again.

The edited document was finally reviewed by 6 project members (SOD, JFM, CMA, LOG, TG, AB). The questionnaire ($n = 75$ questions) was then uploaded to an online survey software platform—Survey Monkey (23).

2.2 Risk assessment questionnaire scores and weightings

Scores were generated separately for each of the three sections of the questionnaire [(1) risk of disease entry (2) speed of disease spread and (3) diagnosis of infection] which in turn had to be generated from individual question scores. Section 4 of the questionnaire relating to herd resilience and vaccination was not scored as this section contained questions (e.g., "are health traits within the EBI sub-indices part of selection criteria when breeding animals?") of qualitative or disease-specific value only, as such actions were not deemed to directly affect the general risk of disease entry or spread.

2.2.1 Within-section scores

Scores were assigned to each question within each of the three sections, based on its perceived risk to farm biosecurity. Scores were derived using a best-worst scaling (BWS) approach (24). This method collects paired comparison data, therefore forcing the expert to make compromises in their decisions (25). Using this approach, experts were provided with sets of four management practices at a time relating to one of the three sections and asked to identify the best (lowest risk for biosecurity) or worst (largest risk for biosecurity) practice relating to the biosecurity area (e.g., risk of disease entry). Repeating this process multiple times (12–16 depending on the number of questions per section) with multiple combinations of options, and across multiple users, facilitates the estimation of relative weights for each of the individual responses.

¹ <https://animalhealthireland.ie/about/who-we-are/technical-working-groups/biosecurity-technical-working-group/>

All possible questionnaire responses (attributes) for each question were transformed into statement format for each of the three sections: risk of disease entry: 101 attributes, speed of disease spread: 96 attributes and diagnosis of infection: 22 attributes. Random subsets of four statements were presented each time, along with a question relating to which would have the best (or least detrimental) or worst (or most detrimental) impact on the aspect of biosecurity covered in that section of the questionnaire. The presentation of subsets was repeated multiple times for each respondent to allow accurate ranking of responses.

Scoring was conducted by representatives ($n = 39$) of five preselected veterinary groupings; the research project team ($n = 5$; university veterinarian, research veterinarian, diagnostic laboratory veterinarian, postgraduate PhD student), the Animal Health Ireland (AHI) biosecurity Technical Working Group (TWG) ($n = 8$; see membership detail above), Irish diplomats of the European College of Bovine Health Management ($n = 7$; private veterinary practitioner, Department of Agriculture, Food and the Marine veterinarian, university veterinarian), biosecurity specialists in Department of Agriculture Food and Marine (DAFM) ($n = 14$; veterinary biosecurity officer, veterinary pathologist, veterinary epidemiologist, veterinary inspector, veterinary research microbiologist) and Irish private veterinary practitioners ($n = 5$). Conjointly software (26) was used to gather and analyse the responses from best-worst scaling. Three webinars were hosted in which the questionnaire and the BWS technique were explained, and training was given on how to answer a BWS survey using a demo scenario. The 39 experts were then asked

to complete a separate BWS survey for each of the three sections individually and reminded that their responses were to be agnostic of any single pathogen or disease. All responses were anonymous.

Settings were applied to the BWS system so that the format at which the attributes (statements) would appear, how many times they would appear, and recommended time taken to complete the exercise could all be altered. Therefore, when applying settings to the BWS, the attributes (statements based on farm practices) shown per set at any time were automatic. This meant that the number of times an attribute could appear throughout the BWS was controlled and all attributes appeared an equal number of times. The order at which attributes appeared was randomised, so no two participants received the same set of attributes at any time. Analytical settings were also applied. A confidence interval of 90% was set. A setting to eliminate low quality responses was applied, with warnings appearing on screen when a participant was completing an exercise too fast; this response was deemed low quality. Where this warning appeared, it was suggested that the participant was not giving their honest opinion or full concentration. The process of scoring was carried out within the 3 sections rather than across all sections. This process was repeated for all the statements/attributes in each of the three sections.

The Best Worst Scaling theory, using Maximum—Difference method, allocates values to each of the answer options per question (within each section), thus allocating a score to each answer option (Figure 1). These BWS scores were then reviewed in detail by the authors to identify cases where non-biologically plausible weights

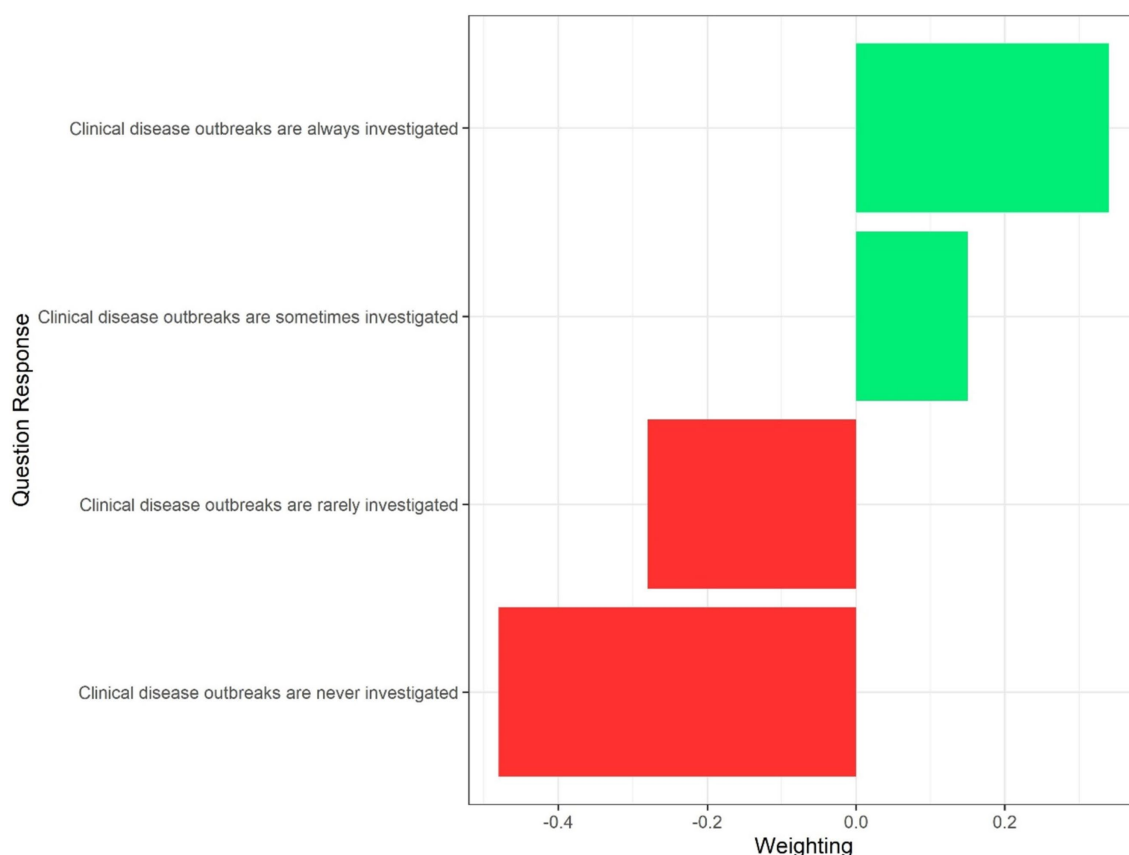


FIGURE 1
Example of best worst scaling weightings for a single question on investigation of a clinical disease outbreak.

had been assigned, that is, weights that placed the ranking of responses in an order which conflicted with biological plausibility. When this occurred, one of four options was followed: When a single biologically higher risk response was assigned a lower risk score than the next response, but the authors believed, on discussion, that the risk was similar from both, both responses were assigned the same weighting, which was calculated as the mean of the weights for the two responses. In contrast, when the authors believed there was a clinically-relevant difference between the risk, for example, if the highest or lowest risk response (from a biological perspective) was assigned a score placing it out of order with the rest of the options, this option would be assigned a value equal to the highest or lowest weighted response. If multiple responses within the question appeared to have biologically inappropriate weightings (relative to the other attributes within that question), but the authors believed these differences to be minor, individual attributes were assigned a weighting of zero and the attribute did not contribute to the overall risk score. Finally, if multiple responses within the question appeared to have biologically inappropriate weightings, and the authors deemed these risks significant, weightings were re-assigned according to the biologically plausible ordering of the risks.

To obtain the total score per section, responses for each survey question were matched to the corresponding BWS weighting and these weightings were added together across all the questions for that section. Then the BWS weighting total per section was expressed as a percentage of the maximum possible score from all questions in that section.

2.3 Cattle introduction tool

Cattle introduction indicators were developed for each farm: in degree, inward strength and secondary inward degree. In degree was defined as the number of cattle moved onto the farm in the previous 3 years, inward strength was defined as the number of herds from which cattle were introduced onto the farm in the previous 3 years, and secondary inward degree extended this to the herds from which source herds introduced cattle from. Herds were categorised using the cattle enterprise classification system recently developed by Brock et al. (27) and benchmarked to other herds within this category according to the median and 10th and 90th percentiles and the overall distribution. Data to compute these metrics reside within the Animal Identification and Movements (AIM) database. Therefore, this section of the score can be populated based on routinely collected data alone and allows each herd to be compared against all herds in the country.

2.4 Farm biosecurity report

Finally, the scores from the biosecurity questionnaire and the cattle introduction tool data were combined in a farm biosecurity report called BioscoreDairy. The automatic generation of the report was coded using R (28). The coding process formulated the farm report by linking the farmer's responses from the questionnaire and the BWS weightings. Farm scores were benchmarked against the records of all other farms that have taken the assessment. For

illustrative purposes, the 33rd and 67th percentiles of the distribution of scores for each section were calculated and a visual plot created to summarise the farmer's score, colour-coded according to their position in the distribution compared to other similar farms (low, medium, high). Farms' sections (e.g., disease diagnosis) with the best scores (lowest risk) one third were coded green, those in the bottom one third were coded red, and those in the middle third are coded amber.

3 Results

3.1 Biosecurity questionnaire and weightings

The final questionnaire is provided in [Appendix 1](#). It consisted of 70 questions across four sections (section one risk of disease entry: $n = 28$; section two speed of disease spread: $n = 21$; section three diagnosis of infection: $n = 12$; section four baseline resilience/vaccination: $n = 9$). The questionnaire took approximately 16 min to complete. This was calculated using the survey platform where the time was recorded from when the link was opened to the time of submission. A sum of all timings was calculated and divided by the number of responses to obtain an average.

The weightings assigned to each biosecurity practice in the three sections subjected to the weighting process (risk of disease entry, speed of disease spread and diagnosis of infection) are shown in [Supplementary Tables S1–S3](#).

3.2 Cattle introduction tool

The number of cattle introductions and number of source herds were analysed for each individual herd and compared with data from comparable herds in the national cattle movement database. [Figure 2](#) shows a plot for an example herd: the red line represents the highest risk (90th percentile), the black line represents the median herd, while the green line represents the lowest risk (10th percentile) position of herds nationally. The position of the example herd is highlighted in yellow.

3.3 Farm biosecurity report (BioscoreDairy)

An example of a farm biosecurity report is provided as [Supplementary material 2](#). The final report summarised the three section scores and categorised the risk of disease entry, speed of disease spread and diagnosis of infection, each separately as low, moderate or high, according to whether their scores were above or below the 33rd or 67th percentiles, respectively ([Figure 3](#)). Risk of disease entry was subcategorised, and scored, into the number of cattle introduced and the sources of these cattle and the farmer's responses to the questionnaire. Speed of disease spread was subcategorised, and scored, as that between sick and healthy cattle, adult to young (pre-weaned calves, weaned calves, yearlings) cattle, young to young cattle and adult to adult cattle, to give an overall farm speed of disease spread score. Answers regarding herd resilience and vaccination were not scored; however, they were recorded for four cattle age categories; pre-weaned calves, weaned calves, yearlings and adults.

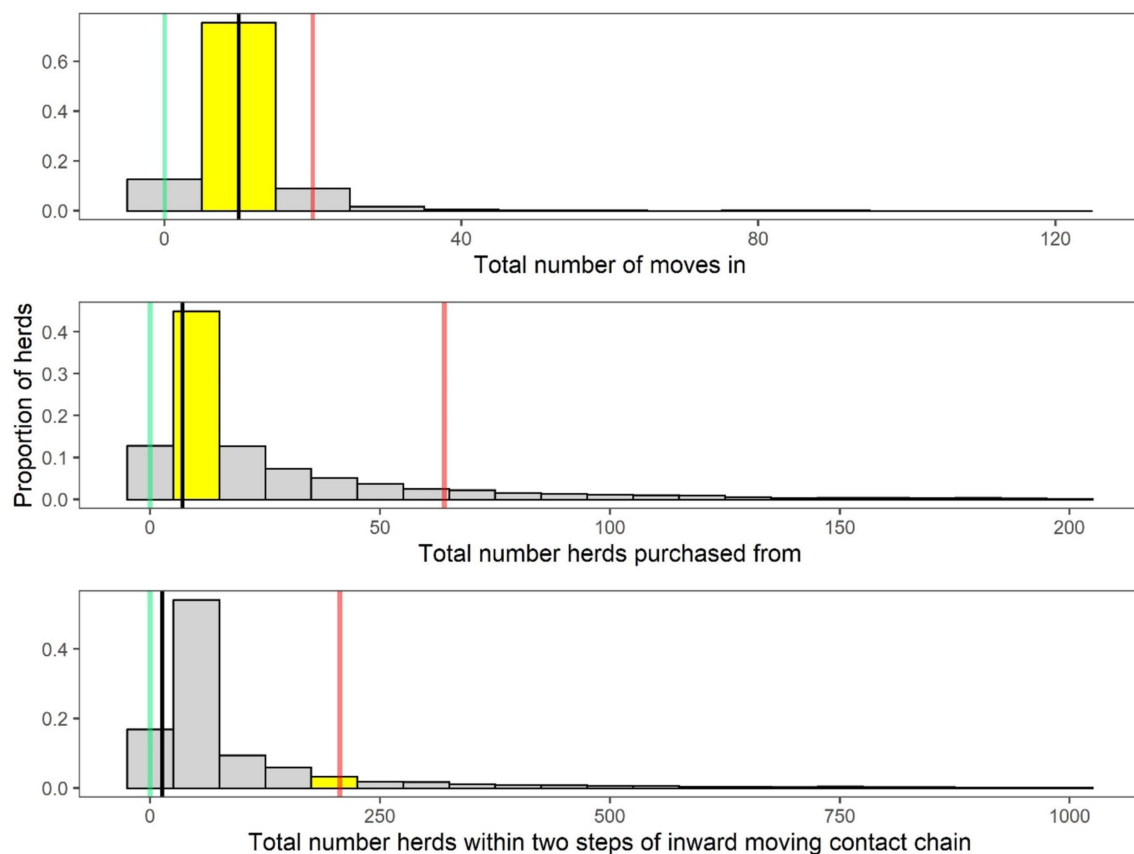


FIGURE 2

An example of an animal introduction summary figure from a BioscoreDairy report. Animal introductions are based on the number of animals bought in (first frame) and the number of source herds (frames two and three). For each farm, these metrics are compared to 50 other similar comparable herds in the same herd category ([27] classification system). The distribution of these data for the comparator herds are shown as grey bars in the three frames. The position of the individual example herd in each graph frame is indicated by the yellow bar. The green line represents the position of the lowest-risk (10th percentile) herds, the black line indicates the position of the average herd, and the red line indicates the position of the highest-risk (90th percentile) herds, nationally.

4 Discussion

This study represents, to our knowledge, the first development of a biosecurity risk assessment tool for pasture-based dairy farms. It makes use of electronic movement data regularly collected in many countries. The tool has been optimised for Irish dairying based on language and phrasing as well as local expert weighting but can easily be adapted as appropriate for a particular country with similar production systems.

Our study is not unique in seeking to develop methods to collect management practices relevant to herd biosecurity. To date, similar studies fall into three broad categories: 1. Questionnaires with a broad biosecurity focus, developed primarily for research use, to capture data relevant to a specific research project or study (29); 2. Questionnaires with a broad biosecurity focus developed for practical or commercial use, for example Biocheck (18) and 3. Questionnaires developed for a specific disease, developed for practical or commercial use, for example the paratuberculosis Risk Assessment (30).

While the underlying data our study seeks to capture are similar to those in tools developed in each of these examples, there are important differences. Firstly, in contrast to comparable

studies, a particular focus of this study was to create a tool which was designed to be ‘farmer-facing’, i.e., for the farmer to complete, rather than an animal health professional. Therefore, we adopted a multidisciplinary approach to questionnaire development including biosecurity experts, social scientists, dairy farmers, dairy advisors, and veterinarians. In this way, question selection and wording were refined through a robust iterative process which including multiple iterations with dairy farmers to ensure language was appropriate, and to reduce any potential for misunderstanding.

Secondly, whilst the weighting and score allocation approach taken in this study can be compared to other scoring systems developed for biosecurity, in non-research settings, two differences in particular are worth noting. Firstly, a strength of the BioscoreDairy approach is the steps taken to weight the responses of farmers according to the level of risk assigned, with particular focus on management practices. The application of BWS is rarely applied to the domain of biosecurity and is a time-efficient approach to assigning objective rather than subjective scores from a large network of experts. The BWS allows for a multitude of expert opinions to be compiled into weights and for these to be converted into percentages or scores. Secondly, whilst the

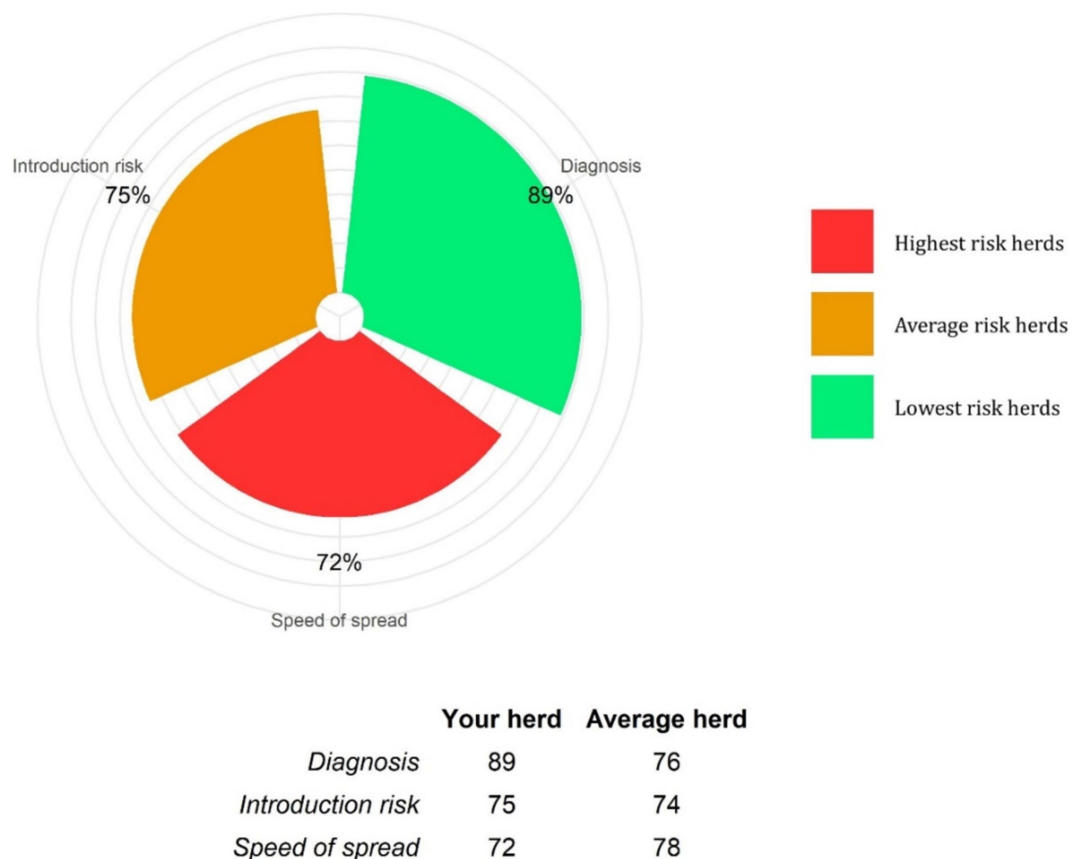


FIGURE 3

An example of a biosecurity score summary, from a farm BioscoreDairy report. The example farm score percentages for disease diagnosis, infection introduction risk and speed of infection spread risk, are a percentage of the maximum score percentage possible for each of these sections. These example farm score percentages are benchmarked against other comparable herds in the BioscoreDairy database. Higher score percentages indicate lower risk. The distribution of score percentages for the comparator herds are colour-coded into low risk (<33rd percentile (green); average risk between the 33rd and 67th percentile (amber); and high risk >67th percentile (red)).

system we developed is developed specifically for pasture-based dairying and therefore useful for dairy farms internationally with similar production methods, the weighting applied to this scoring system is, we believe, context-specific. Our study has outlined how the series of steps taken to tailor the system to the Irish system may act as a robust framework for the development of and refinement of other biosecurity assessments.

Finally, whilst existing disease-specific risk assessments are commonly used in Ireland, our study addresses a particular challenge with these approaches. For example, the Johne's disease Risk Assessment is a widely adopted component of international Johne's disease control programmes (31). However, based on qualitative research of Irish farmers, recent work from our group has argued for integrated disease preventive strategies into group programmes in so far as possible, as opposed to disease-specific programmes (32). The tool developed in this study represents a means by which multiple diseases can be mitigated in an integrated way, as opposed to a disease-specific manner, potentially improving farmer engagement.

Following the development of BioscoreDairy, it is now being utilised on a cohort of Irish dairy farms to both collect biosecurity data and to evaluate its suitability for field use in the Irish NFABS. BioscoreDairy could be used in other countries which

operate a pasture-based dairy system such as New Zealand, United Kingdom, some African countries and parts of Australia, United States of America and Europe (36). However, if BioscoreDairy is used in such countries the weightings of scores may be different due to different expert opinions and the epidemiology of infectious bovine diseases. For example, in many parts of New Zealand calvings occur outdoors (37) in comparison to Ireland where most farms carry out calving indoors (38). There is also potential to develop a BioscoreBeef specifically for pasture-based beef enterprises as they operate in Ireland.

There are some limitations to our study. The BWS resulted in some weightings which "mis-ordered" the question responses. During review, mis-ordered responses were corrected by the project team, by either averaging across responses, or by making the question score neutral. However, this approach is unlikely to have resulted in the 'correct' allocation of weightings for these specific questions. It is unclear why misordering of response weightings may have occurred but may have been a misunderstanding of the statements by some experts for specific questions. Our intended approach to mitigate this risk was to conduct scoring during in an interactive webinar in which experts were free to ask questions where confusion arose. However, this effect may still have persisted for some questions. Another

limitation of this study was the use of an expert opinion approach to scoring. While this is a standard approach in development of such biosecurity scoring audits (33) and trans-disciplinary expertise was enrolled from multiple specialist sources, nevertheless it is a subjective process, though one which also has the advantages of harnessing stakeholders with deep sectoral knowledge of this specialised topic.

Whilst our study makes use of existing databases to ensure robust/objective inputs where possible (e.g., cattle introductions), the questionnaire aspect of the tool relies, like other systems, on farmer responses. There are several reasons why these responses therefore may be an inaccurate reflection of management on the farm. Firstly, there may be a mismatch between what the farmer perceives to be occurring on the farm, compared with actual practices implemented on the farm, the issue of farm-blindness (34); secondly, farmer responses may indicate practice at a particular point in time which may not be reflective of practice if measured over a longer time period. Finally, in many cases, farmers are likely to be aware that the practice implemented on their farm does not conform to best practice, and therefore may give responses which do not reflect their true management practices. In other contexts, the term social desirability bias is often used to describe this effect (35), a recognised approach advocated for addressing this type of bias includes providing the respondent with assurances regarding anonymity and confidentiality. The approach taken with our study, in developing a system that is not delivered by an animal health professional may mitigate this impact since there is no interviewer present at data collection. In addition, like most data collections it is important that farmers are reassured regarding where the data goes, what it is used for and who will be handling the data or personal information. In order to reassure farmers, all data should be anonymised by allocating a response number to their completed survey, and stored in a secure file. Farmers should be provided with a detailed description of how their data would be managed and assessed.

5 Conclusion

Multiple biosecurity risk assessment tools have been developed to audit cattle farms nationally and transnationally, but none were deemed suitable for pasture-based dairy enterprises as they operate in Ireland. The tool developed here, BioscoreDairy, is unique in combining both questionnaire responses and recorded cattle movement data. The co-design methodology adopted in producing the questionnaire and in applying the best-worst scaling method also constituted a novel approach to assigning score weightings across a broad range of experts during the design of a farmer-facing biosecurity risk assessment tool.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

SD: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. CM: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. LO'G: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing. TG: Conceptualization, Methodology, Writing – review & editing. AB: Methodology, Writing – review & editing. M-CM: Methodology, Writing – review & editing. JD: Writing – review & editing. JT: Methodology, Writing – review & editing. JM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2024.1462783/full#supplementary-material>

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Evaluation of the killing effects of UV₂₅₄ light on common airborne porcine viruses

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UV exposure is a common method of disinfection and sterilization. In the present study, the parallel beam test was performed to collect fluids containing infectious viruses using a parallel beam apparatus after UV₂₅₄ irradiation (0, 0.5, 1, 3, 5, 7, 10, or 20 mJ/cm²). The air sterilization test was performed by irradiating the air in the ducts with UV₂₅₄ light (0, 1, 2, 3, 4, or 6 mJ/cm²) to collect airborne particles containing viruses through the air sterilization equipment. Furthermore, viral inactivation was assessed based on cytopathic effect (CPE) detection and immunofluorescent assays (IFA). Both the CPE and immunofluorescence signal intensity decreased as the UV₂₅₄ dose increased. The UV₂₅₄ doses required to inactivate ASFV (10^{7.75} copies/mL), PRRSV (10^{6.29} copies/mL), and PEDV (10^{7.71} copies/mL) in the water were 3, 1, and 1 mJ/cm², respectively. The UV₂₅₄ dose required to inactivate ASFV (10^{4.06} copies/mL), PRRSV (10^{3.06} copies/mL), and PEDV (10^{4.68} copies/mL) in the air was 1 mJ/cm². This study provides data required for biosecurity prevention and control in swine farms.

KEYWORDS

UV radiation, air disinfection, ASFV, PRRSV, PEDV

1 Introduction

China is the world's largest producer and consumer of pork, producing approximately 53% of the global pork supply (1). Furthermore, pork is the main source of high-quality protein for Chinese residents, with the consumption accounting for 62% of total meat consumption (2). Infectious diseases represent a major constraint to pig production (3). Since the first outbreak of African swine fever (ASF) in China in August 2018, ASF, porcine reproductive and respiratory syndrome (PRRS), and porcine epidemic diarrhea (PED) have emerged as the three most serious viral diseases in Chinese pig farms (4). These diseases are highly transmissible and pathogenic, with rapid mutation of the virulent strains, resulting in abortions in sows, growth delay in fattening pigs, and mass mortality among piglets (5, 6). When these diseases occur on pig farms, it is difficult to achieve decontamination because of the labor and resources required to control the spread of the disease in the herd. Notably, ASF virus (ASFV), PRRS virus (PRRSV), and PED virus (PEDV) can be transmitted through the air, further complicating disease prevention and control efforts in the entire Chinese pig farming industry (7–10).

UV disinfection is one of the most commonly used methods for preventing air-mediated microbial disease transmission because of its low cost, simple installation, ease of maintenance,

and significant effectiveness (11, 12). UV light can inactivate pathogenic microorganisms through several mechanisms, such as the formation of cyclobutane pyrimidine dimers in nucleic acids, which ultimately inhibit transcription and replication (13). In addition, the generation of reactive oxygen species (ROS) results in the oxidation of macromolecules such as lipids, proteins, and carbohydrates inside the cells and leads to cell membrane and cell wall damage (14). Table 1 provides a summary of recent studies on the effectiveness of UV in inactivating various viruses. From these references, we can identify that in addition to the UV dose, important factors affecting UV disinfection include the wavelength of the UV light used, the type of virus, the environmental conditions, and the medium through which UV light is transmitted.

Previous studies have shown that UV disinfection is an effective method to inactivate a wide range of pathogenic microorganisms, including various phages and viruses such as SARS-CoV-2 (15–22). This study aimed to evaluate the inactivating effect of UV₂₅₄ light, a UV-C wavelength, on common airborne porcine viruses, providing critical data for the prevention and control of animal diseases.

2 Materials and methods

2.1 Viruses and cells

ASFV, PRRSV, and PEDV were obtained from the National Regional Laboratory for African Swine Fever (Guangzhou) of South China Agricultural University (Guangzhou, China). Porcine primary alveolar macrophages (PAMs) were isolated from the bronchoalveolar lavage fluid of 4-week-old healthy piglets. Marc-145 and Vero cells were obtained via direct passage. Then, 1% porcine erythrocyte suspension was prepared using EDTA-treated fresh porcine blood. Viral stock solutions were diluted to 1×10^6 and 1×10^3 TCID₅₀ using autoclaved ddH₂O for parallel beam UV₂₅₄ experiments. A nebulizer aerosolized 15 mL of virus stock solution for each air sampler operation, with a collection duration of 15 min per sampling. Three replications of each experiment were performed. All viral manipulations in cells were conducted at the BSL-3 laboratory of the College of Veterinary Medicine, South China Agricultural University.

2.2 Parallel beam UV experiment

As shown in Figure 1, compared with traditional UV radiometers, the parallel beam apparatus optimizes beam collimation and uniformity, enabling more precise control and measurement of UV₂₅₄ irradiance, thereby enhancing the reliability of experimental results (23). Parallel beam UV₂₅₄ experiments were performed by fixing the UV₂₅₄ illumination of the light source and using different TCID₅₀ values for viruses and varying durations of UV₂₅₄ irradiation. As presented in Supplementary Table S1, the duration of irradiation using the 36-W UV₂₅₄ lamp (wavelength = 254 nm) were set to 0, 3.5, 6.9, 20.8, 34.6, 48.4, 69.2, or 138.4 s, and the UV₂₅₄ dose was set to 0, 0.5, 1, 3, 5, 7, 10, or 20 mJ/cm². After irradiating ASFV (TCID₅₀ = 1×10^6 /CT = 16.45, TCID₅₀ = 1×10^3 /CT = 29.64), PRRSV (TCID₅₀ = 1×10^6 /CT = 14.36, TCID₅₀ = 1×10^3 /CT = 25.36), and PEDV (TCID₅₀ = 1×10^6 /CT = 16.60, TCID₅₀ = 1×10^3 /CT = 27.47), viral inactivation was detected by assessing cytopathic effects (CPEs) and

performing IFAs to determine the UV₂₅₄ dose required for killing effects. Three replications of each experiment were performed.

2.3 Air sterilization experiment

As shown in Figure 2, the air disinfection experiment was performed by adjusting the UV₂₅₄ illumination intensity and wind speed over a fixed UV₂₅₄ irradiation time. The CT values of ASFV, PRRSV, and PEDV stock solutions were 13.5, 12.36, and 11.01, respectively. As illustrated in Supplementary Table S2, the temperature was set to 26°C. Meanwhile, the power of the UV₂₅₄ light (wavelength = 254 nm) was set to 0, 50, or 150 W; the airflow rates in the air sampler and wind tunnel were set to 1 m/s and 2 m/s, respectively, based on the required UV dose. As shown in Figure 3, the corresponding UV₂₅₄ dose was set to 0, 1, 2, 3, 4, or 6 mJ/cm² based on the simulation. First, the air sampler was used to collect airborne particles containing viruses upstream of the sampling section 30 s after nebulization. Subsequently, similar particles were collected downstream. Each collection lasted 15 min to ensure sufficient capture of airborne particles containing viruses. Note that the air sampler must be replaced after each collection, and the downstream sampler should not be connected while the upstream sampler is in operation. The air collected before and after UV₂₅₄ irradiation was dissolved into the culture medium, and viral inactivation was determined by assessing CPEs and performing IFAs. The end of the ventilation duct was equipped with an exhaust gas treatment unit to inhibit the release of viruses into the environment. Three replications of each experiment were performed.

2.4 Nucleic acid extraction and quantitative qPCR

After treatment, nucleic acids were extracted from ASFV, PRRSV, and PEDV using RaPure Viral RNA/DNA Kit (Guangzhou, China) as per the manufacturer's instructions, and qPCR was performed using the reaction system and procedure described previously (24–26). Three assays were performed for each sample. Regarding the results, negative samples had no CT values, positive samples had CT values of ≤34.0 with typical amplification curves, and suspicious samples had CT values of >34.0 with typical amplification curves. If two samples were considered suspicious, the result of the third sample was used.

2.5 Parameters of the parallel beam UV₂₅₄ meter

The impact of UV₂₅₄ light on pathogenic microorganisms is determined by the UV₂₅₄ dose they receive. The UV₂₅₄ is defined as (27):

$$\text{Dose} = \int_0^t I dt$$

where UV₂₅₄ dose is measured in mJ/cm², I represents the UV₂₅₄ light intensity received by the microorganism at a point on its trajectory (mW/cm²), and t is the irradiation time (s). The average UV₂₅₄ intensity received by microorganisms in the water is defined as (28):

TABLE 1 Killing effect of ultraviolet light on viruses.

Virus type	Killing dose	Virus counting (viability) methods	Ultraviolet length	Inactivation rate constant	Medium	Article
Fr bacteriophage	0.5 J/cm ² 99.99 percent reduction	Plaque infectivity test	405		Viral fluid	(18)
ΦX174 bacteriophage	5 J/cm ² 90 percent reduction					
MS2 bacteriophage	679 J/cm ² 99.68 percent reduction	Plaque infectivity test	365–375		Viral fluid	(19)
PhiX-174 bacteriophage	16.1 mJ/cm ² 99.97–99.99 percent reduction	Plaque infectivity test	280		Viral fluid	(20)
MS2 bacteriophage	16.1 mJ/cm ² 99.97–99.99 percent reduction					
MS2 bacteriophage	143.4 mJ/cm ² 99.99–99.9996 percent reduction					
SARS-CoV-2	1.25 mJ/cm ² 90 percent reduction	TCID ₅₀	254	0.79	Water	(30)
	0.6 mJ/cm ² 90 percent reduction	TCID ₅₀	220	1.5		
Adenovirus	10 mJ/cm ² 99.99 percent reduction	qPCR and Plaque infectivity test	210		Water	(51)
	10 mJ/cm ² 99.9 percent reduction		220			
H1N1 influenza virus	10 mJ/cm ² 99.99 percent reduction	IFA	207–222	1.8	Air	(52)
SARS-CoV-2	10 mJ/cm ² 99.99 percent reduction	IFA	254		Air	(53)
SARS-CoV-2	4 mJ/cm ² inactivation 99.999%	TCID ₅₀	222	12.4	Air	(29)
SARS-CoV-2	2 mJ/cm ² 99.9 percent inactivation	TCID ₅₀ /IFA	222	4.1	Air	(54)
SARS-CoV-2	1,048 mJ/cm ² inactivation 99.999 percent	TCID ₅₀	254		Viral fluid	(55)
SARS-CoV-2	10.25 to 23.71 mJ/cm ² inactivation 99.99 percent	TCID ₅₀	254		Stainless steel, plastic and glass	(56)
SARS-CoV-2	3.7 mJ/cm ² inactivates 99.9 percent	qPCR	254		Water	(57)
SARS-CoV-2	15 mJ/cm ² to inactivate 105 TCID ₅₀ virus solution	TCID ₅₀	253.7		Viral fluid	(58)
SARS-CoV-2	0.28 mJ/cm ² 99.2 percent inactivation	qPCR	254		Air	(59)
SARS-CoV-2	10 mJ/cm ² inactivation	TCID ₅₀ /IFA	222/230		Water and saliva	(60)
SARS-CoV-2	15 mJ/cm ² 99.99 percent inactivation	TCID ₅₀	222		Viral fluid	(61)
SARS-CoV-2	7.4 mJ/cm ² inactivation	TCID ₅₀	254		—	(62)
SARS-CoV-2	3.6 mJ/cm ² inactivation	Plaque infectivity test	254		Viral fluid	(63)
SARS-CoV-2	3.5 mJ/cm ² inactivation	IFA	254		Viral fluid	(64)

$$E_{\text{ave}} = 0.98 \left[\frac{E_0}{L} \left(\frac{(T)^L - 1}{\ln[T]} \right) \right]$$

where E_{ave} represents the average illuminance in the water (mW/cm²), E_0 represents the incident irradiance (mW/cm²), L is the depth of the solution irradiated by the collimated beam (cm), A is the UV₂₅₄ absorbance at a 1-cm light range, and $T = 1 - A$. Considering all irradiated pathogenic microorganisms as a collective group, the total UV₂₅₄ dose received can be calculated as: Dose = $E_{\text{ave}} \times t$ (28).

2.6 Air sterilization parameters

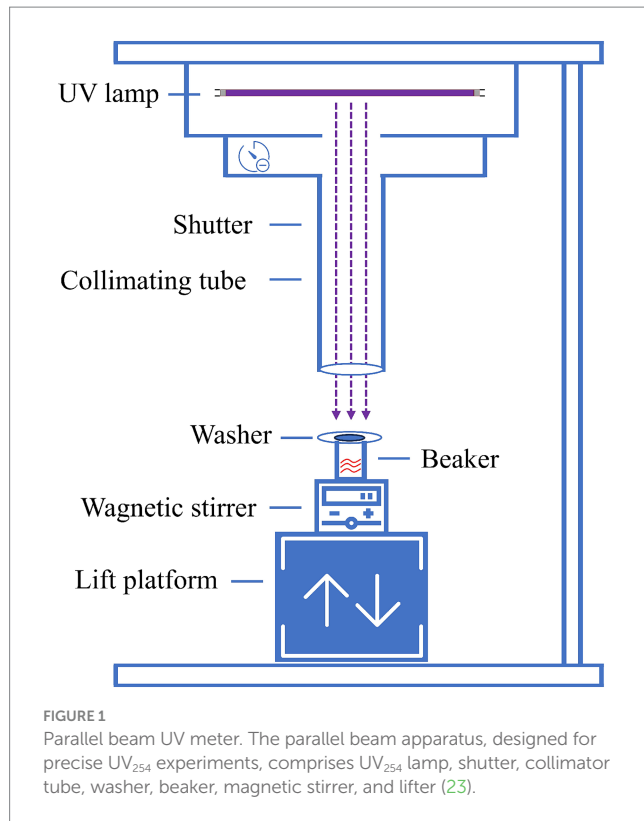
The UV₂₅₄ radiation dose received by a pathogenic microorganism in the reactor is determined by its path and exposure time. The

relationship between microbial inactivation efficiency and UV₂₅₄ dose is defined as (29):

$$-\lg \left(\frac{N}{N_0} \right) = A \times F + B$$

where F is the UV₂₅₄ dose (mJ/cm²); N_0 and N represent the microbial content before and after irradiation, respectively; and A and B are the disinfection kinetic parameters measured using a parallel beam meter. By determining the UV₂₅₄ dose received by each microcluster at the reactor's exit, the corresponding inactivation rate can be calculated. The overall inactivation rate is the combined effect of all microclusters (29):

$$\left(\frac{N}{N_0} \right)_{\text{total}} = \frac{\sum 10^{-(A \times F_i + B)}}{T}$$



where F_i represents the UV₂₅₄ dose received by each microcluster at the exit (mJ/cm²) and T is the total number of microclusters. From this, the total effective dose (RED) is defined as (29):

$$\text{RED} = -\frac{\left(\lg \left(\frac{N}{N_0} \right)_{\text{total}} + B \right)}{A}$$

2.7 Determination of virus infectivity

ASFV samples treated with different UV₂₅₄ doses were used to infect PAMs. Similarly, treated PRRSV samples were used to infect Marc-145 cells, and treated PEDV samples were used to infect Vero cells. Virus infectivity was determined by assessing CPEs and performing IFAs. In brief, PAMs, Marc-145 cells, and Vero cells were inoculated into 96-well plates, and viral suspensions (ASFV diluted in RPMI-1640 containing 10% FBS, PRRSV diluted in Dulbecco's modified Eagle medium [DMEM] containing 2% FBS, and PEDV diluted in DMEM containing 7 μg/mL trypsin) were added to the plates at a 10-fold gradient (1×10^{-1} to 1×10^{-10}), with columns 1 and 12 serving as controls. Viral infectivity was confirmed via the IFA using antibodies specific for ASFV, PRRSV, and PEDV, and the TCID₅₀ was determined using the Reed and Muench method.

2.8 *In vitro* biological characterization of viruses after irradiation

PAMs, Marc-145 cells, and Vero cells were infected with ASFV, PRRSV, and PEDV, respectively, following UV irradiation, and viral

infectivity was confirmed by assessing CPEs and performing IFAs. In brief, PAMs, Marc-145 cells, and Vero cells were inoculated into 96-well plates, and viral suspensions were added to the plates at a 10-fold gradient (1×10^{-1} to 1×10^{-10}), with columns 1 and 12 serving as controls. Three replications of each experiment were performed. Viral fluids were collected at 6-h intervals to construct *in vitro* growth curves using GraphPad Prism 8 software (GraphPad, San Diego, CA, United States).

2.9 Data analysis

The UV₂₅₄ dose responses based on UVC at 254 nm were evaluated using a pseudo first-order inactivation kinetics model in the log₁₀ scale as follows (30):

$$\log_{10} I = \log_{10} \left(\frac{N_0}{N} \right) = k \times D$$

where log₁₀ I represents the reduction in infectivity on the log₁₀ scale; N_0 and N represent the infectivity of virus samples before and after UV₂₅₄ exposure, respectively; D represents the UV fluence in mJ/cm²; and k represents the pseudo first-order inactivation rate constant in cm²/mJ computed using a log₁₀-scale kinetic model. The log₁₀ scale inactivation rate constant was used, which facilitated the calculation of log inactivation using the rate constant.

3 Results

3.1 Viral nucleic acids were not degraded by UV₂₅₄ irradiation at different doses

The ASFV, PRRSV, and PEDV solutions were irradiated with different UV₂₅₄ doses (0, 0.5, 1, 3, 5, 7, 10, and 20 mJ/cm²), as presented in Figures 4A–C. The copy numbers of ASFV, PRRSV, and PEDV did not differ significantly among the treatment groups. Further, ASFV, PRRSV, and PEDV were nebulized and then irradiated with different UV₂₅₄ doses (0, 1, 2, 3, and 6 mJ/cm²). As shown in Figure 4D, the copy numbers of the viruses were not altered by nebulization. This suggests that low-dose UV₂₅₄ irradiation does not lead to significant nucleic acid degradation in ASFV, PRRSV, and PEDV.

3.2 Low-dose UV exposure reduces the abundance of infectious virus in the samples

ASFV, PRRSV, and PEDV (TCID₅₀ = 1×10^6) were irradiated at different UV₂₅₄ doses (0, 0.5, 1, 3, 5, 7, 10, and 20 mJ/cm²) and used to infect PAMs, Marc-145 cells, and Vero cells, respectively. As presented in Figure 5A, the fluorescence intensity of ASFV treated with UV₂₅₄ doses of 0.5 and 1 mJ/cm² was significantly lower than that of untreated ASFV, and no fluorescence was observed for ASFV treated with an external UV₂₅₄ dose of 3 mJ/cm². The fluorescence intensity of PRRSV treated with a UV₂₅₄ dose of 0.5 mJ/cm² was significantly lower than that of untreated PRRSV, and no fluorescence was observed for PRRSV treated with an external UV₂₅₄ dose of 1 mJ/cm². The fluorescence intensity of PEDV treated with a UV₂₅₄ dose of 0.5 mJ/cm² was

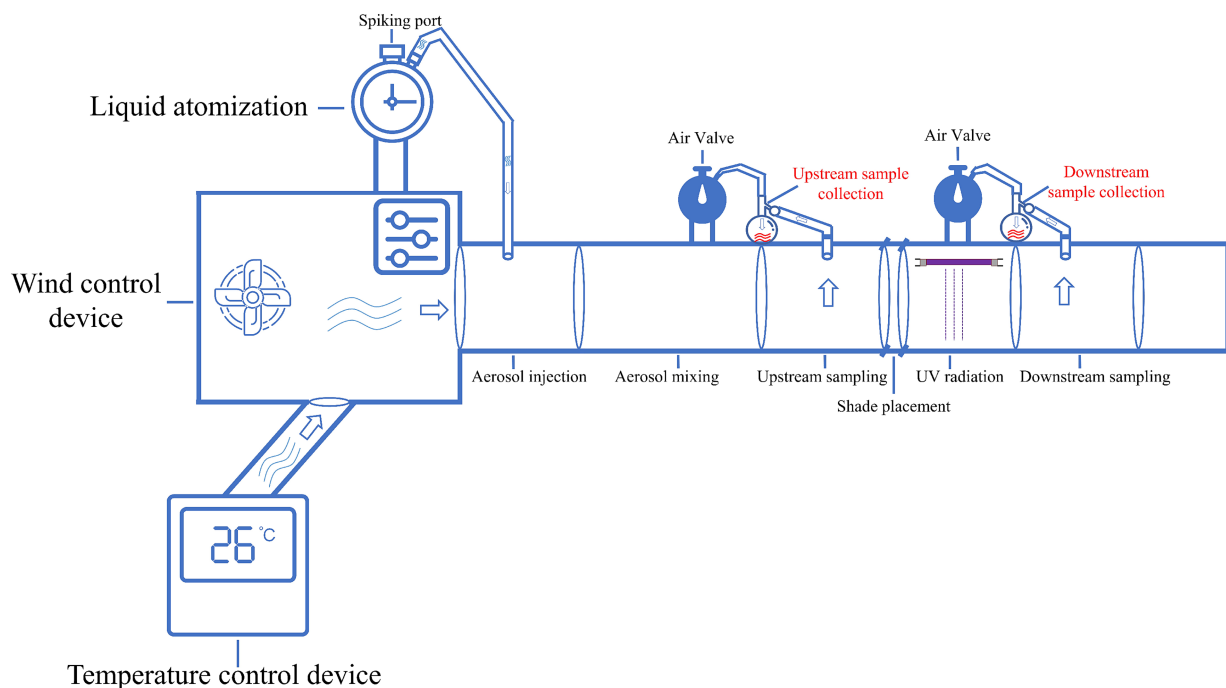


FIGURE 2

Equipment for air sterilization in a duct. The air disinfection equipment contained a temperature regulation device, wind speed controller, nebulizer (with liquid gasification function), air sampler (with gas liquefaction function), UV₂₅₄ device, and ventilation duct to simulate UV₂₅₄ disinfection of the air (50).

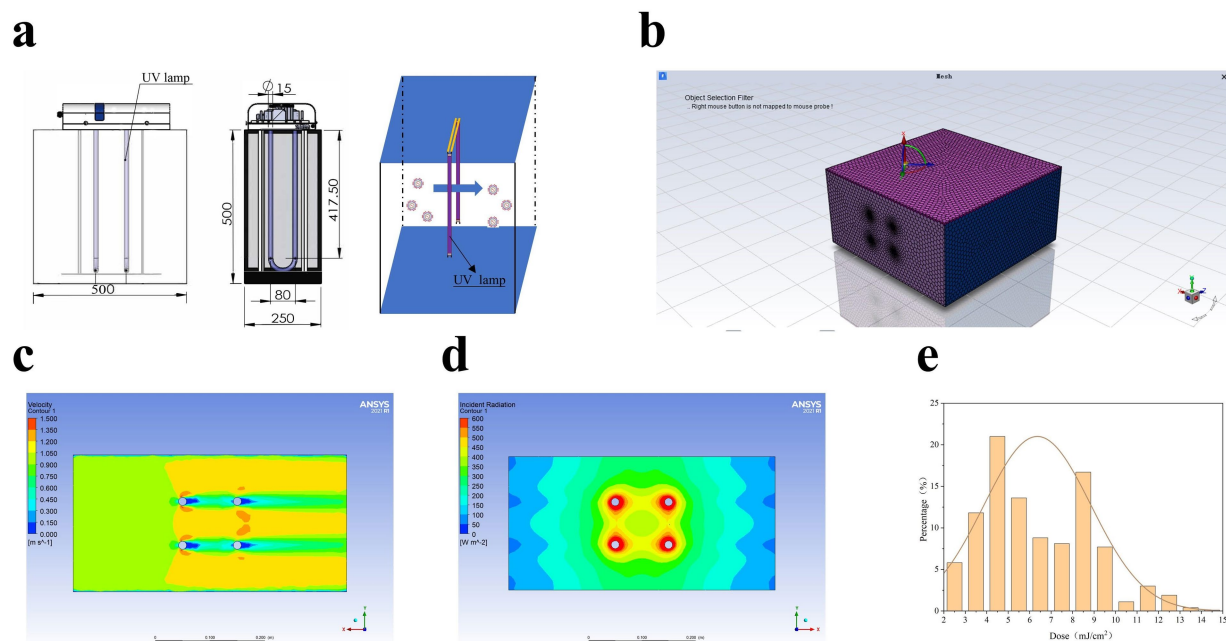


FIGURE 3

Duct calculation method. **(A)** UV₂₅₄ sterilization equipment. The equipment included a closed pipeline disinfection chamber with a cross-section of 500 × 250 mm² and a total length of 500 mm. Two built-in power sources (75 W each), and Kewei brand U-shaped low-pressure, high-intensity UV light (100 mm apart) with a UVC efficiency of 32% placed perpendicular to the wind direction. **(B)** Grid schematic. The structured grid shown in the figure was used to divide the sterilized area for simulation. A total of 288,738 grid cells were applied in the study. **(C)** Velocity field distribution. With an inlet wind speed of 1 m/s, the internal velocity field exhibited an axisymmetric distribution. Due to the bypassing effect of the lamps, the minimum velocity appeared in the downstream region of the light. However, the velocity variation across the flow field was minimal, resulting in a relatively uniform particle residence time in the range of 0.4–0.6 s. **(D)** Radiation intensity distribution. The distribution of internal radiation intensity indicated that the highest intensity occurred near the lamps, gradually decreasing along the radial direction from the light surface. **(E)** UV₂₅₄ dose distribution. The radiation dose of particles flowing through the UV₂₅₄ disinfection equipment is shown in figure. Based on the DPM model, 1,000 particles were injected simultaneously, and statistical analysis calculated the effective dose of the model as 6.086 mJ/cm².

significantly lower than that of untreated PEDV, and no fluorescence was observed for PEDV treated with an external UV₂₅₄ dose of 1 mJ/cm². As shown in Figures 5B–D, the infectivity of the viruses decreased significantly with increasing UV₂₅₄ doses, and ASFV was more resistant to UV₂₅₄ irradiation than PRRSV and PEDV. These results indicated that low-dose UV₂₅₄ irradiation can reduce the infectivity of viruses in cells.

3.3 Quantification of UV₂₅₄-induced inactivation of ASFV, PRRSV, and PEDV

Water and air containing ASFV, PRRSV, and PEDV were irradiated with different doses of UV₂₅₄ and were subsequently used to infect PAMs, Marc-145 cells, and Vero cells, respectively. Figure 6A, linear regression analysis revealed a rate constant of 4.308 cm²/mJ (95% confidence interval = 3.943–4.674) for ASFV, which corresponds to a 90% inactivation dose (D₉₀) of 0.23 mJ/cm². In addition, the rate constant for PRRSV was 9.167 cm²/mJ (95% confidence interval = 8.704–9.629), which corresponds to a D₉₀ of 0.11 mJ/cm². Further, the rate constant for PEDV was 8.333 cm²/mJ (95% confidence interval = 7.871–8.796), corresponding to a D₉₀ of 0.12 mJ/cm². Figure 6B, linear regression analysis revealed a rate constant of 3.167 cm²/mJ (95% confidence interval = 2.461–3.872) for ASFV, which corresponds to a 90% inactivation dose (D₉₀) of 0.32 mJ/cm². In addition, the rate constant for PRRSV was 2.958 cm²/mJ (95% confidence interval = 1.985–3.932), which corresponds to a D₉₀ of 0.338 mJ/cm². Further, the rate constant for PEDV was

2.538 cm²/mJ (95% confidence interval = 1.396–3.681), corresponding to a D₉₀ of 0.394 mJ/cm².

3.4 UV₂₅₄ doses exceeding 1 mJ/cm² inactivate ASFV, PRRSV, and PEDV in the air

ASFV, PRRSV, and PEDV were collected through an air sampler after irradiation with different UV₂₅₄ doses (0, 1, 2, 3, and 6 mJ/cm²) and used to infect PAMs, Marc-145 cells, and Vero cells, respectively. As presented in Figure 7, ASFV, PRRSV, and PEDV irradiated with a UV₂₅₄ dose of 1 mJ/cm² lost the ability to infect cells, whereas untreated viruses caused obvious lesions in the cells within 48 h after inoculation. The IFA and growth curves indicated that the untreated viruses showed normal replication in the cells.

4 Discussion

The ASF outbreak in China in August 2018 led to major changes in pig farming patterns in China, including the introduction of biosecurity prevention and control (31, 32). Previous studies have revealed that the positivity rates of various swine diseases decreased significantly with the establishment of biosecurity prevention and control systems in Chinese pig farms (33). Disinfection is an important part of the biosafety system (34). Currently, chemical disinfection is commonly used in pig farms because of its ease of use and obvious inactivate effects against pathogenic microorganisms

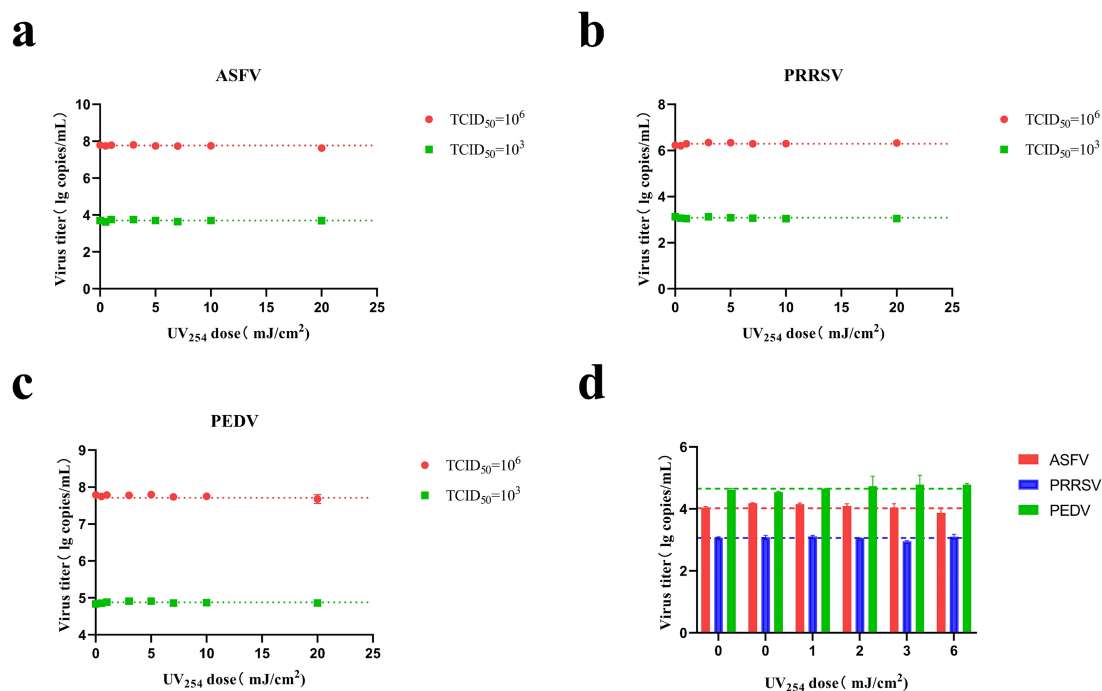


FIGURE 4

Changes in the CT values of ASFV, PRRSV, and PEDV after irradiation with different UV doses. (A) Irradiation of ASFV solution (TCID₅₀ = 1 × 10³ and 1 × 10⁶) using a parallel beam UV device. (B) Irradiation of PRRSV solution (TCID₅₀ = 1 × 10³ and 1 × 10⁶) using a parallel beam UV device. (C) Irradiation of PEDV solution (TCID₅₀ = 1 × 10³ and 1 × 10⁶) using a parallel beam UV device. (D) Irradiation of aerosolized ASFV, PRRSV, and PEDV in air disinfection ducts.

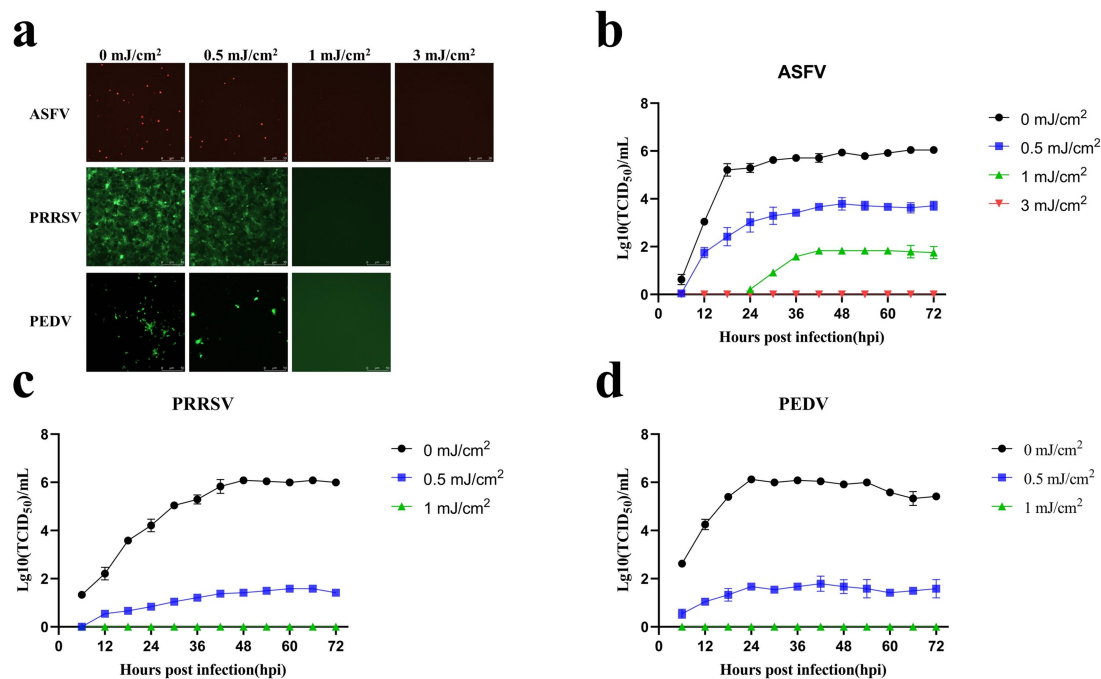


FIGURE 5

Low-dose UV₂₅₄ irradiation reduces the abundance of infectious virus in the samples. (A) Changes in the fluorescence signals of ASFV, PRRSV, and PEDV after treatment with different UV doses. (B) Growth curves of ASFV after treatment with different UV₂₅₄ doses. (C) Growth curves of PRRSV after treatment with different UV₂₅₄ doses. (D) Growth curves of PEDV after treatment with different UV₂₅₄ doses.

(35, 36). However, this disinfection method is associated with various problems, such as the presence of residual chemicals, secondary pollution, and formation of toxic disinfection by-products (DBPs). In addition, the types and usage of disinfectants applied on different objects are diverse, and some disinfectants are prone to cause damage to feed, food, and electronics. Therefore, chemical disinfection methods cannot be used in all scenarios in pig farms (37–42).

UV₂₅₄ treatment is a physical disinfection method, and the use of the UVC band for UV₂₅₄ irradiation leads to photochemical damage and ROS generation in pathogenic microorganisms, which affects the replication and transcription of genetic material and cause cell membrane and cell wall damage, ultimately leading to the death of microorganisms (13, 14, 27, 38, 43). Compared with chemical disinfection, UV₂₅₄ disinfection is characterized by short disinfection time, high efficiency, broad germicidal spectrum, simple structure, small footprint, easy maintenance, and the absence of DBP production, resulting in its widespread use in multiple applications, such as air disinfection, water purification and wastewater treatment, food preservation, and medical applications (11, 12, 44, 45). The effectiveness of UV-mediated inactivation depends on the type of pathogenic microorganism and operating conditions, such as UV wavelength, UV intensity, and duration of irradiation. Moreover, environmental conditions can also affect the efficacy of UV-based inactivation (11, 46).

ASFV, PRRSV, and PEDV are the three most serious viral diseases that can be transmitted through the air to pig farms in China. Similar to SARS-CoV-2 in humans, these viruses can cause widespread and rapid damage in infected pigs if their spread is not controlled, as observed during the ASF outbreak in China in 2018 (31, 47–49). It is

well known that UV₂₅₄ treatment has a strong killing effect. Currently, although UV₂₅₄ disinfection is widely used in pig farms, research on its killing effects on these three viruses is less extensive than that on SARS-CoV-2. Water and air are two important media for viral transmission. In the early stage of experimental designing, we reviewed a large number of studies on the killing effects of UV₂₅₄ disinfection. We revealed that UV₂₅₄ treatment has a stronger effect on viruses in the air than in viruses in the water. A UV₂₅₄ dose of <1 mJ/cm² can inactivate 99.9% of SARS-CoV-2 virions, and the killing effect of UV₂₅₄ is stronger in pure water than in culture medium. Compared with other wavelengths, UV₂₅₄ irradiation at a wavelength of 254 nm has a stronger killing effect (31, 47–49).

We investigated the UV₂₅₄ dose required to inactivate ASFV, PRRSV, and PEDV in pure water using a UV₂₅₄ parallel beam meter and then assessed its effects on viruses in the air using air sterilization equipment. We used primers and probes specific to ASFV-B646L, PRRSV-ORF6, and PEDV-M genes to detect the viral nucleic acid abundance of ASFV, PRRSV, and PEDV, respectively, before and after irradiation with different UV₂₅₄ doses (parallel beam UV₂₅₄ system: 0–20 mJ/cm²; air sterilization duct: 0–6 mJ/cm²). Further, we assessed viral infectivity by measuring CPEs and performing IFAs. The results revealed that low-dose UV₂₅₄ irradiation did not significantly degrade viral nucleic acids or suppress viral infectivity. In addition, ASFV, PRRSV, and PEDV treated with UV₂₅₄ doses of 3, 1, and 1 mJ/cm², respectively, these viral fluids were found to be infectivity-incompetent. To more intuitively demonstrate the relationship of the UV₂₅₄ dose with ASFV, PRRSV, and PEDV inactivation, the inactivation rate was quantified as the ratio of TCID₅₀ before and after UV irradiation. ASFV was more resistant to UV₂₅₄ irradiation than PRRSV and PEDV, probably because ASFV consists of a four-layered

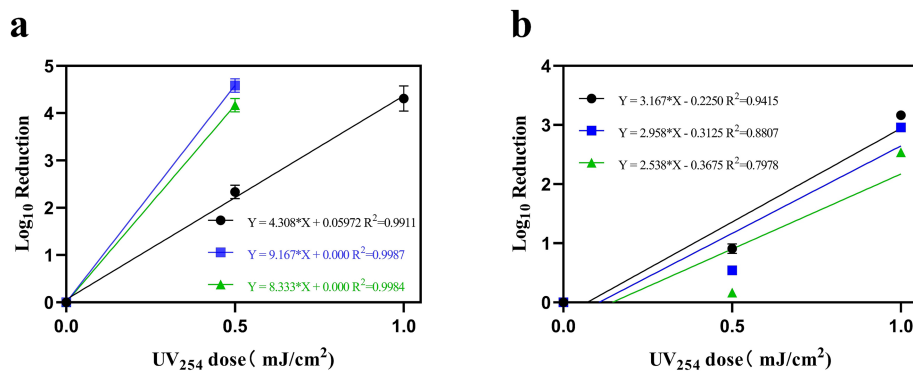


FIGURE 6

The relationship between the inactivation of ASFV, PRRSV, and PEDV in water (A) and air (B) with UV₂₅₄ dose, measured by TCID₅₀ relative to untreated virus controls. (Black indicates ASFV; blue indicates PRRSV; and green indicates PEDV).

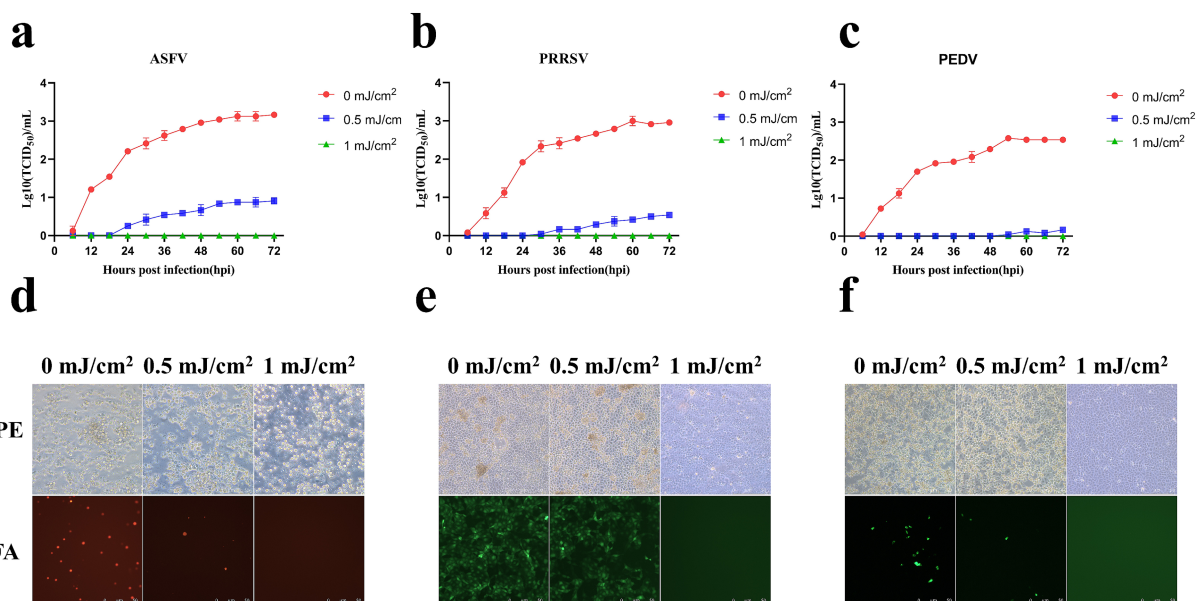


FIGURE 7

Replication of ASFV, PRRSV, and PEDV after UV₂₅₄ treatment at a dose of 1 mJ/cm². (A–C) Growth curves of ASFV, PRRSV, and PEDV. (D–F) CPEs and IFA data for ASFV, PRRSV, and PEDV.

protein shell and an internal genome, which is apparently more complex in structure than the internal genomes of PRRSV and PEDV. The air sterilization experiment revealed good cell growth, no cell lesions, and no fluorescence in the 1 mJ/cm² treatment group, suggesting that this dose is sufficient to inactivate ASFV, PRRSV, and PEDV. The stronger killing effects of UV₂₅₄ in the air than in the water are likely attributable to the fact that UV₂₅₄ can directly contact viruses in the air, whereas water refracts UV₂₅₄ light. This experiment was performed under ideal conditions where in UV₂₅₄ irradiation was applied directly to the viruses, resulting in killing effects at low doses. In real-world situations, the environment is intricate, and the number and size of dust particles in water and air can affect the efficiency of UV₂₅₄ disinfection. Therefore, it may be necessary to increase the UV dose in practical applications. In summary, we believe that UV₂₅₄

disinfection can be used in air filtration devices and other joint applications to detoxify air.

5 Conclusion

This study revealed that low-dose (0–20 mJ/cm²) UV₂₅₄ irradiation significantly reduces viral infectivity without causing nucleic acid degradation. Using parallel beam UV₂₅₄ apparatus, the UV₂₅₄ doses required to inactivate ASFV, PRRSV, and PEDV were preliminarily determined to be 3, 1, and 1 mJ/cm², respectively. The air disinfection experiment illustrated that a UV₂₅₄ dose of 1 mJ/cm² was sufficient to eradicate ASFV, PRRSV, and PEDV. These findings may provide a reference for the design and application of UV₂₅₄ equipment in pig

farms and lay a foundation for further research and development regarding viral disinfection.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding authors.

Author contributions

YQ: Conceptualization, Writing – original draft, Software. QL: Resources, Software, Writing – review & editing. WZ: Data curation, Supervision, Writing – review & editing. HC: Project administration, Validation, Writing – review & editing. JW: Supervision, Validation, Writing – review & editing. QG: Supervision, Visualization, Writing – review & editing. QZ: Formal analysis, Resources, Visualization, Writing – review & editing. GZ: Formal analysis, Project administration, Writing – review & editing. LG: Conceptualization, Data curation, Writing – original draft. LW: Conceptualization, Validation, Writing – review & editing.

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Conflict of interest

JW was employed by Foshan Comwin Light & Electricity Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Application of chlorous acid water for disinfection of surgical site in dairy cows

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Disinfection is crucial for preventing surgical site infections. Recently, the effectiveness of sanitizers using chlorous acid (HClO₂) under conditions rich in organic matter has been reported, and chlorous acid water (CAW) has been approved as a food additive. This study evaluated the potential of CAW as a new presurgical disinfectant for cattle. The experiments were performed on the paralumbar fossa of cattle in Sapporo during March (winter to spring) and August (summer). Colony-forming units (CFUs) of standard plate count bacteria (SPCB), *Enterococcus faecalis* (EF), *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus* spp. (SP) were analyzed as indicators of bacterial load. SPCB and SP were abundantly detected, exceeding 6 log₁₀ CFU/100 g on clipped hair and 6 log₁₀ CFU/100 cm² on the skin immediately after clipping, with no significant seasonal differences. The bacterial load on the skin was evaluated at three time points: after clipping, cleansing, and disinfection. Clipping and cleansing with liquid soap were common procedures, following this, either the standard disinfection protocol using 7.5% iodine scrub for 1 min, 10% povidone-iodine for 5 min, and 70% alcohol for 5 min (SPA), or a modified protocol using CAW with contact times of 15, 10, or 5 min (CAW15, CAW10, CAW5) were performed separately. The cleansing procedure significantly reduced the SPCB, EF, and SP on the skin after clipping, and all disinfection methods significantly decreased the SP after cleansing. Draping significantly enhanced the disinfection efficiency of the SPA, CAW10, and CAW5 protocols. The CAW procedure did not alter skin histology in the paralumbar fossa or udder compared to 10% povidone-iodine or 70% alcohol. Our data suggest that the disinfection method using CAW is useful and comparable to routine disinfection methods and might reduce the time required for presurgical disinfection in farm fields.

KEYWORDS

chlorous acid water, disinfection, dairy cows, surgical site infection, veterinary medicine, skin, paralumbar fossa, farm animals

1 Introduction

Disinfection of the surgical site is crucial for reducing bacterial contamination that can result in surgical site infection (SSI). In both human and veterinary medicine, povidone-iodine (PVP-I), chlorhexidine gluconate, alcohol, and their combinations are commonly used for general skin disinfection before surgical procedures (1, 2). PVP-I, chlorhexidine gluconate, and alcohol exhibit bactericidal effects through the oxidizing action of iodine ions, bacteriolytic

action, and protein-coagulating action, respectively. In human cases, the day before or the day of surgery, the patients take a bath or shower, or wipe the skin to remove dirt and ensure adequate cleansing. Surgical clippers are occasionally used to remove hair, but shaving is currently avoided because of the risk of skin damage leading to SSI, and shaving or short clippers are not recommended in animals for the same reasons (3–5). Finally, in humans, the surgical site is wiped with a cotton ball filled with a disinfectant. To prevent SSI, these procedures are performed in the operating or disinfection room where microbiological cleanliness is maintained.

In veterinary medicine at present, the disinfection control performed is equivalent to that performed in human medicine, especially for surgeries. In particular, well-equipped operating or disinfection rooms are maintained in veterinary hospitals for dogs, cats, and racehorses. Unlike humans, almost all animals have abundant hair coats carrying abundant bacteria, and the hair of farm or wild animals are dirty with soil or excrement ($6 \log_{10}$ colony-forming units [CFU]/cm² in cow-clipped hairs) (6); therefore, it is important for surgical treatment to clean and disinfect the skin after clipping (5). In veterinary medicine, a human hand disinfection method based on the traditional brushing methods by Fürbringer, Grossich, or their modified procedures, has been used for disinfection of the surgical site (7). Although the disinfectant and the time of exposure differ among animals, veterinarians, and hospitals, the surgical site is cleansed with a surfactant, flushed with water, washed with a surgical scrub containing PVP-I or chlorhexidine, and then sprayed with PVP-I and/or an alcohol-based reagent (8–10).

Farm animal surgery is performed in standing or dorsal recumbency in the operating room (11, 12). In addition, several cases are performed using treatment stalls in conventional rearing spaces in the field. In cattle, the paralumbar fossa is a common site for abdominal surgery and is surgically incised for cesarean section, abomasal displacement, and other gastrointestinal or urogenital diseases (11, 12). The paralumbar fossa is disinfected using a brushing method based on Fürbringer's or Grossich's procedures, similar to other animals (8). Brushing methods require time and staff; therefore, it is important to consider a quicker and easier disinfection method with high disinfection efficiency to reduce the contamination risk of falling bacteria and the burden on animals as well as veterinary staff. Several veterinarians have tried to reduce the operating time by changing the exposure time to disinfectants (8, 9, 13); Bourel et al. reported a disinfection method comprising two 90-s periods of cleansing and scrubbing, with 3 passages of 0.5% chlorhexidine gluconate in 70% in isopropyl alcohol solution (approximately, total 4 min) (8).

The present study focused on the usefulness of chlorous acid, HClO₂, for disinfecting animal skin, because HClO₂-based sanitizers have been reported to be more stable than NaClO under organic-matter-rich conditions. They contain chlorinated oxides such as HClO₂ or dissolved chlorine dioxide (ClO₂) which exhibit microbicidal activity. Recently, chlorous acid-based sanitizers have been used in food and environmental sanitation, and other studies have reported their microbicidal effects on a wide range of microorganisms, including yeast and spore-forming bacteria, such as *Escherichia coli* (EC), *Staphylococcus aureus*, *Campylobacter jejuni*, *Clostridium difficile* spores, *Candida albicans*, spore-forming *Bacillus*, and *Paenibacillus* species, as well as human norovirus and feline calicivirus (14–16). Chlorous acid water (CAW) was approved as a food additive by the Ministry of Health, Labor, and Welfare of Japan in 2013, and HClO₂-based disinfection was classified as

a second-class OTC drug in 2019. Thus, HClO₂-based sanitizers, especially CAW, would have the potential to be effective disinfection reagents; however, the effectiveness of animal skin disinfectants is unclear, especially for future applications in clinical procedures in veterinary medicine.

Therefore, the present study evaluated the potential of CAW as a presurgical disinfectant in cattle. Our data suggest that disinfection using CAW is useful and comparable to routine disinfection methods, and might lead to a reduction in the time required for presurgical disinfection in farm fields.

2 Materials and methods

2.1 Animals and environment

Female Holstein cows were maintained at the experimental farm of the Field Science Center for Northern Biosphere, Hokkaido University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval no. 22-0110, 3/17/2023). The experiments were performed in March 2023 (temperature $11.5 \pm 0.7^\circ\text{C}$, humidity $54.4 \pm 2.2\%$) and August 2023 (temperature $27.5 \pm 0.5^\circ\text{C}$, humidity $74.1 \pm 0.5\%$) at cattle housing, considering seasonal effects. Monthly changes in temperature and humidity were based on information published by the Japan Meteorological Agency (<https://www.jma.go.jp/jma/indexe.html>; Tokyo, Japan).

2.2 Routine disinfection procedure based on Fürbringer's or Grossich's method

Figure 1 summarizes the experimental procedure, including routine disinfection using scrubbing, povidone-iodine, and alcohol (SPA). All experimental procedures were conducted within the cattle housing. The cows were randomly assigned to the trial without specifically considering their lactation status, encompassing both dry and lactating periods. The experiments were performed during specific time frames: in March from 10:00 a.m. to 4:00 p.m., and in August from 9:00 a.m. to 4:00 p.m. The cows were tied to a stall and their tails were secured with a string to prevent movement. The left paralumbar fossa (width, 63 cm; height, 60 cm) was clipped using clippers (Xperience, Heiniger; Herzogenbuchsee, Switzerland) equipped with blade no. 53a-23 (1 mm after clipping; Heiniger). Then, the skin surface of the paralumbar fossa was cleaned with 20 mL of liquid soap using a disposable polypropylene brush ($7.5 \times 10 \times 5$ cm) and a small amount of tap water for 1 min. The soap bubbles were rinsed with tap water, and the clipped area was wiped with sterilized gauze (30×30 cm, Iwatsuki; Tokyo, Japan) to prevent contamination from the unclipped area. The clipped area was washed with a new polypropylene brush using 7.5% iodine scrub solution (88.5 g, Shionogi; Osaka, Japan) for 1 min. The scrubs were rinsed with tap water and the clipped area was wiped with sterilized gauze (Iwatsuki). In some cases, the clipped area was covered with a sterile surgical drape (90×90 cm; Nissho Sangyo; Tokyo, Japan). Then, 10% PVP-I (55 mL, Fujita Pharmaceutical Co., Ltd.; Tokyo, Japan) was sprayed and contacted for 5 min; then, 70% alcohol (55 mL, Japan Alcohol Corporation; Tokyo, Japan) was sprayed and contacted for 5 min. To neutralize the disinfectants, 0.1 mol/L sodium thiosulfate

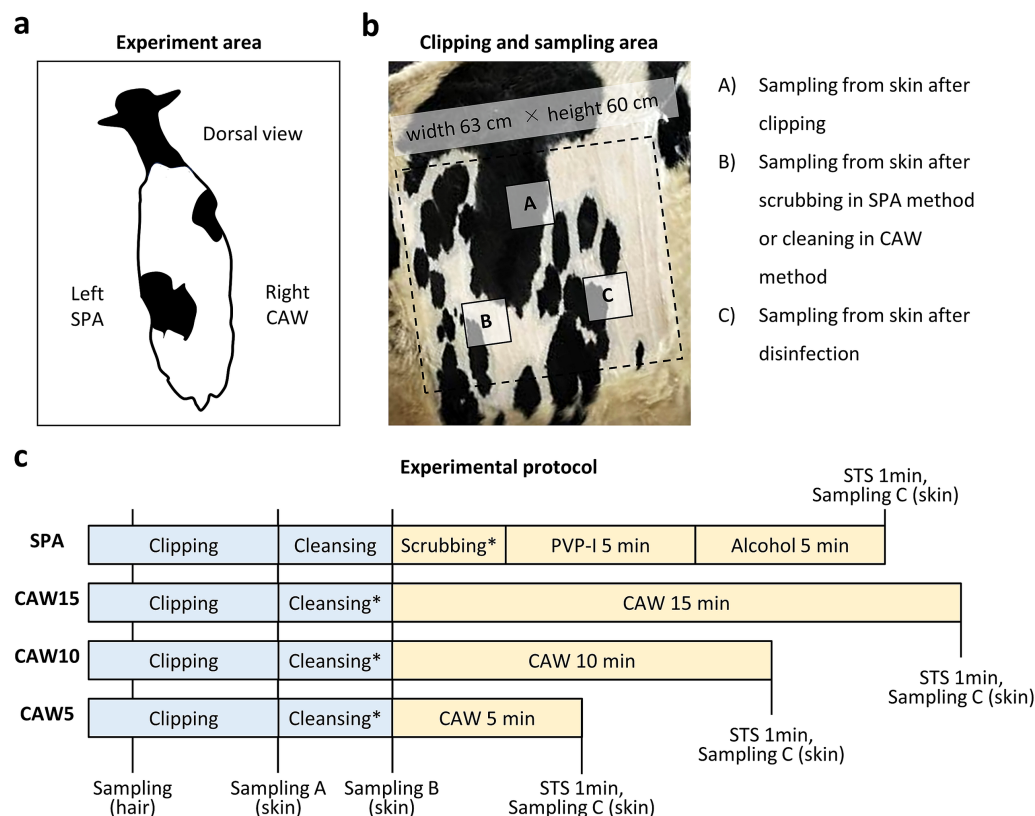


FIGURE 1

Experimental protocol in this study. (a) Dorsal view of the cow in the present experiment. Left or right paralumbar fossae are used for standard disinfection protocol using scrubbing, povidone iodine, and alcohol (SPA) or modified protocol using chlorous acid water (CAW), respectively. (b) Clipped area for bacteria sampling. Areas (A), (B), and (C) are used for sampling from skin after clipping, scrubbing using the SPA method or cleaning using the CAW method, and disinfection, respectively. (c) Experimental protocol. Clipping and cleansing using liquid soap is commonly performed in each protocol. After washing with tap water in cleansing or scrubbing, the experimental area is wiped with sterile gauze. Sampling A–C is corresponding to area A–C in panel (b). After disinfection, 0.1% mol/L sodium thiosulfate (STS) is sprayed on each area. *: Draping is performed in the experiment show in Figure 4. PVP-I, povidone iodine.

(STS) was sprayed and contacted for 1 min. Clipping and cleansing were performed by 2 experimenters, whereas sampling and disinfectant spraying were performed by 1–2 other experimenters.

2.3 Modified disinfection procedure using CAW

As summarized in Figure 1, the procedure was the same as that in the routine SPA method, but we sprayed 1,000–8,000 ppm (free available chlorine = 25–200 mg/L) of Klorus disinfectant water (55 mL, PURGATIO Inc., Tokyo, Japan) after washing with liquid soap, rinsing with tap water, and wiping with sterilized gauze on the right paralumbar fossa of the same cow used in the SPA method. The concentration used was determined through a preliminary experiment (Supplementary Figure S1). The contact times of the CAW were examined at 5, 10, and 15 min. In some cases, the clipped area was covered with a sterilized surgical drape (90 cm × 90 cm; Nissho Sangyo) after spraying with CAW. To neutralize the disinfectants, 0.1 mol/L STS was sprayed and contacted for 1 min. Clipping and cleansing were performed by 2 experimenters, whereas sampling and disinfectant spraying were performed by 1–2 other experimenters.

2.4 Sample collections

Hair or bacteria were collected from the skin surface layers using rayon cotton swabs (Wipe Check II; Eiken Chemical; Tokyo, Japan). The swab area was measured using a sterile frame (10 × 10 cm; AS ONE Corporation; Osaka, Japan). As shown in Figure 1, sample collections of swabbing were performed for 3 times; (A) after clipping, (B) after wiping with gauze after liquid soap cleansing, and (C) after spraying the STS for both the SPA and CAW methods. Samples were collected from different areas (A), (B), and (C).

2.5 Microbiological examination

All samples were appropriately diluted for saline, and 0.1 mL of diluted samples were used.

2.5.1 Standard plate count bacteria

Diluted sample was spread on standard method agar (Nissui Pharmaceutical Co. Ltd.; Tokyo, Japan) using sterilized glass beads and incubated at 35°C for 24–48 h to enumerate the surviving bacteria.

2.5.2 *Enterococcus faecalis*

Diluted sample was spread on EF agar base (Nissui Pharmaceutical Co. Ltd.) with 0.0015% 2,3,5-triphenyltriazolium chloride using sterilized glass beads and incubated at 35°C for 24–48 h. Colonies with colors ranging from pink to dark brown were enumerated as surviving EF.

2.5.3 *Pseudomonas aeruginosa*

Diluted sample was spread on NAC agar (Eiken Co. Ltd.; Tokyo, Japan) using sterilized glass beads, and incubated at 35°C for 24–48 h; yellow-greenish fluorescent colonies were enumerated as the surviving PA.

2.5.4 EC

Diluted sample was spread on X-MG agar medium (Shimadzu Diagnostics Corporation; Tokyo, Japan) with sterilized glass beads and incubated at 35°C for 18–22 h; blue colonies were enumerated as the surviving EC.

2.5.5 *Staphylococcus* spp.

Diluted sample of the diluted sample was spread on mannitol salt agar (Nissui Pharmaceutical Co. Ltd.) with added egg yolk using sterilized glass beads and incubated at 35°C for 24–48 h; colonies on the medium were enumerated as the surviving *Staphylococcus* spp.

2.6 Evaluation of environmental factors

The experimental site, Sapporo, experiences significant seasonal variations in temperature and humidity levels between summer and winter (Figure 2). Generally, bacteria thrive in the warmer summer conditions. Consequently, this study evaluated these environmental factors and their impact on bacterial growth. To assess the potential bacterial contamination of the experimental procedure due to environmental factors, we examined SPBC or coliform bacteria. To evaluate airborne bacterial contamination, standard method agar plates were placed at four different locations within the farm, and this

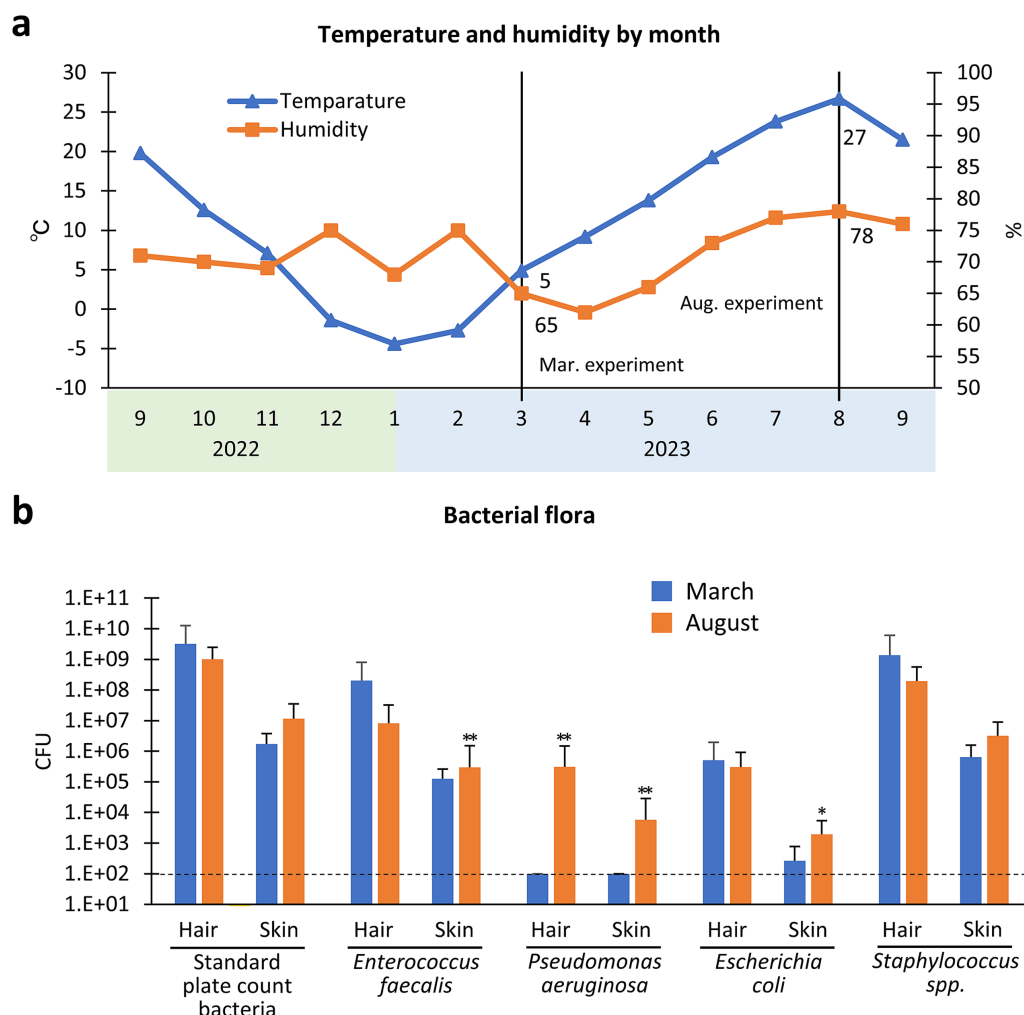


FIGURE 2

Seasonal differences of bacterial flora in the hair and skin of cows. (a) Changes in temperature and humidity according to month (from September 2022 to September 2023) in Sapporo city, Japan. The experiments are conducted in March 2023 and August 2023. Values = mean. (b) Bacterial flora of hair and skin in March or August 2023. *, **: Indicates significances with respect to March in hair or skin, as determined using the Mann–Whitney *U* test ($p < 0.05$, 0.01, respectively). Values = mean $\pm$ standard deviation. CFU, colony forming unit/100 g hair or/100 cm² skin. Dotted line indicate the detection limit (DL). $n = 30$ samples from 15 cows in March, $n = 48$ samples from 23 cows in August.

process was repeated four times. The plates were exposed with their lids open for 5 min, and the resulting bacterial colonies were counted after incubating the plates at 35°C for 24–48 h. For the tap water analysis, the samples were appropriately diluted using saline solution, and 1 mL of each dilution was transferred to a sterile plastic petri dish. Subsequently, 20–25 mL of sterilized medium, either deoxycholate agar (Nissui Pharmaceutical Co. Ltd.) for coliform bacteria or standard method agar (Nissui Pharmaceutical Co. Ltd.) for SPCB, was poured into the dishes. The mixtures were gently agitated, allowed to solidify, and then incubated at 35°C for 18–48 h to count the proliferating microorganisms.

2.7 Histological analysis of skin exposed to disinfectant

For histological analysis, one adult cow that was euthanized in a separate experiment was used, and the skin of the paralumbar fossa was incised within minutes after euthanasia. Alcohol (70%), PVP-I, or CAW was sprayed onto the skin surface or incised area and contacted for 15 min. The skin was collected and fixed in a mixture of formalin, acetic acid, and absolute ethanol (volume ratio, 10:5:85) for 24 h at room temperature. After dehydration using alcohol, tissues were embedded in paraffin and cut into sections (4 µm thick), including the region exposed to disinfectants. The deparaffinized sections were stained with hematoxylin–eosin (H&E).

2.8 Statistical analysis

Results were expressed as the mean with or without standard deviation and analyzed statistically with nonparametric methods using IBM SPSS Statistics 28.0.1.0 (142) (IBM; Armonk, NY, United States). For bacterial analysis, colony-forming units (CFU) were expressed as CFU/100 g in the hair and CFU/100 cm² in the skin. The reduction ratio of CFU in the skin samples after cleansing or disinfection to those immediately after clipping was also calculated. The Mann–Whitney *U* test was used to compare the two populations ($p < 0.05$). The Kruskal–Wallis test was used to compare the three populations, and multiple comparisons were performed using Scheffé's method when a significant difference was observed ($p < 0.05$).

3 Results

3.1 Overview of experimental protocol and time required for each procedure

Figure 1 illustrates the experimental procedures used in this study. The left and right paralumbar fossae were used for SPA and CAW disinfection, respectively (Figure 1a). Both experimental areas were clipped, and skin swab sampling for bacterial analysis was performed at different timing according to each purpose as shown in Figure 1b; sampling from skin after clipping (A), cleansing (B), or reaction termination of disinfects with STS (C). Figure 1c shows the experimental procedure from clipping to disinfection and reaction termination by STS for each time course. Clipping and cleansing were common in all procedures; however, the CAW method could skip

scrubbing. Furthermore, CAW10 and CAW5 (contact times of 10 and 5 min, respectively) were shorter than those of the SPA method, which required scrubbing and exposure to PVI-I and alcohol (5 min each). CAW15 (contact time of 15 min) was the longest procedure in this study.

3.2 Seasonal differences of bacterial flora in the clipped hair and skin of cows

The present experiments were performed in March (winter to spring) and August (summer), 2023 in Sapporo city, Japan. Figure 2a shows the monthly changes in temperature and humidity in Sapporo based on data published by the Japan Meteorological Agency. Actual temperature and humidity of experimental farm in Hokkaido University was $11.5 \pm 0.7^\circ\text{C}$, $54.4 \pm 2.2\%$ on March and temperature $27.5 \pm 0.5^\circ\text{C}$, $74.1 \pm 0.5\%$ on August during this experiment.

Figure 2b shows the CFU differences in bacterial flora cultured from clipped hair and skin between the March and August experiments (note the difference in value calculation, CFU 100 g hair and/or 100 cm² skin). Hair collected in August showed significantly higher CFU in PA than hair collected in March ($p < 0.01$). Furthermore, the CFU in skin samples were significantly higher in August than in March for EF ($p < 0.01$), PA ($p < 0.01$), and EC ($p < 0.05$). Thus, these data indicate that EF, PA, and EC on the skin surface increased in summer, but SPCB and *Staphylococcus* spp. on the skin, which showed higher CFU (over 6 log₁₀ CFU/cm²) than other spp., were comparable between the two seasons.

3.3 Effect of disinfection protocols on skin bacterial flora

The effective concentration of CAW was examined in a preliminary study (Supplementary Figure S1). CFU or reduction ratio of CFU in skin samples after cleansing or disinfection to those immediately after clipping (reduction % vs. skin) were compared between SPA and modified protocol using CAW100% (8,000 ppm), CAW50% (4,000 ppm), CAW25% (2,000 ppm), and CAW12.5% (1,000 ppm). For SPCB, CAW 100% showed comparable disinfection ability to SPA. In the condition below 50% of CAW, CFU of SPCB tended to be higher than that of SPA. For *Staphylococcus* spp., in the conditions below 25% of CAW, CFU tended to be higher compared to SPA. Based on these results, we proceeded with the experiment using 100% to guarantee the highest disinfection performance of CAW.

As shown in Supplementary Figure S2, the CFUs of PA and EC were lower, and the differences in each disinfection method were not clear; therefore, we focused on other bacteria, as shown in Figure 3. The disinfection efficiencies of SPA, CAW15, CAW10, and CAW5 were compared. CFU or reduction % vs. skin was also evaluated for each examined bacterial species using the combined data obtained in March and August. For SPCB, all methods reduced the CFU of skin samples after cleansing or disinfection, and statistically significant reductions were observed for SPA and CAW10 ($p < 0.01$). For the reduction % vs. skin in SPCB, significant differences were observed in skin samples after cleansing or disinfection for all methods ($p < 0.01$). Furthermore, no significant differences between cleansing and disinfection were observed for any of the methods. For EF, all methods

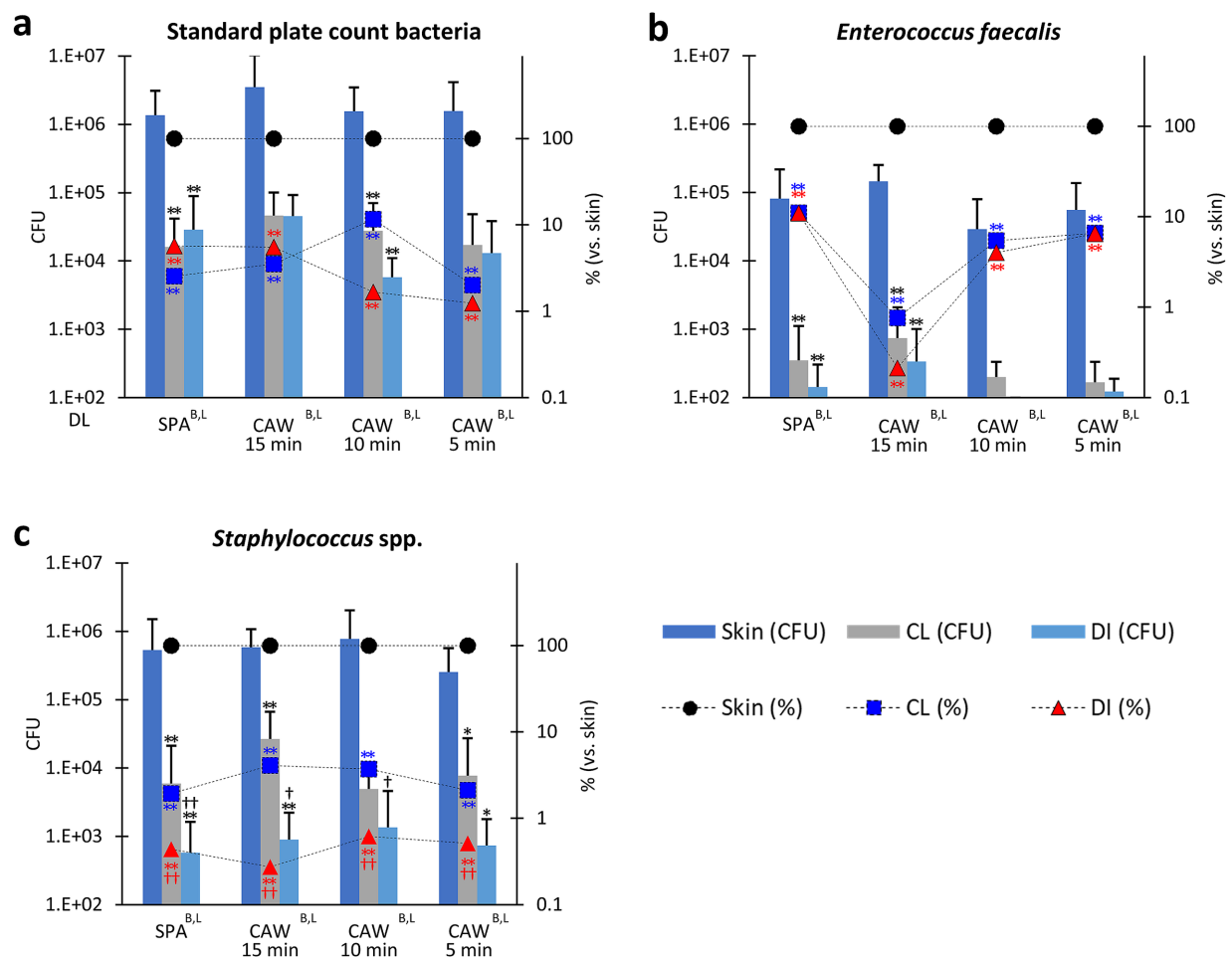


FIGURE 3

Bacterial flora in the skin of cows. (a) Standard plate count bacteria. (b) *Enterococcus faecalis*. (c) *Staphylococcus* spp. Standard disinfection protocols using scrubbing, povidone iodine, and alcohol (SPA), and a modified protocol using chlorous acid water for 15, 10, and 5 min (CAW15, 10, and 5) were compared. Bar graphs represent colony-forming units (CFU). The line graph shows the ratio of skin samples after clipping to those after cleansing (CL) and disinfection (DI). B, L: Significance determined using the Kruskal-Wallis test ($p < 0.01$) in bar and line graphs, respectively. *, ** (black): Indicates significances with respect to skin CFU, as determined using Scheffé's method ($p < 0.05$, 0.01 , respectively). ** (blue): Indicates significances with respect to skin CL (%), as determined using Scheffé's method ($p < 0.01$, respectively). ** (red): Indicates significances with respect to skin DI (%), as determined using Scheffé's method ($p < 0.01$, respectively). †, †† (black): Indicates significance with CL (CFU), as determined using the Mann-Whitney U test ($p < 0.05$, 0.01 , respectively). †, †† (red): Indicates significance with CL (%), as determined using the Mann-Whitney U test ($p < 0.05$, 0.01 , respectively). Bar graph: values = mean $\pm$ standard deviation. Line graph: values = mean. The dotted line indicates the detection limit (DL). $n = 28$ samples from 20 cows in SPA, $n = 8$ samples from 7 cows in CAW15, $n = 9$ samples from 8 cows in CAW10, $n = 9$ samples from 6 cows in CAW5.

reduced the CFU of skin samples after cleansing or disinfection, and statistically significant reductions were observed for SPA and CAW15 ($p < 0.01$). Furthermore, disinfection tended to reduce CFU compared to cleansing. For the reduction % vs. skin, significant differences were observed in samples after cleansing or disinfection for all methods ($p < 0.01$), and significant differences between cleansing and disinfection were observed for SPA ($p < 0.05$). These data indicate the importance of cleansing to reduce SPCB on the cow skin surface.

For *Staphylococcus* spp., all methods significantly reduced the CFU of the skin or the ratio to skin samples after cleansing or disinfection, with statistical significance for SPA, CAW15 ($p < 0.01$), and CAW5 ($p < 0.05$). Furthermore, reduction % vs. skin also significantly decreased after cleansing or disinfection in all methods ($p < 0.01$). In particular, disinfection reduced both CFU and reduction % compared to cleansing, and statistical significance was detected in the CFU of SPA ($p < 0.01$), CAW15, and CAW10 ($p < 0.05$), and in the

reduction % in all methods ($p < 0.01$). These data indicate that disinfection using either SPA or CAW was effective in reducing *Staphylococcus* spp. after cleansing.

3.4 Effect of draping to disinfection efficiency in the farm field

As shown in Figure 4a, numerous colonies, including *Bacillus* spp., derived from airborne bacteria were observed in the samples collected from the different area (265 ± 213 colonies, $n = 11$, 5 min exposure, 24–48 h). As the experiments shown in Figure 3 were performed without draping, we verified the effect of draping on the skin bacterial flora during the disinfection protocol (Figure 4b). Draping with the disinfection protocol using SPA, CAW15, CAW10, and CAW5 decreased the ratio of CFU in skin samples

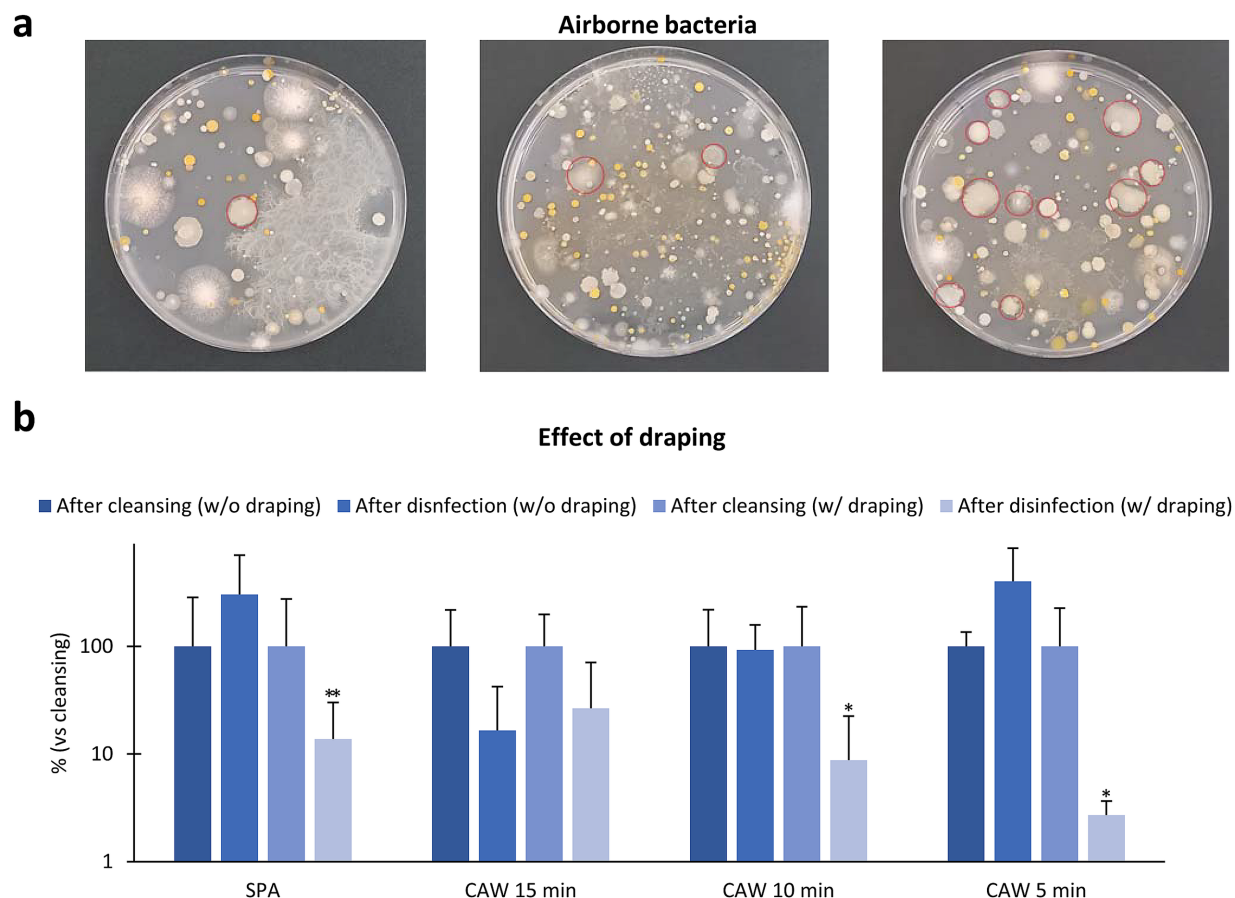


FIGURE 4

Effect of environmental factors. **(a)** Bacteria samples collected from three different area in farm fields. The plates are exposed with their lids open for 5 min, and the resulting bacterial colonies are counted after incubating the plates at 35°C for 24–48 h. Numerous colonies are observed, and red circles indicate *Bacillus* spp. colonies. **(b)** Effect of draping to skin bacterial flora. Data represents the ratio change of standard plate count bacteria in skin sample after disinfection without or with draping to those after cleansing. *, **: Indicates significances with respect to skin DI (%), as determined using the Mann–Whitney *U* test ($p < 0.05$, 0.01, respectively). Values = mean $\pm$ standard deviation. $n = 12$ samples from 10 cows in SPA, $n = 4$ samples from 4 cows each in CAW15 and CAW10, $n = 4$ samples from 3 cows in CAW5, without draping condition. $n = 12$ samples from 12 cows in SPA, $n = 4$ samples from 4 cows each in CAW15, CAW10, and CAW5, with draping condition.

immediately after clipping, and significant differences were observed in SPA ($p < 0.01$), CAW10, and CAW5 ($p < 0.05$), emphasizing the importance of draping during the disinfection procedure.

3.5 Histology of skin after experimental procedures

In Figure 5, we examined the histological features of skin in the paralumbar fossa or udder after exposure to 70% alcohol, PVP-I, or CAW for 15 min; these procedures were performed without scrubbing. As shown in the panels, skin histological structures, including the cornified stratified squamous epithelium and dermis, did not change after any of the procedures (Figures 5a–c). We also examined the histology of the incised skin after direct exposure to each reagent, but no clear histological changes due to reagent contact were observed in the incised area. Furthermore, we applied these methods to udders, assuming the application of CAW for disinfection during milking. The epidermis of the udder was thicker and more highly cornified than

that of the paralumbar fossae, and no histological changes were observed upon exposure to each reagent.

4 Discussion

In this study, we evaluated the potential of CAW as a pre-surgical disinfectant for cattle skin. Our data suggest that disinfection using CAW is useful and comparable to routine veterinary methods that use a combination of scrubbing, PVP-I, and alcohol. Importantly, our disinfection method using CAW could lead to a reduction in the operation time required for presurgical disinfection in farm fields, as it eliminates the need for scrubbing. Furthermore, our data emphasize the importance of clipping, cleansing, and draping for effective disinfection of animal skin.

First, we examined seasonal changes in the bacterial flora in the hair and skin of cattle. In general, hair contains numerous bacteria as reported previously (6). Several studies have indicated that animal hair provides a favorable environment for bacteria growth (17). High variability in bacteria was also observed between different skin regions

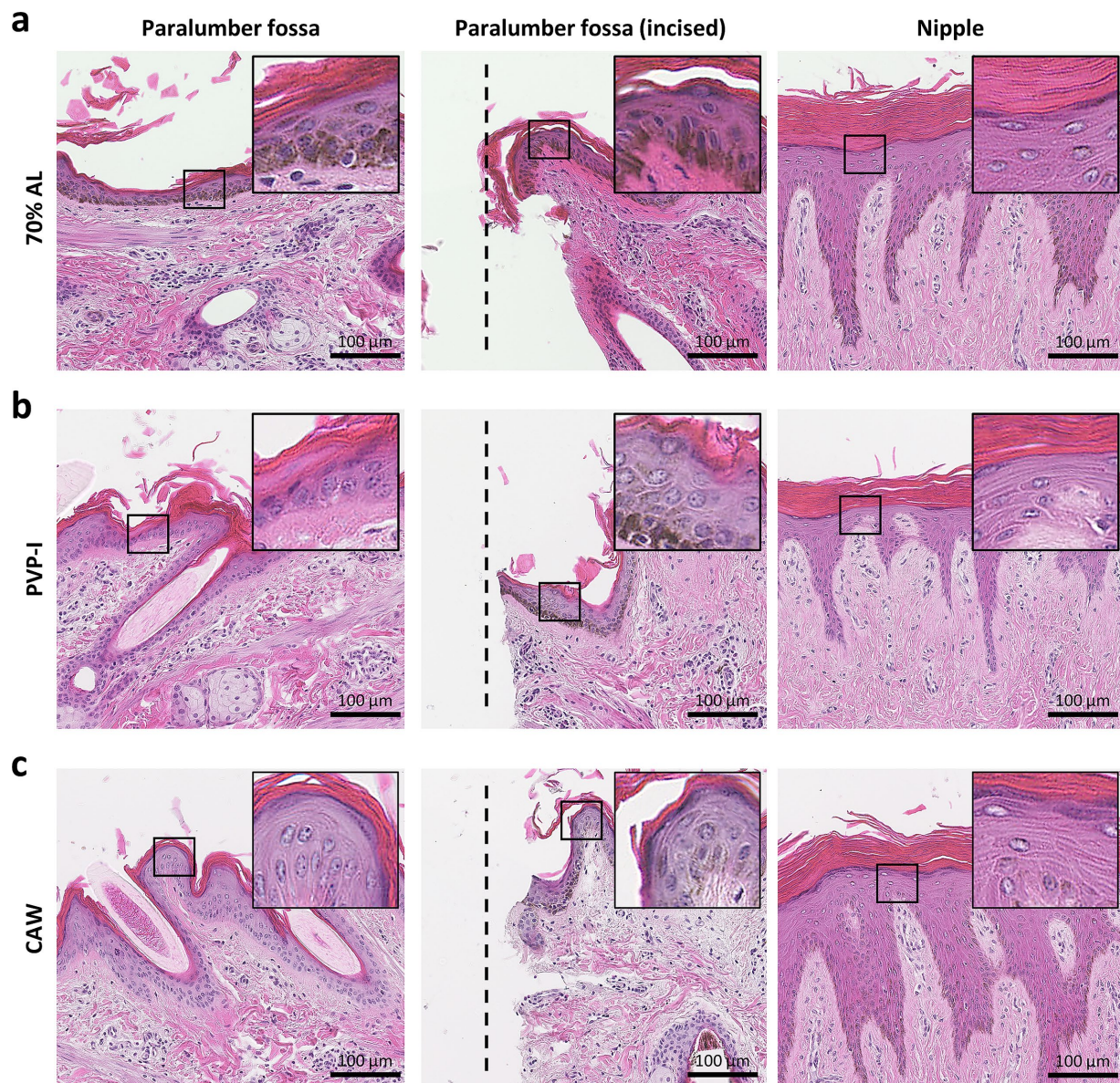


FIGURE 5

Histology of skin after experimental procedures. **(a)** Histology of the skin area sprayed with 70% alcohol (AL). **(b)** Histology of the skin area sprayed with povidone iodine (PVP-I). **(c)** Histology of the skin area sprayed with chlorous acid water (CAW). Samples were collected from each area 15 min after spraying. In the incised area of the paralumber fossa, each disinfectant was sprayed directly onto the incision wound using a scalpel. On histological examination, skin structures, especially those of the epidermis, such as the cornified stratified squamous epithelium, were well-preserved without remarkable structural changes. The insets magnify the squared areas. Hematoxylin and eosin staining.

within the same dog, with a higher number of bacterial species observed on haired skin than on poorly haired skin or mucocutaneous junctions (18). The examined bacterial species, including SPCB, EF, and *Staphylococcus* spp. (over $6 \log_{10}$ CFU/g), were more abundant than PA and EC (under $6 \log_{10}$ CFU/g) in clipped cattle hairs. These data also strongly suggest that hair clipping is a crucial first step in skin disinfection in cattle, although shaving or short clippers are not recommended for animals because of the risk of skin damage leading to SSI (3–5). Cattle skin also contained abundant SPCB and *Staphylococcus* spp. (over $6 \log_{10}$ CFU/100 cm²) compared to others (under $6 \log_{10}$ CFU/100 cm²), and this tendency was similar to that of hair bacterial flora. However, the CFU of EF, PA, and EC were significantly higher in August than in March, and PA in hair showed

significantly higher CFU than in March. Bacterial populations on the human skin are significantly affected by high-temperature and high-humidity environments compared to moderate-temperature and low-humidity environments (19). These data emphasize the importance of hair clipping and skin cleansing and disinfection according to environmental changes, especially under high temperature or humidity conditions.

Among all examined procedures, cleansing with liquid soap and a polypropylene brush, followed by rinsing with tap water, significantly decreased the CFU on cattle skin. In fact, the CFU counts of the examined bacteria, except for *Staphylococcus* spp., were comparable between the samples after cleansing and disinfection, regardless of whether SPA or CAW was used. Regarding the concentration of CAW,

we determined that 8,000 ppm (free available chlorine = 200 mg/L) would be stable for the quality of presurgical disinfectant because 4,000 ppm (free available chlorine = 100 mg/L) decreased the disinfection efficiency of SPCB compared to 8,000 ppm in a preliminary study (see [Supplementary Figure S1](#)). Regarding the exposure time of the CAW, there was no remarkable difference among 5, 10, and 15 min of exposure for the examined bacterial CFU, and their disinfection efficiency was comparable with that of the SPA. Furthermore, disinfection using CAW can eliminate the scrubbing procedure required for SPA. The disinfection efficiency of CAW can be realized based on the chemical properties of HClO_2 in CAW, which can act under organic-matter-rich conditions (14–16). Therefore, the use of CAW (8,000 ppm) for 5 min after hair clipping and liquid soap cleansing can shorten the operation time for pre-surgical disinfection.

In the present study, the CFU of *Staphylococcus* spp. were significantly decreased in cattle skin disinfected with SPA and CAW compared to those cleansed with liquid soap. *Staphylococcus* spp. currently comprises 81 species and subspecies, with most members of the genus being mammalian commensals or opportunistic pathogens that colonize niches, including the skin (20). Several *Staphylococcus* spp. can cause serious pathological problems in human and veterinary medicine. *Staphylococcus epidermidis*, a normal component of the epidermal microbiota, can lead to biofilm contamination of medical devices (21). Especially, in dairy cows, *S. aureus* is a major cause of mastitis, resulting in significant economic losses. Importantly, *S. aureus* infections are major risk factors for SSI in animals (20); therefore, CAW disinfection before surgery can contribute to reducing the risk of SSI, similar to the routine SPA method.

In the present study, we demonstrated the effectiveness of draping the surgical area (22), which can protect against bacterial contamination. In veterinary medicine, draping the surgical area as soon as possible after cleansing and disinfection is crucial in practical on-site disinfection scenarios such as on farms. Importantly, the CAW method, with 5 min of exposure, can reduce the chance of contamination from falling bacteria because it can skip the scrubbing time. In fact, a 15 min exposure to CAW did not result in a more effective disinfection efficiency compared to 5 min. This could be explained by the increased chance of contamination from the environment, such as falling bacteria or dripping water from hair surrounding the surgical area. Therefore, the CAW disinfection method can increase its disinfection efficiency by immediately draping and wiping off the surrounding area using a sterile gauze.

5 Limitations

CAW has been applied in food and environmental sanitation, and the microbicidal effects on a wide range of microorganisms, including yeast, EC, *S. aureus*, *C. jejuni*, *C. difficile* spores, *C. albicans*, spore-forming *Bacillus*, and *Paenibacillus* species, as well as human norovirus and feline calicivirus have been reported (14–16). Because cattle can contract dermatophytosis, it is important to evaluate their susceptibility to fungi. Furthermore, the present study demonstrated the usefulness of CAW for skin disinfection. No histological changes were observed in the skin of the paralumbar fossa and udders of cattle after exposure to CAW for 15 min. However, the residual time in tissues has not yet been evaluated because the reaction was stopped with STS to guarantee

an accurate reaction time. Furthermore, stopping the experimental reaction with STS cannot be used to evaluate the sustained effects of the CAW, which might cause an underestimation of its disinfection efficiency. For example, a recent study showed that 1 h after application, the bacterial reduction was better sustained with chlorhexidine than with ethanol, but no difference was found between chlorhexidine and isopropyl alcohol (23). For further applications of CAW in veterinary medicine, such as multiple spraying in the surgical area, cattle teat disinfection, or fogging of farm areas, the residual time in each region should be accurately evaluated in future studies. Effects on skin when used with electrocautery should also be evaluated (24).

In conclusion, the present study suggests that disinfection using CAW is useful and comparable to routine SPA disinfection methods and might lead to a reduction in the operation time required for presurgical disinfection in farm fields.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of Hokkaido University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

OI: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. TeN: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – review & editing. MH: Formal analysis, Investigation, Writing – review & editing. TaN: Formal analysis, Investigation, Writing – review & editing. MR: Formal analysis, Investigation, Writing – review & editing. TU: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing. MA: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing.

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Conflict of interest

TU and MA were employed by Furukawa Sangyo Kaisha, Ltd.

The authors declare that this study received funding from Furukawa Sangyo Kaisha Ltd. The funder had the following involvement with the study: support of data collection and manuscript submission.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pathological and molecular investigation of infectious bronchitis in broilers: analyzing the impact of biosecurity lapses

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Introduction: Infectious Bronchitis (IB) is an acute, highly contagious disease of poultry that leads to significant economic losses in intensive production systems. Preventive biosecurity measures are essential to control its spread, particularly in broiler farms. This study aimed to investigate the relationship between IB outbreaks and biosecurity practices on a broiler farm.

Methods: The farm, housing 96,000 broilers, experienced increased mortality (over 11%) during two consecutive production cycles. Consequently, serological, pathological, molecular and biosecurity investigations were conducted.

Results: Despite a vaccination program using two types of live vaccines (Massachusetts serotype and serotype 793B), serological testing revealed elevated antibody titers against the IB virus, suggesting exposure to a wild viral strain. Necropsy revealed various lesions, including hemorrhagic tracheitis, pulmonary hyperemia, fibrinous pericarditis, splenomegaly, and ascites. Histopathological findings showed necrotic tracheitis, multifocal hepatitis, and purulent bronchopneumonia. By PCR IB viral RNA was detected in all 24 swabs and tissue samples. Biosecurity evaluation revealed significant deficiencies in both external and internal measures, including improper cross-contamination prevention, inadequate flock management, and insufficient vaccination strategies.

Discussion: These biosecurity deficiencies, coupled with the inadequate selection of vaccines not tailored to the prevalent serotypes in the local area, allowed for the introduction and spread of wild IB virus strains. This highlights the critical importance of robust, well-implemented biosecurity protocols in preventing IB on poultry farms.

KEYWORDS

infectious bronchitis, biosecurity, broiler, pathology, farm

Introduction

Infectious bronchitis is a multisystemic disease that primarily affects the respiratory system, but also impacts the urogenital system, leading to kidney dysfunction and decreased egg production, resulting in substantial economic losses in intensive farming (1, 2). According to global economic estimations, infectious bronchitis is one of three diseases that have claimed the largest numbers of losses of animals of different species. Avian infectious bronchitis and LPAI, which caused significant losses, continued to see an increase in losses over time, while none of them exhibited particularly high mobility (3),

due to its effects on egg production, shell quality, and hatchability. In broilers, the disease leads to reduced weight gain and increased feed conversion (4, 5).

The causative agent is a virus classified under the family *Coronaviridae*, genus *Gammacoronavirus*, order *Nidovirales*. Around 30 serotypes of the IB virus (IBV) are known globally, and they differ in their virulence or pathogenicity for respiratory organs, kidneys, or oviducts. Four structural proteins are present in the IBV virion: nucleocapsid, membrane, small membrane, and spike proteins (6). The spike glycoprotein on the virus's surface contains epitopes related to serotype variation and neutralizing antibody binding and is critical for virus attachment and entry into host cells (7). IBV is prone to frequent genetic changes, leading to the continuous emergence of new strains with increased virulence, different tissue tropism, and expanded host range (4). Transmission occurs through respiratory secretions and feces of infected birds. Contaminated equipment and facilities can contribute to spreading the virus from one flock to another if adequate hygiene measures are not implemented (8).

All age categories of poultry are susceptible to infection, and the severity and intensity of the disease are more pronounced in young chicks, with resistance to infection increasing with age (9). The clinical signs depend on which organ systems are affected. Respiratory infection can lead to clinical signs such as gasping, sneezing, lethargy, ruffled feathers, and nasal discharge. Decreased growth and clustering of individuals around heat sources are also observed. In some cases, conjunctivitis, excessive tearing, edema, and cellulitis of the periorbital tissue may occur. The clinical signs in broilers infected with nephropathogenic strains of IBV include depression, watery feces, and excessive water intake. Infection of the reproductive system, particularly damage to the oviduct, results in reduced egg production and quality. Eggs may appear deformed, with rough shells or soft shells containing watery yolks (2). In flocks already infected with immunosuppressive viruses (avian adenovirus, chicken anemia virus, infectious bursal disease virus), the course of IB infection is prolonged, and the clinical signs are more severe. In such cases, the virus can persist in the environment for an extended period, facilitating the development of new genotypes and virus variants (5). Secondary infections with *E. coli* or *Mycoplasma* spp. are common findings in broilers with IB, leading to airsacculitis, increased mortality (10–60%), and higher carcass rejection rates at slaughter (5).

Epidemiological studies have shown that the infection caused by the avian infectious bronchitis virus is endemic in Serbia. Phylogenetic investigations conducted during 2016 and 2017, based on partial S1 protein sequences, revealed the circulation of strains in Serbia classified into the D274 genotype, QX genotype, and 4/91 genotype (10).

Vaccination programs and other biosecurity measures achieve IB control in commercial poultry production. In Serbia, effective live attenuated and inactivated vaccines are available and widely used in practice. However, the virus's tendency to mutate frequently poses a challenge. Many wild IBV and vaccinal strains create an ideal situation for new virus variants (11). To effectively control the situation in a specific area, IB detection, genotyping, and continuous surveillance need to be performed. Factors that predispose IB to increased variability include the introduction of

wild genotypes, incorrect vaccine selection and administration, and immunosuppressive diseases (5).

A farm's biosecurity plan includes clearly defined measures aimed at reducing the risk of introducing and spreading pathogenic microorganisms (11, 12). Measures are referred to as external and focused on reducing the risk of pathogen introduction to the farm via humans, equipment, vehicles, wild animals, pets, and other animals, while internal measures aim to reduce the spread of pathogens already present on the farm (12, 13). The occurrence of disease on commercial farms is often associated with failures in the implementation of biosecurity plans, the emergence of new virus serotypes, or improperly executed vaccination programs.

This study aims to investigate the pathological and molecular aspects of an infectious bronchitis case in broiler chickens. Additionally, it seeks to identify failures in biosecurity preventive measures that may have contributed to the outbreak.

Materials and methods

Broiler farm

The study was conducted on a broiler farm with a capacity of 96,000 broiler chickens, distributed across four houses. There are no other poultry farms within a 1 km radius of this farm. It is located 2 km away from an artificial lake (fishpond). The farm was built in 2021, and throughout the year, all four houses undergo 5.79 production cycles (batches) successively, with new birds introduced every 63 days. The broilers are normally slaughtered at the age of 38 to 45 days, therefore, each house has a downtime of 10–12 days, but the location where all four houses are situated (the farm) has no rest days until the new chicks are settled in. According to the farm's immunoprophylactic program, broiler chicks were vaccinated on the first day after being delivered on the farm using the spray method with live attenuated vaccines containing two different infectious bronchitis virus serotypes (classic – Massachusetts serotype, strain H-120, GI-1 lineage, and variant—serotype 793B, GI-13 lineage). Depending on the established level of maternal antibodies, the chicks were vaccinated during the second week of life against Newcastle disease virus. The broilers were also vaccinated twice, seven days apart, against the infectious bursal disease virus.

Since the beginning of the 2024 year, during two successive production cycles, high increase of mortality has been observed in two houses of the farm, leading to significant economic losses. The onset of clinical symptoms such as a decrease in food consumption and increased mortality started in the birds aged 2–3 weeks. At the end of the first cycle, mortality in those houses was 11.12%, and at the end of the second, 11.51%. In the other two houses of the farm, the mortality was 7.15% and 7.05%. The total mortality on this farm, or at this location for all four houses, was 9.21%, of which 5.62% of the total number of settled chicks died after 28 days. In this study, ethical approval was not required as it involved routine diagnostic procedures; however, the farm owner consented to publish the results without disclosing the farm's name.

Clinical examination

During the farm visit, the broiler flock was clinically examined using the standard clinical inspection method (regularly checking the birds for visible signs of illness, injury, or abnormal behavior, that includes assessing their overall health, body condition, posture, and the condition of their feathers, eyes, and respiratory system).

Serological testing

For diagnostic purposes, serological testing was performed on 40 blood samples from two houses (20 samples per house) collected from the wing veins of 4-week-old chickens using the indirect ELISA method (ID Screen Infectious Bronchitis Indirect Elisa kit, ID Vet, Montpellier, France) during the first and second production cycles. According to the manufacturer's instructions, the antibody titer threshold for a positive-negative result is 1,625, with titers $>1,625$ considered positive and $\leq 1,625$ considered negative.

Necropsy and histopathological investigation

The carcasses of 12 recently deceased birds, which had previously shown clinical signs of disease, were collected from the second production cycle for necropsy. Following necropsy, trachea, liver, kidney, and proventriculus tissue samples from 4 birds were subjected to histopathological examination, fixed in 10% buffered formalin, routinely processed, and embedded in paraffin blocks. Paraffin sections $\sim 5 \mu\text{m}$ thick were stained using the hematoxylin-eosin (HE) method.

Detection and molecular identification of IBV

During the second production cycle, 20 pharyngeal swabs were collected (10 samples per house) from birds exhibiting clinical symptoms for detection and molecular identification of IBV. After necropsy, four tissue samples (trachea, liver, spleen, proventriculus, lungs, kidneys) were also subjected to molecular analysis. Standard bacteriological analysis confirmed presence of *E. coli* in tissue samples (data not shown).

Tissue samples were homogenized using a TissueLyser (Qiagen, Hilden, Germany). Pharyngeal swabs were immersed in 1 ml of sterile PBS and thoroughly vortexed. The tissue and swab suspensions were centrifuged for 1 min at $1,000 \times g$, and the supernatants were used for RNA extraction (Bioextract Superball extraction kit, Biosellal, Dardilly, France). Real-time RT-PCR for the detection of IBV was performed according to the protocol described by Callison (14) using Luna Universal Probe RT-qPCR Master Mix (NEB, Ipswich, Massachusetts, USA) with a final primer concentration of $0.2 \mu\text{mol}$ and a probe concentration of $0.1 \mu\text{mol}$. Amplification was performed in an AriaMx instrument (Agilent, Santa Clara, California, USA) with the following thermal profile: reverse transcription for 10 min at 55°C , initial

denaturation for 1 min at 95°C , and 45 cycles of denaturation at 95°C for 15 s, followed by annealing at 60°C for 1 min.

Part of the hyper-variable S1 gene of IBV was used for Sanger sequencing to determine the genotype of the virus using the Nested RT-PCR protocol described by Worthington (15). The first round of RT-PCR was performed using Luna Universal Probe RT-qPCR Master Mix (NEB, USA) with a final concentration of SX1+ and SX2- primers of $0.2 \mu\text{mol}$. Amplification was performed in 2,720 Thermal cycler (Applied Biosystems, USA) using thermal profile: reverse transcription 10 minutes at 55°C , initial denaturation 1 min at 95°C , and 30 cycles of denaturation at 95°C 30 s, annealing at 50°C 1.5 min and elongation at 72°C 2 min, and single step of final extension at 72°C . Second round PCR was performed using OneTaq® Quick-Load® 2X Master Mix (NEB, USA) with a final concentration of SX3+ and SX4- primers of $0.2 \mu\text{mol}$. Amplification was performed in 2720 Thermal cycler (Applied Biosystems, USA) using a thermal profile: 30 s at 94°C , and 40 cycles of denaturation at 94°C 30 s, annealing at 48°C 1.5 min and elongation at 68°C 2 min, and single step of final extension at 68°C .

The PCR products were visualized by staining in ethidium-bromide, after electrophoresis in 2% agarose gel. Products with the size of 394 bp were excised from the gel, and purified using a mi-Gel extraction kit (Metabion, Germany), and only one was sent for Sanger sequencing in a commercial company. The consensus sequence was obtained using Chromas lite software.

The molecular testing of samples for the presence of the infectious laryngotracheitis (ILT) virus genome was performed through differential diagnostic procedures. The TaqMan-based real-time PCR was conducted using the commercial kit Quanti Tect Multiplex PCR Kit (Qiagen, Valencia, CA, USA) with the primers and probes that targeted the conserved area of gC gene part of the ILT virus genome, as described by Callison et al. (16).

Biosecurity assessment

A farm observation and assessment of biosecurity measures and their implementation were carried out. A biosecurity assessment was performed using checklists for broiler farms. The checklist consists of 79 questions divided into 11 categories. External biosecurity was evaluated across eight subcategories: purchase of one-day-old chicks, broiler depopulation, feed and water supply, removal of manure and carcasses, visitors and farm workers, material supply, infrastructure and biological vectors, and farm location. Internal biosecurity was assessed with questions from 3 categories: disease management, cleaning and disinfection, and materials and measures between compartments. Each category was scored from 0 (indicating a complete lack of biosecurity on the farm) to 100 (indicating full implementation of biosecurity measures). The study used the Biocheck.UGent risk-based scoring system (<http://www.biocheck.ugent.be>) to describe biosecurity assessment on broiler farms. Overall biosecurity was calculated as the average of external and internal biosecurity scores. Veterinarian and farm owner were briefed on the study's goals and procedures before filling the questionnaire. Farm owner provided written informed consent for data collection, sharing, and publication. During the farm visit, we were able to compare the attending

veterinarian's and farm owner responses with the actual conditions on the farm and input the correct answers into the questionnaire.

Results

Clinical examination

Clinical examination of the flock revealed reduced uniformity, clustering of chickens, and ruffled feathers. Respiratory disease symptoms such as sneezing and nasal discharge were noticed.

Serological testing

Blood samples revealed a high mean antibody titer against the IBV during two successive production cycles in two farmhouses, indicating that the birds had been exposed to a wild strain of the virus (Table 1).

According to the general titer baseline of the producer of the ELISA test, the expected mean titer value for a single application of live vaccine (classical plus variant strain), is 4,000–8,000, with a coefficient of variation of 40–80%. During the first cycle, 65–90% of birds (Figure 1) had titer values of more than 8,000, and during the second cycle 35–90% (Figure 2).

Pathological lesions

Gross pathological examination of the broiler carcasses from the second production cycle revealed the following lesions: catarrhal hemorrhagic tracheitis, pulmonary hyperemia, fibrinous pericarditis and perihepatitis, adhesive airsacculitis, splenomegaly with pinpoint splenic hemorrhages, hepatomegaly, nephromegaly, proventriculus dilation, pododermatitis, hemorrhages of the ileocecal tonsils, and ascites (Figure 3).

Histopathological examinations revealed desquamative necrotic tracheitis, multifocal lymphohistiocytic hepatitis, purulent bronchopneumonia, reactive splenitis, renal hyperemia and hemorrhages with tubular necrosis, and pyogranulomatous proventriculitis (Figure 4).

Molecular investigation

The genome of the infectious bronchitis virus (IBV) was detected in all 20 pharyngeal swab samples and 4 tissue samples tested, representing 100% of the samples. By comparing the consensus of partial S1 gene nucleotide sequence product with the size of 394 bp with the sequences from the GenBank, it was determined that 100% homology with previously deposited IBV vaccine strain 4/91, GI-13 lineage (NCBI GenBank, Acc. No. KF377577.1). The result of molecular analysis for the detection of the ILT virus genome was negative.

Biosecurity assessment

Assessing the biosecurity, it was noted that the farm has a biosecurity plan in place, with defined measures related to both external and internal biosecurity. The biosecurity assessment results are presented in Table 2.

Critical points in external biosecurity that were identified through observation and assessment include infrastructure, farmworkers and visitors, partial depopulation, and carcass storage.

(a) Crossing of clean and dirty pathways: According to the check list, the farm fulfills all requirements concerning infrastructure. Poultry do not have access to the outside. Wild birds or vermin cannot enter in the house (air inlets are protected). The farm is fenced, the surrounding is clean and paved. Rodent and pets' control is present. However, there was cross-contamination between clean and dirty areas. No distinction between the areas that use external vehicles (e.g., for feed delivery, manure, carcass removal, external transport, etc.) and internal farm movement zones. The farm lacks a place intended for cleaning and disinfection of external vehicles. The farm is fenced, but the gate is always open, without entrance control (no sign that stop the entrance). The external trucks may entrance undisinfected and unwashed.

(b) Farmworkers and visitors: There was no clear notification limiting access to the poultry houses for people (employees and visitors) without prior registration—It is implied, but it is not written or clearly stated anywhere, and it also does not mean that it is always followed. The number of employees with direct access to the poultry was minimized (two persons per shift manage the flocks in two houses—four people for the whole farm). However, there was no zoning of the barn anteroom in relation to changing boots, clothing, and hand sanitation, nor a well-organized sanitary area between different houses where employees could change clothes, shoes, and wash their hands before entering. The anteroom is designed so that workers can follow all the outlined steps for prevention, but the reality indicates a low level of hygiene and adherence to the established rules. Furthermore, we observed based on the field visit no consistency enforce the protocol for visitors and employees when transitioning from one house to another.

(c) Partial depopulation of broiler: The flocks in each house were partially depopulated in three to four steps. Workers involved in the poultry depopulation process (farmworkers and time-part paid workers from outside) are not provided with specific clothing, shoes, or gloves—there are no measures in place at the beginning of the process. The loading truck arrives empty, cleaned, and disinfected, but the catching is performed by a large number of people who do not apply biosecurity measures for the occasion.

(d) Carcass storage area: Although equipped with cooling and fully enclosed, the carcass storage area is not located in a clearly defined dirty zone of the farm and is not sufficiently distant from the farm's production units. Workers handling carcasses either were not provided with protective gloves or did not use them.

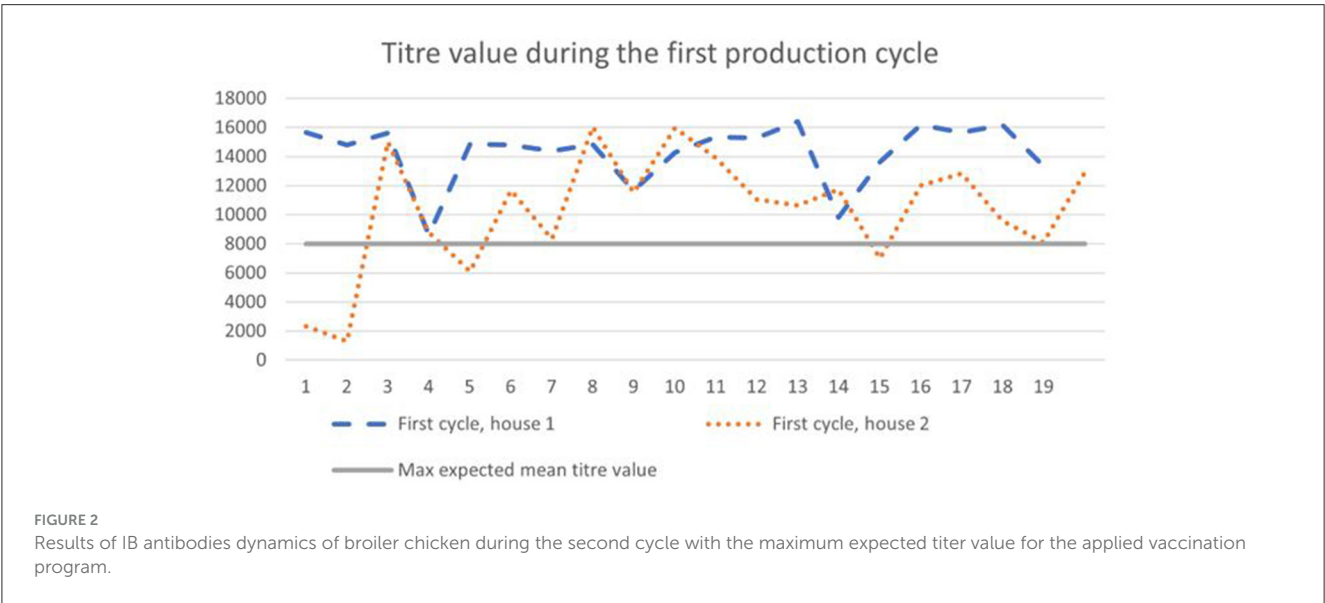
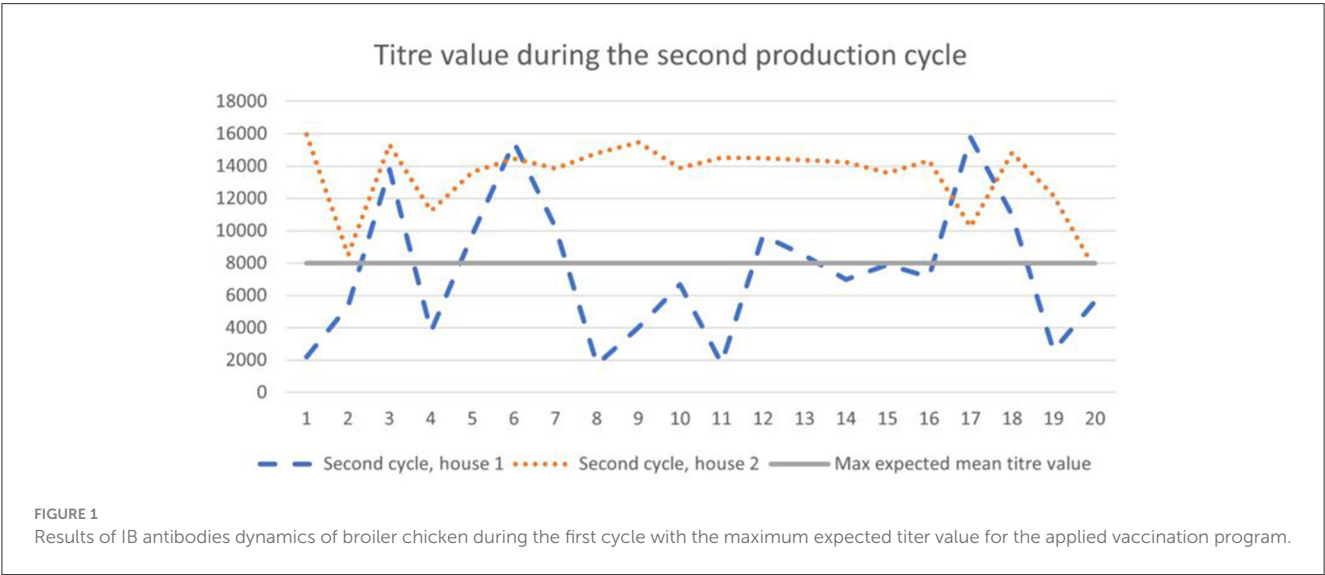
Identified shortcomings in internal biosecurity included the presence of animals of different ages on the same farm, as well as inadequate cleaning and disinfection procedures.

(a) Different ages of poultry: Chickens of different ages are housed in four separate units on the farm. Disease monitoring and

TABLE 1 Results of IB antibodies dynamics of broiler chicken blood samples in two houses during two successive production cycles (the downtime between two production cycles is <10 days).

	Number of samples	Mean titer value	Number of suspect samples*	Highest antibody titer	Coefficient of variation (%)
1st, House 1	20	10,202	18 (90%)	15,951	20
1st, House 2	20	10,338	13 (65%)	16,022	37
2nd, House 1	20	7,494	7 (35%)	15,777	56
2nd, House 2	20	13,372	18 (90%)	15,940	16

*Suspect samples are those with antibody titers indicating possible exposure to the wild strain of the IB virus.



vaccinations are conducted regularly. Carcasses of dead birds are removed multiple times a day, but weak and clinically ill birds are not regularly separated, no quarantine. Stocking density ranges from 33 to 39 kg/m².

(b) Cleaning and disinfection protocols: The farm has a specific protocol for cleaning and disinfecting the premises after each production cycle, but the effectiveness of this process is rarely verified. The downtime between two production cycles is <10 days

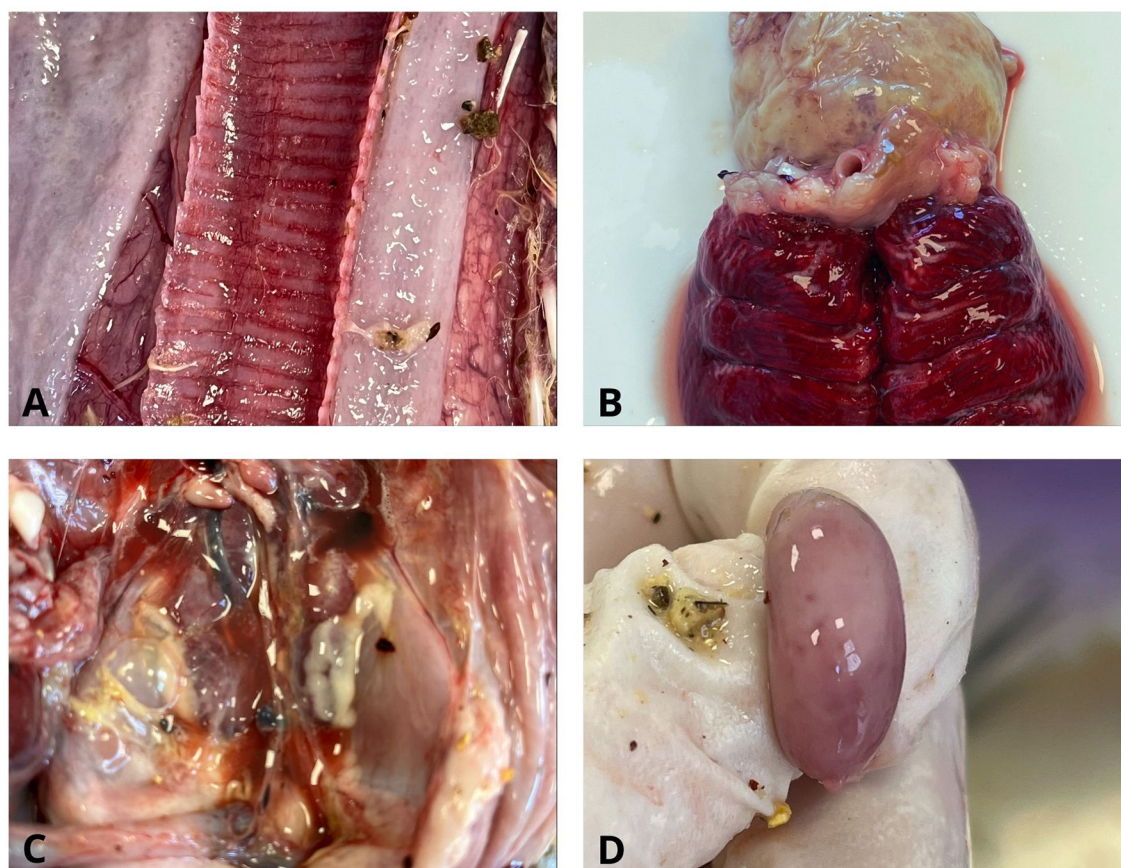


FIGURE 3

Gross pathological lesions in chickens. **(A)** Trachea. Catarrhal hemorrhagic tracheitis; **(B)** Lung and heart. Pulmonary hyperemia and fibrinous pericarditis; **(C)** Pleuroperitoneal cavity. Nephromegaly, ascites, and fibrinous pleuroperitonitis; **(D)** Splenomegaly with pinpoint splenic hemorrhages.

(from the moment of completed disinfection to the moment of placing a new flock).

Discussion

Infectious bronchitis (IB) is a significant endemic viral respiratory disease that spreads between farms and between different houses of farm, both in vaccinated and unvaccinated poultry (17, 18). Immunoprophylactic measures are usually carried out according to the vaccination programs in commercial poultry flocks in Serbia (19). Despite the use of live and inactivated vaccines, the occurrence of IB is nearly constant in regions where the infection has already been diagnosed (20).

This study indicated that the farm had repeated IB infection in two subsequent production cycles, which caused huge economic costs for the owner. The IBV's ability to persist in the intestinal tract and feces for several weeks or months, and its shedding through the respiratory system (aerosol) and feces (8, 21), can help to understand the persistence and transmission in the observed farm. On the studied farm, routine monitoring was not performed, and vaccination was provisorily done. Regular monitoring of the local situation establishes baseline antibody levels, which are

then used for comparison. It is also essential to monitor and record data from specific environments (e.g., farms, and regions) to understand the normal range of antibody levels, as antibody levels in the blood can vary due to different rearing conditions (21). Enzyme-linked immunosorbent assays (ELISAs) represent an appropriate methodology test for routine IB monitoring due to their cost-effectiveness and quick turnaround of results (22). ELISA tests also are the preferred diagnostic approach for the detection of antibodies in poultry flocks resulting from either infection or vaccination (23, 24). High, uniform, and long-lasting antibody titers indicate a well-conducted and adequate vaccination. Low, uneven, and short-lasting titers suggest that vaccination was unsuccessful, likely due to improper application or poor vaccine quality. According to the obtained results, the mean level of antibodies was almost twice as much as expected for the applied vaccination program and the coefficient of variation was below 20%, which means it is uniform in the flock and that we can expect similar results for all birds. If titers are significantly higher than expected, a field infection should be suspected, meaning that the detected antibody levels in the blood are not the result of a regular vaccination program but rather exposure to a wild field strain of the virus (25, 26). According to studies by Leerdam (27) and Bhuiyan (28), average titer values after infection should increase

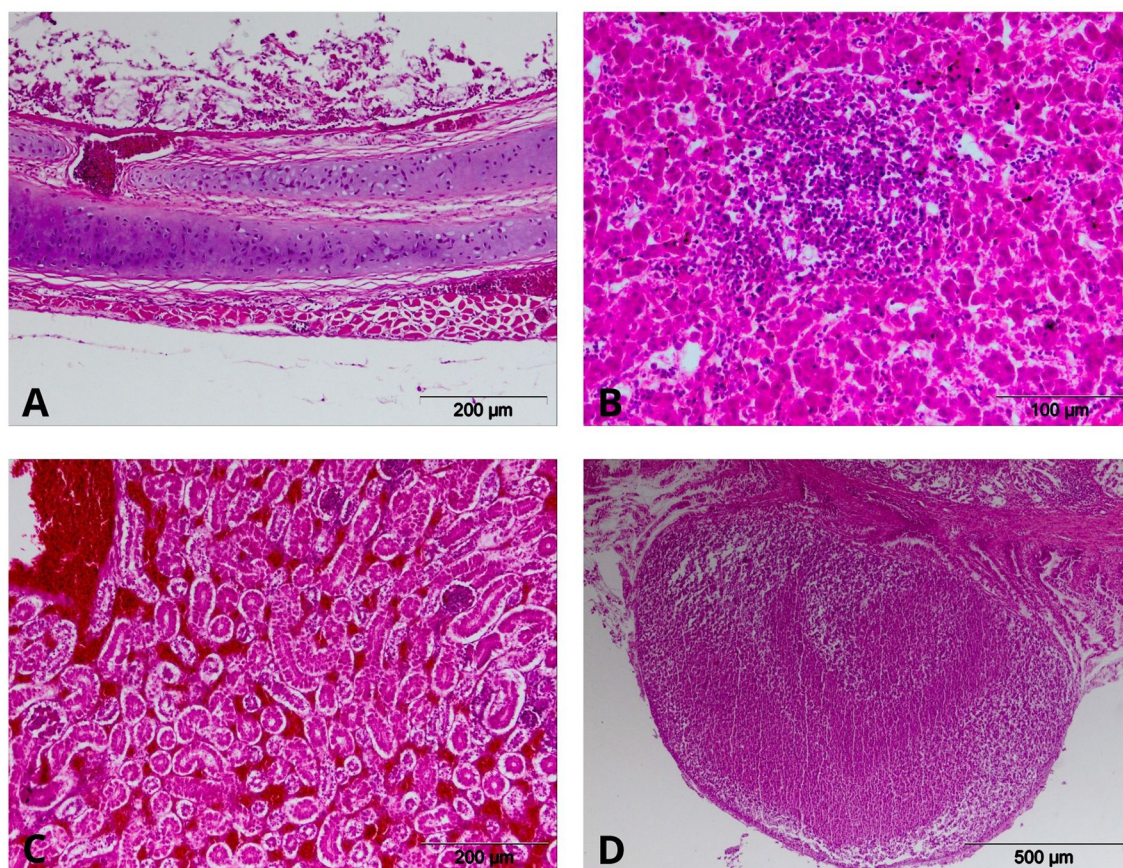


FIGURE 4

Histopathological lesions in chickens. (A) Trachea. Desquamative, necrotic tracheitis; (B) Liver. Multifocal lymphoplasmacytic hepatitis; (C) Kidney. Hyperaemia, hemorrhages, and tubulonecrosis; (D) Proventriculus. Pyogranulomatous proventiculitis (H&E).

significantly, by at least twofold, compared to post-vaccination levels or pre-infection baseline titers. It is common for antibody titers to rise sharply 3–4 weeks after the onset of infection (28). According to previous investigations, smallholder farms in Serbia are facing a high prevalence of IBV. The worrying fact is that only 5% of non-vaccinated flocks were negative for IBV (19). In the present study, the level of antibody titers, the number of suspect samples, and the coefficient of variation (Table 1) may indicate that the birds had been in contact with a wild virus strain.

In this study, the wild-type virus could have been present, causing increased clinical signs. The IBV genome was detected using molecular methods, and *in silico* compared with wild strain. We were not able to prove presence of wild type virus due to sample size, which is a limitation of this study. In this case, a wild strain was likely present in the samples, although it was not identified through sequencing. Had we sequenced a larger number of different samples, maybe it would have been detected. Also, another limitation in this case was the limited length of 394 bp of the S1 gene that was observed which cannot reflect the whole molecular characteristics of the virus (29). Furthermore, the observed gross lesions and histopathological findings, combined with serological analysis of blood samples and detection of the virus genome in the swab samples and internal organs, consistent with findings

from other researchers (26), suggest a strong confirmation that the birds were infected with IBV during the second production cycle. Detected pathological lesions are very suggestive for IB, however impact of other infectious agents such as *Mycoplasma* cannot be excluded. Given that serological analysis of blood samples from both houses during the first cycle also showed significantly high average antibody titers, it can be suspected that the birds had been exposed to a wild strain during the previous cycle, exposing the facility to the IBV.

Controlling IB and other infectious diseases in broilers can be enhanced through good farm management, appropriate stocking densities, quality air, and extended downtime between production cycles (30). The cornerstone of preventive measures in the fight against IB is biosecurity. This involves the strict implementation of external and internal biosecurity measures to regulate the movement of animals, people, materials, and waste (31). Assessing biosecurity protocols on broilers' farms is a useful tool for identifying potential risks, preventing the introduction or spread of diseases, and improving overall flock health and productivity. Some pathogens can serve as a biomarker for the efficiency of the implemented biosecurity protocols (32). According to the regulation, in Serbia, owners and animal keepers ensure animal health and wellbeing, taking measures to prevent the

TABLE 2 Biosecurity evaluation results on the observed farm.

Biosecurity assessment	Farm (%)	National average* (%)	Global average (%)
One-day-old chick purchasing	69	62	67
Broiler depopulation	43	48	65
Feed and water supply	67	60	62
Manure and carcass removal	66	39	67
Farm workers and visitors	84	73	76
Material supply	56	81	70
Infrastructure and biological vectors	97	81	82
Farm location	81	73	68
External biosecurity score	71	65	70
Disease management	74	77	80
Cleaning and disinfection	52	54	71
Materials and measures between compartments	82	71	75
Internal biosecurity score	66	66	75
Overall biosecurity score	70	65	72

*National Average - taken from the Biochek.Ugent database, average obtained by completing 46 questionnaires. Bolded values are below the global average.

spread of infectious diseases, including biosecurity and good farming practices. Critical biosecurity points include: the farm location, physical visibility of the farm and its separation from the surroundings, movement of people, movement of animals and vehicles within and outside the farm, food and medications brought in and used, animal reproduction using artificial insemination or natural mating, handling of animal carcasses, handling of waste water and manure from the farm, pest control, and the conditions of the facility, equipment, and microclimate (33). In this study, the assessment of biosecurity measures on the farm identified critical points in external biosecurity measures (cross-contamination of clean and dirty pathways, thinning management, protocols for staff and visitors, procedures for manure and carcass disposal), as well as internal biosecurity measures (immunoprophylactic programs, stocking density, the presence of different age groups of chickens at the same location). Given the IBV transmission pathways and their ability to survive for extended periods, along with the identified deficiencies in biosecurity measures, it can be assumed that these factors significantly contributed to virus transmission between houses and production cycles. In four different houses (1 to 4), the farm has the presence of different age groups of chickens. In one moment, they may have the depopulation of house number 4 and one-day chicken introduction in house number 1. This increases the risk of the virus spreading. Also, stocking density on farms affects the risk level of virus transmission between farms. However, it has not been determined whether this facilitates virus transmission via air due to proximity or due to shared risk factors (horizontal contacts or environmental conditions) (25, 28). The severity of early-age IB infections can be controlled by reducing extreme amounts of ammonia, carbon dioxide, and hydrogen sulfide, and by maintaining environmental temperature according to the technology prescribed for that age and provenience. The ammonia concentration in the poultry houses above 25 ppm can cause damage in productive performance, impair immune response

(34) and disturb the respiratory system of broilers (35, 36) so the broiler may become more susceptible to viral infections (28, 37). Also, it is reported that high level of ammonia can lead to low breast muscle and carcass composition in broilers (38). Studies showed that the downtime period between production cycles should be not <14 days and the proper performing of procedures of cleaning, washing, and disinfection are essential to reduce the risk of infection in the next production cycle (39, 40).

Concerning IB infection, vaccination as one of the measures of internal biosecurity, is considered the most effective and widely used preventive measure. Although vaccination cannot completely prevent infection, it can reduce clinical symptoms and infection pressure (41). Due to the short life of broilers, they are vaccinated once or twice against IB. In practice, broilers are usually vaccinated with spray vaccines containing 1–3 serotypes of live attenuated vaccines immediately after hatching, or still in the incubator before transport to the farm. For broilers whose production cycle lasts longer than 49 days and in facilities with an increased risk of infection, an additional dose of live attenuated vaccine is administered through drinking water between the 14th and 18th days of age to extend immunity (5, 42, 43).

Eradicating IB remains a challenging goal. The results obtained showed the internal biosecurity score was lower than the external biosecurity score. This is not a common finding for broiler farms (31, 44, 45). Enhancing internal biosecurity frequently requires the implementation of fundamental interventions within the flock, including the establishment of stringent hygiene protocols and adherence to appropriate operational procedures (46). Previous studies showed that broiler farms with better overall biosecurity programs and management practices have a lower risk of transmission of immunosuppressive pathogens (47). Field efforts should be directed toward optimizing the application of well-established, effective measures. Additionally, continuous

monitoring, based on objective criteria and knowledge of the local epidemiological status, is crucial (25).

Conclusions

In this study, infectious bronchitis (IB) was confirmed through clinical, serological, molecular, and pathological examinations, highlighting notable deficiencies in biosecurity measures. The failure to properly implement the biosecurity program, combined with the use of vaccines not based on continuous monitoring of the most prevalent serotypes in the local area, may have contributed to the outbreak, potentially due to the introduction of wild IB virus strains, as suggested by the findings. However, it is important to note that the study is based on a single farm, and the authors cannot be entirely certain about the role of wild strain infection. Since no vaccine can offer complete protection without effective biosecurity practices, it is crucial to provide farmers with proper guidance on the implementation of preventive measures. Additionally, regular monitoring of major viral diseases should be emphasized. Based on these observations, a tailored vaccination strategy for each farm is recommended to improve disease control.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

In this study, ethical approval was not required as it involved routine diagnostic procedures; however, the farm owner consented to publish the results without disclosing the farm's name. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JM: Conceptualization, Investigation, Methodology, Writing – original draft. NJ: Formal analysis, Investigation, Writing – review

& editing. LS: Investigation, Writing – review & editing. BM: Formal analysis, Visualization, Writing – review & editing. AV: Writing – review & editing. JK: Formal analysis, Visualization, Writing – review & editing. BK: Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Gen AI was used in the creation of this manuscript.

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A scoping review of knowledge, attitudes, and practices in swine farm biosecurity in North America

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Pork is one of the most popular consumer meat choices globally, second to poultry. In the past two decades, the rising demand in pork, has seen pig farming move toward intensive farming methods, characterized by high pig densities which is a risk for swift spread of disease necessitating proper and strict biosecurity adherence to facilitate disease-free conditions and business continuity. North America is the second largest pig producer globally. We conducted a review of available peer-reviewed original publications to scope for available data on the knowledge, attitudes, perceptions, and practices concerning biosecurity among swine producers in North America from the year 2011 to 2022 using the PRISMA-ScR guidelines. Out of the 323 papers that fit our search criteria, we present insights from the 18 papers that were relevant to our study. We summarize key findings on biosecurity practices and propose critical practices for biosecurity adherence. We also present our findings on the complexities that influence producers' adoption of biosecurity plans and note variations in biosecurity strictness between states and how these are influenced by farm size and perceived disease risk. In conclusion, this review highlights the need for updated assessments of biosecurity practices, leveraging technology particularly machine learning, for risk assessment, and acknowledges the role that demographics and risk perception play in biosecurity adoption. Ultimately, effective biosecurity measures are imperative for safeguarding North American swine production systems against disease threats especially foreign animal diseases like the African swine fever (ASF), foot and mouth disease (FMD) and classical swine fever.

KEYWORDS

swine biosecurity, knowledge – attitude – practice, North American pig farming, biosecurity adoption, critical biosecurity practices, foreign animal disease (FAD), risk assessment

1 Introduction

Global consumption of pork has increased greatly, making it the second most consumed meat, representing >34% of global meat consumption (2022 estimates) (1, 42). Studies reveal a 77% growth in pork demand from 1990 to 2022 (2) and with the growing world population and economic growth, the demand is likely to continue growing (2, 3). Consequently, global pig production has grown by about 140% from 1990 to 2021 (42). The USA, Mexico, and Canada ranking among the top 10 pig-producing countries globally (2). This puts North

America as the second largest pig producer globally after Asia. With the ever-increasing threat of foreign animal diseases (FADs), such as African swine fever virus (ASFv), and the continued impact of production-limiting effects of endemic diseases, such as porcine reproductive and respiratory syndrome in North American swine populations, a continuous review and reflection on biosecurity practices and threats is crucial to support optimal production, avert disruption of business and protect the global protein value chain.

Global demand for pork has led to intensification in pig production (23, 41). The high swine densities in farms coupled with increased trade in swine and swine products create an environment conducive to rapid disease transmission. Locally, endemic diseases as well as transboundary diseases are threats to the swine industry and associated commercial trade in addition to human health in some instances (3–5).

Biosecurity is the implementation of measures intended to prevent/reduce the risk of the introduction and spread of disease-causing agents. Biosecurity is guided by 3 principles which include bio-exclusion, bio-management, and bio-containment (6). Bio-exclusion refers to the prevention of disease from entering the farm and spreading, and relies on external biosecurity measures to keep pathogens out. Bio-management and bio-containment rely on internal biosecurity measures to stop pathogens from spreading within the facility. Bio-management relates to measures taken to prevent spread of infectious disease to uninfected animals within the same barn. These include but are not limited to surveillance, all-in, all-out model, rodent control, cleaning and disinfection of equipment and premises, separation and quarantine of sick and symptomatic swine and adjunctive measures like vaccination etc. On the other hand, bio-containment relates to measures that prevent spread of infectious disease to other barns in the same farm and from potentially spreading to other farms (pathogen exit) like shower in- shower out, cleaning and disinfection of shared equipment like skid loaders etc.

Swine production like other livestock industries is threatened by infectious diseases which result in direct losses to production through mortality, loss of productivity, resulting in food insecurity and loss of business continuity through supply chain disruptions, reduced market value and trade restrictions. Biosecurity helps prevent the need for extreme measures needed to control disease spread. While there are recommended biosecurity standards and principles in North America, implementation by farmers is voluntary (7). Some of the biosecurity guidelines are presented in documents such as the secure pork supply (SPS) plan for ensuring continuity of business in case of the introduction of a FAD into the United States. The SPS plan provides a checklist for pork producers and swine veterinarians to use as a guide for implementing biosecurity measures with the ultimate incentive being the guarantee of continuity of business for the industry in the event of an FAD event (8). Other similar guidelines are in place such as the swine industry foreign animal disease preparedness and response plan (FAD-PReP) manual created by the USDA and APHIS (9). Similarly, the Canadian Pork Council has a guidance document for swine biosecurity in its repository (10).

Effective biosecurity is best achieved through the proactive adoption of preventive attitudes and behaviors among all relevant stakeholders, supported by a well-structured biosecurity plan. Preventive attitudes involve anticipating risks and implementing measures to mitigate them before a biosecurity breach occurs, while behaviors include actions such as cleaning and disinfecting equipment,

quarantining new animals, and monitoring for disease symptoms (11). A biosecurity plan serves as a framework outlining specific protocols for disease prevention, detection, and containment, tailored to the species, disease prevalence, and management practices (12, 13). Collaboration among stakeholders—farmers, veterinarians, transporters, and policymakers—is critical for ensuring shared responsibility in the implementation of biosecurity measures (14, 15). The success of biosecurity initiatives heavily depends on fostering compliance through behavioral change, as described by the Theory of Planned Behavior, which emphasizes the role of attitudes, perceived behavioral control, and subjective norms in driving actions (16). Challenges such as resistance to change, lack of knowledge, and perceived costs can hinder compliance, but education, training, and demonstrating the economic and health benefits of biosecurity can effectively address these barriers (17, 18).

In this scoping review, we collate information that is available on the knowledge, attitudes, and practices of the swine industry in North America. We assume that published research is representative of how much focus and funding has been applied to the topic within the region. Sources of funding play a role in shaping research focus and will often tend to concentrate efforts on topics involving high-impact biosecurity. Regular assessment of the biosecurity threats and risks for biosecurity breaches is key to sustaining a disease-free status of individual farms and the subsequent continuation of business in the region. To the best of our knowledge, there is limited information on the assessment of biosecurity status in North America that has been publicly published since 2019. The objective of this paper is to provide a review of biosecurity knowledge, attitudes, and practices (KAPs) within the swine industry, the willingness of players to adopt enhanced biosecurity practices, and potential barriers to adoption. Our work also identifies critical biosecurity practices (CBPs) required for the prevention of disease introduction into North American swine farms.

1.1 Review questions

The primary question of our review was to identify the current biosecurity practices of swine producers, including the distribution/implementation of these practices amongst swine producers in North America. Secondly, was to ascertain producer attitudes toward the implementation of biosecurity and biosecurity protocols, and lastly to identify key areas for improvement of biosecurity amongst swine producers within North America.

2 Methods

2.1 Protocol

This scoping review was conducted according to the framework created by Arksey and O'Malley (19). The steps followed include (1) identifying the research question; (2) identifying relevant studies; (3) selecting studies using inclusion and exclusion criteria; (4) charting the data to extract data from each study; and (5) collating, summarizing, and reporting an overview of the results and lastly; reporting follows the guidelines of the Preferred Reporting Items for

Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-SCR) (20). The PRISMA-SCR checklist is attached as [Supplementary data 1](#).

2.2 Inclusion and exclusion criteria

We conducted a review of available peer-reviewed publications to scope for available data on biosecurity practices among swine producers in North America from the year 2011 to 2022 using the Covidence platform (21), this was our defined population, concept, and context (PCC) of our research. The papers also needed to be original publications and contain information on farmer practices and attitudes toward biosecurity, the production system involved, as well as information on their profile. A comprehensive list of our inclusion and exclusion criteria is presented in [Table 1](#).

2.3 Search and review process

A comprehensive search strategy was developed by an experienced librarian at the University of Minnesota in collaboration with subject experts on the research team to identify all available research on swine biosecurity in the United States. After several test searches, we settled on using PubMed/MEDLINE and center for agriculture and bioscience (CAB) Abstracts (via Ovid) as together these would provide a sufficiently representative sample of the published literature on the topic.

The final search terms used were:

- PubMed: *(pigs or swine) and (farmer* or producer) and biosecurity (all fields)*.
- CAB Abstracts: *((pigs or swine) and (farmer* or producer) and biosecurity).af*, where 'af' stands for 'all fields'.

Searches were run on July 12, 2022 and included a publication date limit of 2011 to current (2022), a 10 year study period. Results were imported into Endnote for de-duplication and then transferred to the Covidence platform (21) where they went through filtration of titles and abstracts followed by full-text review and decisions to keep or exclude was based on the inclusion and exclusion criteria described. A total of 6 reviewers were involved in the process with agreement between at least two reviewers being needed for a paper to move to the next step of the review process. Any discrepancies were resolved

either by discussion or review by a third reviewer. Charted data was also extracted by at least two reviewers and agreement was discussed and assessed during analysis. For all the studies that fit our criteria of selection, we looked at what their research aims were, the type of swine production systems they studied, what study design they used to answer their questions, and outlined the internal and external biosecurity practices flagged in their study. The search strategy is outlined in [Supplementary data 2](#).

2.4 Content analysis

Data on CBPs as well as risk factors for disease introduction into swine farms was tabulated and tallied by frequency of mention of either exact factor or synonyms of factor, e.g., truck/vehicle/trailer were considered to refer to the same mode of transportation. In the line-by-line analysis, we teased out what the papers were saying about the highlighted factors; this data can be found in [Supplementary Table 1](#).

3 Results

3.1 Search results and study characteristics

In PubMed, our search strings produced 211 results and CAB Abstracts produced 220 results, respectively. After deduplication in Endnote, 337 records remained. Covidence was able to remove an additional 14 records, leaving a total of 323 papers. These went through a filtration and review process yielding a total of 18 studies relevant to our review with most of the papers being excluded for being outside our geographical scope (i.e., conducted outside N. America) $n = 203$. Other reasons for exclusion were non-original papers ($n = 15$), data on KAPs not reported ($n = 6$), not about biosecurity ($n = 60$), non-research papers ($n = 3$), and not in English ($n = 1$), wrong study design ($n = 6$). The PRISMA summarizing this is in [Figure 1](#).

3.2 Distribution of studies

While North America comprises Canada, the USA, Mexico, Central America, and the Caribbean, the majority of the included studies originated from the United States ($n = 15$). Canada and the USA are top

TABLE 1 Inclusion criteria used for selection of papers to be included in the scoping review.

Inclusion criteria	Exclusion criteria
1. Focuses on swine production systems	Non-swine production systems
2. In English	Not English
3. Study on Biosecurity and Disease Management in Swine Production systems	No mention or focus on biosecurity or disease management
4. Published between 2011 and 2022	Published before 2011
5. The study was carried out in North America	The study was carried out outside of North America
6. Contains information on practices, production systems, the profile of producers, attitudes of producers toward biosecurity, or recommendations about biosecurity	Does not have information on practices, production systems, profile of producers, attitudes of producers toward biosecurity, or recommendations about biosecurity
7. Original research papers	Non-research papers (e.g., reviews, letters to the editor, case reports, etc.)

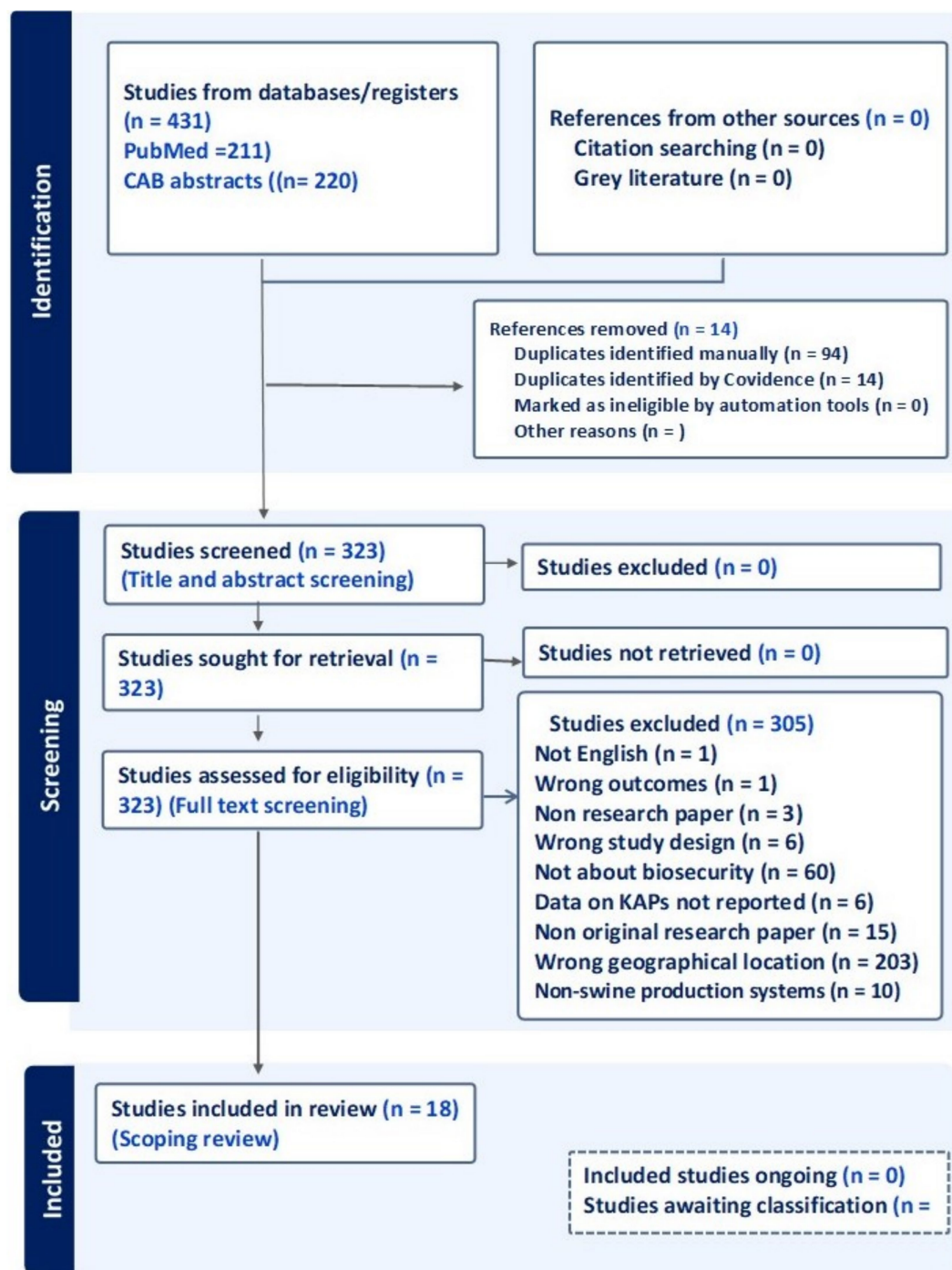


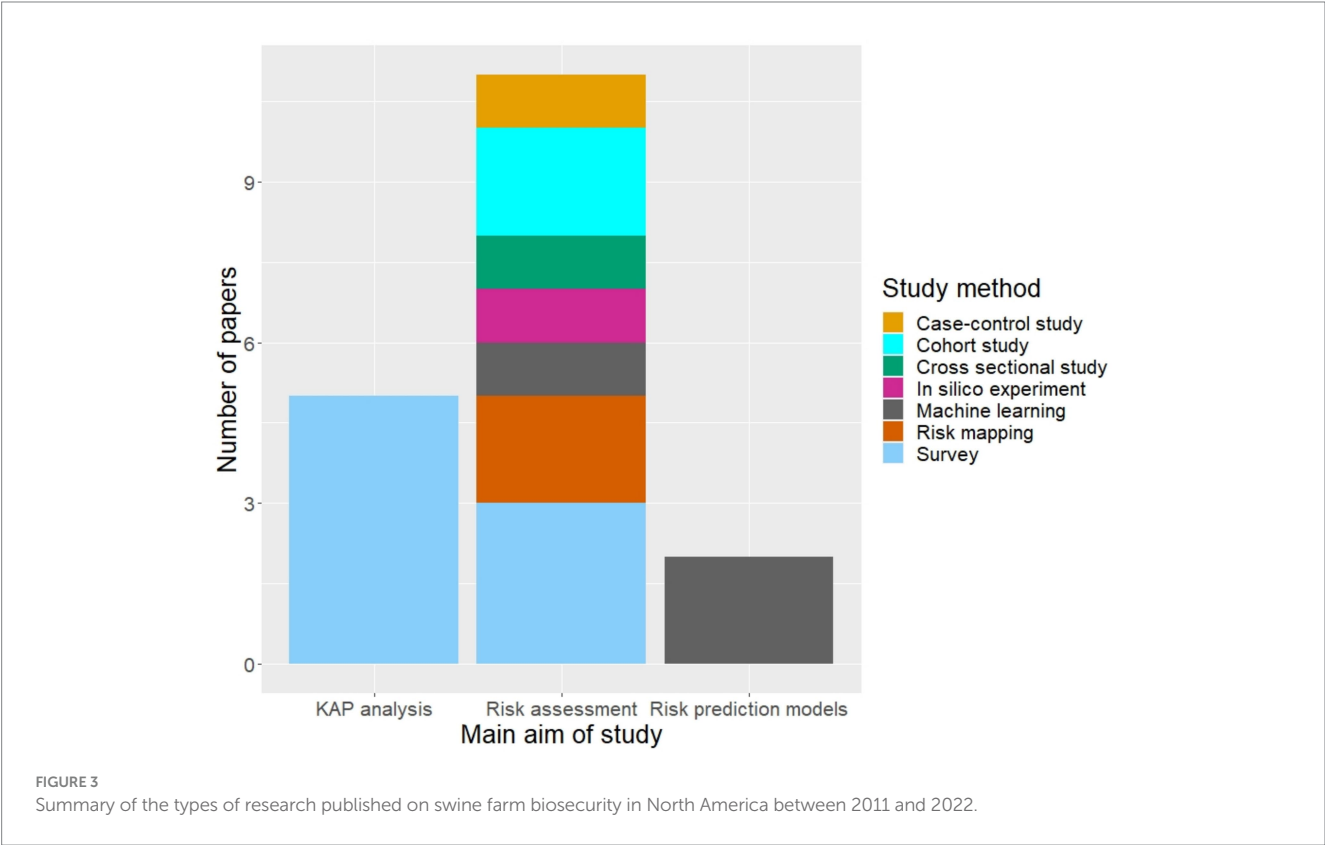
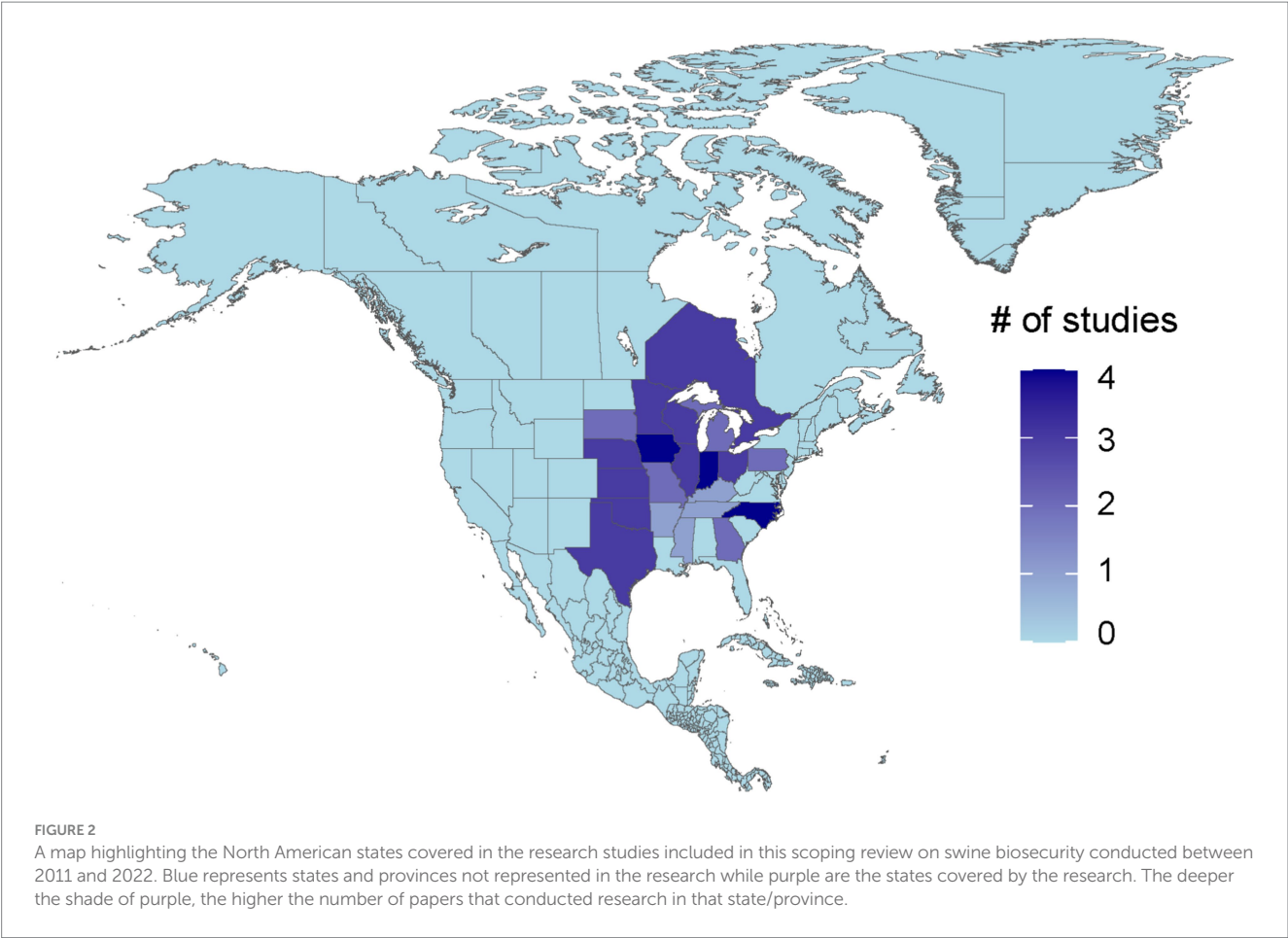
FIGURE 1

PRISMA flow chart of the paper selection process showing the number of papers in each step and the representation of reasons why excluded papers were left out leading to the final 18 selected.

global swine producers, Mexico's role is growing with increasing export to neighboring countries and the Caribbean and Central America keep swine largely for domestic market consumption. There were a total of three studies (16.7%) conducted in Canada and no relevant papers were found from Mexico and the other regions. While inclusion of only English-language papers may have excluded Spanish and French studies from the region, a limitation stemming from the team's constraints in resources, funding, and translation expertise, we attempted to address

this limitation by utilizing multilingual databases such as PubMed and CAB Abstracts. Figure 2 maps the geographic distribution of research on swine biosecurity across North America.

The 18 papers included in this review primarily covered KAP analyses ($n = 6$) and disease-specific biosecurity risk assessments ($n = 9$), such as risk mapping and machine learning simulations to predict swine farm vulnerability to disease (Figure 3). These studies were largely observational, including surveys,



cross-sectional, cohort, and case-control studies, with some employing computer-based experiments, such as *in silico* and machine learning models.

3.3 Knowledge

Knowledge about biosecurity practices varied across studies, with 61% (11/18) of the research focusing on mixed systems of swine farming (e.g., small or medium-scale indoor, medium-scale outdoor, indoor or outdoor extensive). However, few studies emphasized greater knowledge gaps about biosecurity and its implementation among backyard or hobby or small scale farmers. Medium-sized farms were defined as having an inventory of 750–2,499 swine each weighing 55 pounds or more, or 3,000–9,999 swine each weighing less than 55 pounds. Farms with inventories smaller than this were classified as small farms, while those with larger inventories were considered large farms (22).

The swine production industry in North America predominantly consists of commercial swine raised in strict indoor confinement systems (23). Despite this, knowledge gaps remain regarding critical aspects of biosecurity, particularly in areas like small-scale or backyard farming, where there is less access to structured biosecurity protocols.

3.4 Attitudes and perceptions

Producer attitudes and perceptions significantly influence the adoption of biosecurity measures. The most important factors that influenced a swine producer's decision to either take up a biosecurity practice or not, were: producers' perceptions of the risk of disease; personal experience with a disease outbreak on their farm or the neighbor's farm(s); and effectiveness of a biosecurity practice in reducing the perceived risk. A study by Wu et al. (24) reported that 65.8% of swine producers in the United States of America were willing to adopt biosecurity practices to reduce disease risk to their farms and their neighbors' farms; however, this percentage reduced to 56% after considering the high cost of implementation. Experience is indeed the best teacher; (24) also found that experiencing an FAD with significant impact on animal health, trade and food security swine disease either personally or on the neighboring farms was the biggest incentive for swine producers to adopt biosecurity protocols.

Additionally, government incentives or payouts partially encouraged adoption of biosecurity practices. Availability of more educational resources on a topic was the least effective in encouraging national adoption of biosecurity practices among swine producers suggesting that while educational materials are important in farmer education, merely increasing the quantity of resources without considering the alignment of these resources with practical needs of farmers may hinder the effectiveness of the tools.

The role backyard farming may play in disease introduction, spread and establishment is often understated and underestimated. Nicholson et al. (23) observed that backyard swine farmers in Pennsylvania had varying perceptions of biosecurity. While they were relatively knowledgeable about highly infectious diseases like swine influenza due to recent education campaigns, they lacked awareness of zoonotic diseases or those with public health

implications. However, despite the mentioned campaign, biosecurity practices among backyard farmers in the state were very lax, with the majority having no biosecurity protocols or requirements for visitor access to their animals (23). This highlights the importance of targeted education campaigns tailored to specific producer demographics and risks. Consideration for inclusion of small farmers in education campaigns etc., may potentially benefit the entire industry.

The strictness of biosecurity implementation by swine producers varies by several factors including geographical location, density of swine farms and the associated perceived feasibility of implementation. While biosecurity measures have been implemented to varying degrees, farmers' attitudes are largely unknown with regards to how they would prioritize adopting these measures and how this impacts the goal to have continuity of business in the event of introduction of a foreign pig disease into the region. The risk of certain FADs getting established in the region is also relatively higher in some states than others. Taking ASFv as an example; Wormington et al. (25) describe California, Oregon, Nevada, Arizona, New Mexico, Oklahoma, Texas, and Florida as having potential risk of ASFV spillover from the sylvatic cycle due to the co-occurrence of the tick vector, feral swine, and domestic swine. While direct swine-to-swine transmission can also occur in these states, the authors list North Carolina and Oklahoma as being of particularly high concern for direct swine-to-swine virus transmission particularly due to high swine farm densities.

3.5 Practices

Practices associated with biosecurity measures were evaluated based on their frequency of mention and criticality. The most frequently mentioned practices were vehicle movement management (27.8%; 5/18) and personnel management (16.7%; 3/18) (26). Other practices included the sourcing of animals and semen, manure management, feed handling, pest and wildlife control, and air and water filtration, each cited in 11.1% (2/18) of the reviewed papers (Table 2).

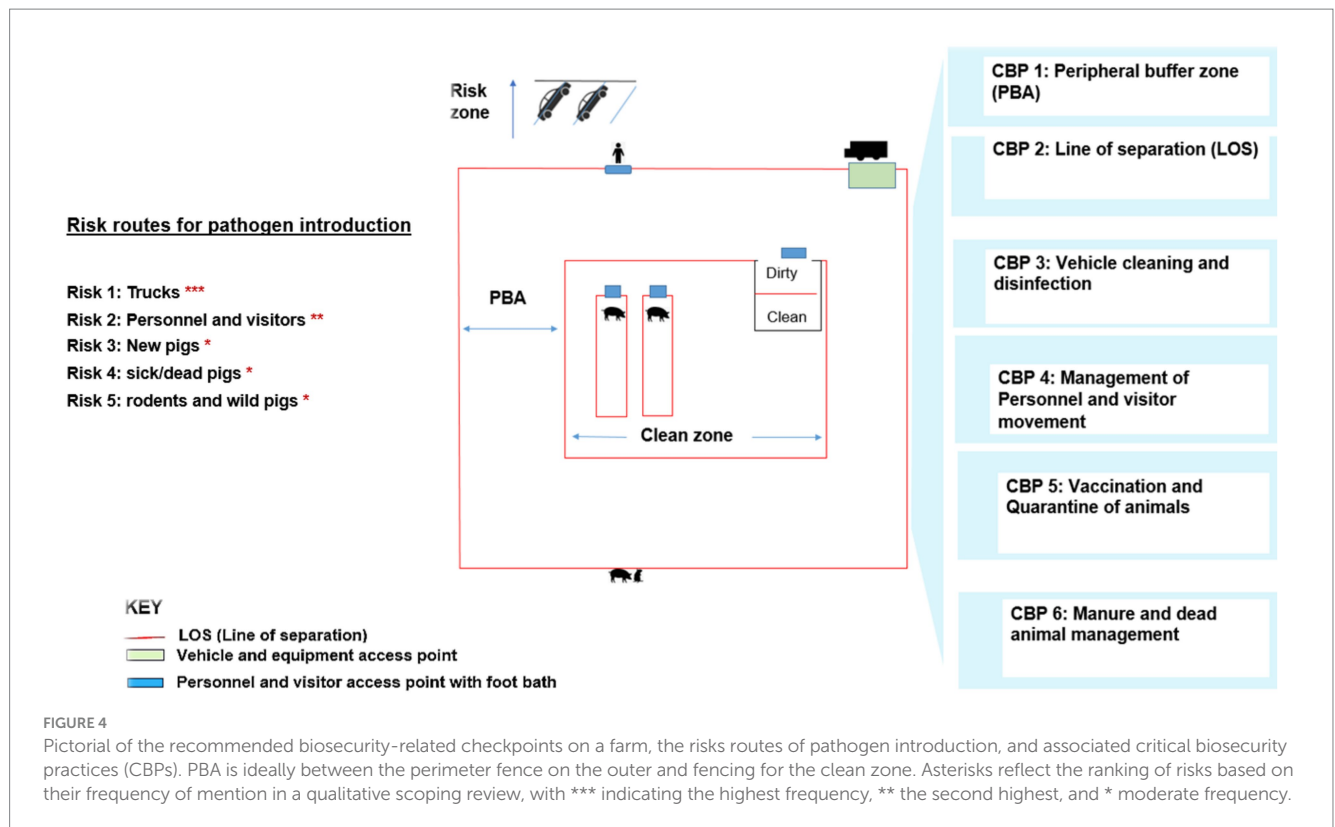
The reviewed literature emphasized specific biosecurity practices that are critical and warrant attention to mitigate pathogen transmission risks. These included controlling vehicle and personnel movement, managing the removal of dead animals, implementing defined, clearly marked and with specified protocols for crossing the designated access points to biosecurity zones such as the Line of Separation (LOS) and the Peripheral Buffer Area (PBA), and ensuring the proper handling of feed and manure. Biosecurity practices discussed included vehicle disinfection, PPE protocols, and quarantine facilities to mitigate risks associated with pathogen introduction and spread. Vehicle disinfection, PPE protocols, and quarantine facilities to mitigate risks associated with pathogen introduction and spread. We used the frequency if the mentioned practices in our reviewed papers as metrics to identify practices and points in a swine farm's biosecurity infrastructure and protocols that are crucial for prevention or mitigation of pathogen transmission risks. We refer to these as critical biosecurity practices (CBPs) of biosecurity in swine farms (Figure 4) and borrow our recommendations from the SPS protocol.

TABLE 2 Summary of the different swine farm biosecurity practices highlighted in the reviewed publications.

Biosecurity practice	Principle	Specific practices
Vehicle movement	Bio exclusion	- Cleaning and disinfection of trucks in manned designated areas. - Restriction of different vehicles to specified zones, e.g., visitor vehicles park outside PBA.
Disposal of dead animals	Bio-management and bio-containment	- Method of disposal. - Directionality of removal of dead animals from barn. - Location of rendering bin in respect to barns and access to rendering truck.
Manure Management	Bio-management and bio-containment	- Frequency of manure removal - Location of lagoons in respect to swine housing. - Directionality of removal from housing.
Management of feed	Bio-exclusion	- Audit and verify feed suppliers. - Feed holding time. - Clean and disinfect feed trucks. - Cleaning feed spills
Perimeter buffer area	Bio-exclusion	- Clearly demarcated PBA with specified entry points. - Cleaning and disinfection of personnel and trucks at PBA entry points.
Restriction of visitors and staff movement	Bio-exclusion and bio-containment	- Presence of specified, monitored entry points. - Cleaning and disinfection of personnel and PPE at entry points. - Clear defined and marked LOS.
Sourcing of animals and genetic material	Bio-exclusion	
Line of separation (LOS) and shower-in facility	Bio-exclusion and bio-management	LOS and shower-in facility specified at entry to each animal barn and entry to farm where feasible.
Filtration of air and water in swine housing	Bio-exclusion and bio-containment	Filter air coming into and going out of the barn.
Animal-animal contact/separation	Bio-management	
Restriction of movement within the facility	Bio-management and bio-containment	- Restriction of staff movement between animal housing. - Directionality of flow of staff, animals and equipment, i.e., designated entry and exit of opposite sides.
Presence of defined clean and dirty areas with a defined LOS	Bio-exclusion and bio-containment	Defined LOS for personnel, animals and equipment.
Wearing of personal protective equipment (PPE)	Bio-exclusion, bio-management and bio-containment	- PPE should be changed before entry and before exiting each housing/facility. - Cleaning and disinfection or disposal of PPE between each animal housing.
Disinfection of pig housing	Bio-management	- Should be done between each swine group after all in- all out occupancy. - Disinfection of floors during cleaning.

We outline here the specific critical biosecurity practices (CBPs) identified through this review:

1. Defined Peripheral Buffer Areas (PBAs): A well-demarcated PBA restricts unauthorized access and minimizes contact between farm operations and external risk factors like vehicles and wildlife.
2. Line of Separation (LOS): Clearly defined zones that separate clean and dirty areas. These are monitored and include biosecurity guidelines for crossing.
3. Vehicle Cleaning and Disinfection: Vehicles used for transport of swine, feed, or equipment should be disinfected at the farm entry point. Shared vehicles between farms present a significant risk of pathogen transmission (26).
4. Personnel and Visitor Movement: Risks of disease introduction by personnel and visitors can be mitigated by footbaths, controlled access points, and mandatory use of personal protective equipment (PPE). A shower-in, shower-out system with designated clean and dirty areas further enhances biosecurity (26, 27).
5. Vaccination and Quarantine: New animals should undergo vaccination and quarantine to reduce the risk of disease introduction. Testing incoming animals for pathogens is also recommended to complement vaccination, especially for diseases without effective vaccines.
6. Manure and Dead Animal Management: Proper handling and disposal practices minimize disease spread within and outside the farm. Disinfection of rendering trucks and



placement of rendering boxes outside the PBA are essential measures (26).

The reviewed studies underscored the importance of combining multiple CBPs to achieve effective biosecurity. For instance, LOS and PBAs provide physical and procedural barriers, while practices like vaccination, testing, and personnel management reduce the likelihood of pathogen introduction and spread.

The studies also identified aspects that act as barriers to implementation of biosecurity practices in swine industry in North America. One significant challenge is ensuring active engagement from swine producers in adopting biosecurity measures and implementing risk assessments. Without their full cooperation and trust, even advanced technological solutions may have limited impact. Another barrier is access to comprehensive and reliable data. In vertically integrated or large-scale production systems, much of the necessary data remains proprietary or inconsistently recorded, hindering its use in comprehensive risk analyses.

4 Discussion

Here we discuss the salient points that emerge from the 18 relevant papers included in our review on knowledge, attitudes, perceptions, and practices of swine farmers in North America. We summarized both, macro and micro-trends in biosecurity.

The application of machine learning and data science as risk assessment and prediction tools remains an area in need of research. Machine learning is being used in the region to predict the exposure of individual farms to disease in the event of an outbreak (26). It would be beneficial to the industry's producers and veterinarians to have the

prediction of the vulnerability of farms in the region to diseases like ASF, which have crippled entire production systems, should it spread to regions where it is not endemic (28, 29). The machine learning models can benchmark and measure which biosecurity practices and farm demographic factors are contributing to the risk of exposure, thus identifying weak points within a farm's biosecurity plan for disease introduction. The use of such technology has the advantage that it can be run at intervals to give a longitudinal assessment of farm vulnerability, essentially aiding the management of virus outbreaks over time (26, 30). With the ever-increasing accessibility to technology and technological advances that can support better disease prevention and risk mitigation, research in the application of different technologies in swine biosecurity management and their role in improving compliance with biosecurity standards and principles in current swine production systems would be beneficial.

While machine learning models provide a promising tool to identify gaps in biosecurity and monitor farm vulnerabilities over time (26, 30), several practical challenges must be considered. One significant challenge is ensuring active engagement from swine producers in adopting biosecurity measures and implementing risk assessments. Without their full cooperation and trust, even advanced technological solutions may have limited impact. Therefore, fostering an understanding of the value these tools bring to farm management is essential for their success (24).

Additionally, access to comprehensive and reliable data presents another significant hurdle. In vertically integrated or large-scale production systems, much of the necessary data remains proprietary or inconsistently recorded, hindering its use in comprehensive risk analyses. Key information, such as farm demographics, movement patterns, and compliance behaviors, may not always be readily available, making it difficult to fully leverage machine learning

capabilities. Collaborative efforts to establish data-sharing frameworks and standardized biosecurity metrics are critical to address this gap and ensure these technologies can be effectively applied in practice.

It is evident that the strictness of biosecurity implementation by swine producers varies by several factors including geographical location, density of swine farms and the associated perceived feasibility of implementation. However, factors like swine density could have mixed effects on implementation of biosecurity practices. For instance, farms in higher swine farm density areas in Ontario, Canada were more likely to adopt higher biosecurity practices; the reverse was reported in Iowa, USA (40). The high pig density in Iowa was found to be associated with decreased rates of on farm biosecurity adoption an observation that (31) refer to as the 'Iowa variable' in their paper. This has been linked to producer perception that disease exclusion using biosecurity measures was not realistically achievable in the high farm density and integrated system of production in Iowa (31). Despite this difference, both studies (31, 40) agreed that larger farms implemented tighter biosecurity, suggesting that the scale of losses a farmer risks is an incentive for the adoption of preventive biosecurity practices.

The role of swine density also extends beyond an individual farm's biosecurity practices to its broader impact on the industry. High-density farms, although capable of tighter biosecurity, carry a greater risk of amplifying disease spread if biosecurity measures fail. A single outbreak in a high-density farm has the potential to result in substantial financial losses for the producer and disrupt the industry by serving as a disease reservoir, exposing smaller neighboring farms to heightened risks (26). Conversely, smaller farms, while posing a lower risk for catastrophic industry-wide impacts, may lack the resources to implement comprehensive biosecurity measures, making them vulnerable to initial introductions of pathogens.

As exemplified here, adherence to biosecurity standards and principles is differential and this variability could be influenced by, among other factors, inventory and farm size, and perceived risk for disease based on farm location. Essentially there is a gradient of biosecurity practices even among large-scale producers (who may or may not be in the same integrated system) underscoring the need for regular assessment of behaviors associated with risks and how these should be factored in when addressing inter and intra-system transactions. Simply, the traditional one-size-fits-all approach may no longer be tenable and a consideration of biosecurity guidelines within which production systems can operate with some flexibility to refine the biosecurity may be more efficacious and sustainable. Similarly farmer education on existing, new and potential risks and the recommended biosecurity practices to mitigate their impact is important and critical but needs to be adapted to specific farmer demographics, operational needs and particular risks (24).

Additionally, given that the North American swine production landscape is punctuated by a mix of backyard and medium to large commercial production systems, the studies showed that farmers care about both their risk and the risk of their neighbors and could be considered reason enough to implement inclusive biosecurity awareness initiatives and develop guidelines applicable to the different production types.

However, farm attributes notwithstanding, the risk of spread of disease and losses from a disease varies with each infection,

depending on the transmission modalities that put a farm at risk, the virulence of a pathogen, and herd susceptibility (based on breed, age, etc.) among other factors. Although the swine industry in the USA has been consolidating over time with 86% of the country's inventory coming from farms with more than 2000 animals (32), a study by White et al. (33) showed that risk of a farm's exposure to a disease like Influenza A was the same for all farms having above 200 animals. This means that at least for this pathogen, biosecurity measures to prevent its introduction and spread are necessary regardless of farm size or inventory. Moreover, we underscore the importance of including small and mid-sized swine farms in the risk assessment of FADs and important endemic diseases.

From the published literature, the most important external biosecurity practices discussed were the use of trucks (26, 27, 30, 34, 40) and the sourcing of replacement animals or genetic material (26, 27, 30, 33, 40). The risk is usually higher with trucks shared by several farms, a common and economical practice, thus necessitating good on-farm disinfection protocols and/or PBAs with clear LOS as well as quarantine protocols for any incoming animals and products (26, 27, 30, 34, 40). Farms that share trucks had higher chances of exposure to disease (26). This practice can be influenced by knowledge gaps regarding the role of shared vehicles in disease transmission, as highlighted by Silva et al. (30). Without adequate understanding of how pathogens can persist on fomites like trucks, farm operators may underestimate the risks associated with shared transportation.

It was also hypothesized that farms with many employees were associated with lower incidence of PRRS due to increased attention to personnel biosecurity protocols (26). This observation suggests that attitudes toward risk management may play a role; farms with larger workforces might have stronger incentives to invest in biosecurity measures to safeguard their operations and maintain continuity of business. However, the perception of biosecurity feasibility might differ based on farm size, as large farms tend to have operational connections to multiple sites, which increases the likelihood of pathogen transmission despite strict biosecurity protocols (26, 27). Furthermore, the higher likelihood of exposure to PRRS in large pig inventories (26) underscores the need for knowledge-driven interventions to mitigate risks in these high-density settings. For instance, tailored biosecurity education targeting large-scale producers may help align biosecurity practices with the unique challenges posed by operational complexity and interconnectivity across sites (26).

For internal biosecurity practices to manage and prevent the spread of disease within a farm, restriction of movement combined with clear and enforced biosecurity protocols for visitors and staff at the LOS were the measures most described in the published papers (23, 31, 35, 36). Pudenz et al. (31) in their assessment of the adoption of the SPS plan on biosecurity by swine producers in the USA, discussed the complexities involved in a producers decision to adopt or not to adopt a biosecurity plan. They found that producers who had site specific biosecurity plans have higher adoption of PBA and LOS as described in the SPS plan, compared to farmers who did not have defined site specific biosecurity plans. Overall, a producer's perception of the feasibility of implementation, risk attitudes, and demographics played a significant role in adoption of biosecurity practices.

Practices like shower-in shower-out, monitoring of movement patterns between animal rooms, and well-maintained foot baths were described with varying levels of implementation on swine

farms. Thirdly, manure and dead animal management were important factors in both bio-management and bio-containment practices to minimize the risk of disease spreading from infected farms. We highlight these practices as possible focus areas in future studies, especially since they are not novel practices or concepts to most farmers. While these biosecurity measures have been implemented to varying degrees, farmers' attitudes are largely unknown with regards to how they would prioritize adopting these measures and how this impacts the goal to have continuity of business in the event of introduction of a foreign pig disease into the region.

The risk of establishment of FADs like the ASF in America is higher in the southwestern states as well as California and Florida due to the presence of both the vector of interest and feral swine that could potentially complete the sylvatic cycle (25). Primarily, the risk of exposure and spread of FADs in the swine industry is defined by the factors influencing the transmission and spread of a particular disease. For the case of ASF, there is concern that increases in temperature and humidity that comes with global warming may influence the spread of tick vectors to new territories where they previously could not survive (37–39). This coupled with reports of feral swine in Canada and some USA states poses a real risk for introduction and successful establishment of ASF in the colder high swine producing mid-western states where swine density is highest. Although at the time of Wormington's study the risk was low, there is need for biosecurity protocols and regulations that would potentially limit the spread of the disease vectors to the high-producing region of the Midwest; and mitigate the risk for swine-to-swine transmission among swine herds as posited for North Carolina (25).

5 Conclusion

No single biosecurity practice is sufficient on its own; a functional biosecurity system relies on the integration of multiple practices. For instance, quarantine measures must be supported by clearly defined Lines of Separation (LOS), proper employee directionality to minimize cross-contamination, and protocols such as shower-in/shower-out and change of clothing between newly quarantined and resident swine. This underscores the importance of a comprehensive, multi-layered approach to biosecurity.

Key findings from this review highlight critical gaps and opportunities for enhancing biosecurity in North American swine farms. These include a scarcity of recent data on biosecurity practices and farmer attitudes, emphasizing the need for updated assessments, particularly given the persistent threat of transboundary swine diseases. Producers' perceptions of risk and the feasibility of biosecurity measures play a pivotal role in determining the adoption of practices, which suggests that improved outreach and education could drive greater compliance.

By prioritizing critical biosecurity practices (CBPs) such as access controls at the Perimeter Buffer Area (PBA) and LOS, farms can strengthen their disease prevention and containment efforts. However, adoption of these practices remains voluntary, with varying levels of implementation driven by farmers' perceived incentives and farm-specific demographics. Emerging technologies, such as machine learning, offer exciting possibilities

for identifying vulnerabilities and optimizing biosecurity strategies.

Ultimately, the synthesis of existing research provided in this paper serves as a foundation for future studies, while offering actionable insights for swine producers, policymakers, and researchers. Investing in up-to-date data collection, targeted interventions, and tailored education programs can bolster biosecurity measures across the industry. A collaborative and proactive approach is essential to safeguard the health of swine populations, protect livelihoods, and ensure the sustainability of North American swine production systems.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding author.

Author contributions

MCC: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. DM: Data curation, Project administration, Validation, Visualization, Writing – review & editing. CY: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. CC: Conceptualization, Funding acquisition, Writing – review & editing. MC: Conceptualization, Writing – review & editing. AP: Conceptualization, Funding acquisition, Writing – review & editing. MP: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. AN: Methodology, Software, Validation, Writing – original draft, Writing – review & editing. MM: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of *Frontiers*, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2025.1507704/full#supplementary-material>

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Streptococcal infection and its antimicrobial resistance profile associated with bovine mastitis in Ethiopia: a systematic review and meta-analysis

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Background: In Ethiopia, bovine mastitis is a major problem affecting production, welfare, and public health. *Streptococcus* is a key pathogen that causes mastitis and is often treated with antimicrobials, which can lead to antimicrobial resistance. Nevertheless, the administration of antimicrobials can unintentionally facilitate the emergence of antimicrobial resistance. Thus, this study aimed to systematically review and estimate the pooled prevalence of streptococcal infection in bovine mastitis in Ethiopia, along with associated antimicrobial resistance profiles, to provide a comprehensive understanding of the current situation and guide effective treatment this bacteria.

Methods: This systematic review was carried out according to the PRISMA guidelines. To estimate the pooled proportion and resistance, a random effects model was utilized with R software. The databases used included SCOPUS, PubMed, HINARI, Web of Science, Google, and Google Scholar.

Results: Twenty-five articles were included in this meta-analysis. The overall pooled proportion of mastitis associated with *Streptococcus* spp. was 20% (95% CI: 17–23%). Significant heterogeneity was observed in the studies included ($I^2 = 87\%$; $p < 0.01$). Among the regions, the highest proportion was reported for South Nation, Nationality of Peoples Region (SNNPR) at 26%, followed by Amhara (24%), Oromia and Addis Abeba (19%), and Tigray (15%). The highest proportion of *Streptococcus* isolates was found in patients with clinical mastitis (24%). Among the major *Streptococcus* spp., *Str. agalactiae* had the highest pooled prevalence at 13%. The greatest prevalence of resistant *Streptococcus* was observed against penicillin (52%), followed by streptomycin, tetracycline, and ampicillin (42, 38, and 35%, respectively). According to the information provided by this meta-analysis, evidence-based risk management measures should be established to prevent and control streptococcal infection in dairy cattle. Monitoring and reporting of streptococcal mastitis and antimicrobial resistance are needed in Ethiopia's different regions. To minimize resistance, stricter guidelines should be implemented for antimicrobial use in dairy cattle, with a particular focus on reducing penicillin use.

KEYWORDS

antimicrobial resistance, bovine mastitis, meta-analysis, prevalence, *Streptococcus*

Introduction

Ethiopia has the largest livestock population in Africa, with an estimated total of approximately 70 million cattle. Cows constitute 55.9% of the country's cattle population, with approximately 20.7% of the entire cattle population being composed of milking cows (1). The milk derived from dairy cows serves as an essential dietary resource for the majority of the urban and peri-urban population (2). A total of 85–89% of the overall national milk production is attributed to cattle (3). However, the quantity of milk falls significantly below the national demand owing to various factors that contribute to diminished milk production (4). Bovine mastitis is a major and serious disorder that has a significant effect on dairy production and is a high public health threat. It causes substantial economic losses due to reduced milk yield, treatment costs, the discarding of milk with antimicrobials, the lower price of poor-quality milk, and death from severe inflammation (5). The estimated economic losses associated with clinical mastitis are between \$69 and \$110 per cow on farms worldwide (6).

Mastitis can be classified by clinical signs, duration, and epidemiology. Clinical mastitis ranges from mild udder infection to severe systemic illness, with approximately 10% of cases resulting in mortality (7, 8). It presents with rapid onset, swelling, and redness of the affected quarter. In contrast, subclinical mastitis often remains undiagnosed because of the absence of visible changes in milk (9). In terms of duration, mastitis can be acute, sudden, or chronic and is characterized by a prolonged inflammatory process and the gradual development of fibrous tissue (10). Epidemiologically, mastitis can be classified into environmental and contagious forms, each caused by various agents (11). Globally, bovine mastitis affects 30 to 50% of cows annually (6).

Mastitis is caused by various pathogens, including bacteria, fungi, and viruses, with approximately 150 agents identified, with bacteria being the most common (12). In bovine mastitis, *Staphylococcus*, *Streptococcus*, and coliform bacteria are particularly harmful to the udder (13). *Staphylococcus* and *Streptococcus* are responsible for 85–95% of bovine mastitis cases (14, 15). *Streptococcus* accounts for 10–30% of cow mastitis cases (16). The pathogenicity of *Streptococcus* is reliant on its capacity to transfer (or acquire) a range of virulence factors through gene exchange (17). *Streptococcus* demonstrates proportions strong adsorption and ant phagocytic activity. Its virulence factors include neuraminidase, lipoteichoic acid, capsular polysaccharide antigen, pyrogenic exotoxin, M protein, CAMP factor, and hemolysin (18, 19). Different virulence factors are linked to specific genetic markers, such as the α -antigen and β -antigen, which are encoded by the *bac* and *bca* genes (20). *Str. agalactiae* is a leading cause of bovine mastitis and has significant economic impacts. It can persist in bovine mammary glands by forming biofilms and is strongly linked to subclinical mastitis (17). Typically, *Str. agalactiae* is beta-hemolytic and is responsible for most mastitis infections in Africa (49%) and Asia (40%).

Several epidemiological studies have examined streptococcal infections in dairy cattle in Ethiopia. *Streptococcus* occurrence in clinical and subclinical bovine mastitis ranges from 6% (21) to 37%

(22). The prevalence of bovine mastitis associated with streptococcal infection varies between 1 and 26% at the species level (23, 24), with *Str. uberis* and *Str. agalactiae* being the most commonly identified isolates. Additionally, a study (25) reported the presence of *Str. agalactiae* in 10.3% of mastitis milk samples in the Haramaya district of eastern Ethiopia.

Antimicrobial agents are the primary treatment for bacterially induced bovine mastitis in most African countries, including Ethiopia, despite increasing antimicrobial resistance (AMR) globally (26, 27). If unchecked, AMR could cause more than 10 million deaths annually by 2050 and cost more than \$100 billion (28). Common antimicrobials in Ethiopia include penicillin, sulphonamide, ampicillin, cloxacillin, oxy-tetracycline, penicillin-procaine, streptomycin, and intra-mammary ampicillin-cloxacillin combinations (22). The regulation of antimicrobial utilization and veterinary practices in livestock production plays a critical role in addressing mastitis, a prevalent and economically significant disease in dairy cattle. Examining the legal framework surrounding anti-mastitis therapy is particularly important in Ethiopia, where challenges such as limited access to veterinary care, inadequate enforcement of antimicrobial regulations, and unregulated drug distribution impact treatment choices (29, 30). Ethiopia has national guidelines on antimicrobial use in livestock, but enforcement remains inconsistent, leading to the misuse of antibiotics and the emergence of antimicrobial resistance (AMR) (31, 32).

Streptococcus resistance can be phenotypic or genotypic, with genes such as *tet(M)* and *tet(O)* for tetracycline resistance and *erm* for macrolide resistance (33–35). The cure proportions for mastitis vary from 64 to 91% (36) and are influenced by pathogen resistance and virulence (37). In Ethiopia, resistance is high: 20% for *Str. agalactiae*, 40% for *Str. dysgalactiae*, and 33.3% for *Str. uberis* to penicillin; 40% for *Str. agalactiae* and 42.9% for *Str. uberis* to ampicillin (25, 38); 73.3% for *Str. dysgalactiae* to oxy-tetracycline; and 50% for *Str. agalactiae* to streptomycin (22, 39). The growing concern over Antimicrobial resistance further complicates this issue, as it limits the effectiveness of conventional treatments, leading to persistent infections and increased transmission risks. A survey in Ethiopia revealed that 31.8% of individuals consume raw milk (40), indicating health risks, as raw milk supports microorganism growth. *Streptococcus* spp. can cause severe human infections (41). The Ethiopian dairy sector is growing, with efforts to increase productivity and address animal diseases through epidemiological data. However, raw milk consumption and inadequate hygiene practices are concerns (42, 43). A One Health approach is essential for managing AMR and ensuring health outcomes. This review was prompted by repeated mastitis cases at the University of Gondar Veterinary Dairy Farm, in which *Streptococcus* spp. were isolated in 45% of 20 mastitis cases, 6 (30%) with *Staphylococcus*, 2 (20%) with *E. coli*, and 3 (10%) were unidentified. Therefore, this systematic review and meta-analysis aimed to estimate the pooled prevalence of streptococcal infections in bovine mastitis patients and their antimicrobial resistance profiles in Ethiopia. The findings will offer evidence-based recommendations for improved management practices, which are essential for enhancing dairy production, safeguarding animal health, and ensuring the sustainability of Ethiopia's dairy industry. Additionally, this research

will advance the understanding of the epidemiology of these infections, underscore the need for targeted interventions, and support the development of effective treatment protocols and monitoring systems for responsible antimicrobial use in veterinary medicine.

Methods

Search strategy

The literature review was conducted from January 12–20, 2024, using the PRISMA checklist (44). This systematic evaluation of *Streptococcus* spp. in bovine mastitis and antimicrobial resistance involved seven key stages: suitability assessment, information sources, search strategy, outcome variables, data extraction, study quality evaluation, and data synthesis with statistical analysis. A comprehensive search was conducted using several databases, including PMC, SCOPUS, PubMed, HINARI, Web of Science, Google, and Google Scholar. Study selection was performed independently by two authors (M.D.F and A.S.M). The research question addressed the proportion, prevalence, and antimicrobial resistance of *Streptococcus* spp. causing bovine mastitis in Ethiopia. Meanwhile, the key words used were *Streptococcus* spp. OR *Streptococcus* infection, epidemiology OR prevalence OR infection proportion, cattle OR bovine OR animals, resistance proportion OR antimicrobial resistance AND (mastitis) AND Ethiopia. A restriction was placed on the language of publication as English. All identified studies were imported into EndNote 20 software to remove duplicates and citations of the references.

Description of the study settings

The meta-analysis was conducted in Ethiopia, which is located in the Horn of Africa between 3°00′–15°00′ N latitude and 32°30′–48°00′ E longitude. Covering 1.04 million square kilometers, Ethiopia is Africa's second most populous country, with 123 million people. The country supports significant agricultural production, with approximately 70 million cattle, 52.5 million sheep, and 42.9 million goats (1). Its diverse topography includes highlands above 2,300 m, a.s.l. proportion transition zone between 1,500 and 2,300 m, and lowlands below 1,500 m.

Study eligibility criteria

Inclusion criteria

To avoid reviewer bias, the search was carried out by three subject matter experts in veterinary clinical medicine, veterinary pharmacy, and veterinary public health and epidemiology. All of the primary descriptive studies that had been published in English and that showed the presence of *Streptococcus* spp. in dairy cattle were included in this meta-analysis. Articles that provided a precise estimate of the percentage of each bacterial isolate were required to meet the inclusion criteria. The research needed to come from observational studies, and the cause of bacterial mastitis in cows had to be determined from clinical or subclinical cases. The study animals were limited to

domestic cattle, or cows, which are commonly raised for their milk. It was necessary to gather samples from animals that had not been exposed to an experimental infection. The geographical location of the bacterial isolates had to be Ethiopia, and the isolates were identified at least down to the genus level. The overall quantity of *Streptococcus* spp. investigated and the quantity of isolates that were resistant or sensitive may or may not have been disclosed. In cases where the scientific papers presented findings from identical sample times and methodologies but with varying *Streptococcus* spp., each occurrence was documented as an individual investigation within our database. Consequently, one scientific article could encompass multiple studies.

Exclusion criteria

Studies focusing on milk from camels and other non-cattle species were excluded from the analysis. Additionally, studies that failed to provide clear and comprehensive estimates of the proportion of each bacterial species in relation to the affected host were not included. Review articles, duplicate studies, publications containing only abstracts, qualitative research, and studies based solely on KAP (knowledge, attitudes, and practices) questionnaires, book chapters, case reports, editorials, short communications, opinion pieces, and studies without original data were excluded. Furthermore, intervention studies that did not include baseline data on the association between animal exposure and disease were excluded from the meta-analysis.

Definition of outcome variable

In this review, we have two outcome variables: first, the pooled proportion/magnitude of *Streptococcus* spp. among the bacteria causing mastitis, and second, the antimicrobial resistance (AMR) profile of *Streptococcus* spp. In the first case, the proportion of mastitis-associated *Streptococcus* infection was estimated by considering the number of *Streptococcus* spp. isolates in the milk sample relative to the total number of bacterial isolates. In the second case, the resistance proportion of mastitis-associated *Streptococcus* isolates was calculated by determining the number of AMR isolates of *Streptococcus* spp. relative to the total number of isolates.

Data extraction

Two investigators (B.A.M and M.G.) extracted the data independently. Data extraction, both quantitative and qualitative, was performed via two tables and an Excel spread sheet from the included studies. The primary author's name, the year the work was published, the region, the total number of bacterial isolates, the number of isolates of *Streptococcus* spp. (the main outcome of interest), diagnostic procedures, data collection, and ethical considerations were included in the extracted components. Information was extracted from each article and entered into a database, including the antimicrobial susceptibility testing methodology (disc diffusion or minimum inhibitory concentration (MIC) estimation), MIC methodology (broth dilution method, agar dilution method, or other), number of *Streptococcus* isolates analyzed, number of resistant isolates, and type

of mastitis (clinical or subclinical). Conflicts were settled through discussion and advice from a third author.

Study quality assessment

To confirm the review's methodological quality, a quality assessment was carried out by two independent authors (Y.D and A.S.M). The AXIS quality tool (45) was used to evaluate the included studies' quality. The study design, sample size justification, sample representativeness, target population, use of validated measures, diagnosis of statistical methods, sample selection, sample frame, and discussion of nonresponse bias, funding reporting, and conflicts of interest are just a few of the items included in this quality assessment tool.

Data synthesis and statistical analysis

R software was used to perform a meta-analysis via the “metaprop” function from the “meta” package version 4.1.3–0 (46) and “metafor” in R Studio (47). The pooled proportion and 95% confidence intervals (CIs) were estimated via a random effects model based on the restricted maximum likelihood (REML) method, which computes within-and between-study variability. It was applied to the resistance proportion, heterogeneity, overall effect size, and weight of each study in the meta-analysis. Furthermore, the pooled prevalence and resistance of bovine mastitis associated with streptococcal infection are illustrated via graphs and tables. The resulting variable is binary (i.e., the only parameter available to measure effect size for single groups (e.g., *Streptococcus* positive or negative); resistant/sensitive to the antimicrobial agent) was the raw proportion with 95% confidence intervals (48). In accordance with (49), a logistic-normal random-effect regression model was used to estimate pooled proportions via logit transformation, whereas a mixed effect logistic regression model was employed for subgroup analysis.

Investigation of heterogeneity

The sources of heterogeneity were evaluated via the Cochran's Q test (reported as the p value), τ^2 (variance between studies), and the inverse variance index (I^2), which indicates the proportion of total variation observed between studies as opposed to heterogeneity as a result of chance. According to (50), the I^2 index was calculated to correspond to values of 25, 50, and 75%, respectively, and was estimated to represent low, moderate and high heterogeneity. When the Q test produced a p value of less than 0.10 and the I^2 value was greater than 50%, heterogeneity was deemed statistically significant. A forest plot was used to evaluate the level of heterogeneity among the studies. Each study's weights, effect sizes, and 95% confidence intervals are displayed in a forest plot diagram.

Subgroup sets

A subgroup analysis of the proportion of *Streptococcus* spp. in bovine mastitis was carried out on the basis of the study year,

study location or region, species of bacteria, and level of mastitis (clinical and subclinical) to ascertain specific between-study variability.

Publication bias assessment

Publication bias is typically assessed via Egger's test, Begg's rank test, and a funnel plot, which allows for the visual assessment of asymmetry (48). Therefore, Egger's regression test and funnel plot diagrams were used to evaluate publication bias.

Sensitivity and influential analysis

A sensitivity analysis of the studies was performed to evaluate the effect of each individual study (51). The results revealed that the studies were the prime determinants of the pooled result.

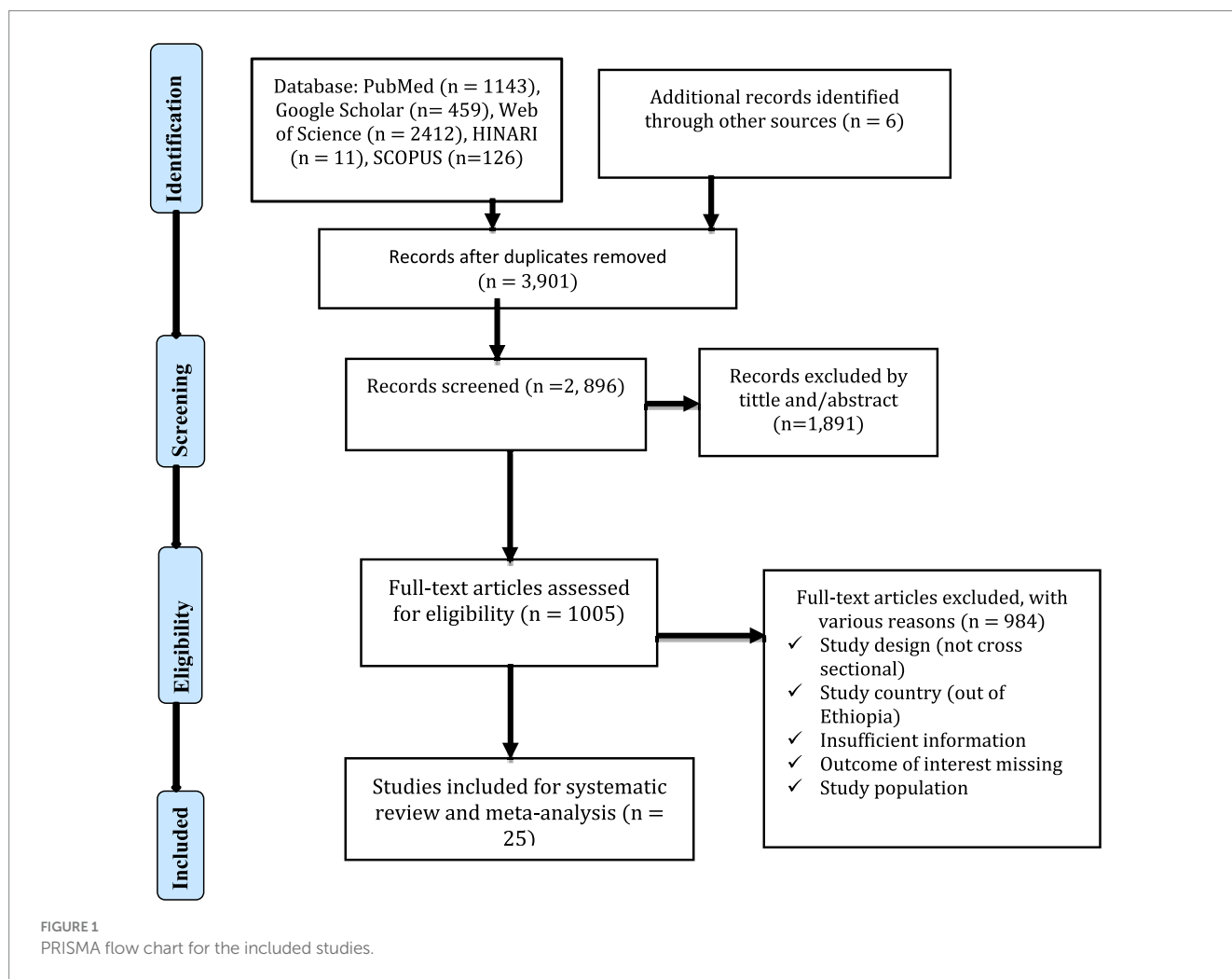
Results

Search results

A comprehensive search was performed in several databases, yielding 4,151 articles, along with 6 additional records identified from other sources (Figure 1). After removing duplicates, 3,901 records remained. Of these, 2,896 records were screened, and 1,891 were excluded based on their title and/or abstract. A total of 1,005 articles were assessed for full-text eligibility, with 984 excluded for various reasons. In the end, twenty five studies ($n = 25$) were included in the meta-analysis.

Overview of the included articles

The characteristics of studies on *Streptococcus* spp. isolates are detailed as follows. The study subjects were lactating dairy cattle. A total of 25 articles were analyzed for the proportion of *Streptococcus* spp. associated with mastitis, and 54 articles were reviewed on the basis of species isolates. We identified the following isolates: *Str. agalactiae* ($n = 18$; 33%), *Str. dysgalactiae* ($n = 13$; 24%), *Str. uberis* ($n = 11$; 20%), *Str. faecalis* ($n = 4$; 7%), and unidentified *Streptococcus* spp. ($n = 8$; 14.8%). The studies included were conducted in various regions of Ethiopia between 2008 and 2024, predominantly in the southern (Oromia) and central regions. Diagnostic methods included CMT and bacterial culture, following procedures described by (52). The regional distributions of studies were as follows: Oromia (11 studies, 44%), Addis Ababa (7 studies, 27%), SNNPR (2 studies, 8%), Amhara (4 studies, 16%), and Tigray (2 studies, 8%). The minimum sample size was 79 cattle, and the maximum sample size was 1,019 (53). In this review, 7,073 dairy cows were evaluated. The prevalence of bovine mastitis associated with *Streptococcus* infection ranges from 1 to 26% (24, 54), with *Str. uberis* and *Str. agalactiae* being the most prevalent. Of the 25 studies, 18 focused on subclinical mastitis, and 15 focused on clinical mastitis. Three studies (55–57) addressed only subclinical mastitis. The detailed characteristics of the included studies are presented in Table 1.



Quality assessment results

In this review, a spectrum of studies was evaluated with respect to their quality, which ranged from low to medium proportion. None of the 25 quantitative studies met the criteria set by the AXIS tool, which encompasses details pertaining to risk factors and outcome variables. A majority of the articles, specifically 22 out of 25 (88%), utilized the simple random method procedure outlined by (58). Moreover, 20 studies (80%) successfully obtained a sample frame from a suitable population that closely resembled the target or reference population being investigated. Among the total number of studies, 17 (approximately 68%) fulfilled the requirements for six out of the 20 questions, namely, aims/objectives, definition of target/reference population, internal consistency of results, authors' justification of the results, sample size justification, analysis of appropriate techniques in the methods and conflicts of interest, and description of the statistical methods used.

Meta-analysis, heterogeneity testing, and bias assessment

The meta-analysis included 25 articles investigating *Streptococcus* species associated with mastitis. Importantly, some articles were

referenced multiple times because of their relevance in similar years but involved investigations of different bacterial strains. The studies included in this analysis showed a substantial level of heterogeneity ($I^2 = 87\%$, $\tau^2 = 0.203$; $p < 0.01$). The estimated pooled proportion of *Streptococcus* species associated with mastitis among all bacterial isolates was 20% (95% CI: 17–23%; Figure 2). The variability between studies was statistically significant ($Q = 386.5$, $DF = 25$, $p < 0.001$). The funnel plots (Figure 3) and Egger's regression asymmetry did not suggest the presence of publication bias ($p > 0.05$).

Subgroup analysis

Because of high degree of heterogeneity, sub analyses were conducted on the basis of the study location or region, study year, degree of mastitis, and type of *Streptococcus* spp., as shown in Table 2 and Supplementary Figures 1–4. Significant heterogeneity between studies was found in the sub analysis by region-wise ($p < 0.001$). The subgroup analysis of *Streptococcus* bacteria associated to bovine mastitis by region revealed that SNNPR had the largest pooled proportion of mastitis-associated *Streptococcus* isolates (26%), followed by Amhara (24%), Oromia (19%), AA (18%), and Tigray (15%). However, Oromia region had the highest heterogeneity ($I^2 = 91\%$; $p < 0.01$) across studies. A sub analysis was conducted

TABLE 1 Characteristics of the studies included in the meta-analysis ($n = 25$).

First author	Study year	Region	Study design ST	Sample taken	<i>Str.spp</i>	TAE	TBI	No. Str	Proportion
Dereje et al. (57)	2014–2015	AA	CS, PS	Milk	<i>Str. agalactiae</i>	186	97	5	0.052
Dereje et al. (57)	2014–2015	AA	CS, PS	Milk	<i>Str. dysgalactiae</i>	186	97	5	0.052
Dereje et al. (57)	2014–2015	AA	CS, PS	Milk	<i>Str. uberis</i>	186	97	12	0.124
Etifu and Tilahun (21)	2011–2012	Oromia	CS, SR	Milk	<i>Str. Sppp</i>	111	138	8	0.058
Zenebe et al. (89)	2011–2012	Tigray	CS, SR	Milk	<i>Str. agalactiae</i>	322	698	142	0.203
Moges et al. (22)	2009–2010	Amhara	CS, SR	Milk	<i>Str. agalactiae</i>	322	164	26	0.159
Moges et al. (22)	2009–2010	Amhara	CS, SR	Milk	<i>Str. dysgalactiae</i>	322	164	23	0.140
Moges et al. (22)	2009–2010	Amhara	CS, SR	Milk	<i>Str. uberis</i>	322	164	11	0.067
Kumbe et al. (59)	2017–2018	Oromia	CS SR	Milk	<i>Str. Sppp</i>	330	155	33	0.213
Ararsa et al. (74)	2009–2010	AA	CS, SR	Milk	<i>Str. agalactiae</i>	90	180	22	0.122
Ararsa et al. (74)	2009–2010	AA	CS, SR	Milk	<i>Str. dysgalactiae</i>	90	180	13	0.072
Ararsa et al. (74)	2009–2010	AA	CS, SR	Milk	<i>Str. uberis</i>	90	180	5	0.028
Ararsa et al. (74)	2009–2010	AA	CS, SR	Milk	<i>Str. faecalis</i>	90	180	5	0.028
Boggale et al. (53)	2009–2010	Oromia	CS, SR	Milk	<i>Str. agalactiae</i>	1,019	1,493	192	0.129
Adane et al. (90)	2010–2011	Oromia	CS, SR	Milk	<i>Str.spp</i>	460	641	160	0.250
Tegegne et al. (91)	2015–2016	Amhara	CS SR	Milk	<i>Str. agalactiae</i>	303	187	27	0.144
Tegegne et al. (91)	2015–2016	Amhara	CS, SR	Milk	<i>Str. dysgalactiae</i>	303	187	11	0.059
Fesseha et al. (92)	2018–2019	Oromia	CS, SR	Milk	<i>Str.spp</i>	283	144	16	0.111
Getahun et al. (38)	2007	Oromia	CS, SR	Milk	<i>Str. agalactiae</i>	500	195	28	0.144
Getahun et al. (38)	2007	Oromia	CS,SR	Milk	<i>Str. dysgalactiae</i>	500	195	6	0.031
Getahun et al. (38)	2007	Oromia	CS SR	Milk	<i>Str. uberis</i>	500	195	20	0.103
Girma et al. (39)	2010–2011	Oromia	CS, SR	Milk	<i>Str. agalactiae</i>	384	121	24	0.198
Girma et al. (39)	2010–2011	Oromia	CS,SR	Milk	<i>Str. agalactiae</i>	384	121	7	0.058
Girma et al. (39)	2010–2011	Oromia	CS, SR	Milk	<i>Str. uberis</i>	384	121	7	0.058
Megersa et al. (54)	2009–2010	Sidama	CS, SR	Milk	<i>Str. agalactiae</i>	245	200	53	0.265
Mekonnen and Tesfaye (93)	2009	Oromia	CS, SR	Milk	<i>Str. agalactiae</i>	206	95	11	0.116
Mekonnen and Tesfaye (93)	2009	Oromia	CS, SR	Milk	<i>Str. dysgalactiae</i>	206	95	6	0.063
Mekonnen and Tesfaye (93)	2009	Oromia	CS, SR	Milk	<i>Str. uberis</i>	206	95	3	0.032
Mekonnen and Tesfaye (93)	2009	Oromia	CS, SR	Milk	<i>Str. faecalis</i>	206	95	10	0.105
Mekibib et al. (94)	2008–2009	AA	CS, SR	Milk	<i>Str.spp</i>	107	153	11	0.072
Wubshet et al. (95)	2012–2013	AA	CS,SR	Milk	<i>Str. agalactiae</i>	28	72	10	0.139
Wubshet et al. (95)	2012–2013	SNNPR	CS,SR	Milk	<i>Str. dysgalactiae</i>	28	72	4	0.056
Wubshet et al. (95)	2012–2013	SNNPR	C.SR	Milk	<i>Str. uberis</i>	28	72	5	0.069
Yohannes and Alemu (96)	2017–2018	SNNPR	CS,SR	Milk	<i>Str. agalactiae</i>	245	51	9	0.176
Yohannes and Alemu (96)	2017–2018	SNNPR	CS,SR	Milk	<i>Str. dysgalactiae</i>	245	51	4	0.078
Tefera et al. (97)	2019–2021	AA	CS,PS	Milk	<i>Str.spp</i>	203	72	12	0.167
Bitew et al. (98)	2009–2010	Amhara.Bdr	CS.Srsm	Milk	<i>Str. agalactiae</i>	302	79	7	0.089
Bitew et al. (98)	2009–2010	Amhara	CS.Srsm	Milk	<i>Str. dysgalactiae</i>	302	79	4	0.051
Bitew et al. (98)	2009–2010	Amhara	CS.Srsm	Milk	<i>Str. uberis</i>	302	79	2	0.025
Haftu et al. (99)	2009–2010	Tigray	CS,SR	Milk	<i>Str. agalactiae</i>	305	128	9	0.070
Haftu et al. (99)	2009–2010	Tigray	CS,SR	Milk	<i>Str. dysgalactiae</i>	305	128	4	0.031
Zeryehun and Abera (100)	2015–2016	Oromia	CS,SR	Milk	<i>Str. agalactiae</i>	384	187	32	0.171
Zeryehun and Abera (100)	2015–2016	Oromia	CS,SR	Milk	<i>Str. dysgalactiae</i>	384	187	12	0.064

(Continued)

TABLE 1 (Continued)

First author	Study year	Region	Study design ST	Sample taken	<i>Str.spp</i>	TAE	TBI	No. <i>Str</i>	Proportion
Zeryehun and Abera (100)	2015–2016	Oromia	CS.SR	Milk	<i>Str. uberis</i>	384	187	7	0.037
Melse et al. (101)	2010–2011	Oromia	CS.SR	Milk	<i>Str.spp</i>	217	61	10	0.164
Redeat et al. (24)	2019–2021	AA	CS.PS	Milk	<i>Str. agalactiae</i>	203	86	8	0.093
Redeat et al. (24)	2019–2021	AA	CS.PS	Milk	<i>Str. dysgalactiae</i>	203	86	2	0.023
Redeat et al. (24)	2019–2021	AA	CS.PS	Milk	<i>Str. uberis</i>	203	86	1	0.012
Birhanu et al. (55)	2015–2016	AA	CS.SR	Milk	<i>Str.spp.</i> , <i>unidentified</i>	262	153	43	0.281
Megersa et al. (54)	2009–2010	Oromia	CS.SR	Milk	<i>Str. agalactiae</i>	245	200	53	0.265
Yusuf and Husen (56)	2021	Oromia	CS.SR	Milk	<i>Str. agalactiae</i>	56	112	8	0.071
Yusuf and Husen (56)	2021	Oromia	CS.SR	Milk	<i>Str. uberis</i>	56	112	3	0.027
Yusuf and Husen (56)	2021	Oromia	CS.SR	Milk	<i>Strp. faecalis</i>	56	112	3	0.027

AA, Addis Abeba; CS, cross-sectional; ST, sampling technique; CMT, California mastitis test; SR, simple random sampling; Srsr, systematic random sampling; BC, bacterial culture; TAE, total animal examination; TBI, total bacterial isolation; No. Str, number of *Streptococcus* isolates; PS, purposive sampling.

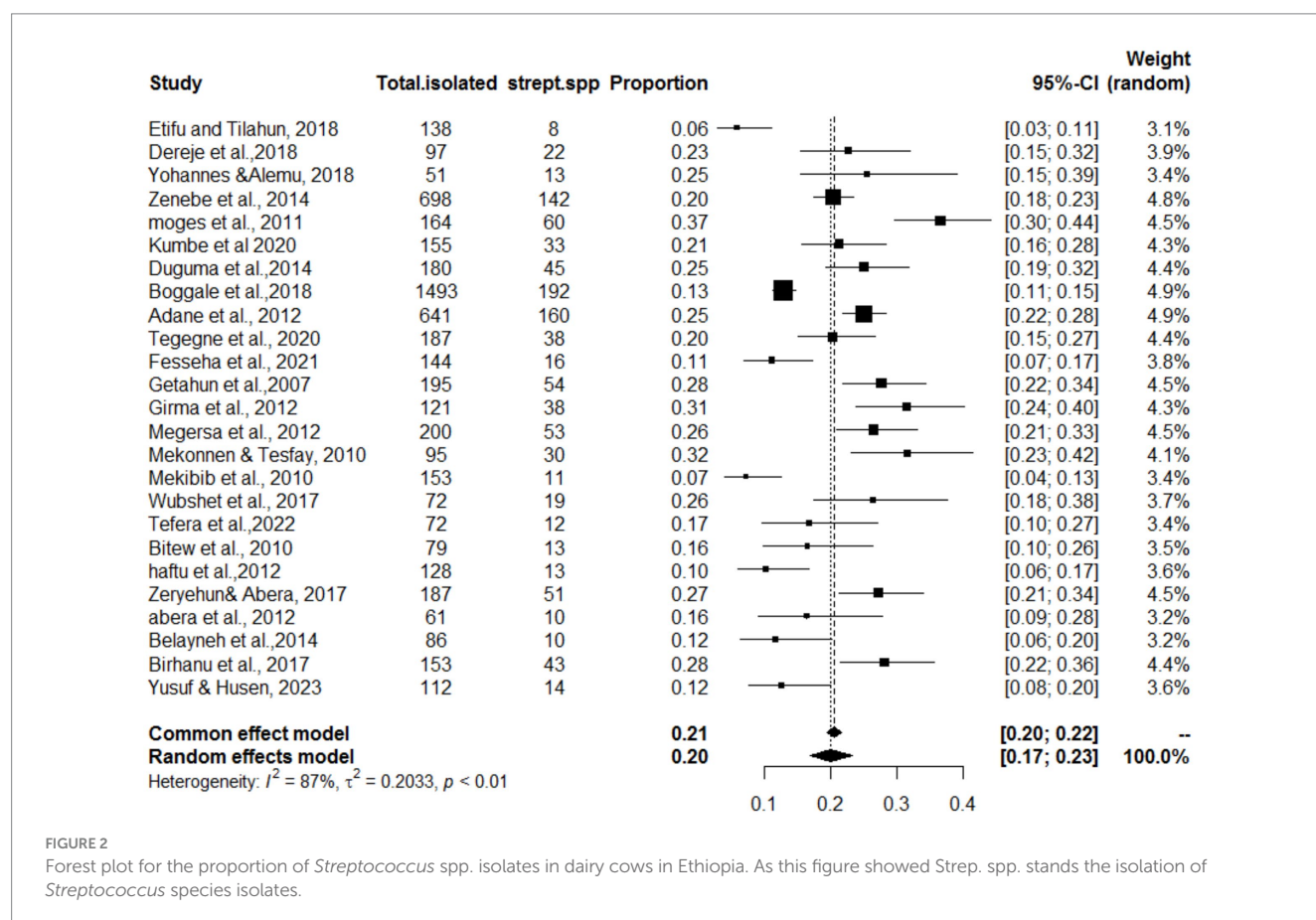


FIGURE 2

Forest plot for the proportion of *Streptococcus* spp. isolates in dairy cows in Ethiopia. As this figure showed *Strep. spp.* stands the isolation of *Streptococcus* species isolates.

regarding the study year (studies grouped into before 2013 and studies after 2013). The magnitude of heterogeneity were $I^2 = 83\%$ and $I^2 = 86\%$, respectively. The highest sub pooled proportion of *Streptococcus* isolates associated to mastitis (20%) occurred prior to 2013. The subgroup difference test results ($Q = 0.18$; $DF = 1$; $p = 0.743$) indicated the absence of a statistically significant group effect.

A sub-analysis on the basis of the degree of mastitis also revealed that, with study weights of 40 and 59.5%, the percentage of

Streptococcus isolates at the species level was greater in clinical mastitis at 24% (95% CI; 16–34%) than in subclinical bovine mastitis at 18% (95% CI; 14–24%). Both the clinical and subclinical mastitis categories experienced significant study variability across studies ($I^2 = 89\%$; $p < 0.01$) and ($I^2 = 87\%$; $p < 0.01$), respectively. Five groups were formed from the sub-analysis of the included studies on the basis of the types of *Streptococcus* species: *Str. agalactiae* ($n = 18$), *Str. dysgalactiae* ($n = 13$), *Str. uberis* ($n = 11$), *Str. Faecalis* ($n = 4$) and

unknown *Streptococcus* spp. ($n = 8$). Several publications did not identify the species level (21, 55, 59, 60). In the sub-analysis of the proportion of *Streptococcus* associated with mastitis by species type, there were notable differences. According to the subgroup analysis proportion shown in Supplementary Figure 4, the pooled proportion of unidentified spp. was the next highest at 15% (95% CI: 10–22%) and ($I^2 = 87\%$; $\tau^2 = 0.370$; $p < 0.01$) for *Str. agalactie*. 13% (95% CI: 11–16%) and ($I^2 = 79\%$) ($\tau^2 = 0.146$; $p < 0.01$). In the present meta-analysis, the proportion of *Str. dysgalactiae* was found to be 6% (95% CI: 5–8%) with low heterogeneity ($I^2 = 49\%$; $\tau^2 = 0.126$; $p = 0.02$).

In all included studies, the Kirby-Baur disc diffusion method was used as an antimicrobial sensitivity test. The present studies on antimicrobial resistance in *Streptococcus* for the treatment of mastitis

in cattle are depicted in Table 3. Only studies accurately proportionally identifying *Streptococcus* species to the species level were included in the meta-analysis. This approach helped to exclude studies that may have misclassified or grouped different species, which could have had varying resistance profiles.

Pooled estimates of antimicrobial resistance

The highest prevalence of resistant *Streptococcus* was against penicillin (pool estimate = 52, 95% CI = 38–67%), followed by streptomycin, tetracycline, and ampicillin (pool estimates = 42, 38, and 35%, respectively). Gentamycin and erythromycin presented the lowest overall prevalence of resistance (pool estimates = 16 and 19%, respectively) (Table 3). In general, the I^2 values were highest for the streptomycin antimicrobials tested ($I^2 = 80\%$). The I^2 of amoxicillin, Co-trimazole and gentamycin were equal to zero since they were used in any of the included studies; therefore, there was no variability (Table 4).

Discussion

This meta-analysis, comprising twenty-five observational studies, revealed that 20% of bovine mastitis cases in Ethiopia were associated with *Streptococcus* spp. Among the various species studied, the prevalence of *Str. agalactiae* was 13%, followed by *Str. dysgalactiae* at 6% and *Str. uberis* at 5%. *Str. agalactiae* had the highest prevalence, likely because it is a highly contagious obligate pathogen of the bovine mammary gland (61). The overall prevalence (20%) is similar to

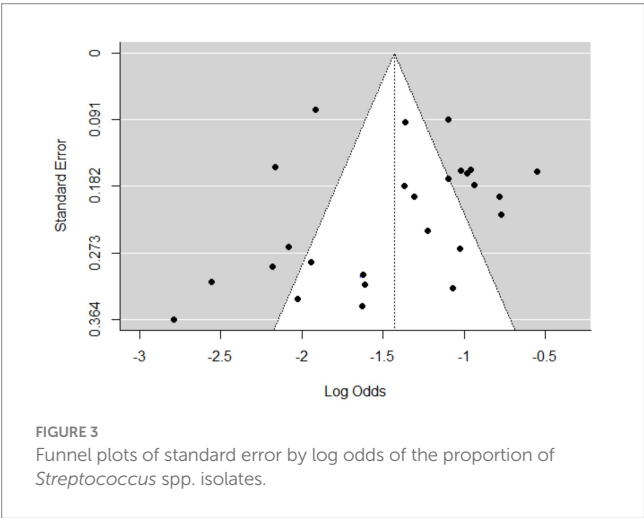


TABLE 2 Pooled estimates of *Streptococcus* spp., stratified by subgroups.

Moderators	K	Category	N	Case	ES (95%CI)(RE)	Heterogeneity			Test for subgroup differences (RE)	
						I^2 (%)	τ^2	p value	Q	p value
Pooled.ES	25	Overall	5,662	1,100	0.20 (0.17;0.23)	87	0.20	<0.01	207.9	<0.0001
Species-wise	13	<i>Str. dysagalcte</i>	1,642	101	0.06 (0.046; 0.079)	49	0.12	0.02	46.79	<0.0001
	16	<i>Str. agalactie</i>	4,791	712	0.13 (0.109; 0.161)	79	0.14	<0.001		
	11	<i>Str. ubreis</i>	1,388	76	0.055 (0.04; 0.080)	60	0.25	<0.01		
	3	<i>Str. Faecalis</i>	387	18	0.046 (0.018; 0.116)	78	0.57	0.01		
	8	Un-identified	1,517	293	0.15 (0.103; 0.224)	87	0.37	<0.01		
Level mastitis	18	Clinical	1823	625	0.24 (0.116; 0.306)	95.9	162	<0.001	0.45	0.5023
	18	Subclinical	3,839	735	0.18 (0.101; 0.231)	96.8	1.08	<0.001		
Year-wise	12	Post-2016	2, 809	463	0.19 (0.150; 0.270)	83	0.14	0.00	0.11	0.74
	13	Pre-2016	2, 853	637	0.20 (0.029; 0.192)	86	0.39	<0.01		
Region-wise	11	Oromia	3,342	606	0.19 (0.1419; 0.25)	91	1.25	<0.01	5.56	0.021
	3	Amhara	430	111	0.24 (0.143; 0.375)	87	1.12	<0.01		
	2	Tigray	826	155	0.15 (0.075; 0.283)	86	1.35	<0.01		
	7	AA	813	162	0.19 (0.131; 0.264)	79	0.27	<0.01		
	2	SNNPR	251	66	0.26 (0.212; 0.321)	0	0	0.88		

K, Number of included studies; N, Total number of isolates; Case, *Streptococcus* spp. isolates; SNNPR, South Nations, Nationalities and peoples Region; AA, Addis Ababa; ES, Effect size; RE, random effect.

TABLE 3 Summary of included studies on antimicrobial resistance.

Author	Antimicrobial	Group	Str. spp	Total	No. resistance	Proportion
Boggale et al. (53)	Amoxicillin	Beta lactam	<i>Str. agalactiae</i>	192	41	0.214
Boggale et al. (53)	Amoxicillin	Beta lactam	<i>Str. faecalis</i>	38	11	0.289
Girma et al. (39)	Amoxicillin	Beta lactam	<i>Str. agalactiae</i>	16	1	0.063
Girma et al. (39)	Amoxicillin	Beta lactam	<i>Str. dysgalactiae</i>	7	2	0.286
Girma et al. (39)	Amoxicillin	Beta lactam	<i>Str. uberis</i>	5	1	0.200
Moges et al. (22)	Ampicillin	Beta lactam	<i>Str. agalactiae</i>	20	8	0.400
Moges et al. (22)	Ampicillin	Beta lactam	<i>Str. dysgalactiae</i>	15	3	0.2000
Moges et al. (22)	Ampicillin	Beta lactam	<i>Str. uberis</i>	7	3	0.429
Etifu et al. (102)	Ampicillin	Beta lactam	<i>Unidentified Str</i>	8	2	0.2500
Boggale et al. (53)	Ampicillin	Beta lactam	<i>Str. agalactiae</i>	192	52	0.271
Boggale et al. (53)	Ampicillin	Beta lactam	<i>Str. faecalis</i>	38	18	0.474
Girma et al. (39)	Ampicillin	Beta lactam	<i>Str. agalactiae</i>	16	8	0.500
Girma et al. (39)	Ampicillin	Beta lactam	<i>Str. dysgalactiae</i>	7	4	0.571
Girma et al. (39)	Ampicillin	Beta lactam	<i>Str. uberis</i>	5	2	0.4
Getahun et al. (38)	Ampicillin	Beta lactam	<i>Str. agalactiae</i>	13	2	0.154
Getahun et al. (38)	Ampicillin	Beta lactam	<i>Str. dysgalactiae</i>	3	0	0.000
Getahun et al. (38)	Ampicillin	Beta lactam	<i>Str. uberis</i>	19	7	0.368
Boggale et al. (53)	Cloxacillin	Beta lactam	<i>Str. agalactiae</i>	192	69	0.359
Boggale et al. (53)	Cloxacillin	Beta lactam	<i>Str. faecalis</i>	38	23	0.605
Girma et al. (39)	Cloxacillin	Beta lactam	<i>Str. agalactiae</i>	16	2	0.125
Girma et al. (39)	Cloxacillin	Beta lactam	<i>Str. dysgalactiae</i>	7	1	0.143
Girma et al. (39)	Cloxacillin	Beta lactam	<i>Str. uberis</i>	5	1	0.200
Dereje et al. (57)	Cotrimoxazole	Sulphonamide	<i>Str. agalactiae</i>	5	1	0.200
Etifu et al. (102)	Cotrimoxazole	Sulphonamide	<i>str. spp</i>	8	1	0.125
Dereje et al. (57)	Cotrimoxazole	Sulphonamide	<i>Str. dysgalactiae</i>	5	1	0.200
Dereje et al. (57)	Cotrimoxazole	Sulphonamide	<i>Str. uberis</i>	12	4	0.333
Dereje et al. (57)	Erythromycin	Aminoglycosides	<i>Str. agalactiae</i>	5	2	0.400
Moges et al. (22)	Erythromycin	Aminoglycosides	<i>Str. agalactiae</i>	20	4	0.200
Moges et al. (22)	Erythromycin	Aminoglycosides	<i>Str. dysgalactiae</i>	15	3	0.200
Moges et al. (22)	Erythromycin	Aminoglycosides	<i>Str. uberis</i>	7	4	0.571
Etifu et al. (102)	Erythromycin	Aminoglycosides	<i>Unidentified/strep</i>	8	0	0.000
Dereje et al. (57)	Erythromycin	Aminoglycosides	<i>Str. dysgalactiae</i>	5	0	0.00
Dereje et al. (57)	Erythromycin	Aminoglycosides	<i>Str. uberis</i>	12	0	0.00
Getahun et al. (38)	Erythromycin	Aminoglycosides	<i>Str. agalactiae</i>	13	1	0.077
Getahun et al. (38)	Erythromycin	Aminoglycosides	<i>Str. dysgalactiae</i>	3	1	0.333
Getahun et al. (38)	Erythromycin	Aminoglycosides	<i>Str. uberis</i>	19	0	0.00
Dereje et al. (57)	Gentamycin	Aminoglycosides	<i>Str. agalactiae</i>	5	0	0.00
Etifu et al. (102)	Gentamycin	Aminoglycosides	<i>Unidentified strep</i>	8	0	0.00
Boggale et al. (53)	Gentamycin	Aminoglycosides	<i>Str. agalactiae</i>	192	30	0.156
Boggale et al. (53)	Gentamycin	Aminoglycosides	<i>Str. faecalis</i>	38	7	0.184
Dereje et al. (57)	Gentamycin	Aminoglycosides	<i>Str. dysgalactiae</i>	5	0	0.00
Dereje et al. (57)	Gentamycin	Aminoglycosides	<i>Str. uberis</i>	12	2	0.167
Girma et al. (39)	Gentamycin	Aminoglycosides	<i>Str. agalactiae</i>	16	2	0.125
Girma et al. (39)	Gentamycin	Aminoglycosides	<i>Str. dysgalactiae</i>	7	2	0.286

(Continued)

TABLE 3 (Continued)

Author	Antimicrobial	Group	Str. spp	Total	No. resistance	Proportion
Girma et al. (39)	Gentamycin	Aminoglycosides	<i>Str. uberis</i>	5	0	0.00
Moges et al. (22)	Oxytetracycline	Tetracycline	<i>Str. agalactiae</i>	20	5	0.25
Moges et al. (22)	Oxytetracycline	Tetracycline	<i>Str. dysgalactiae</i>	15	11	0.733
Moges et al. (22)	Oxytetracycline	Tetracycline	<i>Str. uberis</i>	7	0	0.00
Boggale et al. (53)	Oxytetracycline	Tetracycline	<i>Str. agalactiae</i>	192	49	0.255
Boggale et al. (53)	Oxytetracycline	Tetracycline	<i>S. faecalis</i>	38	15	0.395
Girma et al. (39)	Oxytetracycline	Tetracycline	<i>Str. agalactiae</i>	16	7	0.438
Girma et al. (39)	Oxytetracycline	Tetracycline	<i>Str. dysgalactiae</i>	7	4	0.571
Girma et al. (39)	Oxytetracycline	Tetracycline	<i>Str. uberis</i>	5	3	0.600
Getahun et al. (38)	Oxytetracycline	Tetracycline	<i>Str. agalactiae</i>	13	4	0.308
Getahun et al. (38)	Oxytetracycline	Tetracycline	<i>Str. dysgalactiae</i>	3	0	0.000
Getahun et al. (38)	Oxytetracycline	Tetracycline	<i>Str. uberis</i>	19	2	0.105
Dereje et al. (57)	Pencillin	Beta lactam	<i>Str. agalactiae</i>	5	1	0.200
Boggale et al. (53)	Pencillin	Beta lactam	<i>Str. agalactiae</i>	192	89	0.464
Boggale et al. (53)	Pencillin	Beta lactam	<i>Str. faecalis</i>	38	30	0.789
Dereje et al. (57)	Pencillin	Beta lactam	<i>Str. dysgalactiae</i>	5	2	0.400
Dereje et al. (57)	Pencillin	Beta lactam	<i>Str. uberis</i>	12	4	0.333
Girma et al. (39)	Pencillin	Beta lactam	<i>Str. agalactiae</i>	16	12	0.750
Girma et al. (39)	Pencillin	Beta lactam	<i>Str. dysgalactiae</i>	7	6	0.857
Girma et al. (39)	Pencillin	Beta lactam	<i>Str. uberis</i>	5	4	0.800
Getahun et al. (38)	Pencillin	Beta lactam	<i>Str. agalactiae</i>	13	2	0.154
Getahun et al. (38)	Pencillin	Beta lactam	<i>Str. dysgalactiae</i>	3	2	0.667
Getahun et al. (38)	Pencillin	Beta lactam	<i>Str. uberis</i>	19	7	0.368
Moges et al. (22)	Sulphonamide	Sulphonamide	<i>Str. agalactiae</i>	20	0	0.000
Moges et al. (22)	Sulphonamide	Sulphonamide	<i>Str. dysgalactiae</i>	15	5	0.33
Moges et al. (22)	Sulphonamide	Sulphonamide	<i>Str. uberis</i>	7	0	0.00
Getahun et al. (38)	Sulphonamide	Sulphonamide	<i>Str. agalactiae</i>	13	2	0.154
Getahun et al. (38)	Sulphonamide	Sulphonamide	<i>Str. dysgalactiae</i>	3	0	0.00
Getahun et al. (38)	Sulphonamide	Sulphonamide	<i>Str. uberis</i>	19	6	0.316
Dereje et al. (57)	Tetracycline	Tetracycline	<i>Str. agalactiae</i>	5	1	0.200
Moges et al. (22)	Tetracycline	Tetracycline	<i>Str. agalactiae</i>	20	8	0.400
Moges et al. (22)	Tetracycline	Tetracycline	<i>Str. dysgalactiae</i>	15	9	0.600
Moges et al. (22)	Tetracycline	Tetracycline	<i>Str. uberis</i>	7	3	0.429
Etifu et al. (102)	Tetracycline	Tetracycline	<i>Unidentified</i>	8	1	0.125
Dereje et al. (57)	Tetracycline	Tetracycline	<i>Str. dysgalactiae</i>	5	0	0.00
Dereje et al. (57)	Tetracycline	Tetracycline	<i>Str. uberis</i>	12	4	0.333

findings from the United States (20.8–23.3%), Bangladesh (28.75%) (62), and the Netherlands (25%) but lower than reports from China (36.23%) (63), Nigeria (56.7%), Egypt (38.3%), Tanzania (75.5%) (64), Europe (38%), Australia (50%), France (42.11%), and New Zealand (58.66%) (65, 66). This variability may be due to differences in climate, knowledge levels, management systems, cow breeds, laboratory facilities, and housing styles across countries (67). The pooled prevalence of *Str. agalactiae* (13%) and *Str. dysgalactiae* (6%) was lower than that in Bangladesh, where the prevalence of *Str. agalactiae* was 19.86% and that of *Str. dysgalactiae* was 17.81% (68), and in Egypt,

where the prevalence of *Str. dysgalactiae* was 23% and that of *Str. agalactiae* was 20.1% (69). These disparities could be due to differences in geographic location, livestock rearing, husbandry, and hygiene practices (70–72). Subgroup analysis by region in Ethiopia revealed the highest prevalence of streptococcal infection in SNNPRS (26%) and the lowest in Tigray (15%). This variation could be due to differences in agroclimatic conditions, sampling methods, farm management practices, and cow-related factors. In terms of mastitis severity, this meta-analysis revealed a greater occurrence of streptococcal infection in clinical mastitis cases (24%) than in subclinical mastitis cases (18%)

TABLE 4 Overall pooled estimate of the prevalence of *Streptococcus* AMR to specific antimicrobial agents.

Type of antimicrobial	Total isolates	No. resistance isolates	Pooled resistance (95%)	Heterogeneity%(I^2)	p value	Tau ²
Amoxicillin	258	56	22 (18–28%)	0	0.52	0.000
Ampicillin	343	102	35 (28–43%)	26	0.19	0.077
Cloxacillin	258	96	33 (18–52%)	71	<0.01	0.4731
Cotrimazole	30	7	22 (12–44%)	0	0.75	0.000
Erythromycin	107	15	19 (10–33%)	31	0.16	0.3934
Gentamycin	288	43	16 (12–20%)	0	0.96	0.000
Penicillin	315	159	52 (38–67%)	66	<0.01	0.5339
Streptomycin	72	26	42 (26–61)	80	<0.01	1.0550
Tetracycline	335	111	38 (25–52%)	17	0.30	0.1060
Oxytetracycline	335	100	35 (24–47%)	59	<0.01	0.3429
Sulphonamides	77	13	21 (11–36%)	22	0.27	0.1883

in CMT-positive cows). Our findings show that clinical mastitis caused by *Streptococcus* is more prevalent than subclinical mastitis, contrary to some reports (59, 60, 73), but consistent with others (38, 39, 74).

In recent years, the proportion of streptococcal infection in bovine mastitis has decreased, likely due to increased awareness through scientific training, research, technological advancements, and the implementation of biosecurity measures on farms. Furthermore, studies from China have demonstrated that subclinical mastitis is a significant issue in smallholder dairy farms, with a variety of bacterial pathogens, including *Streptococcus* spp., playing a major role in infections (75). The molecular characterization of antimicrobial-resistant pathogens such as *Staphylococcus haemolyticus* in dairy herds of Northwest China indicates that the dairy environment can act as a reservoir for resistant bacterial strains, further complicating treatment strategies (76).

The second aim of this review was to determine the antimicrobial resistance (AMR) profile of *Streptococcus* spp. in bovine mastitis. The pooled resistance proportion to penicillin was 52%, which was much higher than that reported in Uruguay (28.6%) (77), France (21%) (78), and Argentina (27.6%) (79). The antimicrobials that have been showing the greatest resistance were aminoglycosides, streptomycin, and penicillin, likely due to long-term and repeated use on dairy farms. The resistance proportions for erythromycin and tetracycline were 19 and 38%, respectively, similar to findings in France (20 and 38.5%) (80, 81) but lower than the resistance proportions reported in the USA and Europe, which ranged from 20 to 50% (82, 83). The resistance of *Streptococcus* spp. to gentamycin (16%) and tetracycline (38%) was greater than the proportions reported by (81) (2.4 and 18%, respectively). The 38% resistance proportion to tetracycline in this study is lower than the proportion reported in Denmark between 2002 and 2004 (84, 75.5, and 84.8%) (84, 85). Recent findings highlight that dairy farms are a potential reservoir for antibiotic-resistant bacteria and virulence genes, particularly among *Escherichia coli* strains that carry resistance to aminoglycosides and beta-lactam antibiotics (86). The One Health approach to AMR suggests that the transmission of resistant bacteria is not confined to animals but extends to the broader ecosystem, including farm workers and the surrounding environment. The rise of multidrug-resistant strains in dairy environments underscores the need for stringent antimicrobial stewardship (87).

The variation in antimicrobial resistance across regions may be attributed to differences in medication practices, with improper use of antimicrobial drugs being a significant contributor to the development of resistance (88). Our findings indicate a high prevalence of both contagious and environmental *Streptococcus* spp. in bovine mastitis in Ethiopia. Therefore, an extended ten-point mastitis control plan should be implemented, with components tailored specifically for Ethiopia, including increased awareness among farmers and milkers. Additionally, targeted interventions for regions with high infection proportions, research into alternative therapeutic approaches, and the development of new antimicrobials are critical measures that must be undertaken.

Limitations of the included articles and this systematic review

Most of the articles describe the frequency of the isolates and the percentage of the resistance isolates however no articles tried to identify the resistance genes. Most of the articles used the convenience method of the sample selection so it may lead to selection biased. This systematic review has several limitations, which must be taken into consideration. First, the review is focused exclusively on a single genus, namely, *Streptococcus*. Second, few studies were included in the analysis of antimicrobial resistance. Third, no studies were included in some regions. Finally, the protocol was not registered in the PROSPERO database.

Conclusion and future perspectives

The present meta-analysis showed the overall pooled proportion of mastitis associated with *Streptococcus* spp. at 20% (95% CI: 17–23%). The highest proportions were found in SNNPR (26%), followed in Amhara (24%), Oromia and Addis Ababa (19%), and Tigray (15%). Clinical mastitis had the highest proportion of streptococcal isolates (24%). *Str. agalactiae* had the highest pooled prevalence at 13%. Resistance was highest against penicillin (52%), followed by streptomycin (42%), tetracycline (38%), and ampicillin (35%). Specifically, *Str. agalactiae* accounted for the highest proportion of

bovine mastitis-causing *Streptococcus* spp. infections. This particular species of *Streptococcus* falls under the category of a contagious group of bacterial pathogens, indicating that a notable proportion of contagious *Streptococcus* and the udder of infected cows serve as a significant reservoir. Among the commonly employed antimicrobials, the highest pooled resistance proportion of *Streptococcus* spp. was observed against penicillin. The data presented in this report will facilitate informed decision-making processes aimed at controlling and preventing bovine mastitis within the context of Ethiopia. The findings will benefit stakeholders and policymakers in enhancing the dairy industry. Increased monitoring and reporting of streptococcal mastitis and antimicrobial resistance across Ethiopia will improve the understanding of prevalence and resistance patterns. The implementation of stricter protocols for Antimicrobial use, especially those that reduce the reliance on highly resistant antimicrobials such as penicillin, is essential. Developing targeted interventions for regions with relatively high infection proportions, promoting research into alternative therapies, and innovating new antimicrobials are also critical steps.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding author.

Author contributions

MF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. MG: Data curation, Methodology, Supervision, Writing – review & editing. AM: Data curation, Investigation, Methodology, Writing – original draft. BM: Methodology, Visualization, Writing – original draft, Writing – review & editing. YD: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2025.1503904/full#supplementary-material>

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Stakeholders' perspectives on communicating biosecurity to encourage behavior change in farmers

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Effective communication is crucial for strengthening collaboration and ensuring the successful implementation of biosecurity measures against infectious diseases. A collaborative approach, where farmers and veterinarians play a central role in decision-making, may have a greater impact on promoting the implementation of biosecurity practices compared to a top-down approach. The objective of this study was to explore the perspectives of researchers, official services, and industry on the preferred communication methods between farmers and various on-farm stakeholders. Data were collected through four simultaneous focus groups conducted within the framework of the COST Action BETTER project: three involving researchers, and one involving official services and industry people. The data were analyzed using content analysis, which generated three main themes and 13 subthemes: (i) effective methods for communicating biosecurity messages to farmers: direct interaction and practical learning, audio-visual media and support materials, importance of personalization and coordination, and challenges and innovative solutions; (ii) designing an optimal communication system to promote behavioral change in biosecurity: initial strategies for communication: knowledge and trust, integration of technological tools, mandatory programs and coordinated campaigns, continuous training and collaborative learning, and incentives and certifications; and (iii) measuring the success of biosecurity communication programs: evaluation tools and audits, key indicators and benchmarking, measuring attitudes and behavioral changes, and participation and knowledge as

additional evaluation metrics. The findings highlight the need for collaborative, personalized, and sustainable approaches to biosecurity communication. This study provides valuable insights to inform the development and implementation of communication programs that remain effective over time.

KEYWORDS

communication, behavior change, biosecurity, farmers, focus groups

1 Introduction

Communication within animal farming systems, particularly concerning biosecurity, is a complex process involving multiple elements, ranging from message clarity and channel selection to the willingness of participants to engage (1–5). Effective communication not only involves the transmission of information but also depends on factors such as trust among stakeholders, shared perspectives, power dynamics, and accessibility to appropriate communication methods and tools (4–9). Understanding how these factors interact is essential for strengthening collaboration and ensuring the effective implementation of biosecurity measures against infectious diseases, including zoonoses, thereby improving both animal health and public health.

In recent years, various national and international plans have addressed the issue of biosecurity on livestock farms through a top-down approach, whereby stakeholders within the sector often receive mandatory instructions underpinned by regulations, with non-compliance potentially resulting in sanctions (7, 10, 11). However, this strategy has demonstrated limitations, as many stakeholders comply primarily to avoid sanctions or, in some cases, to obtain financial benefits (12). This indicates that a sanction-based approach alone is insufficient to foster genuine and sustained behavioral change in biosecurity practices. Conversely, a collaborative approach may have a greater impact on encouraging effective implementation of biosecurity measures (13).

Two-way communication among various stakeholders, particularly between farmers and veterinarians, has become essential for improving biosecurity practices (3, 4, 10). Both stakeholders play a central role in decision-making on livestock farms, regardless of the type of farming system, and their interactions can significantly influence the implementation of biosecurity measures (14, 15). In light of this, the present study aims to explore the perspectives of researchers, official services, and industry (such as government representatives, official veterinary services, representatives from the industry/private sector (producers), and private veterinarians or consultants) on the communication methods between farmers and various on-farm stakeholders, with the goal of proposing innovative communication strategies to promote behavioral change in biosecurity practices. The findings of this study seek to contribute to the improvement of communication methods and tools used in biosecurity practices.

2 Methods

2.1 Context

This study builds on a previous survey that explored stakeholders' perspectives on biosecurity communication in livestock farming. The survey was conducted among stakeholders involved in two projects: the COST Action “Biosecurity Enhanced Through Training, Evaluation and Raising Awareness” (BETTER) and the Horizon 2020 “Networking European Poultry Actors for Enhancing the Compliance of Biosecurity Measures for Sustainable Production” (NetPoulSafe).

The survey explored both the communication methods and tools that participants believed were preferred by farmers and those they personally preferred for engaging in biosecurity practices (Supplementary Table S1). It was distributed electronically through the networks of researchers of the COST Action BETTER project. Participants provided informed consent to take part in the study.

Through focus group discussions, this research aimed to explore the initial survey findings in greater depth. Both the survey and the focus groups aimed to explore the perspectives of different stakeholders involved in communicating with farmers, as they were more easily accessible through the COST Action network, a collaborative project between researchers.

2.2 Data collection

With some of the same participants, a data exploration was conducted 48 h after the survey through simultaneous focus group discussions (16). The focus groups were held in person during a meeting of the COST Action BETTER project in collaboration with the Horizon 2020 NetPoulSafe project, which took place in Padua, Italy, on 6–7 February 2024. Key survey findings were presented to explore the perspectives of both researchers, as well as official services and industry, prior to the focus group discussions.

Each focus group (facilitated by a leader and supported by at least one note-taker) included up to 20 participants. Groups were formed through the random selection of participants, with the exception of the official services and industry group. The groups engaged in a semi-structured discussion lasting ~1 h, based around a thematic guide with four key questions: (Q1) “You have just seen the results of the survey, what are your thoughts on that? Were you surprised? Do you think differently?” (5 min); (Q2) “What

do you think are the most effective methods of communication with farmers?” (10 min); (Q3) “If you were going to design an optimal system for communicating with farmers that leads to a change in behavior regarding biosecurity, how would you do it? What would that look like?” (10 min); and (Q4) “How would you measure the success of this system or program? Do you have examples from existing improvement programs?” (20 min). To facilitate and stimulate discussion, participants were provided with post-it notes and flipcharts to write down their ideas or reflections, which were then individually presented for brainstorming and debate. At the end of the discussions, a volunteer from each group summarized their group’s results during a presentation (without discussion) in a plenary session to the other groups. During the plenary presentations, volunteers were allocated a maximum time of ~5 min, with optional use of visual aids. These presentations were recorded and subsequently transcribed for analysis.

2.3 Data analysis

Data from the post-it notes, flipcharts, discussion group notes, and plenary presentations, were transcribed and organized by focus group. These documents were analyzed using content analysis to organize and extract significant patterns from the data collected (17). The data were manually coded and categorized in Microsoft Word into themes and subthemes, while maintaining the identification of each focus group. In fact, in the results, the identification has been kept in parentheses to indicate the source of each finding (e.g., FG1 referring to the first focus group). Although there were no differences in the composition of the researcher groups, they were separated in the results, mainly because the sample collection differed between groups. The themes were derived deductively from the thematic guide, while the subthemes, which were recurrent, were identified inductively from different sections of the text (18). The analysis enabled the interpretation of intra- and inter-group trends and relationships, highlighting shared or divergent perspectives between researchers, official services and industry.

3 Results

3.1 Survey

The prior survey was completed by 51 respondents, with 48% identifying as female and 52% as male. Respondents ranged in age from 25 to 67 years, with an average age of 42. The majority (40.78%) were researchers (mainly involved in projects related to biosecurity and therefore linked to animal health issues), followed by government representatives (4.8%), individuals in “other roles” (4.8%), official veterinary services (2.3%), and one (person) private veterinarian or consultant. Participants represented a wide geographic scope, spanning 22 countries.

In terms of interaction with farmers, 62% (31) of respondents reported engaging with farmers multiple times per year, 20% (10) interacted less frequently, and 18% (9) indicated they rarely interacted with farmers. More frequent interactions with veterinarians were reported: 78% (39) of respondents

estimated that, based on their experience, farmers interacted with veterinarians several times a year, 12% (6) believed that these interactions were less frequent, and 10% (5) believed that they were rare.

The results indicated a certain degree of agreement between the communication methods perceived to be preferred by farmers and those that the respondents actually employed when engaging with them (Table 1). The respondents reported that on-site farm visits and face-to-face group meetings were the primary communication methods they used, which also aligned with the methods perceived as mostly preferred by farmers, although the frequency of perceived preference was slightly higher than actual use. Furthermore, the responses to webinars, online seminars, individual online meetings, and online resources (e.g., websites) showed correspondence between their limited use and the perceived farmers’ preferences. However, discrepancies emerged in specific methods, particularly telephone conversations, which were used more frequently than they were perceived to be preferred by farmers. Printed materials, such as leaflets and pamphlets, also showed moderate differences, whereas written correspondence remained consistently low in both use and perceived preference.

3.2 Focus groups

A total of 54 participants, four facilitators, and seven note-takers took part in four focus group discussions. Three groups were composed of researchers, and the other one was composed of official services and industry people. Details of each group and the materials used for analysis are provided in Table 2. The selected records from the post-it notes and flipcharts are presented in Figure 1. In total, 3 themes and 13 subthemes were developed (Table 3).

3.2.1 Effective methods for communicating biosecurity messages to farmers

For effective communication about biosecurity with farmers, participants recommended a multidimensional approach that considered their specific needs, interests, and contexts. Researchers, official services, and industry reached agreement on several aspects, although they put forward nuanced proposals, including: (i) direct interaction and practical learning, (ii) audio-visual media and support materials, (iii) the importance of personalization and coordination, and (iv) challenges and innovative solutions.

3.2.1.1 Direct interaction and practical learning

Researchers (FG2, FG3, and FG4), official services, and industry (FG1) emphasized face-to-face contact, especially through farm visits, as one of the most effective communication methods. These visits were thought to help establish trust (FG1 and FG2). Researchers (FG2) highlighted that addressing on-site issues and finding solutions with farmers strengthens the implementation of biosecurity measures, while official services and industry (FG1) underlined the role of veterinarians and advisors as key stakeholders due to their trusted relationships with farmers. However, farm visits demand resources, in particular time, which

TABLE 1 Results from two sets of questions on methods of communicating with farmers about biosecurity.

Method	What methods do you use/would you use to communicate with farmers about biosecurity?		What communication methods do you think farmers prefer?	
	<i>n</i>	Total	<i>n</i>	Total
I do not engage	9	51	4	51
Printed leaflets or pamphlets	14	51	7	51
Educational videos	20	51	15	51
Written correspondence (letters)	7	51	1	51
Telephone conversations	22	51	12	51
Individual online meetings	10	51	7	51
Webinars or online seminars	14	51	10	51
On-site farm visits	38	51	43	51
Face-to-face group meetings	30	51	35	51
Online resources and websites	13	51	9	51
Other	1	51	1	51
Total responses	178		144	

TABLE 2 Details of focus groups and materials used for analysis.

Focus group (FG)	Group member composition in the focus group discussions			Materials utilized during the focus group discussions			
	Facilitators and note-takers	Number of participants	Gender distribution of participants	Post-it notes	Flipcharts	Discussion group notes	Plenary presentations
FG1: Official services and industry	AB*, CC-G	9	6F/3M	Yes (Q2); No (Q1, Q3-4)	Yes (Q3-4); No (Q1-2)	Yes	Yes
FG2: Researchers	DDM*, HCF, IC, NC	12	6F/6M	Yes (Q1-4)	No (Q1-4)	Yes	Yes
FG3: Researchers	AA*, MLB, SM	16	5F/11M	Yes (Q2-4); No (Q1)	No (Q1-4)	Yes	Yes
FG4: Researchers	MK*, L-MT	17	6F/11M	No (Q1-4)**	Yes (Q2); No (Q1, Q3-4)	Yes	Yes

F, Female; M, Male; Q, Questions; *Facilitators. **At least for one question, they were used but were not found for analysis.

may hamper the effectiveness of this method of communication in practice (FG4).

Additionally, researchers (FG2) pointed to practical learning as a key tool, proposing activities such as exchange visits, hands-on demonstrations (“learning by doing”), and educational games (e.g., case studies and exercises) designed to simulate disease spread. These activities were felt to not only promote knowledge acquisition but also foster mutual understanding among farmers.

3.2.1.2 Audio-visual media and support materials

All stakeholders highlighted the usefulness of audio-visual materials, such as videos, especially those highlighting success stories or providing clear instructions on biosecurity measures. Researchers (FG4) suggested short videos to capture farmers’ attention in an accessible and engaging format, while official services and industry (FG1) mentioned using tools like social media and farmer “influencers” to counteract misinformation.

Researchers (FG3) stressed that the content of any material must be accompanied by clear instructions or step-by-step protocols aligned with global biosecurity standards. However, they warned that methods relying solely on websites or written texts might be insufficient for achieving communication goals.

3.2.1.3 Importance of personalization and coordination

Message personalization was a recurring theme. Researchers (FG3 and FG4), official services, and industry (FG1) noted that a communication plan tailored to farmers’ characteristics and needs is crucial. The groups emphasized the importance of messengers learning to listen, adapting their approach accordingly, and remaining flexible and adaptable. Researchers (FG3) highlighted the importance of reflecting on questions such as, “Why would farmers do this or why wouldn’t they?” to design more effective strategies.

Additionally, researchers (FG4) highlighted that communication should be a coordinated effort among veterinarians

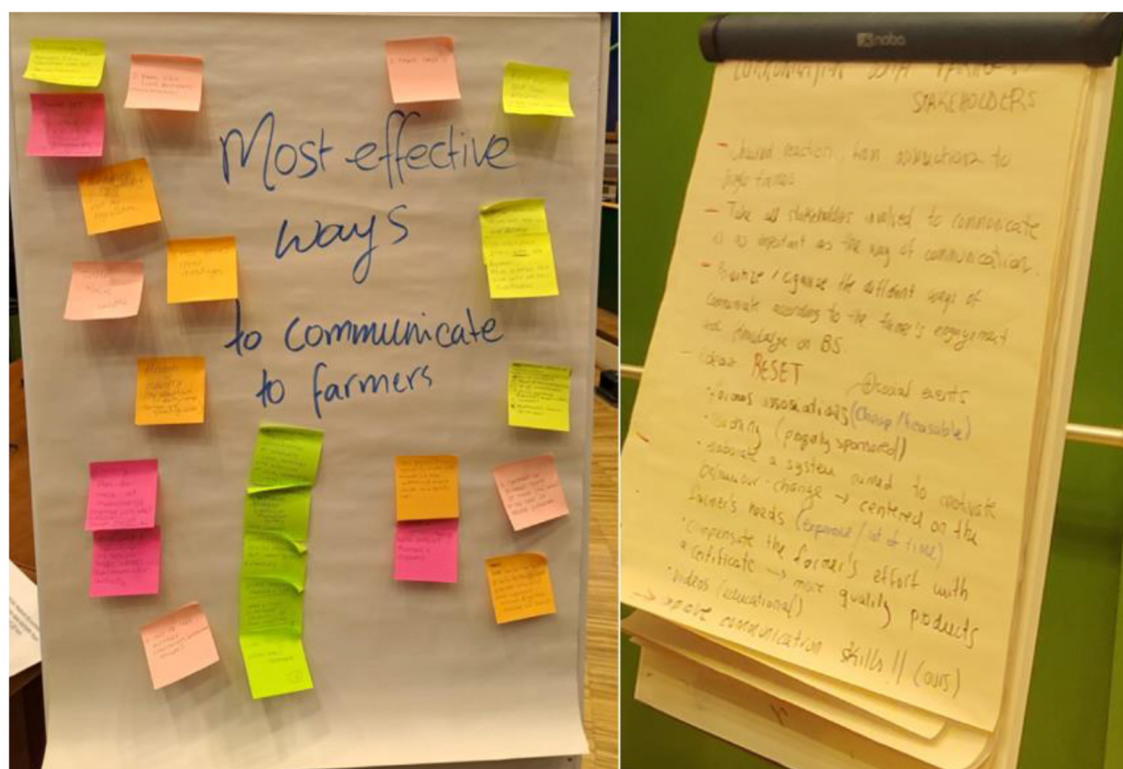


FIGURE 1
Post-it notes and flipcharts created by focus groups 1 and 4.

and other key stakeholders, potentially using frameworks like the RESET (Rules, Education, Social pressure, Economics, and Tools) model to encourage behavioral change (19). They cautioned that inconsistent messages among stakeholders could reduce the effectiveness of biosecurity strategies.

3.2.1.4 Challenges and innovative solutions

A key challenge identified by researchers (FG4) was how to engage farmers during routine circumstances and in the absence of critical situations, such as outbreaks or specific problems. Strategies proposed included creating funded model farms, to serve as examples, and organizing round tables or group discussions to encourage technical exchange and collaborative learning (FG3 and FG4).

Alternative methods such as phone or video calls were also mentioned, which, while considered to be less effective than in-person visits, were reported to help overcome logistical barriers to farm-to-farm visits (FG3 and FG4). However, official services and industry (FG1) stressed that any strategy must be backed by cross-sector learning efforts, involving all stakeholders.

3.2.2 Designing an optimal communication system to promote behavioral change in biosecurity

Discussions on how to design an optimal system for communicating biosecurity messages and driving behavioral

change revealed various complementary approaches from researchers, official services, and industry. These approaches included: (i) initial strategies for communication: knowledge and trust, (ii) integration of technological tools, (iii) mandatory programs and coordinated campaigns, (iv) continuous training and collaborative learning, and (v) incentives and certifications.

3.2.2.1 Initial strategies for communication: knowledge and trust

There was consensus on the need to begin by improving farmers' knowledge of biosecurity. Researchers (FG2) suggested that the first step is to communicate risks and benefits of biosecurity practices more effectively. Proposed approaches ranged from soft strategies based on dialogue and persuasion to more robust ones, including inspections followed by tailored support (FG3). Moreover, researchers emphasized that trust is crucial for farmers to adopt new measures (FG3).

3.2.2.2 Integration of technological tools

The use of technology was widely discussed. Official services and industry (FG1) proposed using artificial intelligence (AI) to allow farmers to input their data and receive personalized biosecurity recommendations, stressing the need to combine this technology with human advice. Researchers (FG4) suggested developing apps with gamified elements, such as biosecurity games, to facilitate practical learning.

TABLE 3 Themes and subthemes developed from the content analysis.

Effective methods for communicating biosecurity messages to farmers
- Direct interaction and practical learning
- Audio-visual media and support materials
- Importance of personalization and coordination
- Challenges and innovative solutions
Designing an optimal communication system to promote behavioral change in biosecurity
- Initial strategies for communication: knowledge and trust
- Integration of technological tools
- Mandatory programs and coordinated campaigns
- Continuous training and collaborative learning
- Incentives and certifications
Measuring the success of biosecurity communication programs
- Evaluation tools and audits
- Key indicators and benchmarking
- Measuring attitudes and behavioral changes
- Participation and knowledge as additional evaluation metrics

3.2.2.3 Mandatory programs and coordinated campaigns

Official services and industry (FG1) discussed the role of mandatory programs, in particular for farmers with substandard biosecurity practices. While recognizing their potential, they warned that they might cause resistance if not handled appropriately. They proposed industry-led programs instead of relying exclusively on legislation, as they were believed this could ease acceptance.

Researchers (FG3) suggested the benefit of coordinated campaigns that deliver a unified message through various media, accompanied by tailored materials for veterinarians and farmers. They suggested an approach of adapting successful public health campaign strategies to animal health biosecurity.

3.2.2.4 Continuous training and collaborative learning

All stakeholders agreed on the importance of continuous training as a cornerstone for behavioral change. Researchers (FG4), official services, and industry (FG1) highlighted the need for group workshops and discussions focused on solving specific problems, promoting knowledge exchange among farmers, such as creating farmer clubs (FG4).

Additionally, researchers (FG3 and FG4) underlined the value of in-person and virtual courses led by recognized farmers, complemented by tools like podcasts, social seminars, and groups on platforms such as WhatsApp or Facebook (FG4).

3.2.2.5 Incentives and certifications

To encourage the adoption of better practices, all stakeholders discussed the importance of incentives. Official services and industry (FG1) suggested adding value to products through

premium pricing or discounts for those implementing good practices. Researchers (FG4) proposed private certification systems, e.g., badges.

3.2.3 Measuring the success of biosecurity communication programs

Researchers, official services, and industry proposed various methods and concrete ideas: (i) evaluation tools and audits, (ii) key indicators and benchmarking, (iii) measuring attitudes and behavioral changes, and (iv) participation and knowledge as additional evaluation metrics.

3.2.3.1 Evaluation tools and audits

Evaluation tools were widely discussed. Researchers (FG2, FG3, and FG4) recommended using systems to measure biosecurity progress through regular audits (FG2 and FG3). However, they warned that audits should be conducted in farmer-friendly environments to facilitate positive engagement (FG2). Researchers (FG2 and FG4), official services, and industry (FG1) suggested self-assessments for farmers to monitor their progress. All stakeholders proposed a step-by-step approach considering each farm's initial situation (FG1 and FG4) and employing the KISS (Keep It Simple, Stupid) principle (FG1) (20, 21).

3.2.3.2 Key indicators and benchmarking

There was consensus on using tangible indicators to evaluate biosecurity progress. Official services and industry (FG1) suggested health parameters, such as reduced antimicrobial use and decreased infectious diseases, and productive parameters. They highlighted benchmarking as a useful tool for comparing progress among farmers and over time. Researchers (FG2 and FG3) expanded this view to include indicators like animal welfare and environmental metrics, such as water quality in aquaculture. They also noted the absence of outbreaks over extended periods as a sign of biosecurity success (FG3).

3.2.3.3 Measuring attitudes and behavioral changes

Social and psychological factors were considered essential for assessing program impact. Official services and industry (FG1) emphasized the importance of delivering repeated messages in various ways. They cited examples such as the milking gloves campaign, which primarily aimed to communicate that farmers must wear gloves as part of behavioral change (22).

Researchers (FG4) proposed the measurement of change in farmers' attitudes toward biosecurity. In addition, a mechanism to capture the perceptions of farmers about the impact of the measures they had implemented.

3.2.3.4 Participation and knowledge as additional evaluation metrics

Researchers (FG4) highlighted the number of farmers participating in biosecurity-related activities as a key indicator of success. They also suggested measuring farmers' level of biosecurity knowledge (i.e., benchmarking) to assess their understanding of key concepts.

4 Discussion

The findings of this study reveal stakeholders' perspectives on biosecurity communication to encourage changes in farmers' behavior. In relation to the focus groups, within the section 'Effective methods for communicating biosecurity messages to farmers,' direct interaction between stakeholders was emphasized by researchers, official services, and industry. It has been previously identified as crucial for the implementation of biosecurity measures within organizational contexts (23), such as on livestock farms. However, depending on the approaches adopted by those involved in the interaction, certain issues may arise. Farm visits conducted without a collaborative focus can lead to rejection from farmers due to the perception of inspection rather than support, as is often the case with government agent visits (10). This study also underscores the importance of ensuring that such interactions promote the co-creation of solutions tailored to the specific context of each farm—an approach that has proven effective in advancing agro-ecological knowledge (24, 25), as well as innovation in farmer field schools (26) and the greenhouse industry (27), among others.

On the other hand, there was consensus on the importance of tailoring biosecurity messages to the farming system and its context. This concern reflects a trend toward the failure of generalized messaging, which often overlooks the specific characteristics and resources of individual farms (28). Furthermore, non-contextualized messages can lead to mistrust and resistance among stakeholders (29), adding an additional barrier to the implementation of biosecurity measures. Designing strategies that incorporate these specificities not only increases the likelihood of implementing biosecurity measures but also ensures that such measures are sustainable in the long term. Selecting the appropriate communication channels for each context is also crucial, considering factors such as farmer demographics, access to technology, and preferred learning styles (30).

Within the section "Designing an optimal communication system to promote behavioral change in biosecurity", one of the key elements identified was the need to improve biosecurity knowledge among farmers. However, there are divergences in the literature regarding the extent of this knowledge. Some studies argue that farmers possess a basic level of understanding that enables appropriate comprehension of biosecurity practices (3), while others suggest significant gaps in their knowledge (31). This disparity could be attributed to differences in farming systems and geographical locations, but also to the communication strategies themselves, such as the content delivered. Therefore, further research is needed to accurately identify which specific areas of knowledge require strengthening, enabling the design of more effective messages.

On the other hand, there was consensus among researchers, official services, and industry that group discussions with farmers, as a form of training designed to address specific biosecurity issues, could be instrumental in promoting behavioral change. While such participatory training approaches can positively impact biosecurity practices (32), the existing literature lacks studies that clearly define the ideal methodology for this specific type of training. Furthermore, existing training programs, such as those promoted by private projects like FarmIQ's "Farm Biosecurity in

Practice" (33) are often designed without the active participation of stakeholders, which may reduce their practical applicability in the field. Therefore, adopting a participatory approach to biosecurity training adds significant value. However, as this approach requires time and a mutual understanding of the needs of all stakeholders, it is essential to combine efforts across all parties, particularly during the design phase, to ensure its efficiency, effectiveness, and sustainability.

Within the section, "Measuring the success of biosecurity communication programs", one aspect discussed was the self-assessment process conducted by farmers. Self-assessment was proposed as a key tool that goes beyond the use of checklists commonly applied in evaluations, which are not always optimal (34). While this tool could include a farm-specific, personalized approach, it could also incorporate a reflective component. This would enable farmers, alongside other stakeholders, to critically identify areas for improvement, thereby increasing their commitment to biosecurity, similar to findings in other fields such as language studies (35).

This study highlighted a difference in the approach adopted by researchers, official services, and industry. While some official services and industry seemed to position themselves as just another stakeholder in the system, others seemed to position themselves differently. Some researchers, particularly veterinarians, often adopt a paternalistic approach, as highlighted in the literature (15, 36), determining what should be done and how it should be done, without actively engaging or collaboratively seeking solutions. This approach has been criticized in various studies, both in animal and human health contexts (36, 37). Recognizing researchers as just another stakeholder within the system could encourage greater integration and collaboration. This approach would enable researchers to work alongside farm stakeholders (official services and industry). By doing so, some researchers, particularly veterinarians, could gradually move away from adopting a paternalistic stance, as is already practiced by some veterinarians in small animal practice (38–40).

Among the limitations of this study, the small number of official services and industry participants in the focus group discussions can be highlighted. It is crucial to involve and correctly identify stakeholders (via e.g., stakeholder mapping (41))—in this case, official services and industry—who are directly engaged with the subject of study as participants, to achieve a greater impact on the implementation of biosecurity measures through effective interventions (42, 43). It is essential that stakeholders from livestock farms themselves propose strategies to improve communication, adopting a non-hierarchical approach in which researchers primarily play a facilitating role. Furthermore, researchers should also be considered as subjects of study, as seen in certain initiatives in animal health, such as work package one of the BIOSECURE project (44), or previous efforts in human health (45).

While this study examined the communication methods and tools used to promote biosecurity, it did not explicitly analyze the nuances of communication style, the specific vocabulary associated with collaborative approaches nor the impact of language barriers in effective communication. In terms of communication style, biosecurity communication strategies could be strengthened by understanding how language choice and tone influence

engagement. In terms of vocabulary, examining how it aligns with collaborative approaches -such as inclusive and participatory language- could improve the effectiveness of biosecurity messaging.

Inclusive language should also consider the written and spoken language skills of other stakeholders involved in the implementation of biosecurity measures, in particular farm workers. Addressing language barriers is essential to ensure effective implementation of biosecurity measures, primarily through adequate training of these stakeholders (46–48). Therefore, inclusivity should be a key element of any communication plan involving all stakeholders.

Future research should explore these aspects in more depth to provide practical insights for stakeholders involved in biosecurity communication.

This study looked at accessibility to appropriate communication methods and tools. However, future research could also explore other aspects of communication not covered in this study, such as trust between stakeholders, shared perspectives and power dynamics, from a collaborative perspective involving all stakeholders, including researchers.

In conclusion, this study offers an initial exploration of the perspectives of researchers, official services, and industry on communication strategies for promoting behavioral change among farmers in relation to biosecurity. It highlights the need for collaborative, personalized, and sustainable approaches to biosecurity communication. However, it does not delve deeply into the various aspects of communication which future studies are recommended to address. This would facilitate the design of communication programs that remain effective over time. While researchers can offer valuable insights and serve as a reference point, these strategies should ultimately be shaped by the perspectives of key stakeholders in livestock farming, including official services, industry, and, crucially, farmers themselves. In fact, this study explored how, from their perspective, biosecurity communication with farmers should be approached by those responsible for education and message dissemination. However, future research should present these ideas directly to farmers, alongside evaluating successful training programs to identify the key elements of effective communication. Additionally, incorporating a participatory action research approach could improve biosecurity communication strategies by promoting co-creation, ensuring that messages are contextually relevant, engagement is more meaningful, and solutions are co-designed to improve uptake and long-term sustainability (25, 49). To build on this, it will be essential to triangulate these perspectives by directly engaging farmers to compare their views on communication with those expressed by researchers and other stakeholders. This would allow for a more comprehensive understanding of how communication strategies can be tailored to meet the needs and expectations of all parties involved.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Scotland's Rural College (No. 141/88099239). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SM: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. KL: Formal analysis, Supervision, Writing – review & editing. MLB: Formal analysis, Supervision, Writing – review & editing. GC: Conceptualization, Methodology, Writing – review & editing. MC: Conceptualization, Methodology, Writing – review & editing. AA: Project administration, Funding acquisition, Writing – review & editing. L-MT: Conceptualization, Methodology, Writing – review & editing. CC-G: Methodology, Writing – review & editing. HCF: Writing – review & editing. MMD: Data curation, Writing – review & editing. TI: Data curation, Writing – review & editing. DDM: Writing – review & editing. MK: Writing – review & editing. IC: Writing – review & editing. AB: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2025.1562648/full#supplementary-material>

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Canine circovirus: emergence, adaptation, and challenges for animal and public health

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Introduction: Canine circovirus (CanineCV) is an emerging pathogen with a significant impact on animal health and potential zoonotic risks. This study addresses its characterization, epidemiology, pathogenesis, and diagnostics, emphasizing its relevance within the One Health approach.

Background: The increasing detection of CanineCV across various species and regions highlights its genetic adaptability and cross-species transmission potential. Furthermore, growing interactions among domestic animals, wildlife, and humans amplify the need to understand its public and animal health implications.

Objective: To analyze the biology, epidemiology, and diagnostic challenges of CanineCV, focusing on its genetic evolution, interactions with co-infections, and implications for control strategies.

Methods: A systematic literature review was conducted, synthesizing data from epidemiological, genomic, and clinical studies. Molecular techniques, such as PCR and qPCR, were evaluated for their efficacy in virus detection and quantification.

Results: Canine circovirus exhibits high genetic variability and has been detected in diverse species and tissues. Co-infections, including parvovirus and adenovirus, exacerbate clinical signs, primarily gastrointestinal, and respiratory. Advances in diagnostics, such as real-time PCR and *in situ* hybridization, have demonstrated increased sensitivity in viral detection.

Conclusion: Canine circovirus poses a growing challenge to animal health and a potential threat to public health due to its genetic plasticity and adaptability to multiple hosts. Continuous research is essential to understand its pathogenesis, develop effective control measures, and mitigate risks in diverse ecosystems.

KEYWORDS

CanineCV, *Circoviridae*, *Porcine circovirus*, public health, emerging, pig disease

Introduction

Pets play a significant role in contemporary society, transcending their historical function as mere guardians or domestic helpers. According to the International Federation for Animal Health (IFAH), “pet” refers to any animal kept by humans for companionship, recreation, or as part of the family unit, encompassing not only dogs and cats but also birds,

reptiles, fish, rodents, and other small mammals (2, 3). The human-animal relationship has evolved over the decades, shifting from a utilitarian interaction to an emotional bond, with documented benefits for the mental and physical health of their owners, such as stress reduction, increased social engagement, and improved quality of life (4). In Brazil, this scenario is widely reflected in households, where 47.9 million families own at least one pet, representing ~46.1% of all national households, according to the Brazilian Institute of Geography and Statistics [IBGE; (1)]. This figure becomes even more remarkable when considering that the country leads globally in the number of small dogs per capita and holds a prominent position in the ownership of cats and other animals. The broad definition of the term “pet” not only reflects the diversity of species that share domestic spaces with humans but also highlights the complexity of human-animal interactions, which are influenced by cultural, socioeconomic, and environmental factors (73). Thus, understanding the concept of “pet” in its entirety is essential to contextualizing health impacts, both human and animal, within the One Health approach (5–7).

Canine circovirus (CanineCV) is an emerging virus with a significant impact, particularly in the absence of vaccines. This virus often displays a variety of clinical signs, which can be further complicated by co-infections, potentially altering the clinical presentation. Additionally, the potential for zoonotic transmission cannot be excluded, as other viruses within the same genus, which includes human circovirus, are being considered for such transmission (8–10).

Canine circovirus belongs to the genus *Circovirus* within the family *Circoviridae*. According to the classification of the International Committee on Taxonomy of Viruses (ICTV), species demarcation within the *Circoviridae* family is based on at least 80% nucleotide identity across the entire genome, along with structural and organizational characteristics. An essential criterion is the location of the replication origin (ori) relative to the coding regions. In members of the genus *Circovirus*, the ori is located on the same strand that encodes the replication-associated protein (Rep), while in the genus *Cyclovirus*, the ori is situated on the strand that encodes the capsid protein [Cap; (10, 11)]. Additionally, the genomes of these viruses exhibit an ambisense organization with two primary open reading frames (ORFs) responsible for encoding the Rep and Cap proteins, with replication occurring through a rolling circle replication mechanism.

The diversity within this family has been significantly expanded through metagenomic sequencing and degenerate PCR methods, revealing a broad distribution among mammals, birds, and even invertebrates. Phylogenetic studies indicate a closer relationship among circoviruses detected in mammals, whereas those found in birds and fish display greater genetic distance, reflecting their complex evolution. These criteria and technological advancements not only facilitate taxonomic classification but also provide a broader understanding of the biology and ecology of these viruses, which are essential for epidemiological and viral evolution studies (12, 13).

Canine circovirus was first identified in 2012 after the extraction of viral nucleic acid from a set of canine serum samples in the United States. In 2013, the complete genome was characterized in California (USA) and, after a year later, the virus was reported in a young dog in Italy. Since its identification,

CanineCV has been associated with conditions such as vasculitis, hemorrhagic gastroenteritis, and diarrhea (57) and has been reported in all continents, except Oceania (14–19).

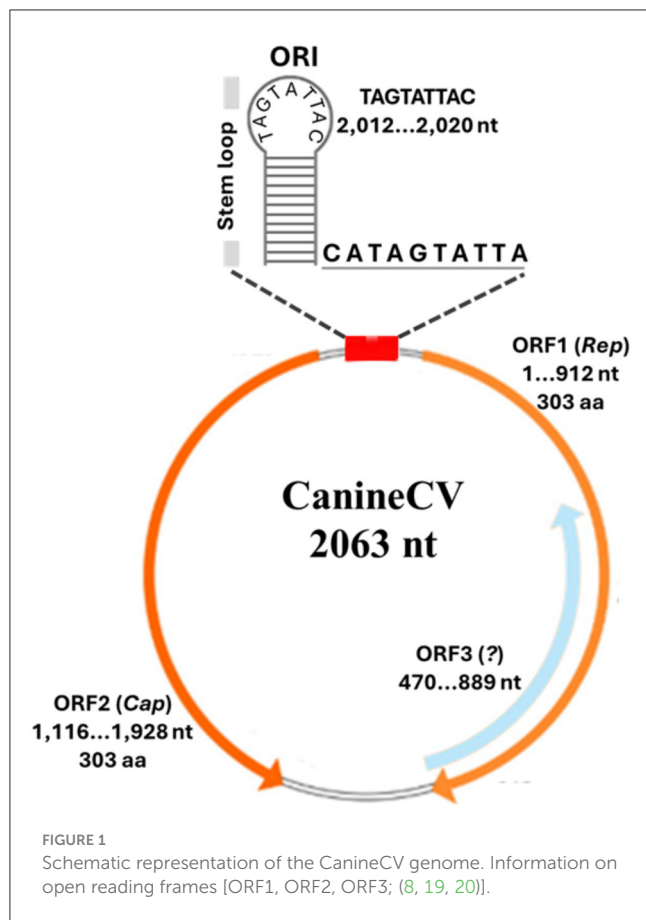
Canine circovirus, like other circovirus species, poses significant challenges in classification due to notable genetic variability. It is crucial to establish common terminology with robust classification criteria, ensuring reproducible results and promoting essential advancements in understanding diseases associated with the virus. This includes assessing the impact of coinfections on clinical signs to comprehend its effects on animal health and potential implications for public health.

Viral characterization and diversity

Canine circovirus are non-enveloped icosahedral viruses with a single-stranded circular DNA genome of ~2 kb. As previously mentioned, they belong to the *Circoviridae* family. The classification of CanineCV as a new species within the circovirus genus occurred because, according to criteria set by the International Committee on Taxonomy of Viruses (10), circoviruses must share more than 75% nucleotide identity across their complete genome and more than 70% sequence identity in their capsid protein sequences to be considered the same species. Despite being genetically closer to porcine circovirus, in the study identifying the complete genome sequence of the first canine circovirus, the capsid (Cap) and replicase (Rep) proteins of CanineCV shared <25% and 50% identity, respectively, with circoviruses from other animals (8).

The CanineCV genome is a circular single-stranded DNA with 2,063 nucleotides (nt) that comprises two open reading frames (ORFs) on complementary strands oriented in opposite directions. ORF1, with 911 nt, encodes the replicase protein (303 amino acids), which is essential for viral replication. ORF2, with 811 nt, encodes the capsid protein (270 amino acids), which has a structural function. The genome also contains two intergenic non-coding regions that are 135 and 203 nucleotides long. At the replication origin (TAG TAT TACA), there is a palindromic sequence of 12 nt pairs and a 10-nucleotide open loop (CAT AGT ATT A). The amino terminus of the proposed capsid protein features a 30-amino-acid arginine-rich region, like those found in other animal circoviruses. Additionally, a third ORF (ORF-3) was identified in the antisense strand of ORF-1 from a Thailand strain, although its function is still unknown [(8, 20); Figure 1].

Although the replication of CanineCV has not been described, we can infer its replication process based on the well-documented replication mechanism of PCVs, especially PCV1 and PCV2, with the following steps (Figure 2): [1] The entry begins with the virus attaching to the host cell surface. This process is mediated by interactions between viral proteins and specific receptors on the host cell membrane. Entry primarily occurs through clathrin-mediated endocytosis, where the virus is engulfed into an endocytic vesicle and transported into the cell. [2] After entry into the cell, the viral capsid is uncoated, releasing the viral genome into the cytoplasm. The uncoating process involves the fusion of the endocytic vesicle with lysosomes, where the acidic pH facilitates the release of the single-stranded circular DNA from the protein capsid, which is transported to the cell nucleus, where replication



occurs. [3] The replication of the virus genome occurs in the host cell nucleus. The Rep protein recognizes and binds to the origin of replication on the viral DNA. The Rep protein has endonuclease activity, which creates a nick in the DNA strand, producing a free 3' OH end essential for new DNA strand synthesis. Using the 3' OH end as a primer, the host DNA polymerase extends the DNA strand, synthesizing a new strand complementary to the original template strand. This results in the formation of a double-stranded replicative form (RF) DNA structure, which serves as a template for the synthesis of new viral single-stranded DNA through the rolling circle replication (RCR) mechanism. Replication is completed when the synthesis of the new DNA strand forms a full circle and meets the original 5' end. The Rep protein makes another nick to release the new single-stranded DNA, which can be encapsulated into new viral particles. [4] The assembly of new viral particles occurs in the host cell nucleus. The capsid proteins (Cap), encoded by the ORF2 gene, are synthesized and transported to the nucleus, where they encapsulate the newly synthesized viral DNA. This assembly process involves forming complete viral capsids that enclose the viral DNA genome, creating new virions. [5] After assembly, the complete virions are transported out of the nucleus and accumulate in the cytoplasm before being released from the infected cell. Release can occur through cell lysis, where the host cell is destroyed, releasing virions into the extracellular environment. Alternatively, virions can be released through exocytosis, where vesicles containing virions fuse with the plasma membrane, releasing virions outside the cell

without causing immediate host cell death. The replication process is carried out by cellular enzymes that are expressed during the S-phase of the host cell cycle (21–26).

There is still a lack of clarity regarding the physicochemical properties, replication process, and pathogenic characteristics of CanineCV. A study was conducted, rescuing a strain of canine circovirus in F81 cells using infectious clone plasmids, and it was discovered that the Rep protein produced by the viral packaging rescue process is associated with cytopathic effects. The Rep protein of CanineCV inhibited the activation of the type I Interferon (IFN-I) promoter, blocking the subsequent expression of interferon-stimulated genes (27).

Like observed in other circoviruses, a high evolutionary rate of 1.21×10^{-3} substitutions/site/year was described (66). This can be confirmed by the different genotypes that have been described since its first identification in 2012 (8, 17–19, 28, 29).

Phylogenetic analyses of the strains reported to date have been conducted using the complete genome sequences. These analyses also incorporate the nucleotide sequences or concatenated amino acids of the Rep and Cap proteins (24, 25, 66). Multiple efforts have been made to establish a classification system that helps understand virus origin and evolution. However, based on most recent articles where sequencing has been performed, the classification into six genotypes, i.e., CanineCV 1 to CanineCV 6, has been the most used and accepted (24, 25, 28, 30–33).

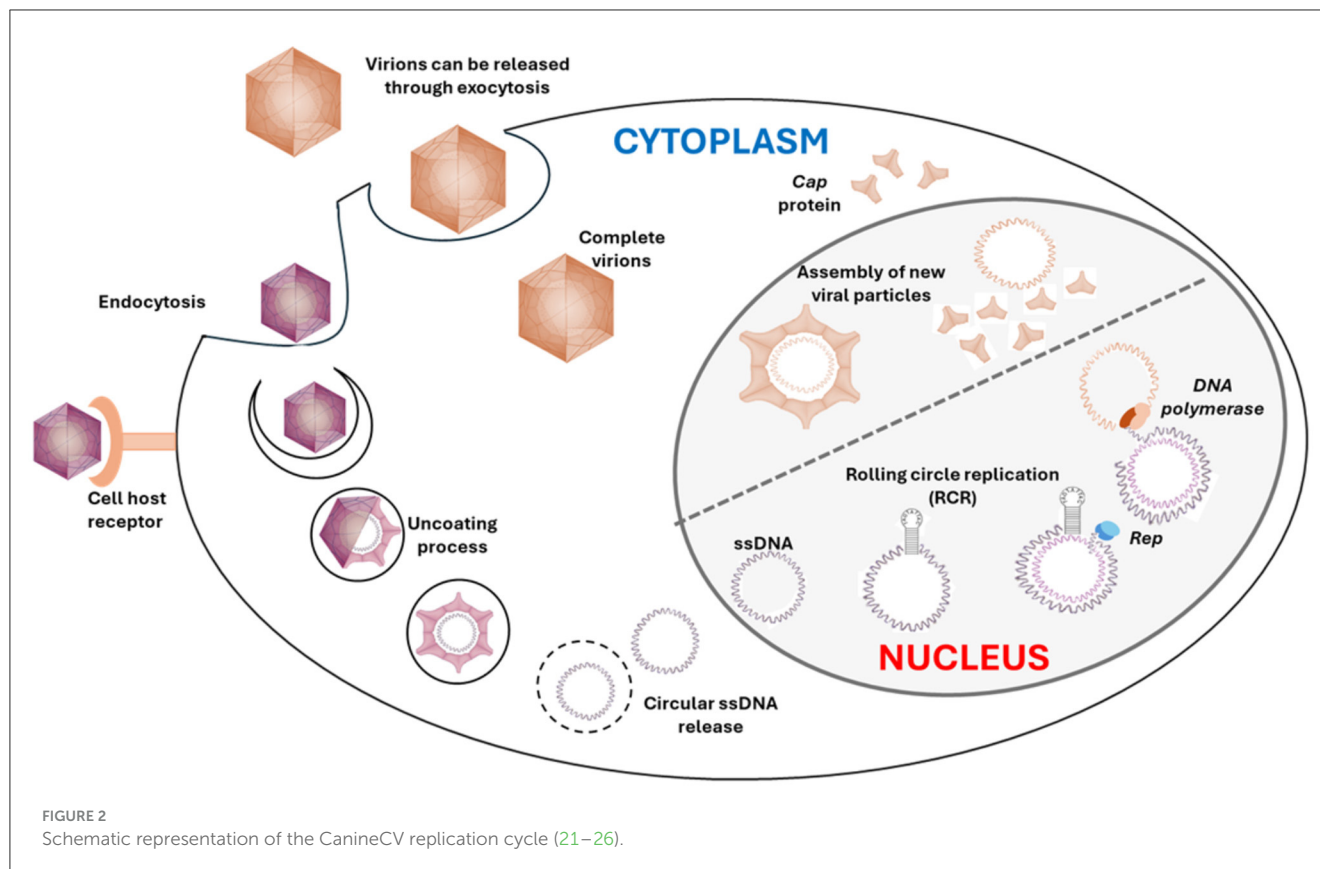
Phylogenetic analyses indicate that CanineCV likely originated from bat circovirus (BatACV). Maximum clade credibility (MCC) and maximum-likelihood (ML) trees constructed from ORF1 gene sequences suggest a close relationship between CanineCV and BatACV strains. This hypothesis is supported by the observation that circoviruses, including CanineCV, often undergo cross-species transmission, a major driver of their evolution. The genetic variations are often reflected in the virus's codon usage patterns, which have been influenced predominantly by natural selection rather than mutation pressure. This natural selection is a significant force shaping the codon usage bias (CUB) of CanineCV, enhancing its adaptability and survival in various hosts (16, 20, 33–36).

Codon adaptation index (CAI) and relative codon deoptimization index (RCDI) analyses have revealed that CanineCV exhibits the highest adaptability to red foxes, followed by domestic dogs and arctic foxes. This adaptability is attributed to the virus's ability to optimize its protein synthesis machinery to align with the host's codon usage preferences, thereby enhancing its replication efficiency and fitness. Interestingly, while CanineCV shows strong ties with wolves based on SiD analysis, the virus has developed the strongest adaptation to red foxes, indicating a complex interplay of host-specific adaptations driven by natural selection (34, 36).

Epidemiology

Canine circovirus has been detected on every continent except Oceania (14–19).

In the United States, the virus was first identified in 2012, followed by Italy in 2014, and the United Kingdom in 2015. Subsequent detections occurred in Taiwan (2016), Germany (2017),



and Thailand (2017). Brazil reported its first case in 2018, with Argentina following in 2019. China, Turkey, and Colombia all recorded their initial detections in 2020. Vietnam identified the virus in 2020, Iran in 2022, and Namibia in 2023. These findings illustrate the widespread and chronological emergence of CanineCV across multiple continents, highlighting its global distribution [(8, 15, 16, 19, 20, 31, 35, 37, 38, 41–43, 57, 60, 67); Figure 3].

Retrospective studies have shown that CanineCV was present in Latin America as early as 2012 (43). In Europe, detection of the virus dates back to samples from 1995, indicating a longer and possibly more widespread historical presence of CanineCV in canine populations across different continents (68).

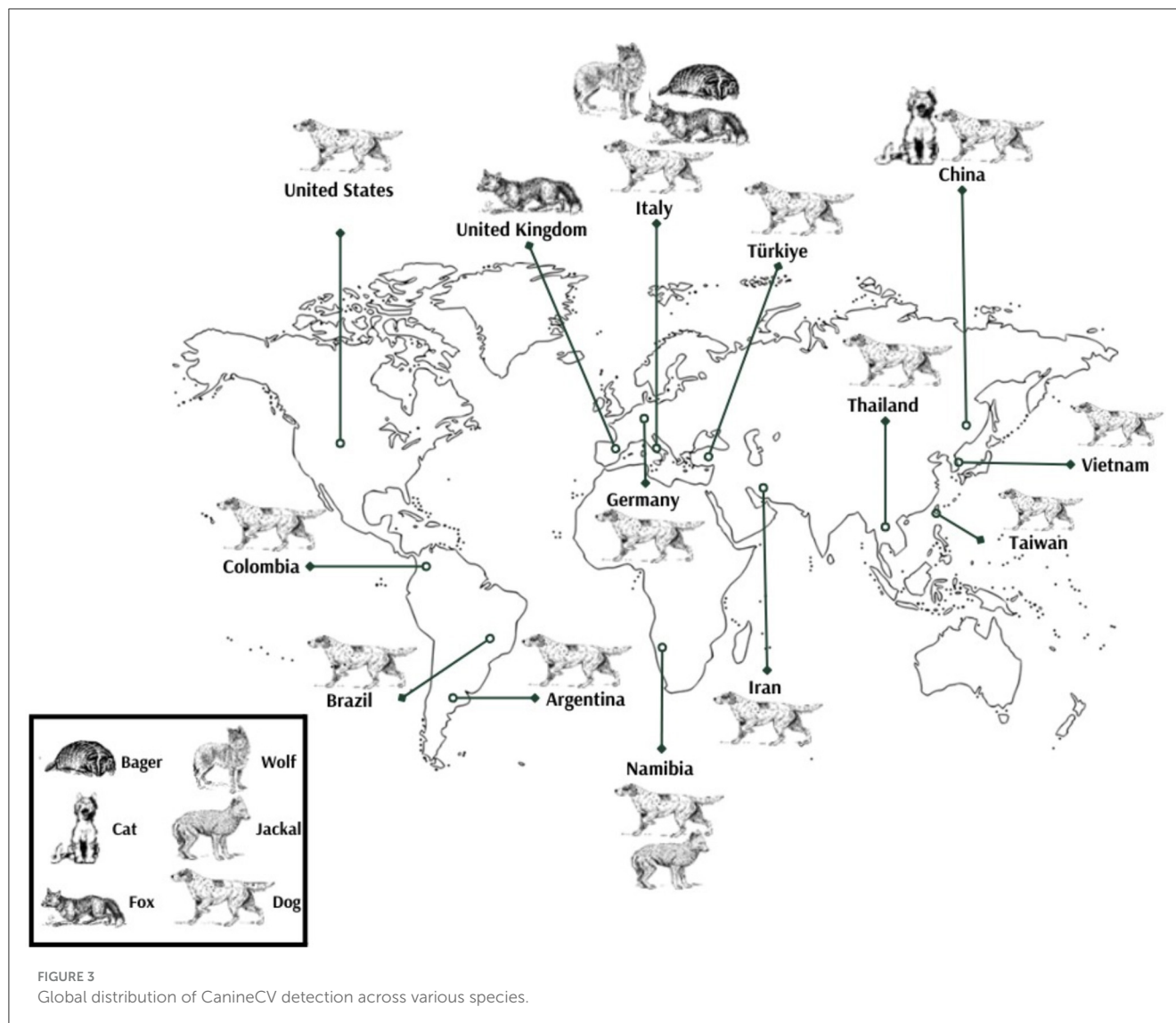
Canine circovirus has been identified in various host species, demonstrating its capacity for cross-species transmission and adaptability. Most detections have been reported in dogs, starting from 2012 [(8, 16, 19, 20, 25, 31, 37–43); Figure 3].

The prevalence of CanineCV in dogs varies widely from 3.6% to 28.0%, depending on the presence and severity of clinical signs. This range indicates that clinical manifestations play a significant role in the detection rates of CanineCV among domestic dogs (30, 44).

In wild carnivores, the prevalence of CanineCV shows considerable variation across different species. In foxes, the prevalence of CanineCV ranged from 0% to 4.3%. In badgers, the prevalence was 18%. In jackals, the prevalence was notably high at 43.7%, while wolves exhibited an even higher prevalence of 50% (15, 18, 30, 45, 46).

The classification into six genotypes, has revealed various geographic and host-specific distributions. CanineCV-1 has been detected in dogs primarily in China, USA, Colombia, Argentina, Italy, Germany, and Vietnam. Additionally, it has been found in wolves in Italy. CanineCV-2 has been found in dogs exclusively in China. CanineCV-3 has been detected in dogs in China, Vietnam, and Thailand. CanineCV-4 has been observed in both wolves and dogs in Italy, as well as in dogs in China, Germany, Argentina, and Colombia. CanineCV-5 has been found in Arctic foxes (*Vulpes lagopus*) and red foxes (*Vulpes vulpes*) in the Arctic, Norway, and the United Kingdom. Finally, CanineCV-6 has been detected in dogs in Iran [(8, 18, 19, 24, 29–33, 47); Table 1].

Canine circovirus has been detected in numerous tissue types, including the brain (dog and wolves), intestine (dog, wolves, and badgers), liver (dog), spleen (dog, wolves, fox, and badgers), lymph nodes (dog and jackals), and lungs (dog, wolves, and jackals). This extensive range of sample types demonstrates the virus's ability to infect and persist in different organs and tissues, contributing to its maintenance and spread within and between species [(8, 15, 28, 30, 45, 46); Table 2]. The detection of the virus in such a wide array of tissues highlights its versatility and pathogenic potential. Understanding the tissue tropism of the virus is crucial for developing effective strategies to control its spread and mitigate its impact on both domestic and wild animal populations. Further research is necessary to elucidate the mechanisms behind the virus's tissue-specific infection and its implications for disease progression and transmission.



Like *Porcine circovirus 3* (PCV3), which infects swine, CanineCV originated from bat circovirus (BatACV). This ancestral virus may have adapted either directly to domestic dogs or through other intermediate hosts, allowing for cross-species transmission. Notably, PCV3 has been detected in ticks, and although CanineCV has not yet been described in ticks, this possibility should be considered. Figure 4 illustrates the potential transmission routes of CanineCV among different environments and species. It highlights direct contact and possible tick-mediated transmission as mechanisms through which the virus may spread among peri-domestic animals such as dogs and cats, and wild animals including foxes, badgers, jackals and wolves (9, 16, 33, 44, 48, 49). The figure underscores the complexity of CanineCV transmission dynamics and the need for further research to understand these interactions and their implications for viral maintenance and spread in diverse ecological setting (Figure 4).

The spillover events should be closely monitored due to the high mutation rate of CanineCV and recent socio-economic

changes that have increased the proximity of companion animals to wild environments. Understanding these dynamics is crucial for developing effective strategies to control the spread of CanineCV and mitigate its impact on both domestic and wild animal populations (50).

The primary route of transmission for CanineCV is fecal-oral, affecting both domestic and wild animals. Viral loads in feces have been detected at 1.8×10^3 copies of target DNA/ μL of extracted DNA in dogs and 8.94×10^4 copies in foxes. Notably, animals without clinical signs also shed high viral loads in their feces, as indicated by a low cycle threshold (C_t) value of 20.7 (31, 32, 45). It must be considered that the high amount of viral particles is an important factor for viral spread in animal populations.

Special attention in the epidemiological chain should be given to the fact that some animals, such as dogs, cat, foxes, jackals, and wolves, may be infected without showing clinical signs, as they can still disseminate the virus. However, it should be considered that, like swine, the viral load shed by asymptomatic animals is lower. This highlights the importance of monitoring both symptomatic

TABLE 1 Global distribution and host range of CanineCV genotypes.

Genotype	Animal species	Countries
CanineCV 1	Dogs	China; USA; Colombia; Argentina; Italy; Germany; Vietnam
	Wolve	Italy
CanineCV 2	Dogs	China
CanineCV 3	Dogs	China; Vietnam; Thailand
CanineCV 4	Wolve; dogs	Italy
	Dogs	China; Germany; Argentina; Colombia
CanineCV 5	<i>Vulpes lagopus</i>	Artic
	<i>Vulpes vulpes</i>	Norway; United Kingdom
CanineCV 6	Dogs	Iran

TABLE 2 Detection of CanineCV in various host species and tissue type.

Species	Year first detection	Samples
Dog	2012	Feces, tissue*, nasal swab, and serum
Cat	2018	Nasal swab and serum
Fox	2010	Spleen
Wolves	2014	Tissue [#]
Badgers	2013	Spleen and intestine
Jackals	2021	Lymph node and lung

*Brain, intestine, liver, spleen, lymph nodes and lungs; [#]Brain, lungs, spleen and intestine.

and asymptomatic carriers to effectively control the spread of CanineCV in various animal populations (15, 28, 30, 51, 52).

While the fecal-oral route is the primary mode of transmission for CanineCV, the potential for respiratory transmission also warrants attention. Although few studies have investigated this route, the virus has been detected in respiratory samples, indicating that respiratory transmission could be a significant pathway for the spread of CanineCV (28, 29). Further research is necessary to understand the extent and implications of respiratory transmission in both domestic and wild animal populations.

The prevalence of CanineCV varied significantly by age group. Among dogs aged 0–1 years, the prevalence ranged from 17.5% to 43.1%, indicating a higher susceptibility in this age group. For dogs aged 1–8 years, the prevalence ranged from 9.6% to 43.1%. In dogs older than 8 years, the prevalence was consistently reported at 0% to 13.8%. Additionally, for dogs with unreported ages, the prevalence was noted to be 13.2%. These findings highlight the significant differences in CanineCV prevalence across age groups, with the highest rates observed in the youngest dogs (14, 15, 29–32, 38, 42, 44, 53).

The prevalence of CanineCV in wolves showed significant variation across different age groups. The overall prevalence was 47.8%, with 43.5% of infected wolves being puppies (<12 months old), 30.4% being sub-adults (13–24 months), and 26.1% being adults (older than 24 months). Specifically, the prevalence

was highest in puppies at 50% (5/10), followed by sub-adults at 42.9% (3/7), and adults also at 50% (3/6). These findings highlight that CanineCV affects wolves across all age groups, with a notable prevalence in both the youngest and oldest age categories, differently from what is seen in dogs (15, 30, 46).

Sex-based analysis of CanineCV infection rates revealed a higher prevalence in female dogs compared to male dogs, although this difference was not statistically significant. The prevalence in female dogs ranged from 57.1% to 67.6%, while in male dogs it ranged from 32.4% to 42.9% (31, 53).

Clinical signs

The virus’s ability to infect and persist in multiple tissues not only aids in its dissemination but also in its pathogenicity, contributing to a range of clinical manifestations in infected hosts. In CanineCV infected animals, most clinical signs are related to the digestive system but are also associated with the respiratory and nervous systems.

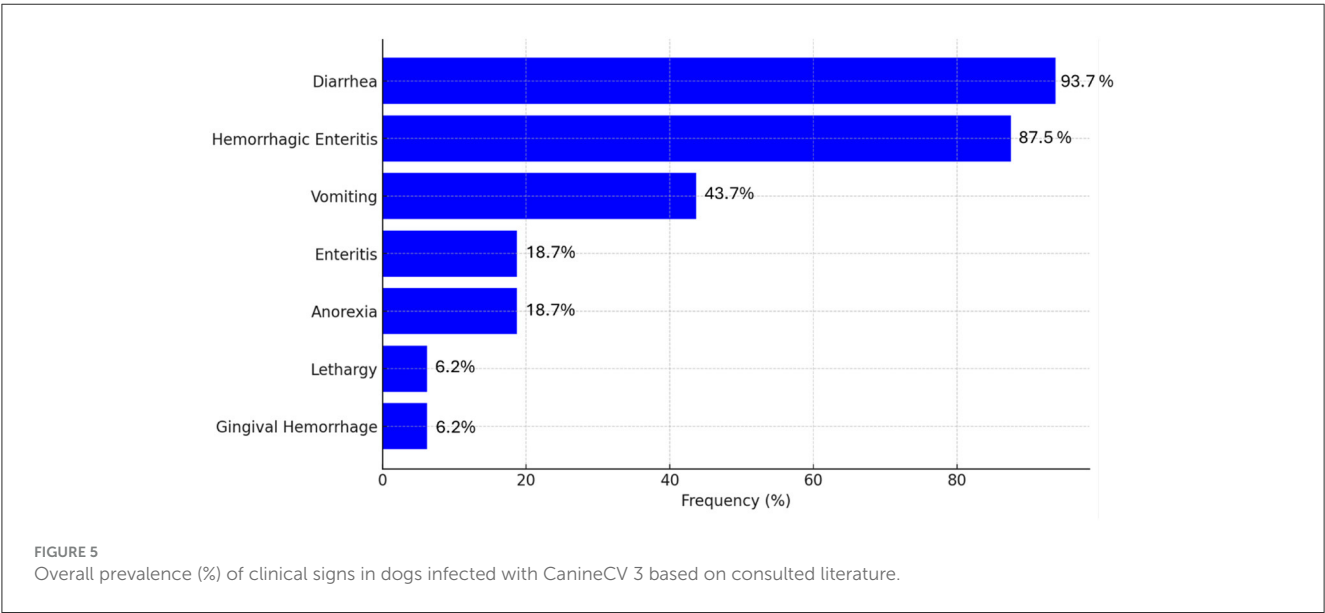
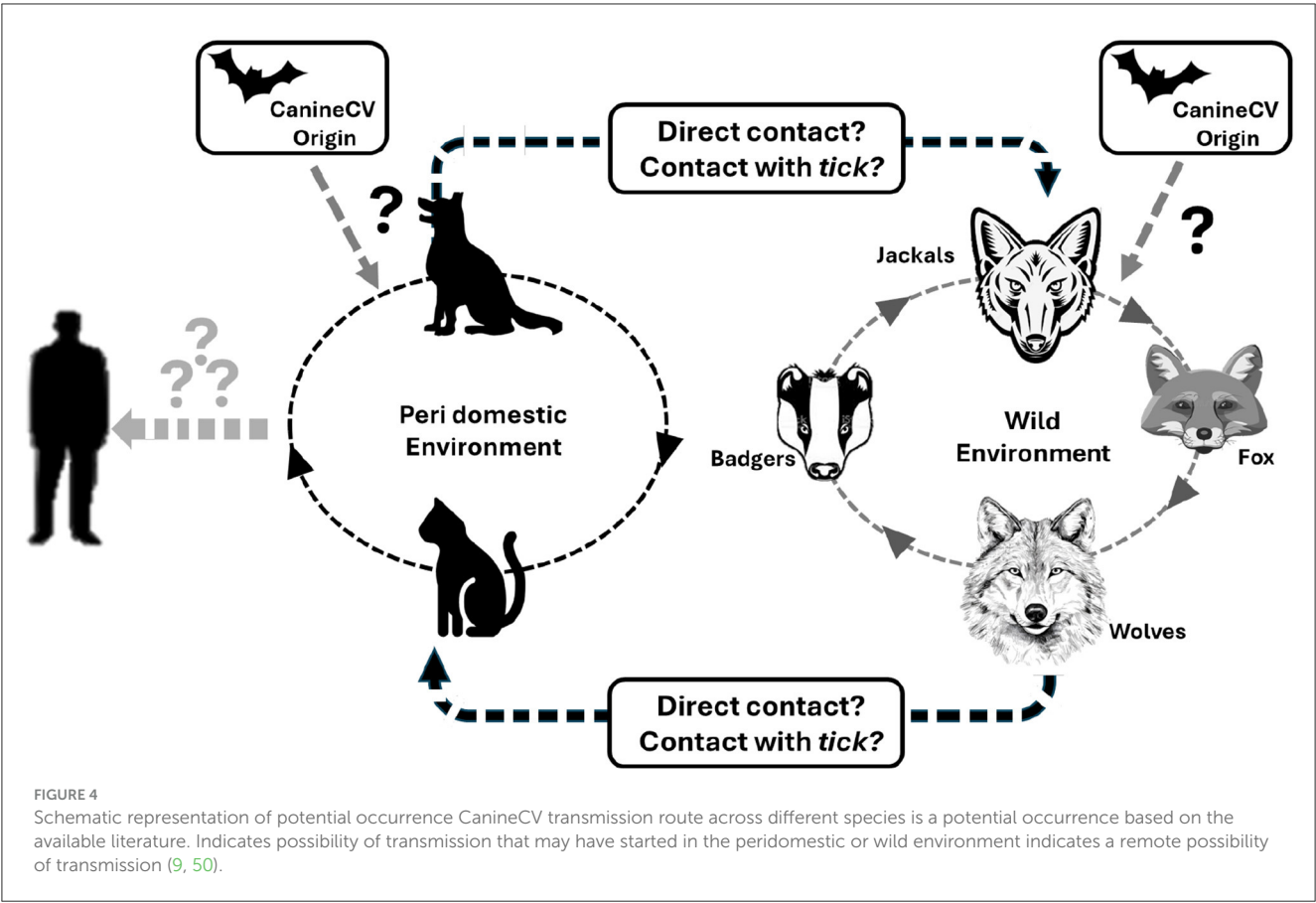
Cats and dogs

The prevalence of CanineCV among symptomatic and asymptomatic animals shows significant variability. On average, 10.6% of asymptomatic animals are infected, with a range between 6.9% and 28.5%. Among symptomatic animals, the average prevalence is higher, at 20.3%, with a range between 6% and 32.8%. In cats, a similar pattern is observed, with a higher prevalence of CanineCV in symptomatic animals (3.6%) compared to asymptomatic ones (1.1%). These findings show that CanineCV infection is present in both symptomatic and asymptomatic populations, with a notably higher prevalence in those showing clinical signs (14, 15, 18, 24, 28, 29, 31, 32, 38, 42, 53, 54).

Considering 16 articles describing clinical signs, a ranking of the most common clinical signs associated with CanineCV, as shown in Figure 5, indicates that enteric disturbances are the most frequently observed. Diarrhea was the most prevalent symptom, observed in 93.7% of cases, followed by hemorrhagic enteritis, which occurred in 87.5% of cases. Vomiting was documented in 43.7% of the cases, while anorexia and enteritis were less frequent, each with a prevalence of 18.7%. Lethargy and gingival hemorrhage were the least common symptoms, each observed in 6.2% of cases [(14, 20, 24, 28, 31, 32, 38, 42, 51, 53–58); Figure 5].

In addition to these more common signs reported in studies, there are studies associating CanineCV with respiratory (28, 59) and nervous signs (58) and lymphadenitis (31).

As observed with CanineCV involvement digestive systems clinical signs, the prevalence of the virus in animals with respiratory illness is also higher, as demonstrated in this study linking respiratory diseases to CanineCV. The overall occurrence of CanineCV infection was 8.95% (17/190), with 2.6% (2/76) in the healthy group and 13.2% (15/114) in the respiratory illness group and several factors can influence its prevalence. Age-wise, juniors (<1.5 years) exhibited the highest positivity rate at 17.5%, compared to adults (1.5–6 years) at 10.5% and seniors (≥6 years)



at 1.3%. Sex-wise, females showed a higher positivity rate at 10.5% compared to males at 5.5% (29).

There are few studies that describe histopathological lesions, most of which are associated with enteric disorders. In a case report from Connecticut, United States, microscopic examination revealed major lesions in the gastrointestinal tract confined to the small intestine. These included random crypt cell necrosis and focal hemorrhage in the lamina propria and submucosa. Vascular changes comprised endothelial cell swelling and sloughing, leading to endothelial disruption. Vasculitis was

noted in small arterioles of the basal mucosa and submucosa, rarely accompanied by thrombi. Hyaline degeneration and fibrinoid necrosis of small vessels were occasionally observed (32, 58, 59).

Histologically, CanineCV-positive dogs with respiratory illness exhibited varying severities of generalized hemorrhagic pyogranulomatous pneumonia, multifocal hemorrhagic pneumonia, and severe diffuse suppurative, hemorrhagic, and necrotic bronchiolitis and alveolitis (29).

Other organs such as the brain, meninges, myocardium, lung, liver, and kidney exhibited severe focal vasculitis with mononuclear cell inflammation. The spleen and lymph nodes showed significant lymphocyte necrosis and hemorrhage. Histological analysis revealed hyaline degeneration, fibrinoid necrosis of small vessels, and marked sinus histiocytosis in lymph nodes (58–60).

As observed in other animal species, such as swine, PCVs co-infected with other viruses or bacteria have been demonstrated to enhance PCV2 and PCV3 replication in target tissues. This co-infection increases the severity of induced lesions and exacerbates the clinical course of the disease. The presence of concurrent infections significantly impacts the pathogenesis, leading to more severe clinical manifestations and challenging disease management (9, 52). Studies have shown that CanineCV can co-infect with various other pathogens, the most common being canine parvovirus (CPV), canine adenovirus (CAV), coronavirus (CCoV), and distemper virus (CDV), generally resulting in severe clinical signs.

In double co-infections, co-infection with CPV varied between 18.7% and 57.7% (54, 61), while CPV2 co-infection rates were 15.6% (15/96) and 16.6% (33, 41). CCoV was found in 12.67% of cases (54). The frequencies of triple co-infections involving CPV, CCoV, and CanineCV ranged from 3.2% to 9.8%. A retrospective study analyzing 95 samples of enteritis caused by parvovirus found that 8 (8.9%) were positive for CanineCV (30).

In addition to viral co-infections, double and triple infections with other agents were also related with enterotoxigenic *Escherichia coli*, Shiga toxin-producing *Escherichia coli*, *Salmonella*, *Cryptosporidium* spp., *C. perfringens* α toxin, *Giardia* spp., *Campylobacter jejuni*, and *Campylobacter coli* were also described, although their frequencies were not reported (59, 60, 62).

Co-infection was also observed in animals in studies investigating the association between CanineCV and respiratory diseases. Among CanineCV-positive dogs, nine (52.94%) were co-detected with other pathogens: canine herpesvirus 1 (CaHV-1; $n = 2$), canine distemper virus (CDV; $n = 2$), canine respiratory coronavirus (CRCoV; $n = 2$), canine parainfluenza virus (CPIV; $n = 1$), canine adenovirus type 2 (CAV-2; $n = 1$), and triple-detected with CaHV-1 and CRCoV [$n = 1$; (29)].

The association between viral load and disease severity has been described in PCV2 infections in swine, where a threshold viral load correlates with clinical signs. There for, 10^7 or greater PCV2 genomic copies per milliliter of serum were associated with severe PCV2-associated disease (PCVAD), and poor prognosis. Consequently, PCR results are reported as negative, positive with no PCVAD ($<10^6$ PCV2 DNA copies), positive with PCVAD suspect (10^6 PCV2 DNA copies), or positive with PCVAD [10^7 PCV2 DNA copies or greater; (52)].

In CanineCV infections, viral loads varied between 3.57×10^1 and 8.37×10^8 (30, 54). Another method used to determine viral load in studies was Cycle Threshold (C_t) values, which are inversely proportional to viral load; lower C_t values indicate higher viral loads. C_t values ranged from <13 to 30 (18, 32). Although there is no standardization of viral load and disease severity for CanineCV, $C_t < 13$ in intestinal samples of three dogs from a study were associated with severe clinical signs such as anorexia, vomiting, and severe bloody diarrhea during outbreaks in a Papillon breeding colony in Michigan in March 2013 and February 2014 (32). The association between viral load and disease severity was also observed, with CanineCV loads generally low, ranging from 3.57×10^1 to 8.37×10^8 (mean of 1.03×10^3) and from 8.60×10^1 to 5.38×10^5 viral DNA copies/ μ L (mean of 2.45×10^2) for clinical cases and control animals, respectively (54).

Wild animals

Different studies have confirmed the presence of CanineCV in wild carnivores, including wolves, foxes, badgers, and jackals, with wolves being the most studied. These studies are concentrated in Italy and Africa. In wolves, the prevalence of CanineCV varies between 26.4% and 50% of the animals tested, using tissue samples such as intestine and spleen. The overall median quantity of CanineCV DNA was 6.8×10^2 copies of the target DNA per microliter of template [range: 8.2×10^0 – 3.7×10^7 ; (18, 30, 46)].

In wolves, co-infection with CanineCV and other pathogens has been reported in only two studies. One study identified co-infection in 47.8% (11/23) of wolves, with 72.7% (8/11) involving Carnivore protoparvovirus 1 and CanineCV. Additionally, 18.2% (2/11) tested positive for three viruses: one case with Carnivore protoparvovirus 1, CAV-2, and CanineCV, and another with Carnivore protoparvovirus 1, CAV-1, and CanineCV (30). Another study found that CanineCV was detected alongside CDV in 77.8% (7/9), CPV-2 in 44.4% (4/9), and *Trichinella britovi* in 22.2% (2/9) of the cases. Co-infection with two or three agents, in addition to CanineCV, was observed in 22.2% (2/9; CPV-2 + CDV), 11.1% (1/9; CDV + *Trichinella britovi*), and 22.2% (2/9; CPV-2 + CDV + *Trichinella britovi*) (46).

In foxes, the prevalence of CanineCV varied from 0% (0/232) to 4.3% (5/115) (17, 66). The viral load ranged from 1.96 to 8.94×10^4 copies of DNA/mL of tissue homogenate (pool of organs or spleen), reflecting variations in viral replication or the stage of infection at the time of sampling. The only CanineCV-positive animal that did not die from trauma (1/5) presented neurological symptoms (46).

The only study involving jackals was conducted on samples (lung and lymph node) collected during predator control operations in 2021, and the prevalence was 18%. Therefore, no clinical signs or diseases were associated with CanineCV infection in this study. The prevalence of CanineCV in jackals was 43.75% [14/32; (15)].

Recently, a novel circovirus was identified in Iberian lynxes (*Lynx pardinus*), one of the most endangered feline species in the world and a symbol of wildlife conservation in Europe. Study

conducted by Castro-Scholten et al. (63) identified the Iberian lynx-associated circovirus-1 (ILCV-1) in 57.8% of spleen samples analyzed, collected from both wild and captive populations. The high positivity rate observed suggests a systemic infection that may have significant implications for the immunological and overall health of this species. Iberian lynxes, which already face substantial challenges due to habitat loss, prey scarcity, and infectious diseases such as bovine tuberculosis and feline leukemia virus, now confront a new potential pathogen that could further complicate conservation efforts (63). The discovery of ILCV-1 highlights the urgent need for additional studies to better understand the epidemiology, clinical impact, and potential transmission mechanisms of this virus, as well as to evaluate management strategies to mitigate the risks associated with its circulation in already vulnerable populations. This identification also broadens our understanding of viral diversity in large felines and underscores the importance of systematic virological investigations in endangered species.

Diagnostic

One of the most widely used techniques to detect the CanineCV genome is the Polymerase Chain Reaction [PCR; (14, 20, 24)] particularly real-time quantitative PCR (qPCR). PCR has also been employed for sequencing purposes (14, 15, 25, 29–31, 41, 45, 53, 57, 60).

The systems utilized for qPCR include SYBR Green (18.5%) and Taqman (81.5%). Several studies have utilized qPCR qualitatively due to its ability to be up to 1,000 times more sensitive than traditional PCR, while others have used qPCR to quantify CanineCV DNA in various types of samples (15, 17, 19, 31, 33, 38, 46, 48, 55, 64).

The advantage of qPCR lies in its ability to establish the absolute quantification of viral nucleic acid. As indicated above, the quantification of CanineCV in canine tissues (18, 29, 30, 32, 57), wolves (30), and foxes (45) allows for determining the target tissues for replication that may be related to the virus's pathogenesis. Additionally, the quantification of CanineCV in feces (32, 43, 54, 68) and nasal secretions (29) lays a significant role in understanding viral dissemination (Table 3).

The primer sets used in both PCR and qPCR reactions target the cap gene (60) the Rep gene (29, 60), and intergenic region (65). The Rep region and intergenic region, due to their lower mutation rates compared to the cap region, should be considered in diagnostics as they enhance the detection of viruses that may have undergone mutations.

Although several PCR techniques are currently in use, considerable research is still being conducted to standardize and validate these methods to optimize the diagnosis of CanineCV.

Hao et al. (27) developed multiplex PCR (mPCR) method demonstrated superior results compared to traditional PCR techniques, offering simultaneous detection of multiple canine viruses, including canine adenovirus type 2 (CAV-2), canine influenza virus (CIV), CD, CPIV, CanineCV, CCoV, and CPV, with high sensitivity and specificity. The mPCR method's detection limit was established at 1×10^4 viral copies for both respiratory and enteric viruses, significantly enhancing diagnostic accuracy

in clinical samples. The ability to detect up to seven different viruses in a single reaction not only streamlines the diagnostic process but also improves the reliability of detecting co-infections. This method, therefore, presents a valuable tool for comprehensive epidemiological surveillance and the rapid, precise diagnosis of canine viral infections.

Still with the aim of diagnosing agents involved in CanineCV co-infections, Wang et al. (36) developed a duplex SYBR Green I-based real-time PCR assay developed for the simultaneous detection of CanineCV and CaAstV demonstrated high sensitivity and specificity. The assay's detection limits were 9.25×10^1 copies/ μ L for CanineCV and 6.15×10^1 copies/ μ L for CaAstV, making it significantly more sensitive than traditional PCR methods. The duplex PCR also showed no cross-reactivity with other common canine viruses, such as CPV, CCoV, CDV, and canine kobuvirus (CaKoV), underscoring its specificity. Additionally, the reproducibility of the assay was confirmed through low intra- and inter-assay variation. This method offers a rapid, reliable, and cost-effective tool for detecting co-infections in clinical samples, significantly improving the accuracy of diagnosis in cases where CanineCV and CaAstV are suspected.

Chip digital PCR (cdPCR) is a cutting-edge PCR method that involves encapsulating nanoliter-sized volumes of liquid in high-throughput microcells or microchannels for PCR amplification, followed by direct interpretation of fluorescence signals. This technique allows for the absolute quantification of nucleic acids without the need for external standards, calibration curves, or Ct values. cdPCR excels in precisely detecting and measuring even very small amounts of DNA, making it especially useful for samples with low DNA concentrations or those that contain inhibitors that could interfere with traditional PCR methods. The technique is also known for its high sensitivity and specificity, significantly minimizing the chances of false positives or negatives. This method was used for the detection of CanineCV and exhibited a detection limit of 6.62 copies/ μ L, making it ~ 10 times more sensitive than qPCR, which had a detection limit of 6.62×10^1 copies/ μ L. This increased sensitivity allows for more accurate detection, especially in samples with low viral loads. Furthermore, the cdPCR method showed excellent specificity, with no cross-reactivity observed with other common canine viruses and demonstrated high repeatability with low intra-assay and inter-assay coefficients of variation (36).

Another widely used technique for viral detection is *in situ* hybridization (ISH), which labels viral DNA within tissue samples, enabling precise localization of the virus. This technique is crucial for identifying target tissues and understanding the lesions associated with viral infections. By determining the exact location of the virus in the tissue, ISH provides valuable insights into the pathogenesis of the infection and its impact on specific tissues. In this technique, the Rep gene has been used as the target for detecting CanineCV DNA within various tissues of infected dogs (29, 32, 59).

In situ hybridization was particularly effective in identifying the presence of viral nucleic acid within specific lymphoid tissues, such as the spleen, mesenteric lymph nodes, and Peyer's patches. The ISH method produced strong positive signals in these lymphoid tissues, especially within epithelioid macrophages

TABLE 3 Summary of methods and sample types used for CanineCV detection across different species and regions.

Method	Country	Species	Sample	References
PCRq*	Africa	Dog and jackals	Lung and lymph node	(15)
PCRq*	Africa	Dog	Serum	(18)
PCRq*	Brazil	Dog	Feces	(56)
PCRq**	Brazil	Dog	Lung, liver, and spleen	(64)
PCR	Brazil	Dog	Feces and fecal swab	(14)
PCRq	China	Dog	Feces	(33)
PCR****	China	Dog and cats	Fecal, nasal swabs, and serum	(28)
PCRq*	China	Dog	Blood samples	(59)
PCRq**	Colombia	Dog	Feces	(41)
PCRq**	Colombia	Dog	Feces	(25)
PCRq*	Germany	Dog	Feces	(55)
PCRq*	Iran	Dog	Rectal swabs	(31)
PCRq*	Iran	Dog	Feces	(53)
PCRq*	Italy	Dog	Liver and intestine	(57)
PCRq*	Italy	Dog, wolfs, foxes, and badgers	Tissue***	(46)
PCRq*	Italy	Dog	Feces and/or rectal swabs	(54)
SYBR Green-based qPCR*****	Italy	Wolfs	Tongue, intestine, and spleen	(30)
SYBR Green-based qPCR*****	Italy	Foxes	Pools of organs	(45)
SYBR Green-based qPCR*****	Italy	Dog	Faces or intestine	(30)
PCRq*	Italy	Wolfs, foxes, and badgers	Spleen and intestine	(18)
SYBR Green-based qPCR*****	Taiwan	Dog	Rectal swabs or feces	(38)
PCR/ISH	Thailand	Dog	Nasal, oral swabs, and tissue samples [#]	(20)
SYBR Green-based qPCR*****/ISH	Thailand	Dog	Nasal swab and lung	(29)
PCRq/ISH	USA	Dog	Feces, serum, and tissue ^{##}	(32)
PCRq*	USA	Dog	Intestine, liver, and spleen	(58)
PCRq Rep and Cap/ISH	USA	Dog	Blood, feces, and tissue	(60)
PCRq***	Vietnam	Dog	Fecal swabs	(19)

*PCRq by Li et al. (60); **PCR by Kotsias et al. (16); ***PCRq by Piewbang et al. (20); ****PCR by Hao et al. (27); *****PCRq by De Arcangeli et al. (82); [#]Brain, lung, liver, kidneys, tonsil, and tracheobronchial lymph nodes; ^{##}Lung, liver, spleen, and intestine; ^{***}Spleen, tonsil, lymph nodes, liver, intestine, lung, kidney, brain.

located in regions of granulomatous inflammation (32, 59). Additionally, ISH located CanineCV DNA within the pulmonary tissues, notably within the alveolar lining cells, endothelial cells of capillary blood vessels, and lymphoid cells within the follicles of the tracheobronchial lymph nodes. The technique demonstrated high sensitivity, successfully detecting viral DNA within the nuclei and cytoplasm of histiocytes and macrophages. This precise localization of the virus in both lymphoid and pulmonary tissues highlights the direct association between CanineCV and the pathological lesions observed in these areas (69). By providing detailed insights into the specific tissues affected and the cellular localization of the virus, ISH proves to be a critical tool in understanding the pathogenesis of CanineCV infections.

One Health

The One Health concept, defined as the collaborative effort of multiple disciplines working locally, nationally, and globally to attain optimal health for people, animals, and the environment, has gained significant recognition. This approach acknowledges the interconnectedness of human, domestic animal, and wildlife health within the broader context of ecosystem health. By providing a holistic framework, One Health facilitates the development of comprehensive solutions to global health challenges. The emergence of infectious diseases, whether novel or known, exemplifies the dynamic interplay between pathogens, hosts, and their environments, highlighting the necessity of an integrated approach to health (70–72).

The proximity between wild and domestic hosts plays a crucial role in the transmission of viruses. As human populations expand and urbanize, the interactions between humans, domestic animals, and wildlife increase, heightening the risk of pathogen transmission and the emergence of novel disease outbreaks. Factors such as wildlife trade and the introduction of domestic species decrease the geographical and behavioral separation between donor and recipient hosts, promoting viral emergence. These interactions create opportunities for cross-host exposures, a critical step in the transference to new hosts, and facilitate the establishment of epidemics by enabling sufficient contact for virus transfer and adaptation (71, 74–76).

RNA and single-stranded DNA (ssDNA) viruses exhibit high mutation and nucleotide substitution rates, allowing rapid evolution and adaptation to new environments. This high variability, coupled with error-prone replication and the lack of a proofreading mechanism, enhances their ability to infect new hosts. For instance, RNA viruses have rapid replication, short generation times, and large populations, which increase the likelihood of adaptation to new hosts. In contrast, most DNA viruses are less variable, often showing virus-host co-speciation. However, ssDNA viruses like *Circoviridae* can exhibit mutation rates like RNA viruses, suggesting their potential for rapid evolution and cross-species transmission. These rapid evolutionary capabilities are particularly concerning when considering the increased interactions between humans, domestic animals, and wildlife (77–80).

The behavior of CanineCV and PCVs highlights the complexities of viral adaptation and cross-species transmission. PCV3, a member of the *Circoviridae* family, has been shown to infect multiple hosts, with a high possibility of infecting baboons, demonstrating its capability for trans-species transmission. CanineCV, with its high mutation rate, can adapt to various hosts, like the behavior observed in PCV (9, 24, 25).

Adaptation to interhost transmission by droplet spread, and fecal-oral transmission, which occur with the CanineCV, represent different adaptational challenges due to host differences and variation in environmental exposure, therefore the capacity of the virus in the environment is very important (75). There is no study that shows the viability of CanineCV in the environment, but there is with PCV. PCV2 were detected in wastewater from manure treatment systems consisting of an equalization tank, a settling tank, an anaerobic reactor, an aerobic reactor, and a secondary settling tank, showing its stability in the environment (81). The survival of the virus in the environment is a crucial factor in the spread of the virus and increases the possibility of the virus contacting new hosts.

Another important factor that must be considered is the dog meat feeding habits in some countries. Additionally, serum from these animals may have a high viral load, with a *Ct* ranging from 28 to 35, making a possible human route of infection, not only through ingestion but also during handling of the animals during slaughter. It should also be noted that depending on the moment of infection, the amount

of virus may be even greater, with a *Ct* range of 13–30 (18, 32).

The detection of CanineCV across these various host species highlights the virus's adaptability and potential for cross-species transmission. Further research is necessary to understand the mechanisms behind this adaptability and the implications for disease management in both domestic and wild animal populations.

Conclusion

In conclusion, CanineCV represents a significant emerging pathogen with the ability to infect various species, including domestic dogs, wild carnivores, and potentially other hosts. The high genetic variability and adaptability of CanineCV, as evidenced by its widespread detection across different regions and host species, underscore the importance of continued surveillance and research. Diagnostic advancements, including the use of techniques such as qPCR, ISH, and multiplex PCR, have significantly enhanced our ability to detect and quantify the virus, thereby improving our understanding of its epidemiology and pathogenicity. These tools, combined with detailed phylogenetic analyses, are crucial in monitoring the virus's evolution and in developing strategies to mitigate its impact on animal health. The observed associations between CanineCV infections and co-infections with other pathogens highlight the complex interplay between the virus and host immune responses, which can exacerbate disease severity.

Therefore, ongoing research into the virus's transmission dynamics, tissue tropism, and interactions with co-infecting agents is essential for developing effective control measures and understanding the broader implications of CanineCV infections for both domestic and wild animal populations.

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Higher biosecurity level was associated with reduced risk of Danish dairy cattle farms becoming test-positive for *Salmonella* Dublin in a nested case-control study

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Salmonella Dublin (*S. Dublin*) is a cattle-adapted bacterium with enzootic occurrence in cattle populations of many countries. Preventing the spread of *S. Dublin* between cattle farms requires an understanding of the local pathways for the direct and indirect transmission of bacteria. Identifying key risk factors is complicated due to the numerous pathways through which the bacteria can be introduced and established on dairy cattle farms. This study aimed to provide new knowledge about the effect of biosecurity in dairy farms in *S. Dublin*-enzootic areas of Denmark. The association between the researcher-assessed biosecurity level and the risk of introducing and establishing *S. Dublin* in farms was investigated by following a monthly recalculated cohort of dairy farms with no test-positive *S. Dublin* surveillance results over the previous 2 years. There were 37 new test-positive farms matched by herd size with 74 control farms that remained test negative in the mandatory *S. Dublin* surveillance programme. A published Biosecurity Assessment Framework for *S. Dublin* (BAF-SD) was used to systematically and semi-quantitatively assess the on-farm biosecurity practices across 12 farm sections. Each section was scored on a scale from 0 (total lack of biosecurity measures) to 100 (excellent biosecurity) based on observations and interviews. Lower biosecurity scores in the sections "entrance area," "pick-up-delivery of calves," "calves < 130 days," "cattle > 130 days," and "storage of feed and feeding" were associated with becoming test-positive for *S. Dublin* at a 90% confidence interval (CI) level in univariable logistic analyses. In the multivariable analysis, a higher weighted biosecurity score across all sections was found to be associated with ($p < 0.05$) with lower odds of becoming test-positive for *S. Dublin* (odds ratio [OR] = 0.64 per 10-unit increase in biosecurity level). None of the study farms had very good (score 80 to <90) or excellent biosecurity (score of 90 or above), highlighting the opportunities for biosecurity improvements on-farm. In conclusion, the current biosecurity levels in Danish farms appear insufficient to resist the infection pressure of *S. Dublin* from the farm surroundings. Hence, biosecurity practices need to be improved, and/or the infection pressure needs to be reduced, to lower the number of new test-positive dairy cattle farms in Denmark.

KEYWORDS

biosecurity, *Salmonella*, one health, control, prevention, cattle, dairy herds

1 Introduction

Salmonella Dublin, a bacterium that is host-adapted to cattle (1, 2), causes losses for the dairy industry (3–6) and is a serious zoonotic hazard (7, 8). Among European cattle, *S. Dublin* is the most frequently reported *Salmonella* serotype, with a reported prevalence of up to 40% test-positive dairy farms in some countries (9, 10). In the late 1990s, Denmark initiated monitoring for *S. Dublin* on cattle farms, which in 2008 evolved into a national control programme aimed at eradicating *S. Dublin* from the Danish cattle population (11, 12). The prevalence of “likely infected” dairy cattle farms declined from above 25 to 7.1% in December 2015 (12, 13). Since then, the prevalence has steadily increased to above 10% in December 2021. Despite progressively tighter biosecurity control measures (including strictly regulated movement of animals out of ‘likely infected’ farms for live purposes), new farms continue to become infected or re-infected, especially in enzootic areas (14–19). This suggests that local direct or indirect transmission pathways drive the *S. Dublin* enzootic occurrence, and a better understanding of these pathways is required to more effectively control the disease. Indeed, with the excretion of *S. Dublin* in faeces and its ability to survive for weeks in slurry and even for years in dried manure (20, 21), transmission by fomites is a likely source of introduction and establishment of *S. Dublin* in cattle farms. Furthermore, the dairy sector is undergoing structural development toward larger, and more complex (multi-site) farms. This increases the frequency of exposure risks through local transmission pathways between the different sites and hence also between farms.

The on-farm biosecurity level is therefore hypothesised to be important for the prevention of *S. Dublin* between-farm transmission. Different studies have identified single factors related to local transmission associated with *S. Dublin* occurrence; however, the majority of the risk factor studies include or concern other *Salmonella* serotypes (22, 23). Furthermore, studies on *Salmonella* spp. have failed to identify specific local transmission pathways (24). This may be due to inconsistencies in the probability of transmission caused by intermittent excretion of low bacterial numbers, particularly for serotype Dublin, or the numerous possible introduction pathways (25–28). Even if the pathogen is introduced to a farm, it may not establish itself in the animals or environment if adequate internal biosecurity measures are in place.

Existing tools that employ quantitative methods to measure biosecurity or conduct risk assessment for cattle diseases have been developed (29–31). However, less attention has been paid to whether a more qualitative or semi-quantitative assessment approach, involving in-depth on-farm investigations, similar to experiences from the field of animal welfare assessment, can offer a better understanding of the on-farm biosecurity and the risk of disease introduction and establishment. A semi-quantitative Biosecurity Assessment Framework (BAF-SD) aimed at the introduction and establishment of *S. Dublin* was developed and described by Pedersen et al. (23). It comes with an electronic tool that can assist trained biosecurity assessors in performing systematic biosecurity assessments on dairy farms by conducting on-farm observations and interviewing the farmer.

The overall purpose of the current nested case–control study was to provide new information that can be used to reduce the introduction and establishment of *S. Dublin* on dairy cattle farms. The specific objectives were to (1) describe the biosecurity levels in Danish

dairy cattle farms situated in *S. Dublin* enzootic areas; (2) analyse the association between on-farm biosecurity and the risk of *S. Dublin* introduction and establishment in Danish dairy cattle farms, and (3) identify the farm sections most relevant for biosecurity improvement.

2 Materials and methods

2.1 Study design and source population

For this epidemiological study, a nested case–control study was designed. From 1 September 2021 to 31 August 2022, a delineated source population of Danish dairy cattle farms at risk of becoming test-positive for *S. Dublin* was followed (see overview of farm and business definitions in Table 1). The source population (cohort) included dairy farms with recorded *S. Dublin*-tested bulk-tank milk (SD-BTM) samples and *Salmonella* level 1 (most likely free from *S. Dublin* infection) during previous 2 years or more, located in enzootic *S. Dublin* areas, defined as areas within a 10-km radius around the farm with at least one test-positive neighbouring cattle farm (see overview of definitions of infections status of farm and areas in Table 1).

The source population was recalculated every month to account for the new dairy farms at risk and included 1 September 2021, at study start, 1,383 of the 2,513 Danish dairy farms (Figure 1A). Dairy farms outside the source population included 744 farms in non-enzootic areas, and 386 were farms in *Salmonella* level 2 (likely-infected with *S. Dublin*) or were farms with a history of level 2 within the last two years (Figure 1B).

2.2 Detection of case and control dairy cattle farms in the source population

Every day during the study period, case farms were designated from the Danish Cattle Database (DCD) when they changed from level 1 to exceed the thresholds in SD-BTM, leading to *S. Dublin* level 2 in the Danish surveillance system (17). Simultaneously, a list of 20 relevant control farms (remaining test negative) were randomly selected, for each case farm, matched by the herd size groups (<100, 100–200, 200–300, 300–500, and >500 cows, measured as mean number of cows during the last year prior to study farm designation) to account for frequency of potential exposure occurrences (see overview of outcome and explanatory variables in Table 2). The first author invited farmers by phone subsequent to the designation. If case farms agreed to participate, matching control farms were contacted from the top of the control list until two controls were included per case. A case–control ratio of 1:2 was decided to accomplish more observations, within the limitation of a 1-year study period and financial resources. A visit date was scheduled respecting restrictions related to farm disease status and farmers’ availability. Farms could only be included as controls once, and they had to remain test negative in the same year and quarter of SD-BTM surveillance.

2.3 On-farm data collection

To collect the primary explanatory variables, information on biosecurity was obtained at a single farm visit by the same trained

TABLE 1 Definitions of cattle farm and business, infection status, and areas.

Term	Definition
Farm	Property located at a specific geographical location identified by a unique number in the Danish Central Husbandry Register (CHR). The farm may include one or more cattle herds with the same or different owners.
Business	One or more herds with the same owner at one or more farms.
<i>Salmonella</i> Dublin (S. Dublin) bulk tank milk (SD-BTM)	Bulk tank milk from dairy farms was tested in an indirect in-house Enzyme-Linked Immunosorbent Assay (ELISA) <i>Salmonella</i> serogroup D test, measured as a corrected test optical density coefficient (ODC%). Dairy cattle farms are tested quarterly according to the Danish National Surveillance Programme.
<i>Salmonella</i> level 1	Farms are most likely free from S. Dublin infection based on antibody surveillance and trade classification. Surveillance: Level 1 criteria for dairy cattle farms are (i) an average SD-BTM ELISA threshold below 25 ODC% for the last four SD-BTM samples with more than 21 days between sampling, and (ii) a maximum increase in percentage point of 20 between the last SD-BTM sample and the average of the previous three (denoted “the jump criterium”). Non-dairy farms level-1 criteria include ELISA <i>Salmonella</i> serogroup D test results below 50 ODC% in blood samples. Level-giving surveillance samples include automatically collected samples from slaughter animals by quarterly designation of farms in the Central Danish Cattle Database or the annual blood sampling of 8 or 16 animals in heifer-replacement farms. Trade classification: When cattle are moved from level-1 farms to other cattle farms, it has no effect on the <i>Salmonella</i> levels of the farms.
Enzootic S. Dublin area	An area of 10-km radius with at least one test-positive neighbouring cattle farm (all types of cattle farms)
Test-positive neighbour cattle farm	Cattle farm with 182 interrupted or connected days or more in <i>Salmonella</i> level 2 and a positive indirect ELISA <i>Salmonella</i> serogroup D test. Both criteria were fulfilled during the last year prior to the monthly recalculated cohort. A positive antibody test was defined as either a bulk-tank milk reaction of ≥ 25 ODC% or a serological sample ≥ 50 ODC%, regardless of monitoring purpose.
<i>Salmonella</i> level 2	Farms most likely infected with S. Dublin based on antibody surveillance, detected salmonellosis, or trade classification. Surveillance: Farms exceed the threshold of level-1 criteria, including a follow-up SD-BTM sample with the same ODC% threshold in dairy cattle farms. Salmonellosis: Farms with clinical signs and positive bacteriological samples for S. Dublin. Trade classification: Ingoing animals from farms with unknown or level-2 status (trade from a level-2 farm is only allowed if the receiving farm is part of the same business or if all animals at the receiving farm is from the same farm).
Local infection pressure	The mean number of cattle during the last year across all test-positive neighbour cattle farms within the S. Dublin-enzootic area.

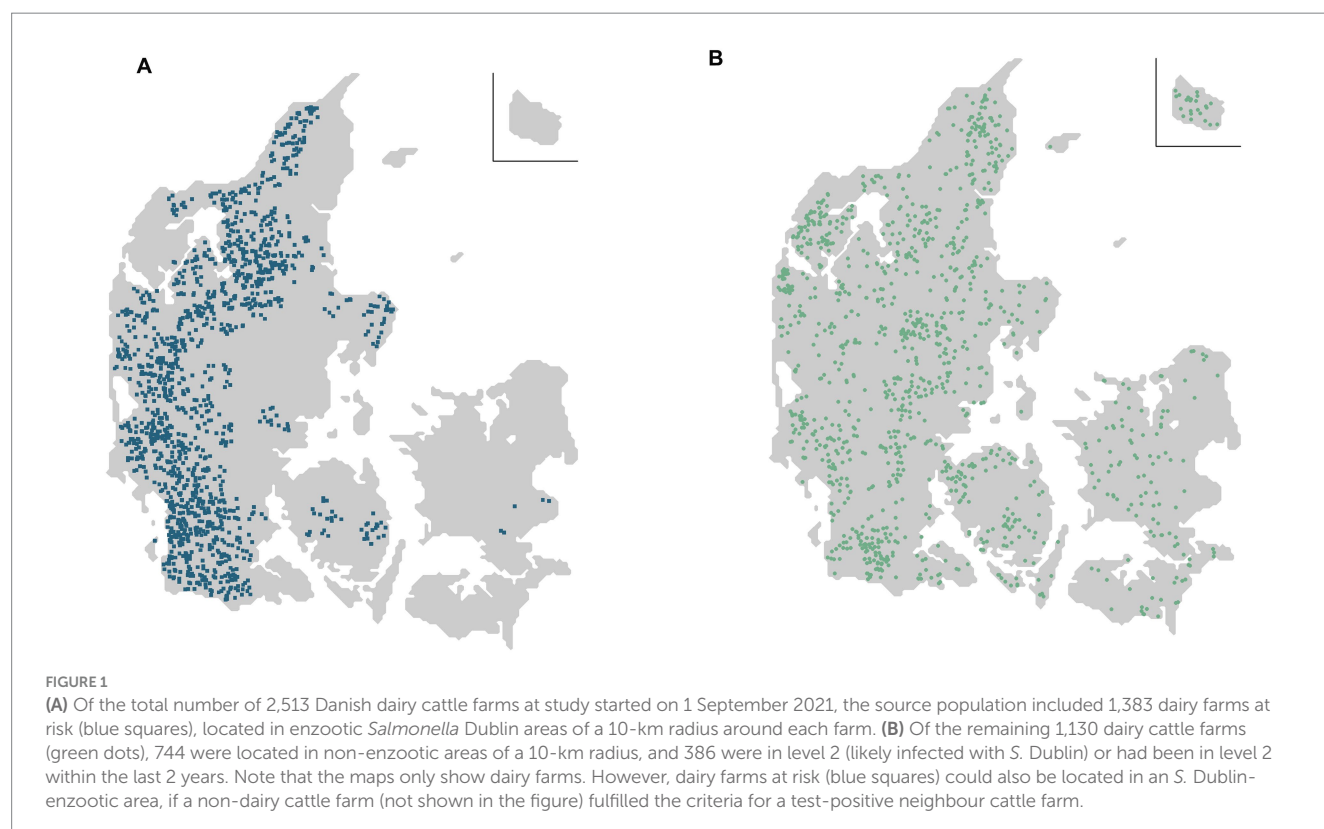


TABLE 2 Overview of outcome and explanatory variables, scale, and data origin.

Role of variable in model	Variable	Scale	Data origin
Outcome	Likely infected in the national surveillance programme for <i>Salmonella</i> Dublin (S. Dublin) due to exceeding the threshold in SD-BTM	Qualitative, dichotomous	Surveillance register data from the Danish Cattle Database
Primary explanatory	Individual biosecurity sections (up to 12 sections)	Quantitative, pseudo continuous	Farm observations and interviews through BAF-SD
	Overall biosecurity score, weighted		
Secondary explanatory	Animals on the pasture	Qualitative, dichotomous	Online register data from the Danish Agricultural Agency homepage
	Production type, organic		
	Ingoing animal movement		Register data from the Danish Cattle Database
	Local infection pressure	Quantitative, continuous	
	Business network	Qualitative, ordinal	

TABLE 3 The 12 biosecurity sections included in the Biosecurity Assessment Framework for *Salmonella* Dublin introduction and establishment in dairy cattle farms (BAF-SD), published by Pedersen et al. (23).

Number	Biosecurity section	Number	Biosecurity section
1	Entrance	7	Manure
2	Pick-up-delivery calves	8	Storage of feed and feeding
3	Pick-up-delivery adults	9	Washing facilities
4	Calving facilities	10	Animals on pasture
5	Calves < 130 days	11	Vermin control
6	Cattle > 130 days	12	Carcass disposal

biosecurity assessor (the first author) using the published BAF-SD (23). Through the 7-step BAF-SD process, a single weighted semi-quantitative score of the farm biosecurity level was obtained for the 1-year risk period prior to the designation of the study farm, serving as a primary explanatory variable. As part of that process, up to 12 biosecurity sections were evaluated on each farm based on information from 56 observations and 109 interview questions posed in an open conversation style to the farmer or herd manager. The 12 sections are listed in Table 3. Each section and the weighted biosecurity level were reported on a scale from 0 to 100, where 100 represented excellent performance of all aspects of biosecurity with only a few minor deficiencies, and 0 represented the worst performance with no or the very minimum level of biosecurity barriers in place. Additionally, regarding the biosecurity section about animals on pasture, the answer was dichotomised into “animals on the pasture” or “no animals on the pasture” as a secondary explanatory variable. During the farm visit, venous blood was randomly sampled from calves between 100 and 180 days for Enzyme-Linked Immunosorbent Assay (ELISA) *Salmonella* serogroup D serological testing at Eurofins Steins laboratory, Department of Milk Testing Denmark, section for serology in Vejen, using the same test as for the surveillance programme (32).

2.4 Register data collection

Data for secondary explanatory variables were obtained from the interviews, the DCD, and the Danish Agricultural Agency (DAA).

Coordinates from the Geographical Information System (GIS), animal and farm records from the Central Husbandry Register (CHR) and *Salmonella* surveillance data was extracted from the DCD for the individual study farm risk period to include proxies for: (i) Local infection pressure, (ii) Business network, and (iii) Ingoing animal movement. The “local infection pressure” was defined as the mean number of cattle during the last year across all test-positive neighbour cattle farms within the enzootic area of the individual study farm (see Table 1). To account for indirect and direct transmission pathways due to multisite business, we identified cattle herds and farms in Denmark with the same ownership as the study unit farm and categorised the number of farms including cattle herds belonging to each study farm as a proxy for “business network.” Additionally, records of animal movement were combined with the study unit business network information and movement of animals from farms outside to inside the business network, including shows and/or common pasture areas, and dichotomised into a proxy for “ingoing animal movement.” Publicly available farm information about ownership and production type, that is, organic or conventional, was downloaded from the DAAs homepage on 3 October 2022 (33).

2.5 Establishment of dataset

The data management was performed in the statistical software R version 3.6.1 (34): (i) the daily information of potential case and control farms were shared with the first author per email in-house at

the SEGES Innovation company (SEGES Innovation P/S, Aarhus, Denmark) during the study period, (ii) processed data for secondary explanatory variables was organised into Microsoft Excel (Microsoft Corporation, Redmond, WA, United States) spreadsheets, and (iii) merged with BAF-SD data collected on-farm on paper and entered into a final Microsoft Excel spreadsheet for analysis together with information about production type.

2.6 Data control and descriptive data analysis

The first author performed the management of BAF-SD data and manually checked for errors by a controller. Using descriptive statistics in the statistical software R version 3.6.1: (i) we carefully inspected data and outliers for incorrect data entry by measures including frequency distribution, summary statistics report, and compared the source and sampled population, (ii) we described explanatory variables by measures including cross-tabulation, standard deviations, percentiles, and graphical illustrations including scatterplots, box-plots, histograms, and violin plots.

2.7 Statistical analysis

Pairs of explanatory variables were initially checked for collinearity ($p < 0.05$) using Fisher's exact test, Spearman's rank correlation coefficient, and point-biserial correlation. If collinearity between pairs of explanatory variables was observed, the one with the highest p -value in the univariable analysis was excluded from the subsequent multivariable modelling.

Thereafter, we analysed data using the R-survival package, in statistical software R version 4.3.1 (35) for conditional logistic regression models stratified by matching pairs with disease status (case or control) as outcome. Initially, we tested the univariable association between each explanatory variable, including the semi-quantitative biosecurity assessment for each biosecurity section (one by one), and the outcome. Thereafter, we included the overall weighted biosecurity score and all secondary explanatory variables and possible meaningful interactions in a multivariable model (Equation 1), which is expressed as

$$\text{logit}(P(Y_{ij} = 1)) = \beta_A A_{ij} + \beta_B B_{ij} + \beta_C C_{ij} + \beta_d d_{ij} + \beta_e e_{ij} + \mu_{Fj} \quad (1)$$

where $P(Y_{ij} = 1)$ represents the probability that a farm i in stratum j is a case; A , B , and C represent the categorical variables "production type, organic," "business network," and "incoming animal movement"; d and e represent the numerical variables "overall biosecurity score, weighted" and "local infection pressure"; μ_{Fj} is the random intercept for farm size group F_j , with $i_{Fj} \sim N(0, \sigma^2)$. The model is stratified by matching pairs j , controlling for confounding at the stratum level.

The model was manually fitted by backward stepwise elimination. Akaike's Information Criteria was used as an elimination criterion and a likelihood-ratio test to evaluate the explanatory variables criterion for inclusion (36). Furthermore, all excluded explanatory variables were reintroduced one by one to the final model to check for

overlooked statistical associations with the outcome and to consider possible confounding defined by more than 20% change in final model estimates when the variables were reintroduced.

2.8 Ethics approval and consent to participate

At the beginning of each farm visit, the objective of the study was repeated for the farmer, and written approval with consent to contribute to data collection using the BAF-SD and to grant access to farm data from registers was obtained from the farmers. It was clear that only anonymised data and results would be made publicly available, and that farmers could withdraw from the study at any time. The study was ethically approved by the institutional Research Ethics Committee of Science and Health at the University of Copenhagen, case number: 504-0306/22-5,000. The National Committee for the Protection of Animals approved the sampling of venous blood from animals, permit number: 2021-15-0201-00946.

3 Results

3.1 Population

In total, 37 case and 74 control farms were included in the study, including two case farms testing serologically positive above threshold (≥ 50 optical density coefficient % [ODC%]) in individual animals immediately before the farm visit and repeatedly being test-positive in random sampling during the farm visit. Ninety-nine included farms (89%) were designated in the third and fourth quarter of 2021, and the remaining 12 farms (11%) in the first and second quarter of 2022. All case farms and 69 control farms were located on the Jutland Peninsula; the remaining control farms were on the islands of Funen and Zealand. Forty farms with a median herd size of 127 cows declined to participate, including five case farms (Table 4), mainly due to lack of time, interest, or the farmer being close to retirement (10 out of the 40 farms were either closed or had stopped milk production within 3 years after designation). Additionally, to the 40 farms declining to participate; (i) it was not possible to establish contact to one case farm and 10 control farms, (ii) fourteen control farms was excluded due to other reasons (recent stop in milk production, no calves, etc.), and (iii) another two case farms were excluded due to a change in ownership leading to a lack of ability to respond to the interview part about the previous year of practices, and due to suspected cross-reactions upon positive culture for *Salmonella* Typhimurium, respectively. None of the tested calves in control farms were serologically positive above the ELISA *Salmonella* serogroup D threshold (≥ 50 ODC%). In 25 out of 37 case farms, at least one calf was serologically positive. Visits to case farms were prioritised to have their biosecurity level assessed close to the outcome of becoming test-positive, and 87.5% were visited within 20 days of confirmed level-2 status. Comparing study farms with our source population at study start, the median herd size was higher among study farms (Table 4). In comparison, farms declining to participate had a smaller median herd size. Additionally, the median number of test-positive neighbour farms was similar among the source population and control farms but higher for case farms.

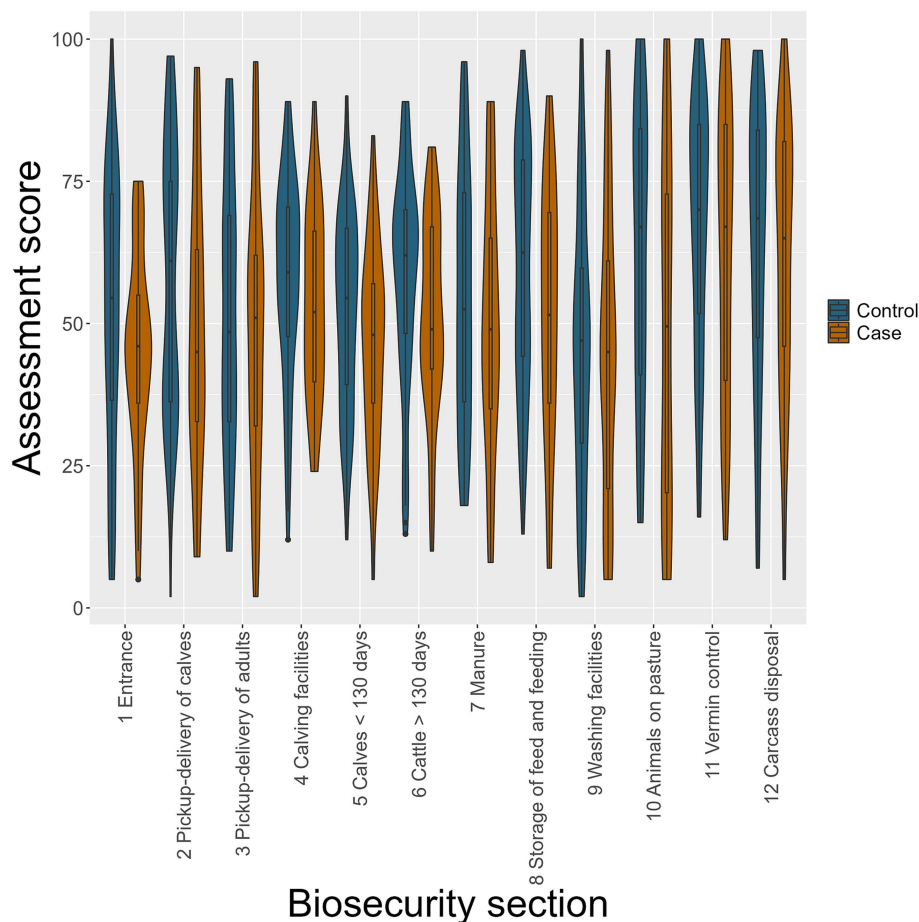


FIGURE 2

Violin and box plots of up to 12 assessed biosecurity sections in 74 control (blue) and 37 case (brown) farms using the Biosecurity Assessment Framework for *Salmonella* Dublin. Scoring from 0–100, 0 is a total lack of biosecurity measures, and 100 is excellent biosecurity.

3.4 Biosecurity regression model for becoming a *Salmonella* Dublin test-positive dairy farm

The results of the multivariable conditional logistic regression model are illustrated in Table 6. The final model included biosecurity level (between 19.6 and 79.2) and local infection pressure as the only significant explanatory variables. Within the same herd size group, the odds for a 10-unit increase in biosecurity level and 1,000-unit increase in local infection pressure in farms becoming test-positive relative to the odds for a 10-unit rise in biosecurity level and 1,000-unit increase in local infection pressure for farms remaining test negative were 0.64 (95% CI: 0.43–0.96, $p = 0.03$) and 1.13 (95% CI: 1.01–1.25, $p = 0.03$), respectively. This demonstrates the importance of improving the overall biosecurity level and lowering the local infection pressure for prevention against *S. Dublin* introduction and establishment in dairy farms.

4 Discussion

In this study, we found that the overall biosecurity level is associated with the risk that dairy farms become test-positive in

the ongoing Danish *S. Dublin* surveillance programme, indicating that they have become newly infected with *S. Dublin*. We assessed biosecurity practices using the published BAF-SD in dairy farms classified as newly test-positive (37 cases) and remaining test-negative (74 controls), respectively. Adjusted for the confounder local infection pressure, approximated by the total number of cattle in test-positive neighbour farms within 10 km, a 10-unit increase in biosecurity level was significantly associated ($p < 0.05$) with reduced odds (OR = 0.64) of becoming a *S. Dublin* test-positive dairy farm within the same herd size group. At the same time, none of the other tested variables were found to be associated with the outcome. While other studies have investigated environmental factors associated with the *S. Dublin* introduction and establishment (22, 23), this is, to our knowledge, the first study quantifying an association between overall measurable biosecurity level and the risk of dairy farms becoming test-positive for *S. Dublin*.

The study also provided insights into the level of biosecurity targeting *S. Dublin* in Danish dairy farms. We did not observe farms with a very good (score 80 to <90) or excellent (score of 90 or above) overall biosecurity level in any study farms. Despite a higher biosecurity level in control farms, we observed considerable room for improvement across all biosecurity sections in many

TABLE 5 Results of conditional univariable logistic analysis of biosecurity assessment and secondary explanatory variables in a nested case–control study with 37 case farms and 74 control farms matched by herd size groups.

Univariable analysis	Conditional logistic regression strata (37 cases and 74 controls)							
Variable	Level	Case	Control	OR	95% CI		SE	p-value
1 Entrance	(Count. increments of 10)	37	74	0.84	0.69	1.02	0.10	0.08
2 Pickup-delivery of calves		34	70	0.84	0.69	1.03	0.10	0.08
3 Pickup-delivery of adults		37	74	0.93	0.78	1.12	0.09	0.44
4 Calving facilities		36	74	0.84	0.64	1.09	0.13	0.17
5 Calves < 130 days		37	74	0.80	0.62	1.03	0.13	0.07
6 Cattle > 130 days		37	74	0.81	0.64	1.02	0.12	0.07
7 Manure		37	74	0.92	0.76	1.10	0.09	0.35
8 Storage of feed and feeding		36	74	0.83	0.68	1.02	0.10	0.07
9 Washing facilities		37	74	0.97	0.83	1.14	0.08	0.74
10 Animals on pasture		28	48	0.89	0.74	1.08	0.10	0.23
11 Vermin control		37	74	0.89	0.74	1.08	0.10	0.25
12 Carcass disposal		37	74	0.96	0.82	1.13	0.08	0.63
Overall biosecurity score, weighted		37	74	0.63	0.43	0.92	0.20	0.009
Local infection pressure	(Count. increments of 1,000)	37	74	1.14	1.03	1.27	0.05	0.008
Business network	1	19	46	Ref.				
	2	11	17	1.69	0.65	4.44	0.49	0.47
	≥3	7	11	1.96	0.52	7.34	0.67	
Ingoing animal movement	No	27	46	Ref.				
	Yes	10	28	0.57	0.23	1.43	0.47	0.23
Production type, organic	No	27	62	Ref.				
	Yes	10	12	1.77	0.72	4.31	0.46	0.22
Animal on the pasture	No	9	26	Ref.				
	Yes	28	48	1.72	0.69	4.25	0.46	0.23

The odds ratio (OR), 95% confidence interval (95% CI), standard error (SE), and significance level (*P*) are given for each explanatory variable. Unsourced biosecurity sections in farms with fewer than 12 sections were excluded from the univariable analysis.

farms. Similar results have been obtained in 50 dairy farms in Belgium using another available biosecurity assessment system, Biocheck[®] UGent (Biocheck.Gent BV, Dentergem, Belgium), where no farms received a score above 83 in external biosecurity and 69 in total biosecurity out of an ideal biosecurity of 100 points (29). Furthermore, the system with different classifications and scores has been used to identify an association between apparently free-BVD status and higher biosecurity score (37). The similarity is interesting. The open structure of dairy farms enables multiple introduction pathways for infectious agents; as described by others, this may add to the failure in identifying of single risk factors (24). Indeed, we did not identify clear associations between biosecurity scores for the individual biosecurity sections and risk of becoming test-positive for *S. Dublin*, although there were some indications that cases had lower scores in five sections than

matched control farms. However, we identified an association with the overall level of biosecurity. One explanation for this could be the need to accumulate all risk factors, as the introduction and establishment of the bacteria can happen through many different pathways over time. Hence, it can be argued that the biosecurity of dairy farms in enzootic areas of Denmark is insufficient to resist the introduction and establishment of a local transmission-driven pathogen, such as *S. Dublin*, and potentially other infectious agents. To some extent, this might also explain the association we found between local infection pressure and the odds of becoming test-positive for *S. Dublin*, with an OR of 1.13 for each 1,000 head increase in the number of cattle in test-positive neighbour farms within a 10-km radius. Local infection pressure is a risk factor repeatedly recognised by similar proxies across countries (38–44).

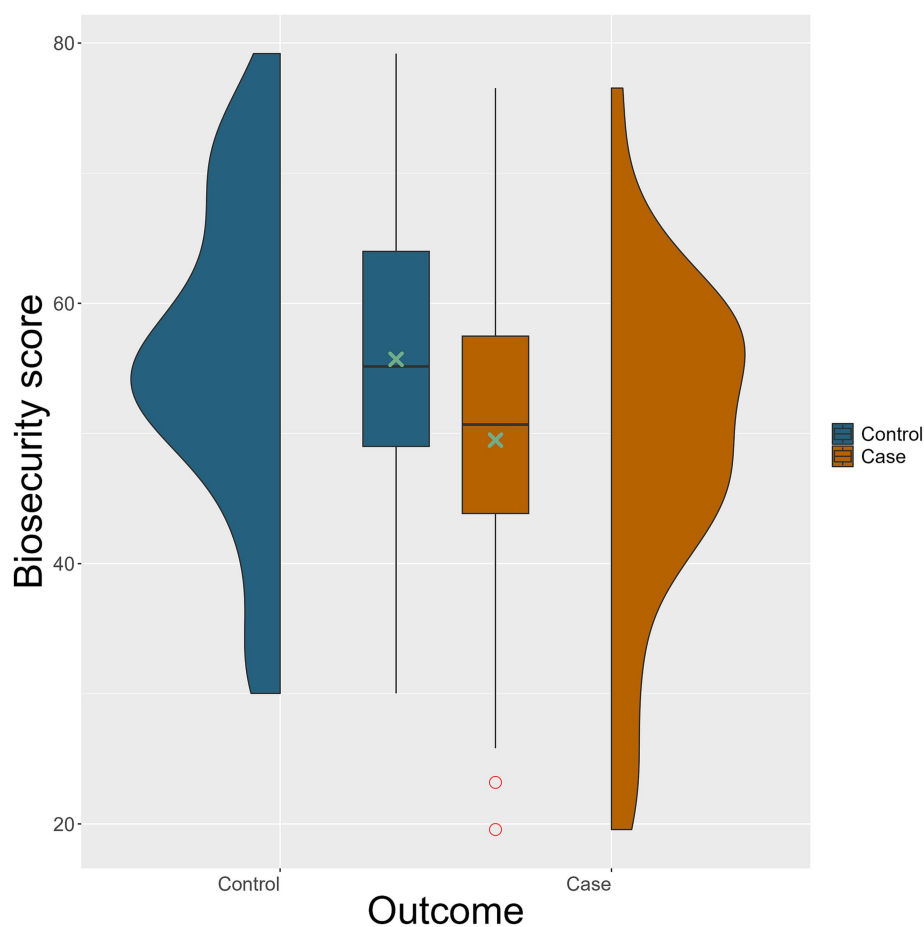


FIGURE 3

Violin and box plots of weighted overall biosecurity score for the 74 control (blue) and 37 case (brown) farms. Green cross (X) indicates the mean biosecurity score for cases and controls, respectively, and circle (O) indicates outliers. Weighted biosecurity assessment scores can range from 0 to 100, where 0 is a total lack of biosecurity measures and 100 is excellent.

4.1 Study limitations

Surprisingly, no interaction between the overall weighted biosecurity score and local infection pressure was identified. This raises the question of whether the used biosecurity framework can fully quantify the true biosecurity level, either because of unknown pathways for introduction and establishment not captured by the framework, assessment reliability, or other study limitations.

A poorly understood pathway might be the risk coming from wild birds. High numbers of migrating birds are recorded periodically on some Danish dairy farms, but the scientific evidence for wild birds as reservoirs or mechanical transmitters for *S. Dublin* is not clear (45–47). In the used framework, the biosecurity section 11, “Vermin control,” was given the lowest weight in the final score by the experts during the development of the framework.

Regarding consistency in assessment score, a single inter-observer reliability test indicated a moderate interclass correlation for the framework’s scores (23). To minimize the observer effect, we used the same trained assessor in all farms. However, intraobserver reliability was never tested due to lack of available resources, and due to expected dynamics in on-farm biosecurity and time needed not to be able to recall assessment scores from previous biosecurity scoring sessions.

Also, the biosecurity level was assessed for 1 year prior to designation as case or control, which introduces potential for recall bias and changes over time that are very difficult to capture and quantify in this type of study.

Another limitation of the study is that, according to the Danish legislation, farmers must inform visitors about their farm’s health status, excluding a blinded study design, with possible introduction of performance bias. However, we consider that the potential bias due to the unblinded study design is negligible due to the inclusion of a scoring guide in the BAF-SD.

Because the cumulated risk combines probability and frequency, another limitation is unmeasured frequency variabilities within each herd size group. In BAF-SD, a separate section on the purchase and replacement of animals is not included but merged into sections 2, “pickup-delivery of calves,” and 3, “pickup-delivery of adults.” Additionally, the risk through animal movement was supported by whether animals had been moved to the business network from other cattle businesses, shows, and/or common pasture, limited by the possibility to include frequency and number of animal source businesses for this study. The “incoming animal movement” variable was overrepresented among the control farms. The lack of association with the risk of becoming test-positive agrees with other

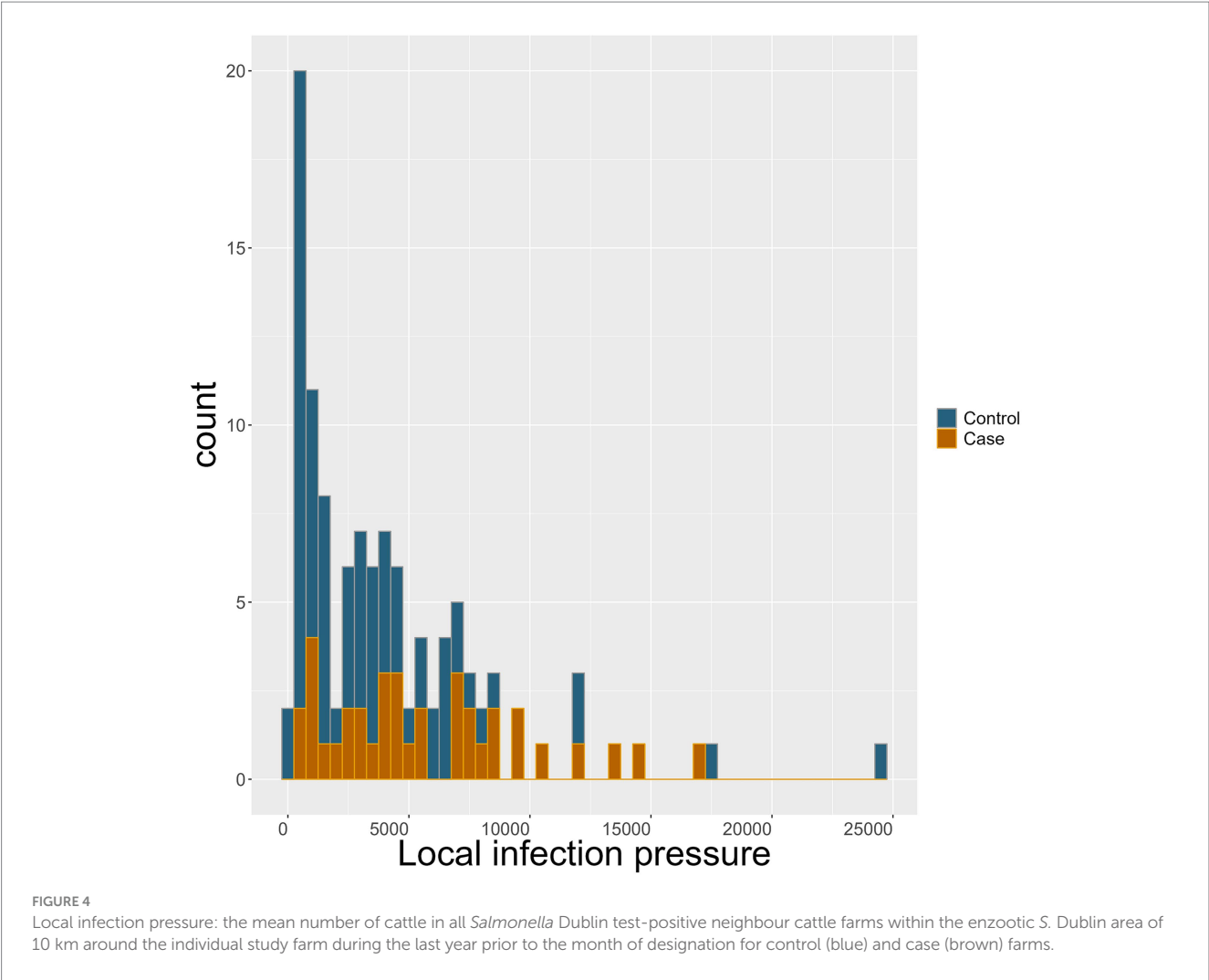


TABLE 6 Significant variables for becoming *Salmonella* Dublin test-positive farms according to the final multivariable analysis comprising 37 case farms and 74 matched control farms by herd size groups in a nested study design by conditional logistic analysis method.

Multivariable analysis		Conditional logistic regression strata (37 cases and 74 controls)				
Variable	Level	OR	95% CI		SE	p-value
Overall biosecurity score, weighted	(Count, increments of 10)	0.64	0.43	0.96	0.20	0.03
Local infection pressure	(Count, increments of 1,000)	1.13	1.01	1.25	0.05	0.03

The odds ratio (OR), 95% confidence interval (95% CI), standard error (SE), and significance level (*P*) are given for each explanatory variable.

studies conducted under the given national movement restriction or movement from test-negative farms in Denmark (15, 42). Interestingly, a recently published network analysis of Danish farms identified movement activity as a predictor for farms becoming classified as infected with *S. Dublin* (17), supporting studies from other countries with less strict *S. Dublin*-related movement restrictions at the time of study (44, 48). The authors of the Danish network analysis (17) suggest that the results about the strong effect of animal movements may be somewhat overestimated due to multisite business structures that were not accounted for in the model. Such movements still occur in Denmark, because animal movements between farms within the same-owner business networks are not as strictly limited as the movement of animals out of test-positive business structures for live purposes under the

Danish legislation. Moreover, farm status changes may occur as an administrative consequence of risky animal movements and not always due to a change in status determined by test results. However, it is likely that *S. Dublin* survives more easily and longer by recirculation between and within multisite farms, as supported by the association with lower release hazard from *Salmonella* restriction in Swedish multisite cattle farms (49). In this study, an association between multisite businesses and the odds of becoming test-positive was not identified, despite a higher proportion of multisite business structures among the case farms. Indeed, a similar tendency was observed for production type, with 27% of the case farms certified as organic, compared to 16% among controls. Organic production has been associated with both being *Salmonella* test-positive and time to recovery, but to our knowledge, not as a risk for introducing

S. Dublin (24, 42, 50). The authors of those studies interpreted the findings as related to the extended period of cow-calf contact before separation and stocking density among calves due to requirements by regulation in infected organic farms. In addition, collinearity was observed between animals on the pasture and organic production, but not with the overall biosecurity score. It seems logical that having animals on the pasture is the biologically plausible explanation between the two variables. However, in the multivariable analyses we only included “production type, organic” as potential interaction or confounder in favour of whether the farm had animals on the pasture, because pasture management was covered in the BAF-SD and organic farmers’ perception of the benefit of biosecurity measures has been measured as lower compared to conventional farmers (51).

Single biosecurity sections were not identified as significant risk factors in this study. An explanation may be that type-II errors are introduced due to a small sample size. In the univariable analyses, five biosecurity sections were significant (at a 90% confidence interval) with the outcome of becoming test-positive (section 1 “entrance,” 2 “pick-up-delivery of calves,” 5 “calves < 130 days,” 6 “cattle > 130 days,” and 8 “storage of feed and feeding”). In general, the number of professional visitors and visitors in contact with animals is higher in cattle farms than in farms with other livestock animals, and with an increasing number with herd size (52). Nevertheless, cattle farms often lack proper entrance biosecurity measures (53–56). Indeed, different single risk factors related to the entrance area, such as the use of protective clothing and a clean parking area for visitors, have been associated with the risk of *Salmonella* introduction (57, 58). Similar to other Scandinavian countries, a separate loading area for animals is not available in many Danish cattle farms, even though *Salmonella* bacteria can be cultured from livestock transport vehicles for cattle (53, 54, 59, 60). Segregation of the haulier and the livestock transport vehicles from the internal farming area is weighted high in the BAF-SD scoring guide for the section 2 “pickup-delivery of calves,” and the tendency toward a significant association with the odds of becoming test-positive is therefore not surprising. Calves are the most susceptible and infectious age group, and a similar tendency was not observed for the biosecurity section 3 “pickup-delivery of adults,” even though older animals were often picked up near feeding tables and thereby potentially led to contamination of feed. Other studies have found that open storage of silage and concentrate is associated with dairy farms being positive for *Salmonella* spp. (58, 61), supporting the findings for the biosecurity section 8 “storage of feed and feeding” in this study. *S. Dublin* is rarely isolated from feed, and the pH value in ensiled forage does not promote the survival of *Salmonella* (62, 63). Most likely, the correlation is linked to contamination of the farm’s feed. Certainly, this introduction and establishment pathway is not unthinkable. Modern feeding procedures involve storage of feed in open silos often located close to contaminated transport driveways or on-farm washing facilities. Moreover, feeding practices related to the total mixed rations may lead to close contact between the equipment, feed, and animals. Therefore, an oral-faecal transmitted pathogen, such as *S. Dublin*, can rapidly be established from point contamination to many animals within the farm. The biosecurity section 9 “washing facilities” was not found associated with the risk of becoming a case farm. This might be explained by an inadequate level of biosecurity in the majority of both case and control farms for this section.

Nonetheless, the results need to be considered with precautions due to both the sample size in this study and the limitations in the accuracy of the BTM test programme. The surveillance has been evaluated with an estimated herd sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) at 15% true herd-level infection prevalence of ~0.95, ~0.96, ~0.80, and 0.99, respectively (64). We improved the accuracy by including dairy farms from source population testing serologically positive on calves between 3 and 6 months, and furthermore testing a randomized sample of calves between 100 and 180 days at farm visit in all study units (65). All control farms were ELISA-negative (<50 ODC%) in blood-sampled calves. However, calves in 12 out of 37 case farms were also serologically negative on the blood samples, which could indicate either recent disease introduction in other barn sections than where the calves are housed, misclassification, or strong segregation and control measures in place to protect the calves at the farm level. Furthermore, graphic evaluation of 5-year BTM profiles of study farms indicates that some farms were misclassified as newly test-positive, but could have been reactivated infection with two years of low serological values on BTM, delayed responses in BTM reactions, or negative follow-up ELISA BTM testing indicating false-positive reaction in the first sample, while some control farms likely were in a recovery period where latent infection could not be completely ruled out.

In conclusion, this study could not identify single biosecurity sections as clear risk factors for the introduction and establishment of *S. Dublin* in Danish dairy farms. Still, the overall expert-weighted biosecurity score was significantly lower in dairy cattle farms that became *S. Dublin* test-positive than in herd size-matched control farms that remained test-negative. Hence, after being adjusted for local infection pressure in a multivariable statistical model, the overall biosecurity level is deemed to have a preventative effect against the introduction and establishment of *S. Dublin*. Moreover, we can conclude that the current level of biosecurity is insufficient to resist the infection pressure from the surroundings. Under the current biosecurity levels, the local infection pressure needs to be reduced to lower the number of new test-positive dairy farms in Denmark. The study illustrates the complicated relationship between infection pressures, biosecurity, and farming practices and structures in intensive dairy farming today.

Data availability statement

The datasets presented in this article are not readily available because we do not have the authority to share the data used for analysis. Requests to access the datasets should be directed to larp@seges.dk.

Ethics statement

The studies involving humans were approved by the Institutional Research Ethics Committee of Science and Health at the University of Copenhagen, case: 504-0306/22-5000. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to

participate in this study. The animal studies were approved by the National Committee for the Protection of Animals, permit number: 2021-15-0201-00946. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. HH: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. ER: Conceptualization, Methodology, Supervision, Writing – review & editing. LN: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The impact of legislative regulation on animal epidemic prevention and control input: evidence from 13 main provinces of pig production in China

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Introduction: The issue of animal epidemic prevention and control has gained significant attention. Regulating and incentivizing farmers' animal epidemic prevention behaviors is vital for safeguarding national biosecurity. Previous studies have focused on the importance of animal disease prevention and control legislation but not examined the incentive of animal epidemic prevention behavior from the perspective of legislation. This study investigates the relationship between legislative regulation and farmers' animal epidemic prevention input, generating critical evidence for refining China's animal epidemic control framework and advancing the high-quality development of animal husbandry.

Methods: Using balanced panel data from 13 main pig-breeding provinces in China from 2006 to 2022, this study employs the Feasible Generalized Least Squares (FGLS) method to: (1) evaluate the impact of legislative regulation on pig farmers' animal epidemic prevention and control input; (2) investigate the changes in epidemic prevention and control input of pig farmers of different scales and the differences in the effects of laws and regulations of different legal hierarchies, and (3) examine the impact of law enforcement practices on the effect of textual legislation.

Results: Legislative regulation significantly increases animal epidemic prevention and control input, with the strongest effect on medium-scale farmers and no effect on large-scale farmers. The input-enhancing effect varies across laws and regulations of different legal hierarchies, with descending order: local administrative rules, central-level administrative regulations and divisional regulations, and local regulations. Heterogeneity analysis indicates that this input-enhancing effect of legislative regulation is only pronounced in regions with higher law enforcement on animal epidemic prevention and control.

Discussion: This study can also provide important inspiration for other developing countries. Governments should intensify legal literacy initiatives, enhance farmers' regulatory awareness, implement regionally differentiated prevention measures, strengthen adaptive enforcement capacities, and ultimately realizing synergistic welfare gains across economic, biosecurity, and animal wellbeing domains.

KEYWORDS

legislative regulation, laws and regulations on pig epidemic prevention and control, animal epidemic prevention and control input, pig farmer, legal hierarchy, law enforcement practice

1 Introduction

“One world, one health”. The realization of biosecurity is the common vision of all countries in the world. Outbreaks of animal diseases such as bird flu, blue ear disease, and African swine fever pose a serious threat to animal husbandry development, food security, and human health (1, 2), and the possibility of animal transmission has not been ruled out in the global COVID-19 epidemic (3). In 2020, Xi Jinping, general secretary of the Communist Party of China Central Committee, stressed that “we should strengthen the protection of rule of law in public health and comprehensively intensify and improve the construction of relevant laws and regulations in the field of public health” at the 12th meeting of the Communist Party of China Central Committee for Comprehensively Deepening Reform. The construction of laws and regulations is an important aspect of the legalization of the prevention and control of animal epidemics (4), which institutionalizes the prevention and control policy of animal epidemics through legalization, making it more stable, continuous, and authoritative. Since the promulgation of the Animal Epidemic Prevention Law in 1997, hundreds of laws and regulations on animal disease prevention have been passed by the Chinese and provincial legislatures, and the Agricultural Law (amended in 2012), the Biosecurity Law (enforced in 2021), and the Animal Epidemic Prevention Law (revised in 2021) have come into force. These laws and regulations cover implementation measures, technical specifications, disease classification, legal liability, and other aspects and stipulate the responsibility of livestock producers for animal epidemic prevention. Farmers play a crucial role in biosecurity as they are the first to notice changes in the health or productivity of their livestock and are on the front lines of animal epidemic prevention (5), determining the effectiveness of the government’s animal disease control system. How to effectively motivate farmers to prevent and control animal epidemics? Particularly, can legislative regulation increase farmers’ animal epidemic prevention and control input (referred to as “animal epidemic prevention input”)? The scientific answers to the above questions have important theoretical value and practical reference significance for improving China’s animal epidemic prevention and control policies and promoting the high-quality development of animal husbandry.

The academic literature on the factors affecting the prevention and control of animal epidemics in farmers is abundant. There have been studies on various aspects of animal epidemic prevention and control, such as vaccine injection (6), decision-making on the resource treatment of sick and dead pigs (7), and the adoption of biological isolation measures such as bird control, rat control, vehicle disinfection, and personnel disinfection (8, 9). In addition, some scholars have measured farmers’ animal epidemic prevention behavior by the number of epidemic prevention measures (10) and medical epidemic prevention expenditure (11). The influence of farmers’ social and demographic characteristics such as gender, age, and education level and breeding characteristics such as income structure, breeding scale and breeding years, disease risk cognition, knowledge of epidemic prevention measures, and policy cognition on animal disease prevention and control behavior has been widely recognized (12, 13). Due to the large externality of animal disease prevention and control, government intervention

is of great significance for animal disease prevention and control (14). Using mathematical modeling, Tian et al. (15) found that increasing punishment could significantly increase the risk faced by farmers in concealing the epidemic and thus drive them to report the epidemic. Si et al. (16) believed that the withdrawal period supervision mainly forced farmers to regulate veterinary drug use by improving their perceived level of loss risk. Some scholars believe that regulatory policies and subsidy policies work in a similar way where they both improve production behaviors by affecting expected revenue and expected cost (17). The government can also reduce the time and labor costs required for farmers to verify disease conditions and implement animal epidemic prevention measures by publicly providing animal disease information and epidemic prevention technical assistance (18, 20).

It is worth pointing out that laws and regulations not only represent command-and-control government regulation but also provide a legal basis and guarantee for administrative penalties and rights compensation, which is the key to promoting the normalization of animal epidemic prevention and control. Qin (19) discussed the compatibility of the newly revised Biosecurity Law with existing legislation on animal disease prevention and control. Yu et al. (20) took the livestock forbidden area policy in the Regulation on the Prevention and Control of Pollution from Large-scale Breeding of Livestock and Poultry implemented by China in 2014 as a natural experiment and found that the policy improved environmental standards of the livestock industry and forced farmers to make resource utilization of livestock and poultry manure. However, there are still some scholars who doubt the importance of textual legislation in China (21) and believe that China’s textual legislation generally has the problem of “incomplete implementation” (22).

The existing research has important theoretical value and practical significance for improving the animal epidemic prevention behavior of farmers, but there is still some room for expansion. First, animal epidemic prevention behavior includes all aspects of preventing pathogens from entering and spreading among animals (23). However, existing literature pays more attention to one link of animal epidemic prevention or one specific epidemic prevention technology and lacks analysis of the comprehensive performance of various epidemic prevention behaviors of farmers under epidemic risk¹. Second, the existing literature has focused on the importance of animal disease prevention and control legislation but has not examined the incentive of animal epidemic prevention behavior from the perspective of legislation. In addition, domestic and foreign scholars mainly regard legislative regulation as command-and-control regulation. However, any law or regulation not merely

1 Measuring animal epidemic prevention and control behavior by a specific epidemic prevention and control measure may lead to two problems. First, in the process of raising pigs, farmers may have different priorities for animal epidemic prevention and control. For example, some regions pay attention to pre-prevention, while others focus on post-control. If a specific epidemic prevention and control measure is used to measure the behavior, it is likely to induce measurement errors. Second, when using whether to implement animal epidemic prevention and control measures to measure animal epidemic prevention and control behavior, it is easy to neglect the difference in the intensity of animal epidemic prevention and control.

contains a kind of policy tool but often comes with financial support and regulatory measures. Therefore, it is imperative to study the entire animal disease prevention and control policy as a system. Third, current studies related to animal epidemic prevention and control policies mainly focus on policy optimization, or the inclusion of this policy as a control variable. The heterogeneity of animal epidemic prevention and control policy on animal epidemic prevention and control behavior remains to be further explored.

Introducing the textual legislative regulation of pig epidemic prevention and control laws and regulations into the research of incentives for farmers' animal epidemic prevention and control behavior, this study employs the balanced panel data of 13 provinces with advantageous pig breeding in China from 2006 to 2022 to analyze the epidemic prevention promotion effect of legislative regulation, to investigate the changes in epidemic prevention and control input of pig farmers of different scales and the differences in the effects of laws and regulations of different legal hierarchies, and to examine the impact of law enforcement practice on the effect of textual legislation. This article aims to provide a reference for incentivizing farmers' epidemic prevention and control decision-making, improving the animal epidemic prevention and control policy, and boosting the high-quality development of animal husbandry. The 13 provinces with advantageous pig breeding are selected on the following basis. The 10 provinces (autonomous regions), namely, Sichuan, Hunan, Henan, Shandong, Hebei, Guangdong, Guangxi, Hubei, Jiangsu, and Anhui, accounted for approximately 65% of the total amount of pigs slaughtered² and are the major pig producing regions. Taking into account the spatial transfer trend of "southern pigs moving northward", the three provinces of Heilongjiang, Jilin, and Liaoning are included in the analysis.

2 Theoretical framework

In this study, legislative regulation refers to textual legislation regulation on pig epidemic prevention and control, which is measured by the number of laws and regulations on pig epidemic prevention and control. The Constitution of the People's Republic of China declares that the legislative body includes central and local levels. So, the laws and regulations on pig epidemic prevention and control refer to the laws and regulations promulgated by the central and local governments with the aim of promoting the development of the pig industry, ensuring public health safety and human health, and acting on the animal epidemic prevention and control behavior of pig breeding individuals or organizations.

The theoretical basis that the laws and regulations on pig epidemic prevention and control affect animal epidemic prevention input is mainly the externality theory. The externalities of pig epidemic prevention and control consist of two aspects: negative externalities of not implementing epidemic prevention and control measures and positive externalities of implementing epidemic prevention and control measures. In terms of negative externalities, if farmers do not take measures to prevent and control epidemics in pig breeding, it will accelerate the spread of the epidemic and cause public health and food health problems. All nearby residents,

including other pig farmers who actively prevent epidemics, will be affected by it. Thus, the epidemic prevention cost that should be borne by individual pig farmers is shared by all, and the marginal private cost is less than the marginal social cost. Due to economic factors, insufficient epidemic prevention often occurs. In terms of positive externalities, when farmers take animal epidemic prevention and control measures and get epidemic prevention benefits, surrounding farmers and even the whole society can enjoy the benefits of reducing animal epidemic risk for free, resulting in the phenomenon of "free riding". The marginal private benefit is lower than the marginal social benefit, which makes them less active in adopting epidemic prevention and control measures. Government intervention is an important way to solve the externality problem. Appropriate government policies cause the marginal private benefit and marginal social benefit to gradually converge to the equilibrium point, thus internalizing the externality (24).

There is no doubt that the law is irresistible and mandatory. According to this feature of law, many researchers have used the number of decrees issued by the government to measure compulsory government regulation (25, 26). Meanwhile, the law also plays a role in information dissemination and guidance (27). Legislative regulation can affect animal epidemic prevention input in two aspects: information dissemination and behavior deterrence. From the perspective of information dissemination, the central and local governments are the main information dissemination sources of laws and regulations on pig epidemic prevention and control. These laws and regulations are seen as important biosecurity information carriers (28), but their content is often obscure as formal institutional texts. According to compensatory control theory, when a law or a regulation on pig epidemic prevention and control is promulgated, farmers lack understanding and awareness of it, and their sense of control will decrease (29). Motivated to compensate for their sense of control, farmers will increase attention to information related to pig epidemic prevention and control laws and regulations (30). Information is the basis of risk judgment and individual decisions on preventive behavior (31). Information attention is an important component of farmers' information awareness, which emphasizes the subjective initiative of the information subject and directly affects farmers' decision-making (32). Therefore, the more attention farmers pay to epidemic prevention information, the more information they receive and search, the stronger their awareness of epidemic prevention, and the more consciously they will carry out animal epidemic prevention measures.

From the perspective of behavior deterrence, laws and regulations grant administrative punishment rights to the administrative department of the Ministry of Agricultural and Rural Affairs. They can impose administrative punishment such as warning, criticism, fines, revocation of licenses, and suspension of production and business on subjects who act inappropriately in pig epidemic prevention and control practices pursuant to the law, and they can even refer cases and personnel involved in disciplinary offenses to the judicial authorities to pursue legal liability,³ which

² <https://www.stats.gov.cn/>

³ Animal Epidemic Prevention Law of the People's Republic of China, <http://society.people.com.cn/n1/2021/0219/c1008-32031242.html>.

not only punishes violators but also has a deterrent effect on other farming subjects. Becker (33) believed that the certainty and the severity of punishment deter crime. The intensive promulgation of laws and regulations on pig epidemic prevention and control will not only convey to farmers that regulation on pig epidemic prevention and control is becoming more frequent but will also raise farmers' attention to the administrative penalties for epidemic prevention violations and make them perceive higher violation costs. These expected ex-post costs will act as ex-ante incentives (34), driving farmers to adjust their epidemic prevention decisions. As a result, the deterrent effect of pig epidemic prevention and control laws and regulations will be significantly enhanced, and pig farmers will be more likely to actively prevent the epidemic and increase epidemic prevention input. Based on this, the following hypothesis is proposed.

H: Legislative regulation on pig epidemic prevention and control can increase farmers' animal epidemic prevention input. Legislative regulation on pig epidemic prevention and control gradually balances the marginal private benefit and marginal social benefit of farmers, thus improving the enthusiasm for epidemic prevention and control and affecting their animal epidemic prevention and control behavior.

3 Sample and empirical strategy

3.1 Data sources

The data used in the study are a balanced panel data of 13 provinces with advantageous pig breeding in China from 2006 to 2022 ($N = 221$). We obtain data on animal epidemic prevention and control input of pig breeding from the National Compilation of Information on Cost and Benefit of Agricultural Products,⁴ with some missing data determined by interpolation. The laws and regulations on pig epidemic prevention and control data are derived from the PKULAW Database,⁵ Data on the education of rural households are from the China Population and Employment Statistical Yearbook.⁶ Data on the proportion of wages in the disposable income of rural households are from the China Yearbook of Rural Household Survey,⁷ the China Yearbook of Household Survey,⁸ and provincial statistical yearbooks. Data on pig market price, the number of employees in township animal husbandry and veterinary stations, and the number of pig breeding households are obtained from the China Animal Husbandry and Veterinary Yearbook⁹ and China Animal Husbandry Information Network.¹⁰ Data on slaughtered pigs, gross domestic product

(GDP), road, railway, inland waterway mileage, and per capita disposable income of rural households are from the China Statistical Yearbook.¹¹ We also make use of the Official Veterinary Bulletin¹² published by the Ministry of Agriculture and Rural Affairs of the People's Republic of China to find pig death and cull data. Sample data processing and regression analysis are performed using STATA 16.0.

Pig farmers, as an important subject in the development of the pig industry, are the primary object of scholars' research on the pig industry. The use of macro-level farm household data to measure the behavior and endowment characteristics of micro-farmers is well documented. Based on the number of farms of different scales in 30 provinces, the transition probability of pig breeding scale structure is measured (25). Rural residents' education level is usually used to characterize the education level of pig farmers when studying the determinants of pig industry development (35, 36). The number of research and development personnel at the provincial level can be an indicator of the scientific and technological input in the hog industry (37).

Compared with previous studies, the data used in the study are from open statistical data published by China's National Bureau of Statistics (CNBS). It reduces systematic errors and keeps the core indicators of the data unchanged and comparable (38). At the same time, the data cover 13 provinces with advantageous pig breeding in China, which can represent the overall level of legislative regulation on pig epidemic prevention and control.

3.2 Data and sample description

3.2.1 Dependent variable-animal epidemic prevention and control input (Input)

The expenditure on animal medical and epidemic prevention is part of the production cost. Although it increases the total cost, it can reduce the risk of epidemic in pigs and guarantee stable long-term returns for farmers (39, 40). Animal epidemic prevention and control behavior is a collection of a series of epidemic prevention measures. Therefore, when exploring the promotion effect of legislative regulation on animal epidemic prevention input, one should not only focus on farmers' input in a specific epidemic prevention measure but also examine the overall epidemic prevention input as a priority. In addition, protecting susceptible animals and treating sick animals are the most common biosecurity measures applied by farmers (23, 41), and the elements involved include vaccines, veterinary drugs, antibiotics, disinfection drugs, and other epidemic prevention and treatment substances, with inconsistent measurement units and product types. Using expenditure on medical and epidemic prevention as a measure of animal epidemic prevention and control input can avoid the aforementioned issue. The natural logarithm of the average expenditure on medical and epidemic prevention of pig farmers of different scales (scattered farmers and small-, medium-, and large-scale farmers) is chosen as a proxy variable for animal epidemic prevention and control input.

4 <https://www.agdata.cn/>

5 <https://www.pkulaw.com/>

6 <https://navi.cnki.net/knavi/yearbooks/YZGRL/detail?uniplatform=NZKPT&language=chs>

7 <https://navi.cnki.net/knavi/yearbooks/YRFTU/detail?uniplatform=NZKPT&language=chs>

8 <https://navi.cnki.net/knavi/yearbooks/YZZD/detail?uniplatform=NZKPT&language=chs>

9 <https://navi.cnki.net/knavi/yearbooks/YZGXM/detail?uniplatform=NZKPT&language=chs>

10 <https://caaa.cn/>

11 <https://www.stats.gov.cn/sj/ndsj/>

12 <https://www.moa.gov.cn/gk/sygb/>

3.2.2 Core independent variable-legislative regulation (LR)

Drawing on Mo et al. (42), the number of laws and regulations on pig epidemic prevention and control at the central level and the local level from 2006 to 2022 is obtained from the PKULAW Database, a professional authoritative database of policies and regulations in China. The laws and regulations are searched by “animal epidemic + pig” and counted yearly.¹³ On this basis, the number of laws and regulations at local and central levels retrieved in the corresponding year is summed up to obtain the number (flow) of new laws and regulations on pig epidemic prevention and control in province *i* in year *t*. Since a piece of legislation is valid for more than 1 year, to examine the level of legislative regulation on pig epidemic prevention and control in province *i* in year *t*, it is necessary to obtain the sum (stock) of all laws and regulations in force in province *i* in year *t*. Therefore, the natural logarithm of the sum of laws and regulations on pig disease prevention and control is taken as a proxy variable for legislative regulation.¹⁴

3.2.3 Control variables

Drawing on the existing literature on the influence of farmers’ animal epidemic prevention behavior, the following control variables are introduced.

3.2.3.1 Education (Edu)

More educated farmers tend to adopt production techniques and management practices that meet biosecurity and institutional needs (23). As there is a lag in the effect of education on farmers’ behavior (43, 44), a lagging

education is used for the regression¹⁵. Education is calculated by Equation 1.

$$Edu = \left(\frac{PP \times 6 + JP \times 9 + SP \times 12 + TP \times 16}{TTP} \right)_{t-1} \quad (1)$$

where PP is the rural population with primary education. JP is the rural population with junior secondary education. SP is the rural population with senior secondary education. TP is the rural population with tertiary and above education. TTP is the total rural population aged 6 years and above.

3.2.3.2 Income structure (IS)

Farmers’ income structure can reflect the degree of farmers’ dependence on pig farming, which is an important factor influencing farmers’ biosecurity behavior (18). Instead, Zhang and Zhang (45) argued that the higher the proportion of farm income, the more likely farmers are to increase epidemic risk exposure and adopt short-sighted behavior in management practices in pursuit of low-cost and high returns. The proportion of wages in the disposable income of rural households is chosen as a measure of income structure.

3.2.3.3 Pig market price (Price)

The market price is the wind vane of the development of the pig industry. In regions with higher pig prices, the economic development is relatively better, the comprehensive quality of farmers is higher, and they are more willing to comply with the requirements of animal disease prevention laws and regulations on epidemic prevention. In addition, given the positive correlation between pig prices and expected return, the higher the pig price, the higher the farmers’ expected return, and they will increase biosecurity precautions to prevent pigs from being infected with the virus (46). As short- and medium-term market prices are more exogenous than long-term market prices and may influence farmers’ behavior (25), the annual average pig price, derived from the monthly pig prices, is used as a proxy variable for the pig market price.

13 First, we do not only count the legislation for preventing and controlling specific pig epidemics, but we also keep laws and regulations that regulate the prevention and control of animal epidemics in general, as well as general laws and regulations referring to farm construction, implementation measure, and technical standard. The reason for this is that pathogens are transmitted by only a few routes, and it is possible to take effective action even if there is a gap in our knowledge of a certain epidemic. The animal epidemic prevention regulation can provide a reference for effective epidemic prevention. Second, according to the Legislation Law of the People’s Republic of China, the animal epidemic prevention legislation contains seven types of laws and regulations, including law, administrative regulation, divisional regulation, local regulation, local government rule, local normative document, and local working document. Finally, the main pig epidemics comprise foot-and-mouth disease, swine vesicular disease, swine fever, porcine reproductive and respiratory syndrome, porcine cysticercosis, anthrax, swine erysipelas, swine plague, and African swine fever.

14 Following the research logic of “having laws to abide by—strict law enforcement”, this study focuses on the effect of the text legislation of “having laws to abide by” on animal epidemic prevention and control input. The subsequent heterogeneity analysis emphasizes the further promotion effect of the integration of “having laws to abide by” and “strict law enforcement” on animal epidemic prevention and control input. So, only the cumulative number of laws and regulations is used as a proxy variable for legislative regulation.

15 The selection of the one-period lagged education level of rural households is grounded in three key considerations. Theoretically, education’s impact on agricultural behavior exhibits a temporal lag as farmers require time to internalize knowledge and apply it to production cycles. Education enhances cognitive skills, technology adoption, and risk management incrementally, aligning with the inertial nature of agricultural decision-making that relies on accumulated knowledge reserves. Empirically, data characteristics confirm sufficient temporal variation in education levels ($SD = 0.3930$, $CV = 5.08\%$, $Within\ SD = 0.2812$), while correlation analysis reveals the strongest association between epidemic prevention inputs and one-period lagged education ($r = 0.4449$ vs. 0.4422 current-period and 0.3853 two-period lag). Methodologically, the xtglm model comparison demonstrates superior performance of the one-period lag specification through minimized pseudo-information criteria (Pseudo-AIC = -855.30 , Pseudo-BIC = -780.54), consistent with established practices in agricultural productivity studies. This approach simultaneously addresses endogeneity concerns while capturing education’s delayed yet cumulative effects on behavioral change.

3.2.3.4 Economic development (Dgdp)

A sound financial guarantee mechanism is an important prerequisite for animal disease prevention and control work (47). The higher the level of regional economic development, the better the financial guarantee mechanism for animal disease prevention and control, and the more the government invests in the construction of animal disease prevention and control infrastructure, which is more likely to improve farmers' enthusiasm for disease prevention and control. This variable is characterized by the natural logarithm of the deflated GDP.

3.2.3.5 Scale breeding (Scale)

Scale breeding is the developing direction of the modern pig industry, which to a certain extent reflects the continuous improvement of the technical level (48). The higher the level of scale breeding, the higher the level of farming technology, and the greater the likelihood that farmers increase their efforts in epidemic prevention and control. From another perspective, the degree of pig scale breeding depends on the number of scale farms (households). The scale farms (households) have rich knowledge reserves of animal epidemic prevention and control, strong production capital, and high awareness of biosecurity, so they can establish a complete biosecurity system by introducing advanced epidemic prevention and control technology and equipment (49, 50). Following the practice of Yang and Wang (49), we use the percentage of scale farms (households) of 500 or more pigs slaughtered annually in the total number of farms (households) to measure it.

3.2.3.6 Convenience of technical service (Conv)

The impact of technical service support on the adoption of biosecurity behavior has also attracted scholars' attention (45). Improved transport conditions facilitate farmers to seek technical guidance from service organizations such as professional veterinarians, animal hospitals, or universities. Referring to Huang et al. (25), the natural logarithm of the sum of road, railway, and inland waterway mileage in each province is used to measure the variable.

3.2.3.7 Pig epidemic shock (Epi)

The more severe the epidemic shock, the more farmers invest in epidemic prevention (51). Empirical studies (49, 50) demonstrate that farmers' animal epidemic prevention and control behaviors exhibit time-dependent adjustments, primarily shaped by their retrospective evaluation of prior outbreak severity. The historical epidemic informs future biosecurity decisions. Pig epidemic shock is calculated by Equation 2.

$$Epi = (\ln(\frac{Death + Culling}{Slaughter} + 1))_{t-1} \quad (2)$$

where Death and Culling are the number of pig deaths and forced culls caused by nine pig epidemics, respectively. Slaughter is the number of pigs slaughtered. To reduce the impact of outliers and heteroscedasticity, we take the natural logarithm of this variable. The inclusion of the "+1" inside the logarithm in Equation 2 is a mathematical necessity to handle non-negative variables that can be zero, ensuring that the argument of the logarithm is always positive, thus avoiding the undefined value

TABLE 1 Descriptive statistics.

Variables		(1)	(2)	(3)	(4)	(5)
		N	Mean	SD	Min	Max
Dependent variable	Input	221	2.9571	0.3810	1.8798	3.7906
Independent variable	LR_stock	221	5.8553	0.8693	3.3322	7.2086
	LR_flow	221	3.9395	0.3139	3.0910	5.2523
Control variables	Edu	221	7.6682	0.4869	4.0853	8.6538
	IS	221	0.3358	0.1107	0.0960	0.5750
	Price	221	15.9675	5.7679	6.5008	37.2033
	Dgdp	221	9.3287	0.5904	8.0792	10.5182
	Scale	221	1.3900	1.5468	0.0220	10.2450
	Conv	221	12.1584	0.3488	11.4015	12.9531
	Epi	221	0.3755	0.7569	0.0000	4.7980

To ensure data comparability, all variables denominated in currencies in the table are measured in 2006 constant prices.

problem. By adding 1, we are effectively shifting the distribution of the variable slightly to the right, which does not fundamentally alter the relationship between the variables but allows us to use the logarithmic transformation for our analysis.¹⁶

The descriptive statistics of the variables are presented in Table 1.

3.3 Empirical strategy

To test the underlying relationship between legislative regulation and animal epidemic prevention and control input, the model is as defined in Equation 3.

$$Input_{it} = b_0 + b_1 LR_{it} + \sum_{i=1}^J w_j Control_{it} + \alpha_i + \gamma_t + \varepsilon_{it} \quad (3)$$

where *i* denotes the region, and *t* represents the year. Input denotes animal epidemic prevention and control input. LR denotes the level of legislative regulation (flow/stock) on pig epidemic prevention and control. Suppose the coefficient *b*₁ of the independent variable LR is significant and positive. In this case, it indicates that the promotion effect of legislative regulation on animal epidemic prevention and control input does exist. Control is a series of control variables affecting animal epidemic prevention and control input. α and γ are vectors of the province and year dummy variables that account for province and year fixed effects, and ε is the error term.

¹⁶ The "+1" transformation is a common practice in econometrics and statistics when dealing with non-negative data that includes zeros. It allows us to work with the logarithm of the variable while preserving the integrity of the data.

4 Empirical results and discussion

4.1 Effect of legislative regulation on animal epidemic prevention and control input

The results of the variance inflation factor (VIF) test for independent variables are shown in Table 2. It shows that the largest VIF value is 4.5400, much <10. Therefore, multicollinearity is proved to be weak. This has reached a basis for the next regression analysis.

There may be interactions between contemporaneous economic activity across regions, so we first perform a modified Wald test for between-group heteroscedasticity, a Woodridge test for within-group autocorrelation, and a Pesaran test for between-group contemporaneous correlation on the panel data. All three tests strongly reject the original hypothesis,¹⁷ indicating that the model developed has between-group heteroscedasticity, within-group autocorrelation, and between-group contemporaneous correlation. The feasible generalized least squares method (FGLS) is known to be more efficient than OLS in the presence of heteroscedasticity, and serial and/or cross-sectional correlation (52, 53). Therefore, we apply FGLS that allows different individual disturbance terms to be contemporaneously correlated and have different variances, while controlling for individual factors that do not vary over time and the effect of time trends.

Table 3 presents the results of the model. Whether LR is measured by the stock or the flow of the number of laws and regulations on pig epidemic prevention and control, its coefficients are all positive and statistically at a 1% confidence level. It indicates that legislative regulation can indeed increase animal epidemic prevention and control input, and the hypothesis is confirmed. This is consistent with previous studies. Both qualitative (39) and empirical analyses (51) show that government regulation has a positive impact on farmers' animal epidemic prevention and control behavior. First, the laws and regulations on pig epidemic prevention and control convey information on pig epidemic hazards, probability of occurrence, and epidemiological status to farmers, which will improve their perception of disease hazards. Second, the issue of pig epidemic prevention and control laws and regulations has aroused their information demand on epidemic prevention and control. Farmers take the initiative to acquire knowledge related to disease prevention and control technology, heighten awareness of disease prevention and control and production efficiency, and reduce uncertainty in behavioral decisions. In addition, the intensive introduction of laws and regulations on pig epidemic prevention and control has enhanced the deterrent effect on farmers' opportunism. It

17 The modified Wald test value is 80.19, the Woodridge test value is 10.817, and the Pesaran test value is 3.631 with a *p*-value of 0.0003.

TABLE 2 Variance inflation factor (VIF).

Variable	Dgdp	IS	LR_stock	Scale	Price	Conv	Edu	Epi	Mean
VIF	4.5400	3.4000	2.7600	2.1600	2.1100	1.8900	1.4700	1.2100	
1/VIF	0.2200	0.2940	0.3630	0.4620	0.4740	0.5300	0.6810	0.8270	2.4400

promotes the probability and penalty cost of farmers' violation of epidemic prevention and strengthens the punishment perception for epidemic prevention violations, which in turn motivates them to increase their epidemic prevention and control input.

As shown in column (2) of Table 3, income structure has a significant negative effect on animal epidemic prevention and control input. It suggests that the higher the proportion of wages, the less farmers rely on pig breeding and are prone to neglect pig epidemic prevention and control. Pig market price exhibits a positive effect on animal epidemic prevention and control input. Generally speaking, the higher the pig price, the stronger the motive of farmers to hide pig disease or secretly sell sick and dead pigs for higher pay. Such behavior may result in farmers facing huge fines

TABLE 3 Baseline regression.

Variables	(1) Input	(2) Input	(3) Input	(4) Input
LR_stock	0.2159*** (0.0283)	0.1431*** (0.0263)		
LR_flow			0.1462*** (0.0201)	0.0970*** (0.0099)
Edu		−0.0010 (0.0090)		0.0116* (0.0068)
IS		−0.4001*** (0.1328)		−0.1976* (0.1181)
Price		0.0058*** (0.0017)		0.0072*** (0.0012)
Dgdp		0.4909*** (0.1006)		0.9255*** (0.0785)
Scale		0.0305*** (0.0059)		0.0264*** (0.0051)
Conv		−0.1198 (0.0792)		−0.1033 (0.0631)
Epi		0.0116** (0.0049)		0.0122*** (0.0040)
Constant	1.3853*** (0.1349)	−0.9558 (1.3305)	1.7495*** (0.1005)	−4.5955*** (0.9625)
Province FE	Yes	Yes	Yes	Yes
Year FE	Yes	Yes	Yes	Yes
N	221	221	221	221

The standard errors are reported in the brackets. ***, **, and * represent significance at the 1%, 5%, and 10% levels.

or even criminal penalties.¹⁸ So, even with rising pig prices, farmers will not risk legal limbo. Areas with higher prices are relatively more economically developed, and farmers in those areas are better qualified and more willing to comply with the requirements of animal disease prevention laws and regulations on epidemic prevention. Economic development promotes farmers' input in animal epidemic prevention and control. The higher the level of economic development, the more funds for animal epidemic prevention and control, which can create favorable conditions for farmers to prevent and control animal epidemics. In areas with a high level of scale breeding, farmers are more aware of biosafety and will be more cautious in increasing epidemic prevention input to avoid diseases in their pigs. Farmers in areas with severe pig epidemic shock have strong epidemic risk perceptions and are more active in animal epidemic prevention and control.

4.2 Addressing endogeneity and robustness check

4.2.1 Addressing endogeneity

The possible existence of a “two-way causality problem” between legislative regulation and animal epidemic prevention and control input raises endogeneity concerns. Specifically, the intensity of legislative regulation on pig epidemic prevention and control may also be influenced by farmers' animal epidemic prevention and control behavior. The instrumental variable method (IV) is employed to address this issue (Table 4). We follow Zhang et al. (54) and use LR_iv as an instrumental variable for LR. LR_iv is measured by the mean value of legislative regulation intensity in neighboring provinces.¹⁹ As can be seen from columns (1) and (2), the F-value of the first stage is >16.38, and the *p*-value is 0.0002. It proves that this instrumental variable is valid and rejects the original hypothesis that there is no endogeneity problem. Column (2) demonstrates that the coefficient of LR_iv is still significant and positive, supporting that legislative regulation helps to enhance animal epidemic prevention and control input.

4.2.2 Robustness check

Robustness tests are conducted in the following aspects, and the specific results are shown in Table 4.

4.2.2.1. Replacing the dependent variable

The percentage of expenditure on medical and epidemic prevention in per capita disposable income of rural households (Input_p) is selected to replace the dependent variable.

4.2.2.2 Replacing the core independent variable

The natural logarithm of the number of legal entries on animal epidemic prevention and control (LR_c) from the China Legal Knowledge Database (CLKD)²⁰ is used as a proxy for legislative regulation.

4.2.2.3 The one-period lagged core independent variable

Given the lag of legislative regulation on pig epidemic prevention and control²¹, we take a lagged period for the core independent variable (LR_stock_1) in regression.

All the regression findings, which correspond to columns (3)–(5) in Table 4, are consistent with the claim that the strengthening of legislative regulation has significantly increased animal epidemic prevention and control input.

4.3 Differential performance of farmers of different farm scales

Studies conducted in the UK (55) and Indonesia (56) found that broiler production systems regulated under the same law differed in biosecurity performance, which attributed to differences in farm characteristics. So, how would the animal epidemic prevention and control behavior of pig farmers of different farm scales in China differ under the legislative regulation? Therefore, this study examines the changes in epidemic prevention and control input of large-scale farmers (annual slaughter of 10,000 head and above), medium-scale farmers (annual slaughter of 3,000–9,999 head), small-scale farmers (annual slaughter of 500–2,999 head), and free-range farmers (annual slaughter of 499 and below), separately.

Table 5 shows that the strengthening of legislative regulation has a significant contribution to the epidemic prevention and control input of medium-scale farmers, small-scale farmers, and free-range farmers, and the incentive effect decreases sequentially. However, legislative regulation has no significant impact on large-scale farmers. Medium-scale farmers may expand to large-scale farmers to implement the scale operation, and their requirement for animal epidemic prevention and control will be more stringent. Small-scale farmers are a high-risk sector (57, 58), they have less access to information and knowledge on biosecurity practices (56), and their biosecurity awareness and epidemic prevention capacity are yet to be improved. Motivated by animal epidemic prevention laws and regulations, they expand the scale of epidemic prevention input, which also fits the view that the smaller the farm scale, the more sensitive the farmers are to policy (25). Under continuous strengthening of legislative regulation on animal epidemic prevention and control, the stable policy expectation of free-range farmers with less fixed investment and free access to the market has

²¹ Policy implementation and production adjustments inherently involve time lags due to information transmission delays and adaptive decision-making processes. As policymakers signal regulatory changes (e.g., enhanced animal epidemic controls), farmers require time to interpret policy credibility, modify production techniques, and overcome the “wait-and-see” inertia characteristic of agricultural cycles. These real-world response delays align with the biological constraints of livestock production cycles and institutional friction in policy enforcement. In addition, this approach follows established econometric practice in panel data analysis (68, 71), where lagged variables help address potential reverse causality and temporal mismatch between policy signals and observable outcomes. Therefore, we conduct a robustness check by using the one-period lagged term of the core independent variable as a proxy for the core explanatory variable.

¹⁸ Source: <https://www.chinanews.com.cn/sh/2019/08-20/8931863.shtml>.

¹⁹ “Neighboring provinces” are defined as provinces bordering each other.

²⁰ <https://lawnew.cnki.net/kns/brief/result.aspx?dbPrefix=CLKD>

TABLE 4 Endogeneity and robustness check.

Variables	(1)	(2)	(3)	(4)	(5)
	First	Second	Replacing Y	Replacing X	Lagged X
	LR_stock	Input	Input_p	Input	Input
LR_iv	0.2157***				
	(0.0436)				
LR_stock		0.3423***	0.0157*		
		(0.0997)	(0.0090)		
LR_c				0.1429***	
				(0.0183)	
LR_stock_1					0.0271*
					(0.0164)
Constant			−0.4710***	0.1429***	−6.4388***
			(0.1013)	(0.0183)	(1.2802)
CV	Yes	Yes	Yes	Yes	Yes
Province FE	Yes	Yes	Yes	Yes	Yes
Year FE	Yes	Yes	Yes	Yes	Yes
N	221	221	221	221	208
R-squared	0.749				
F-value in the first stage	24.48				
p-value	0.0000				
Kleibergen-Paap rk LM statistic		13.665			
p-value		0.0002			
Kleibergen-Paap Wald rk F statistic		24.479			

Columns (1) and (2) are the results of IV. The standard errors are reported in the brackets. ***, **, and * represent significance at the 1%, 5%, and 10% levels. CV represents the control variables. Yes represents all the control variables are added to the model. The regression coefficients presented are significant at the 1% or 10% level, while the regression coefficients not presented are significant at either the 1%, 5%, or 10% level.

evolved into stable benefit expectations, which can encourage their animal epidemic prevention and control input. Large-scale farmers usually adopt standardized management modes, with normative biosecurity management and high levels of epidemic prevention and control, and thus, they are not greatly affected by animal epidemic prevention laws and regulations. However, the possibility of a higher probability of epidemic transmission in scale farms due to animals being housed nearby should not be ignored (59).

4.4 Effect of different legal hierarchy

The animal epidemic prevention legislation contains law, administrative regulation, divisional regulation, local regulation, local government rule, local normative document, and local working document. The association between legislative regulation and animal epidemic prevention and control input is expected to vary with the legal hierarchy. Laws and regulations of different legal hierarchies issued by different subjects have differences in liability, supervision, and applicability. Initiatives that draw on locally situated practices and knowledge of disease are more likely

TABLE 5 Differential performance of farmers of different farm scales.

Variables	(1)	(2)	(3)	(4)
	Large-scale	Medium-scale	Small-scale	Free-range
LR_stock	−0.0211	0.2315***	0.1932***	0.1381***
	(0.0277)	(0.0124)	(0.0227)	(0.0229)
Constant	−4.7344***	−4.8202***	−0.5003	2.6063*
	(1.3770)	(1.0165)	(1.4300)	(1.3395)
CV	Yes	Yes	Yes	Yes
Province FE	Yes	Yes	Yes	Yes
Year FE	Yes	Yes	Yes	Yes
N	221	221	221	221

The standard errors are reported in the brackets. ***, **, and * represent significance at the 1%, 5%, and 10% levels. CV represents the control variables. Yes represents all the control variables are added to the model. The regression coefficients presented are significant at the 1% or 10% level, while the regression coefficients not presented are significant at either the 1%, 5%, or 10% level.

to have an impact on biosecurity (60). Insight into the association between laws and regulations of different legal hierarchies and animal epidemic prevention and control input is, therefore, useful.

TABLE 6 Effect of different legal hierarchy.

Variables	(1)	(2)	(3)
	Central	Local regulation	Local administrative rule
	Input	Input	Input
LR_stock	0.1062*** (0.0271)	0.0036 (0.0118)	0.1720*** (0.0206)
Constant	−1.6370 (1.3702)	−3.9401*** (1.2040)	−1.3278 (1.1459)
Province FE	Yes	Yes	Yes
Year FE	Yes	Yes	Yes
CV	Yes	Yes	Yes
N	221	221	221

The LR_stock of columns (1)–(3) is central-level administrative regulations and divisional regulations, local regulations, and local administrative rules, respectively. The standard errors are reported in the brackets. ***, **, and * represent significance at the 1%, 5%, and 10% levels. CV represents the control variables. Yes represents all the control variables are added to the model. The regression coefficients presented are significant at the 1% level, while the regression coefficients not presented are significant at either the 1%, 5%, or 10% level.

Table 6 gives the results of the effect of central (central-level administrative regulations and divisional regulations), local regulations, and local administrative rules,²² respectively. Positive effects of these laws and regulations on animal epidemic prevention and control input are observed, with descending order: local administrative rules, central, and local regulations.

Although the legal hierarchy of local administrative rules is lower than that of local regulations, the epidemic prevention promotion effect of administrative rules is better than that of local regulations. Local administrative rules on animal epidemic prevention are normative documents promulgated by local governments according to the actual situation and needs (61, 62). They mainly reflect the local government's interests and preferences, and they may be implemented far more efficiently than local regulations in practice. It can be concluded that the local administrative rules on animal epidemic prevention and control have substantial incentive and constraint effects on farmers' pig disease prevention and control behaviors and improve their epidemic prevention and control input. Central-level administrative regulations and divisional regulations are the programmatic documents for local government and departmental administration at all levels (63), and they play an important guiding role in the management of local animal disease prevention and control. In the Internet era, one of the criteria for farmers to quickly select information is whether it is published by official media. When laws and regulations at the central level are introduced, heavyweight central and local official media will report and publicly interpret them several times. More importantly, Chinese citizens give priority to policy signals from the central government compared to those from local governments (69).

²² Local administrative rules here include local government rules, local normative documents, and local working documents.

4.5 Heterogeneity analysis on animal epidemic prevention law enforcement practices

Textual legislation and law enforcement practice are important guarantees for advancing law-based governance. In a situation where textual legislation in China is generally “not fully enforced” in practice (22), the incentive effect of legislative regulation on farmers depends not only on the promulgation of textual legislation but also on the intensity of actual law enforcement. Insight into the differences in the effect of legislative regulation on animal epidemic prevention and control input in areas with different intensities of law enforcement is, therefore, necessary.

According to the Administrative Measures for Rural Animal Husbandry and Veterinary Stations, the primary duties of township animal husbandry and veterinary stations include propagating and implementing guidelines, policies, laws, and regulations for the development of animal husbandry, and supervising animal epidemic prevention of units and individuals that engage in raising or marketing of animals, or production or marketing of animal products. The role of grassroots animal husbandry and veterinary stations in the construction of the “bottom of the net” is crucial in opening up the “last mile” of epidemic prevention.

In consideration of the data availability, the ratio of the number of employees in township animal husbandry and veterinary stations to the number of pig farming households was chosen to measure the intensity of law enforcement. Using the median of law enforcement intensity as the dividing criterion, we divided the sample into two subsamples: areas with greater than the median law enforcement (high law enforcement) and areas with less than the median law enforcement (low law enforcement). The statistical result shows that the average number of township animal husbandry and veterinary staff per 1,000 households in regions with high law enforcement is 11, which is higher than the sample with low law enforcement (3 persons per 1,000 households). Larger regions often possess more complex livestock supply chains, potentially diluting regulatory oversight through fragmented implementation. In the prevention and control of animal epidemic in China, resource-thinning risk in large-scale regions, where fixed enforcement resources are spread thinly across an extensive population, potentially undermines regulatory efficacy.

The impact of law enforcement on textual legislation is displayed in Table 7. In regions with high law enforcement, the coefficient of LR_stock is significant at the 1% level and is 0.1929, that is to say, for every 1% increase in the legislative regulation, farmers' animal epidemic prevention and control input increases by 0.1929%. Meanwhile, the coefficient of LR_stock is not significant in regions with low law enforcement. It suggests that the input-enhancing effect of legislative regulation is greater in regions with high law enforcement than in regions with low law enforcement. According to the *p*-value of the coefficient difference, the positive effect of legislative regulation varies significantly in regions with different law enforcement practices. This means that local law enforcement does play a key role in contributing to the effect of legislative regulation.

In addition, an intriguing finding is uncovered that provinces with a greater number of neighboring regions exhibit a

TABLE 7 Heterogeneity analysis.

Variables	(1)	(2)
	High law enforcement	Low law enforcement
	Input	Input
LR_stock	0.1929*** (0.0320)	0.0894 (0.0563)
Constant	2.3186 (3.0762)	9.0742*** (2.8575)
Province FE	Yes	Yes
Year FE	Yes	Yes
CV	Yes	Yes
p-value	0.0000	
N	119	102

The standard errors are reported in the brackets. ***, **, and * represent significance at the 1%, 5%, and 10% levels. CV represents the control variables. Yes represents all the control variables are added to the model. P-value of coefficient difference is obtained by the Chow test of interaction term model. The regression coefficients presented are significant at the 1% level, while the regression coefficients not presented are significant at either the 1%, 5%, or 10% level.

stronger synergistic effect between legislative regulation and law enforcement in boosting farmers’ animal epidemic prevention input.²³ Areas with numerous neighbors face higher cross-area epidemic risks (64), which may amplify the marginal effect of law enforcement due to inter-jurisdictional externality internalization. Stricter local law enforcement not only reduces local epidemic risks but also mitigates spillover impacts on neighboring regions, thus amplifying the overall benefits of law enforcement.

The basic guideline for constructing the socialist legal system with Chinese characteristics is to have laws to follow and to enforce them strictly. Our study indicates that local law enforcement on animal epidemic prevention and control does play an important role in the effect of textual legislation. The integration of textual legislation and enforcement practice on pig epidemic prevention and control further enhances the promotion effect of legislative regulation. Having laws to follow and enforce them strictly complements each other. Laws and regulations enacted through legislative activities are the basis and prerequisite for ensuring strict law enforcement in the process of the rule of law; strict law enforcement is the focus of comprehensively promoting the rule of law and is the key to maintaining the authority and dignity of the law (65, 66). Since the promulgation and implementation of the Animal Husbandry Law in 2005, China has used the rule of law to promote the transformation and upgrading of the husbandry industry. The National People’s Congress Standing Committee has twice carried out law enforcement inspections on this law. According to law enforcement reports, the animal epidemic prevention system is understaffed. After a new round of institutional reform, some county-level animal husbandry and veterinary departments have reduced their on-the-job personnel by more than 20%, and 65% of them have part-time jobs, even with only one animal husbandry and veterinary management

23 See Appendix for details.

personnel in some provinces.²⁴ It fully illustrates the imbalance and importance of the actual enforcement intensity of animal epidemic prevention and control.

5 Conclusion

This study examines the impact of legislative regulation on farmers’ animal epidemic prevention and control input. The main findings are as follows. First, legislative regulation has significantly increased farmers’ animal epidemic prevention and control input. Farmers of different farm scales respond differently to the legislative regulation, with medium-scale farmers inputting the most in epidemic prevention and control, followed by small-scale farmers, free-range farmers, and no significant response from large-scale farmers. Second, the effect of legislative regulation on animal epidemic prevention and control input varies noticeably due to different legal hierarchies: local administrative rules > central-level administrative regulations and divisional regulations > local regulations. Third, heterogeneity analysis reveals that the input-enhancing effect of legislative regulation has been further strengthened by the integration of textual legislation and enforcement practice. Specifically, the positive effect of legislative regulation is only significant in regions with high law enforcement.

Some policy implications are obtained. First, the government should increase the law popularization and enhance farmers’ awareness of legislative regulation. The government could fully understand the difficulties and realistic needs of farmers in receiving and understanding the laws and regulations on pig epidemic prevention and control through the visits and research activities of local animal epidemic prevention supervision functionaries and accordingly explore more feasible and diversified epidemic prevention and control mechanisms. In addition, it is necessary to adjust the intensity of legislative regulation based on farm scale. Second, the government should attach importance to the differentiated application of laws and regulations, assess pig development situation in various regions, and scientifically and accurately set up appropriate pig epidemic prevention and control laws and regulations. They should flexibly apply local regulations and formulate characteristic and differentiated animal epidemic prevention and control measures that are compatible with local epidemic prevention and control conditions, rather than a “one-size-fits-all” approach. Third, it is necessary to strengthen the implementation of animal epidemic prevention laws and regulations, improve the administrative capacity of supervising agencies for animal epidemic prevention and control, and standardize animal epidemic prevention law enforcement procedures, to achieve a two-pronged situation of “having laws to abide by” and “strict law enforcement” in animal epidemic prevention and control. To implement, joint regional animal prevention and control for neighbor-dense areas is also recommended.

There may be some limitations in this study. First of all, legislative regulation is a comprehensive concept covering legislation, law enforcement, and judiciary (67). Measuring it by textual legislation alone may underestimate its effect. Further research on building a complete legislative regulation index

24 Source: https://www.sohu.com/a/484491008_29936.

system for animal epidemic prevention and control might be more persuasive. Second, since the public data on medical and animal epidemic prevention expenditure are only available at the provincial level, there is a lack of micro-level farmers' motivation-decision response process. In the subsequent study, the latest literature and data will be tracked to supplement and improve accordingly.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

WL: Conceptualization, Data curation, Software, Validation, Writing – original draft, Writing – review & editing. JT: Conceptualization, Funding acquisition, Validation, Writing – review & editing.

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Appendix

TABLE A1 Impact of the number of neighbors on the moderating effect of law enforcement.

Variables	Input
LR_stock	0.3135***
	(0.0366)
Enforce	1.2828***
	(0.1909)
Neighbor	1.1988***
	(0.1249)
LR_stock×Enforce×Neighbor	0.1348***
	(0.0271)
Constant	0.5905
	(1.4991)
Province FE	Yes
Year FE	Yes
N	221

Enforce = 1 for provinces with high law enforcement intensity and Enforce = 0 for low-enforcement regions. Neighbor = 1 for provinces with a neighboring region count above the median and Neighbor = 0 otherwise. The standard errors are reported in the brackets. ***, **, and * represent significance at the 1%, 5%, and 10% levels. CV represents the control variables. Yes represents all the control variables are added to the model. The regression coefficients presented are significant at the 1% level, while the regression coefficients not presented are significant at either the 1%, 5%, or 10% level.



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Using a co-created checklist to improve on-farm biosecurity: an observational pilot intervention with pig farmers and livestock field officers in Sumbawanga, Tanzania

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The Tanzanian pig sector has the capacity to become market-oriented but it is constrained by significant factors like poor husbandry, management practices and disease, like African swine fever (ASF). Good biosecurity is essential to prevent, minimise or even eliminate biosecurity risks on farms. This study aimed to evaluate a pilot intervention based on an innovative, participatory approach to progressively improve biosecurity practices on small- and medium-scale pig farms in Tanzania. An observational study was conducted, where 30 farms were systematically monitored to assess the impact of using a co-created checklist on biosecurity compliance and production parameters. Livestock field officers (LFOs) were trained to provide technical guidance to farmers to implement the checklist. Focus group discussions (FGDs) were also conducted with LFOs, which were coded and thematically analysed. The median compliance score for biosecurity was significantly higher after the pilot intervention (20.0 out of 26 practices or 76.9%) compared to baseline (median of 5.50 out of 26 practices 21.2%). The time spent implementing biosecurity per sow (per day) increased from a median of 7.8–18.6 min by the end of the intervention. Pre-weaning mortality decreased from 28.6 to 25.0% and cost of antimicrobial use per sow (per month) was reduced by 57%. Meanwhile, FGDs revealed that the pilot intervention allowed LFOs to connect with farmers to provide services and collaborate with other LFOs to co-develop solutions for farmers. Despite an initial lack of trust, the relationships between LFOs and farmers were described to have positively transformed. These findings highlight the potential of using bottom-up approaches, combined with sensitisation and capacity-building, to address the unique challenges of biosecurity in low-resource settings.

KEYWORDS

biosecurity, pig production, Tanzania, participatory approach, co-creation, Theoretical Domains Framework, evaluation

1 Introduction

In Tanzania, about 537,000 (3.7%) households raise pigs and there are approximately 3.2 million pigs distributed throughout the country (1). Although the Tanzanian pig sector has huge potential to grow, the sector is challenged by inadequate extension services, poor husbandry and slaughtering practices, limited marketing infrastructure and diseases such as African swine fever (ASF), which represents one of the biggest constraints to pig farming in the country (2).

Many outbreaks of transboundary animal diseases (TAD) and/or production limiting diseases are spread by human actions and indirect transmission, such as the movement of infected animals to the farm, sharing infected breeding animals, using contaminated feed, and poor disposal of infected waste that may be spread by animals like birds and dogs (3). Good biosecurity is essential to prevent, minimise or even eliminate biosecurity risks on farms. For instance, it has been predicted that biosecurity implemented within 14 days of the onset of an outbreak can avert up to 74% of ASF-related deaths in pigs (4).

To curb production-limiting disease outbreaks and more broadly, improve pig farming in Tanzania, the Government of Tanzania and the Food and Agriculture Organization of the United Nations (FAO) have collaborated to implement the 'Progressive Management Pathway for Terrestrial Animal Biosecurity' (PMP-TAB), which is a stepwise approach to improve biosecurity along value chains and ultimately strengthen livestock systems, sustainably (5). The FAO defines the term 'biosecurity' as a strategic and integrated approach to analysing and managing risks to human, animal and plant life and health, and associated risks to the environment. It is a holistic concept that encompasses health policy, regulation and practices to protect agriculture, food and the environment from biological risks (6). The stepwise approach starts with identifying the practices, risks, interests and benefits; improving biosecurity at farm level and after successful piloting, the approach will be scaled up to other value chain nodes and/or livestock systems or to other geographic areas (5). This approach responds to the existing challenge of the poor uptake of recommended biosecurity practices by value chain actors, especially in low resource settings, such as rural areas in low and middle income countries (7).

In fact, improving biosecurity and its uptake is often challenging by due to the "knowledge-action gap." Consistent with broader findings on the intention-behaviour gap in the social and behavioural sciences, simply possessing knowledge of benefits or forming the intention to act are frequently poor predictors of whether farmers consistently implement biosecurity measures (8–15). Consequently, achieving sustainable improvements in on-farm biosecurity necessitates moving beyond solely information-based strategies towards approaches that emphasise meaningful stakeholder engagement and facilitate behavioural change. This has led to increased interest amongst researchers and practitioners to understand the enablers and barriers related to disease control and prevention measures using socio-psychological frameworks (16). These frameworks provide theory driven approaches for identifying and analysing factors influencing behaviours and to design intervention mechanism likely to drive behaviour change. Recognising the importance of systematically understanding these factors for biosecurity uptake in the Tanzanian context, this study incorporated established behavioural science frameworks to explore influencing factors.

This present study takes an innovative approach by utilising the Theoretical Domains Framework (TDF) (17) and the Capability, Opportunity, Motivation-Behaviour (COM-B) Model (18) as well as blended learning concepts with local-level public-private partnerships, participatory approaches, and financial incentives. At the centre at the field level is a partnership between public (government employed) and private livestock officers (LFOs) and farmers who together are responsible for progressively implementing biosecurity practices outlined in an agreed-upon checklist for pig farms. LFOs in Tanzania are trained (at certificate, diploma or bachelor level) in livestock health and production and are responsible for providing extension services at the ward-level.

The aims of this study were to (i) describe the local biosecurity situation of small to medium-scale pig farms in Sumbawanga Municipal Council (MC), Tanzania; (ii) identify perceived challenges, successes and opportunities related to LFOs engaging with farmers to implement the checklist in farms, (iii) identify factors influencing (enabling and disabling factors) farmers' decision-making around uptake of good biosecurity practices and (iv) investigate if a co-created checklist on biosecurity can lead to progressive improvements of biosecurity and production on pig farms.

2 Methods

2.1 Study design

An observational baseline-and-endline on-farm pilot intervention was performed from May to October 2024.

2.2 Study area

This study was conducted in Sumbawanga MC, the United Republic of Tanzania (hereinafter referred to as Tanzania), located in East Africa (Figure 1). Tanzania has 30 administrative regions comprising 184 districts, which are subdivided into divisions. Each division is made up of three to five wards, and the lowest administrative units are the villages. Sumbawanga MC is a district in the Rukwa region and has 19 wards. According to the latest available numbers from a study conducted in Sumbawanga MC in 2017, the population of pigs is 13,010 heads and the majority of the farmers are smallholders with less than 10 pigs per herd (19).

2.3 Study population

The study population included pig farmers in all 19 wards of Sumbawanga MC (Figures 1, 2). Reliable information about the number and location of pig farms (i.e., a reliable sampling frame) was not available prior to this study. Instead, pig farms were identified by LFOs based on local knowledge of their respective wards. Geospatial data related to the farm location was exported from Kobo Collect and imported into QGIS (version 3.26.1) to create maps. The maps were developed using data from GADM (20). A total of 30 farms were purposively selected for monthly



FIGURE 1

Map of the United Republic of Tanzania (to the district level) with the study location, Sumbawanga MC, indicated by the coloured area. Developed using GADM data. Created and reproduced with permission from GADM. Published under CC BY 4.0 licence.

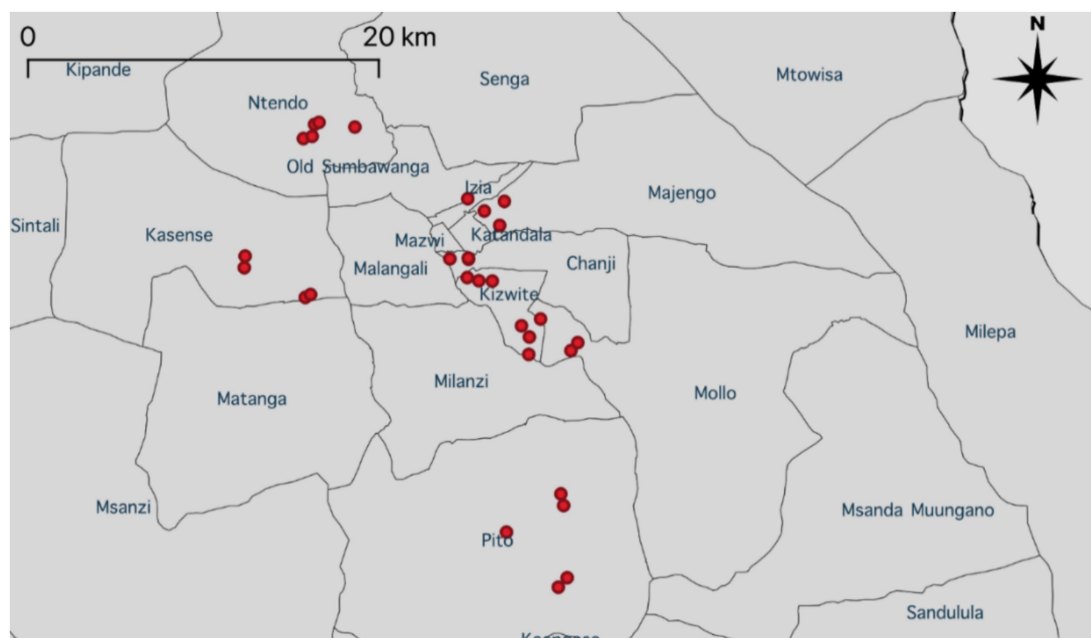


FIGURE 2

Partial map of the United Republic of Tanzania indicating locations of selected farms in red ($n = 30$) and their respective wards in Sumbawanga MC.

systematic monitoring of compliance with the checklist on biosecurity (21). In addition to systematic monitoring, LFOs could conduct voluntary audits to other farms on an ad-hoc basis, provided farmers were open to regular visits and implementing biosecurity measures.

2.4 Pilot intervention

The pilot intervention was centred around the implementation of a co-created checklist on biosecurity with small- and medium-scale pig farmers who expressed a willingness to participate. The checklist

outlining 26 biosecurity practices was previously designed using participatory methods, specifically co-creation (22). Each practice was further detailed with examples of indicators to make sure local stakeholders have a clear understanding of how it is implemented (for instance, the practice related to access control can comprise different elements like a fence, security guard, sign indicating restricted access, etc.). The checklist considers national legislation, includes indicators to measure compliance, and was validated by local stakeholders (specifically, farmers and farm labourers) and subject matter experts to ensure that it is practical and tailored for the local context of Sumbawanga. The methodology used to develop the checklist has been published previously in detail (21). Participatory approaches were utilised given the existing challenges of traditional approaches to implement biosecurity such as a mismatch between central and local levels, neglect of prerequisites, unsuitable standards for small-scale actors and a limited focus on biosecurity in low- and middle-income countries (23). In line with the participatory approach, the pilot intervention activities in Sumbawanga were co-created with relevant stakeholders.

A blended capacity building approach (using in-person and online training methods) was used to train 14 public and five private LFOs to provide extension to pig farmers to implement the biosecurity checklist on their farms (i.e., perform biosecurity audits of farms). All LFOs from Sumbawanga MC were invited to attend the training and participate in the pilot study as enumerators. Training was delivered on a monthly basis from April to July and the training sessions were 3 days in duration. LFOs were then encouraged to voluntarily visit and audit farms. During each audit by an LFO, farmers were encouraged to improve biosecurity by first focusing on a few self-selected practices from the checklist and then, progressively improving adoption of practices. Two sensitisation events were held in Sumbawanga to (i) foster collaboration and knowledge exchange between farmers and LFOs about the checklist and (ii) recognise participating farmers with higher levels of compliance.

2.5 Data collection

2.5.1 Quantitative

2.5.1.1 Systematic monitoring

Monthly systematic monitoring of compliance to the biosecurity checklist was undertaken on 30 pig farms between May and October 2024 (Figure 2). The checklist was uploaded as a survey to Kobo Collect (version 2024.1.3) to use for auditing purposes and a local enumerator was trained by the authors (AA and MH) to ensure a standardised approach to data collection. Kobo Collect is part of the Kobo Toolbox, an open-source and user-friendly platform for researchers looking to streamline their data collection process (24). Likewise, LFOs were requested to collect data using Kobo Collect during ad-hoc farm visits. Data collected from farms was based on direct observation by the enumerator(s), discussion with farmers and farm records (if available).

Each of the 30 selected farmers signed a community contract to signify their commitment to progressive adoption of biosecurity practices on farms and to consent to data collection. This approach leverages evidence from previous studies demonstrating the effectiveness of psychological commitment mechanisms in

encouraging adoption (25). Obtained data was treated anonymously without using any personal identifiable information. As an approved field project under government oversight and focused on implementation evaluation, no further oversight was indicated.

Data relating to compliance to biosecurity practices, production parameters, antimicrobial use and labour time were collected regularly. During the final phase of monitoring (i.e., endline), farmers were also asked survey questions about community and individual level factors influencing uptake of biosecurity practices informed by the Theoretical Domains Framework (TDF) (17). The TDF provides a systematic way to identify determinants of behaviour by synthesising constructs from multiple behaviour change theories into 14 domains. These domains map onto the broader COM-B model (18).

2.5.1.2 Ad-hoc monitoring

In addition to the systematic monitoring, public and private LFOs were also requested to document observations from farm audits carried out on an ad-hoc basis (hereinafter referred to as 'ad-hoc monitoring'). This ad-hoc monitoring was used as a proxy measure for assessing the effectiveness of LFOs to diffuse information about the biosecurity checklist to pig farmers. Those farm visits were entirely voluntary. LFOs were reimbursed 10 USD per month for the duration of the pilot period to cover for the costs of data usage. The study design for data collection is outlined in Figure 3.

2.5.2 Qualitative

Three rounds of focus group discussions (FGDs) were conducted in Kiswahili with the LFOs between May and October 2024 coinciding with the start, midpoint, and end of the intervention. Each round focused on different themes: the first explored perceived challenges and initial reactions to the checklist; the second focused on evolving dynamics, including solutions and adaptations; and the third captured successes, relationship changes, and opportunities for scale-up. Each FGDs lasted on average for 60 min. Discussions were recorded through comprehensive field notes in the local language (Kiswahili), which were then translated into English by the bilingual facilitators. In addition to FGDs, the checklist survey from the systematic monitoring included some open-ended questions on aspects such as feed formulation and disease symptoms; although qualitative in form, these responses were categorised and are presented as quantitative summaries in Table 1.

2.6 Data analysis

2.6.1 Quantitative

Data collected was exported from Kobo Collect and imported into Microsoft Excel. Descriptive analyses were conducted using Microsoft Excel (Version 2,411) by producing numerical summaries for continuous variables and frequency tables for categorical variables. Graphical figures (including histograms and boxplots) were also produced using Excel.

A baseline-and-endline approach was utilised, meaning the data collected at baseline during the first round of monitoring was compared with the data collected during the final phase of monitoring. Here, 'baseline' refers to the initial data collection intended for comparison after introducing the checklist. Where there were issues

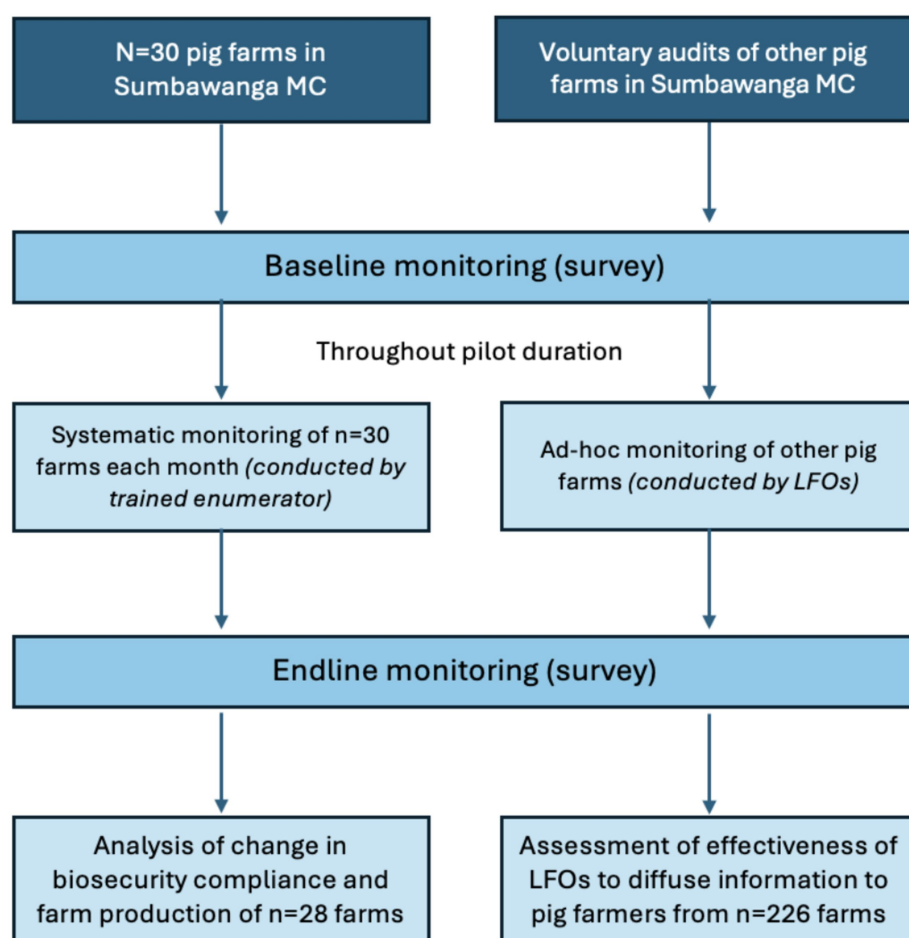


FIGURE 3
Study design used for data collection across pig farms in Sumbawanga MC.

with data incompleteness, missing values were imputed with the value from the subsequent or previous monitoring phase for baseline and endline responses, respectively. This approach was selected to avoid significant deletion of data.

To investigate significant differences in checklist compliance scores and other parameters of interest (e.g., mortality, live weight, time spent implementing biosecurity and antimicrobial use) from baseline to endline, statistical tests were conducted using Excel and Epitools (26). Data were tested for normality using a visual assessment of a histogram and the Shapiro–Wilk test. After test assumptions were assessed, the Wilcoxon signed rank test was used to assess differences in the average biosecurity compliance scores between farms (from baseline to endline), and McNemar’s test was used to assess the difference in proportions between compliance to specific biosecurity practices at the baseline and endline of the intervention. The Wilcoxon signed rank test was used to compare the medians of the production parameters due to violations of the normality assumption.

2.6.2 Qualitative

Qualitative data from the FGDs with the LFOs was analysed using an inductive and deductive approach. Initially, an inductive approach, based on thematic analysis (27), identified codes and

themes using Microsoft Word independently by AA and KL. Subsequently, a deductive approach was employed where relevant identified themes were mapped onto the COM-B framework (18). This mapping aimed to complement and provide deeper contextual understanding to the quantitative findings derived from the TDF informed survey. Due to time and resource constraints, rapid qualitative research methods were utilised. Findings from the FGDs were analysed in conjunction with the quantitative results collected through systematic monitoring of farms to contextualise findings from the quantitative analysis and increase validity.

3 Results

3.1 Systematically monitored farms

A total of 30 farms were visited by the enumerator as part of the systematic monitoring of the pilot project (Figure 2). Two farms dropped out due to going out of business and hence only 28 complete responses were included for further quantitative analysis (i.e., beyond descriptive analysis). Farms scheduled for systematic monitoring were located in nine out of 19 wards (47.4%) in Sumbawanga

TABLE 1 Sample characteristics at baseline of systematically monitored pig farms in Sumbawanga MC ($n = 30$).

Characteristic of farms/farmers	Categories	n (%)
Gender	Male	20 (66.7)
	Female	10 (33.3)
Level of education	Primary	11 (42.3)
	Secondary	5 (19.2)
	Diploma	7 (26.9)
	Bachelors	2 (7.7)
	Postgraduate	1 (3.8)
Herd size	1–5	6 (20.0)
	6–10	5 (16.7)
	11–20	11 (36.7)
	21–30	3 (10.0)
	31–40	3 (10.0)
	41–50	0 (0.0)
	>50	2 (6.7)
>12 months experience with pig farming	Yes	30 (100.0)
	No	0 (0.0)
Pig husbandry system	Confined (in pens) at all times	27 (90.0)
	Not confined (free-range)	3 (10.0)
Outbreak of ASF in the last 6 months before the pilot*	Yes	5 (16.7)
	No	25 (83.3)
Feed formulation**	Homemade feed ¹	30 (100.0)
	Commercial feed (pre-formulated or commercially formulated)	0 (0.0)
	Swill/kitchen scraps	9 (30.0)
	Other	0 (0.0)
Common signs of sickness as per farmers' reports**	Diarrhoea	50 (52.6)
	Fever	2 (2.1)
	Lameness	16 (16.8)
	Skin (dermatological) condition	27 (28.4)
Farm location by ward	Chanji	4 (13.3)
	Kasense	4 (13.3)
	Kizwite	3 (10.0)
	Lwiche	2 (6.7)
	Majengo	3 (10.0)
	Mollo	3 (10.0)
	Momoka	1 (3.3)
	Ntendo	5 (16.7)
	Pito	5 (16.7)

*Based on anecdotal reporting, not laboratory confirmation of ASF infection.

**Multiple responses were selected, therefore total >100%.

¹Homemade feed is formulated using commonly available ingredients like broken maize, maize bran, sunflower meal, soybean meal and pig premixes.

MC. Twenty farmers were male (66.7%), 10 farmers (33.3%) were female and all farmers had over 12 months experience with pig farming. Majority of farmers received primary (42.3%) or secondary education (19.2%). The characteristics of the farms and farmers are presented in [Table 1](#).

3.2 Baseline and endline analysis

3.2.1 Overall biosecurity compliance

The biosecurity compliance score at baseline was on average 6.0 out of 26 practices or 23.1% (median: 5.5 practices, minimum: 1.0,

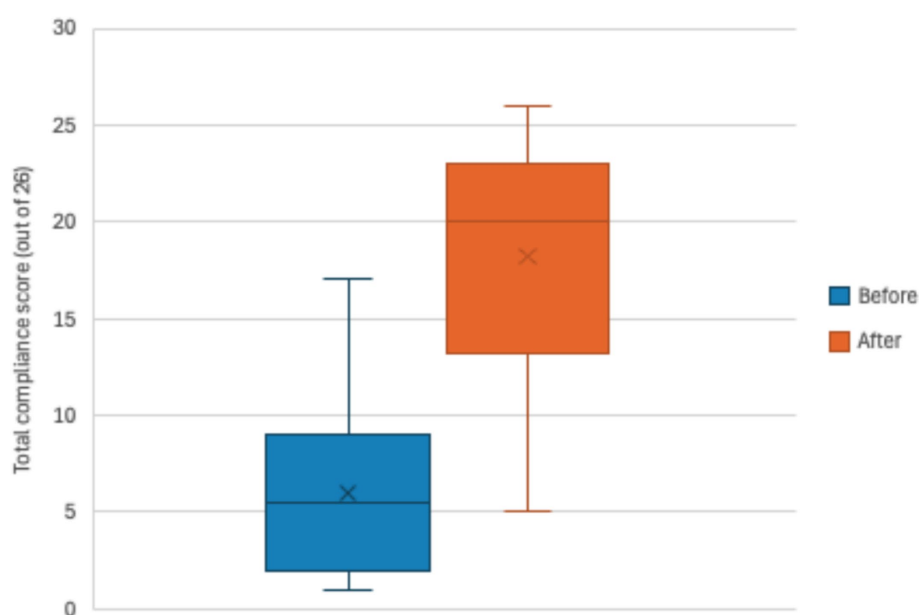


FIGURE 4

Total biosecurity compliance score (out of 26 practices) of pig farms at baseline (blue boxplot) and the endline (orange boxplot) of the pilot intervention ($n = 30$).

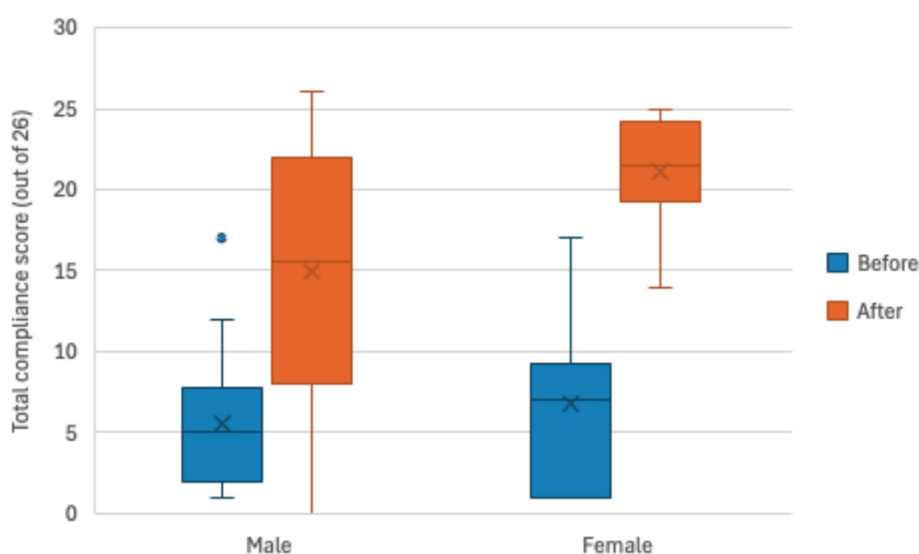


FIGURE 5

Total biosecurity compliance score (out of 26 practices) of pig farms by gender at baseline (blue boxplot) and after the endline (orange boxplot) of the pilot intervention ($n = 30$).

maximum: 17.0) and after the pilot intervention was 18.2 out of 26 practices or 70.0% (median: 20.0, minimum: 5.0, maximum: 26.0) (Figure 4). The median compliance score was significantly higher after the pilot intervention (20.0 out of 26 practices or 76.9%) compared to baseline (median of 5.50 out of 26 practices 21.2%) ($df = 27$, $z = 4.6$, effect size = 0.87, $p < 0.001$).

When looking at the change in compliance by gender of the farmer, at the baseline of the intervention, male farmers had an average compliance score of 5.6 out of 26 or 21.5% (median: 5.0,

minimum: 1.0, maximum: 17.0) while female farmers had an average score of 6.8 out of 26 or 26.2% (median: 7.0, minimum: 1.0, maximum: 17.0). At the endline of the intervention, male farmers had an average compliance score of 16.6 out of 26 or 63.8% (median: 18.5, minimum: 5.0, maximum: 26.0) while female farmers had an average score of 21.1 out of 26 or 81.2% (median: 21.5, minimum: 8.0, maximum: 25.0). On average, female pig farmers adopted more practices than male farmers (Figure 5).

TABLE 2 Compliance of farms to each good biosecurity practice included in the checklist at baseline and endline of the intervention ($n = 28$).

Good biosecurity practice	Baseline (before) n (%)	After n (%)	$\Delta\%$ (95%CI)	p -value*
1. No visitors without permission	6 (21.4)	25 (89.3)	61.9 (50.6, 85.2)	<0.001
2. Pigs confined at all times	25 (89.3)	28 (100.0)	10.7 (0.7, 22.2)	0.248
3. Changing area before pen	1 (3.6)	14 (50.0)	46.4 (28.0, 64.9)	<0.001
4. Change boots and overcoat before pen	1 (3.6)	15 (53.6)	50.0 (31.5, 68.5)	<0.001
5. Segregate by age group	19 (67.9)	28 (100.0)	32.1 (14.8, 49.4)	0.008
6. Good housing structure	6 (21.4)	10 (35.7)	14.3 (1.32, 27.25)	0.134
7. Good housing conditions	8 (28.6)	15 (52.6)	25.0 (9.0, 41.0)	0.023
8. Access to clean water	14 (50.0)	27 (96.4)	46.4 (28.0, 64.9)	<0.001
9. Animals handled with care	12 (42.9)	23 (82.1)	39.3 (21.2, 57.4)	0.003
10. Clean farm area	6 (21.4)	23 (82.1)	60.7 (42.6, 78.8)	<0.001
11. Washing hands	8 (28.6)	16 (57.1)	28.6 (11.8, 45.3)	0.013
12. Cleaning and disinfection	1 (3.6)	17 (60.7)	57.1 (38.8, 75.5)	<0.001
13. No swill feeding	20 (71.4)	28 (100.0)	28.6 (11.8, 45.3)	0.013
14. Protected feed storage	2 (7.1)	17 (60.7)	53.6 (35.1, 72.0)	<0.001
15. Use clean farm equipment	3 (10.7)	23 (82.1)	71.4 (54.7, 88.2)	<0.001
16. Safe reproduction practices	12 (42.9)	13 (46.4)	3.6 (−3.3, 10.5)	1.0
17. Safe and prompt waste disposal	6 (21.4)	26 (92.9)	71.4 (54.7, 88.2)	<0.001
18. Good drainage on-farm	3 (10.7)	14 (50.0)	34.6 (16.3, 52.9)	0.008
19. Safe carcass disposal	4 (14.3)	27 (96.4)	82.1 (68.0, 96.3)	<0.001
20. Purchase disease free, healthy pigs	1 (3.6)	4 (14.3)	10.7 (−0.7, 22.2)	0.248
21. Isolate new and sick pigs	1 (3.6)	15 (53.6)	50.0 (31.5, 68.5)	<0.001
22. No movement or sale of sick pigs	0 (0.0)	19 (67.9)	67.9 (50.6, 85.2)	<0.001
23. Report sick pigs to veterinary services	7 (25.0)	23 (82.1)	57.1 (38.8, 75.5)	<0.001
24. Training on good husbandry	0 (0.0)	26 (92.9)	92.9 (83.3, 100.0)	<0.001
25. Use record keeping system	2 (7.1)	22 (78.6)	71.4 (54.7, 88.2)	<0.001
26. Prudent use of veterinary drugs	0 (0.0)	12 (42.9)	42.9 (24.5, 61.2)	0.001

* p -values <0.05 are bolded.

3.2.2 Compliance by practice

All practices increased in compliance by the end of the pilot intervention (Table 2). The practices most frequently complied with at baseline were ‘keeping pigs confined at all times’ ($n = 25$, 89.3%); ‘segregating pigs by age groups’ ($n = 19$, 67.9%) and ‘no swill feeding’ ($n = 20$, 71.4%). The practices most frequently complied with at the end of the intervention were ‘keeping pigs confined at all times’; ‘no swill feeding’ and ‘segregating pigs by age groups’ (all $n = 28$, 100.0%).

The practices least complied with at baseline were ‘no movement or sale of sick pigs’; ‘training on good animal husbandry’ and ‘prudent use of veterinary drugs’ ($n = 0$). The practices least complied with after the intervention were ‘purchase of disease free, healthy pigs’ ($n = 4$, 14.3%); ‘good housing structure’ ($n = 10$, 35.7%) (see Figure 6) and ‘prudent use of veterinary drugs’ ($n = 12$, 42.9%).

The practices with the greatest improvement throughout the pilot duration were ‘training on good animal husbandry’ (92.9% improvement, $p < 0.001$), ‘safe carcass disposal’ (82.1% improvement, $p < 0.001$) and ‘safe and prompt waste disposal’ and ‘use of record

keeping system’ (71.5% improvement, $p < 0.001$). On the other hand, practices with the least improvement were safe reproductive practices (3.6% improvement, $p = 1.0$), ‘keeping pigs confined at all times’ (10.7% improvement, $p = 0.248$) and purchase of disease free, healthy pigs (10.7% improvement, $p = 0.248$). Practices including ‘pigs being confined at all times’; ‘segregate by age group’ and ‘no swill feeding’ all improved to 100% compliance by the end of the pilot intervention.

3.3 Production-related parameters

The findings of production-related parameters measured at the baseline and endline of the pilot intervention are summarised in Table 3.

3.3.1 Production parameters

The number of sows per herd increased from an average of 3.1–3.6 sows (median increased from 2.0 to 2.5) and the average litter size increased from an average of 6.9–7.0 piglets (median decreased from



FIGURE 6

Example of improved housing structure with baseline image of pig farm pen (left) and after image of improved pig farm pen with raised wall height and structure (right). Improvements include segregation of pigs through installation of a new wall, fixing defects in existing and increasing height of walls to prevent contact of animals between pens.

TABLE 3 Characteristics of participating pig farms monitored (at baseline and endline) as part of the pilot intervention in Sumbawanga MC ($n = 28$).

Characteristic	Baseline		Endline		p -value
	Mean (SD)	Median [Min, Max]	Mean (SD)	Median [Min, Max]	
Total biosecurity compliance score (out of 26)	6.0 (4.3)	5.5 [1.0, 17.0]	18.2 (6.4)	20.0 [5.0, 26.0]	<0.001
Number of sows per herd	3.1 (3.3)	2.0 [0.0, 14.0]	3.6 (4.3)	2.5 [0.0, 22.0]	0.73
Litter size	6.9 (4.1)	8.0 [0.0, 12.0]	7.0 (1.6)	7.0 [5.0, 10.0]	0.69
Herd size	18.0 (19.6)	13.0 [1.0, 91.0]	18.7 (23.4)	12.0 [1.0, 125.0]	0.93
Pre-weaning mortality (%)	33.6 (18.2)	28.6 [12.5, 75.0]	27.6 (8.6)	25.0 [14.3, 42.9]	0.21
Time spent implementing biosecurity per sow (minutes)	13.2 (12.2)	7.8 [2.0, 60.0]	28.0 (24.3)	18.6 [6.4, 120.0]	0.0037
Proportion of herd treated with antimicrobials per month (%)	58.4 (43.7)	68.1 [0.0, 100.0]	37.2 (42.8)	6.7 [0.0, 100.0]	0.08
Cost of AMU per month per sow in TZS (USD)	7,506.5/2.8 (6,863.3/2.6)	7,500.0/2.8 [0.0/0.0, 27,500.0/10.2]	5,035.9/1.9 (6,574.2/2.4)	3,333.3/1.2 [0.0/0.0, 30,000.0/11.1]	0.085

8.0 to 7.0 piglets). The herd size increased from an average 18.0–18.7 heads (median reduced from 13.0 to 12.0), while the average pre-weaning mortality had decreased from 33.6 to 27.6% (median decreased from 28.6 to 25.0%). None of these differences were found to be statistically significant (Table 3). Some variables such as litter size have a minimum value of 0, indicating that the farm did not have any pigs at the time of the visit, which could be attributed to factors such as a recent sale or a disease outbreak. Post-weaning mortality, average daily gain and age when sold for slaughter were also assessed, however, due to incompleteness of data and inadequate counts, no statistical testing was carried out.

3.3.2 Time spent implementing biosecurity

The (self-reported) time spent implementing biosecurity per sow and day increased from an average of 13.2–28.0 min or 71.8% by the end of the intervention (median increased from 7.8 to 18.6) (Table 3).

3.3.3 Antimicrobial use (AMU)

The average proportion of the herd treated with antimicrobials per month decreased from 58.4 to 37.2% animals by the end of the intervention (median reduced from 68.1 to 6.7%). The cost of AMU per sow (per month) also reduced from an average of 7,506.5 TZS

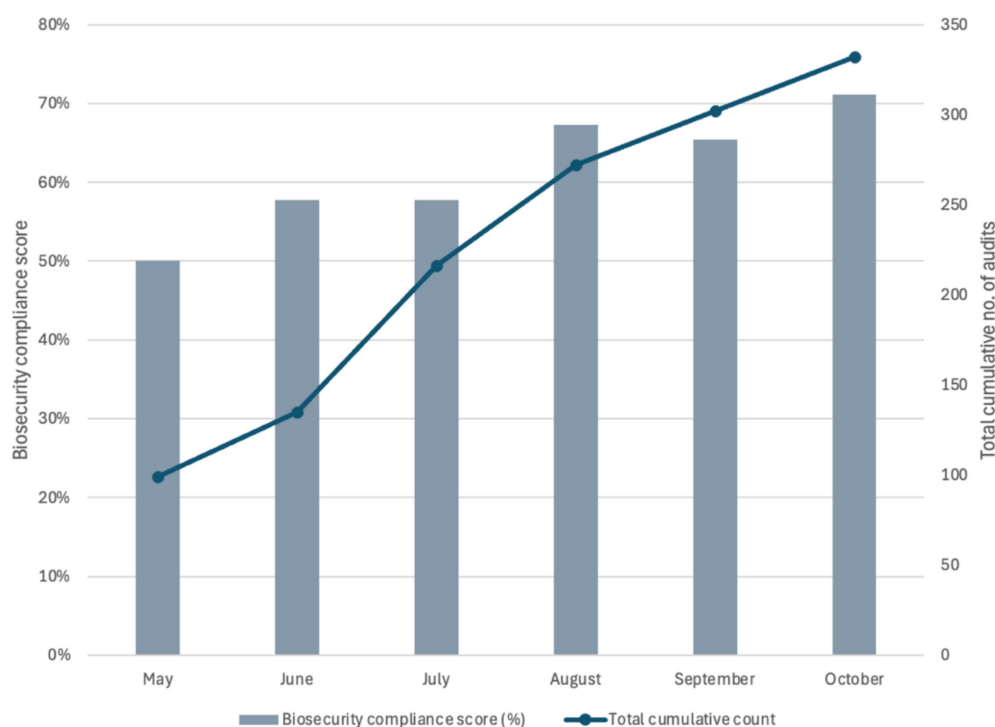


FIGURE 7

Bar chart displaying biosecurity compliance score (%) on primary y-axis and total number of audits completed (including revisits) through ad-hoc monitoring by livestock field officers in Sumbawanga MC by month (secondary y-axis).

(2.8 USD) to 5,035.9 TZS (1.8 USD) (median of 7,500 TZS (2.8 USD) to 3,333.3 TZS (1.2 USD)), i.e., a 57% reduction. However, both of these differences were not found to be statistically significant (Table 3).

3.4 Ad-hoc monitored farms

A total of 226 pig farms were visited (audited) as part of the ad-hoc monitoring conducted by LFOs in Sumbawanga MC from May to October 2024. The majority of farmers were male (61.1%) and most of the farms had a herd size of 1–5 pigs ($n = 100$, 44.2%) or 6–10 pigs ($n = 56$, 24.8%) while only four farms (1.8%) had >50 pigs in the herd.

Occasionally, farms were audited more than once by LFOs—a total of 69 farms (30.5%) were audited more than once and up to six times throughout the pilot duration. The average biosecurity compliance score at baseline was 13.0 out of 26 practices or 50.0% (median: 15.0, SD: 5.3, minimum: 1.0 and maximum: 26.0) and at the end of the intervention was 17.9 out of 26 practices or 69% (median: 18.4, SD: 5.7, minimum: 4.0, maximum: 26.0). The number of pig farms audited per month throughout the pilot duration remained consistent, with small increases in the number of farms audited in August and October (Figure 7). However, it should be noted that the cumulative number of audits depicted in Figure 7 includes revisits conducted on farms already visited by LFOs previously. The farms monitored on an ad-hoc basis by LFOs also showed a similar level of improvement as the systematically monitored farms. Additionally, a steady rate of submissions of auditing data was received throughout the pilot duration (Figure 7).

3.5 Behavioural factors

3.5.1 Enabling and disabling factors associated with implementing good biosecurity practices

A central aim was to understand the behavioural determinants influencing the uptake of biosecurity practices. This was approached through complementary methods during the final phase of monitoring (i.e., at the end of the intervention). Farmers were asked about community and individual level perspectives on barriers and enablers to implement the checklist. Using a survey informed by the TDF (17) (see Table 4). Further analysis has been provided in the Discussion section.

Behavioural determinants extracted during the final monitoring phase were further complemented and contextualised by conducting FGDs, asking LFOs about barriers to implement the checklist or to perform their role as agents of change to improve biosecurity amongst pig farmers. The themes and codes extracted from the FGD conducted are presented in Tables 5–7.

Themes, which emerged during the FGD in terms of challenges and barriers included a general lack of interest from farmers in the checklist approach to improve biosecurity; distrust of working with LFOs and the approach and factors that impacted capacity, such as lack of transport for LFOs to conduct farm audits (Table 5). FGDs were repeated at the midpoint of the pilot, where challenges related to capacity were reiterated. However, at this point, LFOs were able to offer solutions to the problems like simple flyers about the checklist or education (sensitisation) of the entire family or farm staff to enhance understanding. Despite the efforts, it was acknowledged that some capacity issues would only be resolved through

TABLE 4 Perceived influences on the implementation of biosecurity measures: farmer-reported barriers and facilitators assessed at intervention endline.

Good practice	Enabling factors	Disabling factors
1. No visitors without permission	• have some knowledge about why the practice is necessary • somewhat confident in ability but very motivated to continue or begin implementing • feel rewarded when the practice is implemented (by limiting entry of disease) • feel very positive and optimistic that the practice will benefit farming business	• sometimes forget about practice; only done out of habit sometimes • no resources available to implement practice (e.g., gates, fencing) • very uncommon practice amongst pig farmers in the community • only somewhat concerned when the practice is not implemented
2. Pigs confined at all times	• have some knowledge about why the practice is necessary and often done out of habit. Very motivated and very confident in ability to implement • feel rewarded when the practice is implemented (by protecting pigs against spreading disease) • feel very concerned when the practice is not implemented • feel very positive and optimistic that the practice will benefit farming business	• sometimes forget about practice • limited resources available to implement • very uncommon practice amongst pig farmers in the community • limited resources to build improved housing structures for pigs • people whose opinions farmers value are neutral or indifferent about the practice
3. Changing area before pen	• have extensive knowledge about why the practice is necessary and very confident in ability to implement. Never or rarely forget to implement • people whose opinions farmers value strongly encourage the practice • very motivated and feel rewarded implementing the practice (by reducing cost of treatments, preventing deaths, improving safety of pigs) • feel very concerned when the practice is not implemented	• limited resources, time and technical guidance available to implement the practice (build changing area) • very uncommon practice amongst pig farmers in the community
4. Change boots and overcoat before pen	• have some knowledge about why the practice is necessary and somewhat confident in ability to implement • somewhat motivated and feel rewarded when the practice is implemented (because farmers will protect their animals and not be responsible for spreading diseases in their farm) • feel very concerned when the practice is not implemented	• sometimes forget to implement the practice and only done sometimes out of habit • no resources available to implement the practice (i.e., buy an overcoat) • people whose opinions farmers value are neutral or indifferent about the practice • it is an uncommon practice amongst pig farmers in the community
5. Segregate by age group	• have some knowledge about why the practice is necessary and it is often done out of habit. Feel very confident and motivated in ability to implement • people whose opinions farmers value somewhat support implementing this practice • feel rewarded and somewhat optimistic when the practice is implemented (since the farmers is protecting their pigs, which will lead to a more profitable farm)	• sometimes forget to implement the practice • it is an uncommon practice amongst pig farmers in the community
6. Good housing structure	• have extensive knowledge about why the practice is necessary • feel very confident and motivated in ability to implement • feel positive when it is implemented and very concerned when it is not implemented • feel rewarded when implemented (by providing safety to animals and money that has been invested) • strongly encouraged by people whose opinions farmers value	• requires money and resourcing to have good housing structure
7. Good housing conditions	• have some knowledge about why the practice is necessary and somewhat confident in ability to implement • feel rewarded when implementing the practice • it is a common practice in the community and very motivated to implement	• limited resources available to implement • feel as though people whose opinions farmers value are neutral or indifferent about the practice
8. Access to clean water	• have some knowledge about why the practice is necessary and feel confident in ability to implement. Often it is done out of habit • have resources available to implement this practice; it is strongly encouraged and a common practice in the community • feel rewarded when implementing the practice (when pigs' water intake is high, and they are protected from water-borne disease) • feel very concerned when the practice is not implemented correctly	• no challenges reported

(Continued)

TABLE 4 (Continued)

Good practice	Enabling factors	Disabling factors
9. Animals handled with care	• have some knowledge about why the practice is necessary and feel confident in ability to implement • have resources available to implement this practice	• the practice is only somewhat supported in the community • feel only somewhat motivated to begin or continue implementing this practice and it is done sometimes out of practice • do not feel rewarded when implementing this practice
10. Clean farm area	• have some knowledge about why the practice is necessary • it is strongly encouraged and a very common practice in the community. Often the practice is done out of habit • very motivated and feel rewarded when the practice is implemented (since the farm area is clean and the pigs are therefore clean and healthy)	• sometimes forget to implement the practice • have limited resources available to implement the practice—it requires money to buy items to help with cleaning
11. Washing hands	• have extensive knowledge about why the practice is necessary • never forget about this practice and often done out of habit now • feel motivated and rewarded to implement practice (as it will save on money to treat pigs with less sickness and potentially make the farm more profitable)	• can be challenging to have financial resources to purchase or build handwashing station • uncommon practice amongst pig farmers in the community
12. Cleaning and disinfection	• have some knowledge about why the practice is necessary • feel very motivated to begin or continue following the practice and feel rewarded (since it will contribute to having healthier animals or avoiding disease which will make the farm more profitable)	• only somewhat confident in ability to implement the practice • sometimes forget to implement the practice and sometimes done out of habit • have limited resources to implement the practice • people whose opinions farmers value are neutral or indifferent about the practice and it is uncommon in the community
13. No swill feeding	• have extensive knowledge about why the practice is necessary • feel confident in ability to implement and is always done out of habit • strongly encouraged by people in the community and is common • feel rewarded (since pigs will be healthier)	• commercial or pre-formulated feeds can be expensive and so sometimes farmers may opt to feed kitchen scraps/leftovers
14. Protected feed storage	• have some knowledge about why the practice is necessary • feel motivated and rewarded when implemented (since it will mean diseases aren't spread to pigs via feed)	• sometimes forget to implement the practice and somewhat supported by others in the community whose opinion farmers value • uncommon practice amongst pig farmers in the community and only sometimes done out of habit • can be high cost associated with having to build protected area for feed
15. Use clean farm equipment	• have some knowledge about why the practice is necessary • feel motivated and rewarded when the practice is implemented (since farmers will prevent the entry and spread of disease within their pig farm)	• only somewhat confident in ability to implement the practice • sometimes forget to implement the practice • have limited resources to implement the practice • only somewhat concerned when the practice is not implemented
16. Safe reproduction practices	• have good knowledge about why the practice is necessary • feel very confident and motivated in ability to implement • resources are available to implement the practice • feel rewarded when the practice is followed (since diseases through breeding can be avoided but can still access/keep the breeds that the farmer wants)	• people whose opinions farmers value are neutral or indifferent about the practice and it is very uncommon in the community
17. Safe and prompt waste disposal	• have extensive knowledge about why the practice is necessary • feel very confident in ability to implement, motivated and as though all the resources are available • the practice is strongly encouraged by people whose opinions farmers value • it is a very common practice in the community and done out of habit	• challenges include area for solid waste disposal being is far away from pig housing and lacking equipment to transport waste (increasing manual labour required for this practice)

(Continued)

TABLE 4 (Continued)

Good practice	Enabling factors	Disabling factors
18. Good drainage on-farm	- have some knowledge about why the practice is necessary and feel somewhat confident in ability to implement - very motivated to implement and feel rewarded (as it contributes to cleanliness of the farm)	- sometimes forget to implement the practice and somewhat supported by others in the community whose opinion farmers value - limited resources available to implement the practice - only sometimes done out of habit
19. Safe carcass disposal	- have some knowledge about why the practice is necessary and feel very confident in ability to implement - very motivated and feel rewarded when implementing the practice - often done out of habit	- implementing the practice is somewhat supported by others in the community whose opinion farmers value - challenges include space on farm for appropriate carcass disposal
20. Purchase disease free, healthy pigs	have some knowledge about why the practice is necessary	- only somewhat confident in ability to implement the practice - have no resources available to support implementing the practice - people whose opinions farmers value are neutral or indifferent about the practice and it is very uncommon in the community - not at all concerned when the practice is not implemented or overlooked - practice is never done out of habit given the costs involved
21. Isolate new and sick pigs	- strongly encouraged by others in the community whose opinion farmers value - motivated to implement the practice and feel rewarded when it is implemented (since it will maintain healthy animals, prevent the spread of disease and increase productivity)	- have little knowledge about why the practice is necessary - only somewhat confident in ability to implement the practice - have limited resources to implement the practice and often difficult in small farm areas - it is an uncommon practice amongst pig farmers in the community and rarely done out of habit
22. No movement or sale of sick pigs	- feel somewhat motivated to implement the practice - the practice is somewhat commonly done - believe that the practice is somewhat important and they are somewhat optimistic that this practice would improve farming business	- have little knowledge about why the practice is necessary and do not feel confident in ability to implement - the practice is often forgotten about and never done out of habit - do not feel rewarded when the practice is adopted - the practice is somewhat discouraged by people in the community whose opinions farmers value - farmers feel as though they will lose money if they do not sell animals - challenges to implementing the practice are no compensation schemes for sick/dead animals
23. Report sick pigs to veterinary services	- have some knowledge about why the practice is necessary - feel motivated to implement the practice - very common practice amongst pig farmers in the community but only done sometimes out of practice	- only somewhat confident in ability to implement the practice - sometimes forget to implement the practice and report and only somewhat supported by others in the community whose opinions farmers value - do not feel rewarded when the practice is implemented because there is difficulty in getting LFOs to visit (poor availability) and trust issues to effectively treat animals
24. Training on good husbandry	- have extensive knowledge about why the practice is necessary - feel positive and very motivated to implement the practice since it increases their knowledge and awareness on issues related to farming	limited resources or opportunities to implement this practice (there is an issue to be involved in trainings frequently)

(Continued)

TABLE 4 (Continued)

Good practice	Enabling factors	Disabling factors
25. Use record keeping system	• have some knowledge about why the practice is necessary • feel confident in ability to implement, motivated and have the resources available • strongly encouraged by others in the community and somewhat common • feed rewarded when the practice is implemented (since it shows how their farm and business is growing)	• can be challenging and demanding but feasible with guidance and training
26. Prudent use of veterinary drugs	• feel very motivated and rewarded when the practice is implemented (since limiting unnecessary drug use saves on expenditure and improves animal health) • feel positive when the practice is followed	• have little knowledge about why the practice is necessary and only somewhat confident in ability the practice • sometimes forget to implement the practice and it is rarely done out of habit • people whose opinions farmers value are neutral or indifferent about the practice and it is very uncommon in the community • limited resources to build a proper place for storage of vet drugs • difficult to break out of habit to reach for vet drugs whenever something is wrong with animals

commitment of additional resource allocation by the local government (Table 7).

During the pilot design phase, remuneration from farmers had been proposed as a possible solution to sustainably overcome the capacity issues faced by LFOs (as opposed to resourcing directly provided through the project). This could be monetary or non-monetary remuneration. However, the discussions elucidated that public LFOs were unable to charge farmers for transport fees or provision of technical, extension advice. Remuneration can only be provided for provision of veterinary drugs or performing procedures and therefore, was not deemed to be a suitable solution for public LFOs in the long-term.

Conversely, themes highlighting successful factors and experiences included the level of awareness of biosecurity amongst farmers and readiness of some farmers to listen and work with LFOs. Opportunities included connecting LFOs to new farmers to provide services and collaboration between LFOs to co-develop solutions for farmers (Table 7). Despite the initial lack of trust, relationships between LFOs and farmers were described to have transformed throughout the pilot duration, highlighting another success of the pilot intervention. LFOs were trained on effective communication and using gender-sensitive approaches, which were reported to be factors contributing towards working more effectively in partnership with farmers. When LFOs advised farmers that the checklist had been proposed as a solution in response to hearing the problems of farmers, this improved trust and strengthened relationships.

4 Discussion

4.1 Compliance to biosecurity

This pilot intervention found there is utility of a co-created and progressive checklist approach—where farmers are encouraged to prioritise practices to implement based on risks on farms and

feasibility, rather than adopt several practices immediately—as evidenced by the significant improvement in the adoption of biosecurity measures at the farm level by the end of the intervention. The baseline biosecurity compliance was low at 23%, indicating substantial room for improvement. This finding aligns with a recent study conducted in the neighbouring region of Mbeya, Tanzania, where biosecurity was assessed on pig farms using a 25-item biosecurity checklist and the mean score for premises evaluated was 29% (3). The poor baseline biosecurity could reflect the low-input, low-output pig production systems in Tanzania (28); farmers' unwillingness to invest; limited understanding of how to encourage farmers to change and adopt measures and/or a lack of enforcement and/or technical knowledge from LFOs.

The approach used in this pilot intervention is tailored to the local context and has been validated by local stakeholders to ensure it is practical, feasible and affordable. Other approaches, like farmer field schools (FFS), which are also based on a bottom-up participatory approach (29) have reported similar findings, in that FFS participants also reported significantly higher infection, prevention and control (i.e., basic hygiene or biosecurity measures) scores compared with non-FFS respondents (30).

Female farmers were found to adopt a greater number of practices throughout the pilot. Female farmers in Sumbawanga MC have a prominent role in pig farming—many are sole keepers of pigs while others are joint owners with men in the household (31). Training or sensitisation around biosecurity should, therefore, explicitly target both men and women in the households to ensure equal participation and benefit from income-earning opportunities. To facilitate this, LFOs were trained in using gender-sensitive approaches when providing extension services to ensure that female farmers feel comfortable interacting with animal health services, who are responsible for creating opportunities for women to reduce their exposure risk (as female farmers are more often responsible for day to day tasks like, feeding, cleaning and looking after sick pigs which puts them at higher risks of being exposed to or inadvertently

TABLE 5 Themes and codes from FGDs conducted with LFOs during the training delivered in May 2024 in Sumbawanga MC.

Discussion question: <i>What was learnt about implementing the checklist in the first four weeks of the pilot?</i>			
Themes	Theme 1: Awareness of farmers	Theme 2: Farming systems	Theme 3: Distrust of the checklist/pilot
Codes	• Awareness of ASF • Farmers unaware of how checklist prevents pig diseases • Poor economic status hindering willingness	• Pig farming systems not aligned with checklist recommendation • Existing farming systems are porous and not secure	• False information about pig disease and deaths • Inconsistent commitment to visits from LFOs

Discussion question: <i>Were there any challenges or barriers to implementing the checklist?</i>			
Themes	Theme 1: Lack of interest in the checklist/pilot	Theme 2: Distrust of the LFOs; checklist/pilot	Theme 3: Factors impacting capacity
Codes	• Farm owner cannot be located • Farmers not readily available • Not willing to give up time • Farmers not interested • Lack of interest to submit information for audit • Farmers feel like time wasted	• Unwillingness to answer questions • False information provided	• Lack of transport for farm visits • Lack of internet to submit audit data • Persons looking after pigs have poor knowledge to support with checklist audit

Discussion question: <i>Were there any successful experiences? What did farmers respond best to?</i>		
Themes	Theme 1: Level of awareness and trust	Theme 2: Readiness to adopt measures
Codes	• Good level of awareness • Ready to listen to LFOs	• Readiness to adopt measures • Started keeping records • Improved cleaning of pens and farm environment • Use of protective gear • Improved drainage

TABLE 6 Themes and codes from FGDs conducted with LFOs during the training delivered in June 2024 in Sumbawanga MC.

Discussion question: <i>Is there any form of remuneration (monetary and non-monetary) made between LFO and farmers? Under which conditions are livestock field officers usually paid for extension services?</i>			
Theme	Theme 1: Types of remuneration between farmer and LFO	Theme 2: Remuneration for public servants	Theme 3: Remuneration for private business owners
Codes	• Remuneration based on conditions • Monetary remuneration for vet drug administration and procedures (by both public and private sector) • Non-monetary remuneration often in the form of gifts (chicken, eggs, groundnuts, maize)	• Unable to charge transport fees (despite demand for) • Advisory services provided free-of-charge • Charge no consultation fee	• Private employees set costs/fees based on item cost • Charge no consultation fee • Transportation and drug costs charged to farmer

Discussion question: <i>Why is remuneration for extension services provided in some instances and not others?</i>			
Themes	Theme 1: Factors related to the farmer	Theme 2: Factors related to the extension service provider	Theme 3: External factors
Codes	• Willingness of the farmer to provide remuneration • Level of hardship encountered • Demand of service • Level of expertise needed through services	• Level of technical knowledge • Ability to provide services remotely (e.g., telephone consultation) • Demand of service	• Recovering financial costs of drugs and equipment

TABLE 7 Themes and codes from FGDs conducted with LFOs during the midterm meeting in August 2024 in Sumbawanga MC.

Discussion question: <i>What are some of the challenges that you have encountered in the pilot so far?</i>			
Theme	Theme 1: Inconsistent sensitisation and interest	Theme 2: Factors impacting capacity	Theme 3: Solutions to challenges
Codes	• Inconvenience when farm workers are helping with audit (“starting from zero”) • Farmers only interested in the checklist when ASF occurring during the rainy season • Farmers with only a few animals not interested in long-term improvements • Farmers set in traditional way of doing	• Lack of transport allowance for farm visits • Lack of internet to submit audit data • Farmers have limited resources to implement certain practices	• Simple flyers to sensitise • Educate more widely • Commitment from local government through allocation of regular budget • Creativity or innovative solutions to convince those last farmers

Discussion question: <i>How has your relationship with farmers changed during the pilot? (How have you worked in partnership with farmers?)</i>		
Themes	Theme 1: Improved trust	Theme 2: Unintended benefits
Codes	• Checklist framed as a solution from the government, produced after listening to farmers • Trust improved after seeing benefits • Improved recognition of LFOs • Improved communication with farmers following training • Use of gender-sensitive approaches following training	• Gift giving from farmers and some payments • Word of mouth leading to more farmers requesting services • Checklist can be adapted for use in other livestock systems • Usually, only cattle and poultry farmers working with LFOs, now also pig farmers • Improved confidence amongst LFOs

transmitting disease) and empower them to improve their pig enterprises and profitability.

The findings of this study show that different changes in behaviour do not necessarily have the same benefits/enablers and/or barriers/disablers and as such need different approaches and interventions to result in implementation. Despite increased knowledge of biosecurity practices, data indicate that uptake remains low for certain practices, such as ‘good housing structure’, and ‘purchase of disease free, healthy pigs’. Our findings are consistent with other studies investigating biosecurity uptake (8, 32). Key barriers identified through the TDF-informed assessment to implementation of many practices in the checklist, related to the Opportunity component of COM-B, specifically Environmental Context and Resources (e.g., limited finances for housing improvements) and Social Influences (e.g., lack of supportive community norms or encouragement from peers for novel practices). Additionally, challenges related to Psychological Capability, such as Behavioural Regulation or Memory, Attention and Decision Processes, were evident in difficulties establishing habits or remembering specific steps for less routine practices. To address these challenges, in the pilot intervention, there was a great importance paid to practical solutions feasible to implement and progressively improve with existing resources or options to avoid prohibitive costs preventing farmers from investing in practices. Examples include use of jerry cans as handwashing stations (that might be upgraded to proper taps later as illustrated in Figure 8); use of detergent or other cost-effective alternatives like pure vinegar or whitewash (rather than cost-prohibitive disinfectants) and encouraging safe reproductive practices for farmers that cannot afford their own breeding boar and continue to borrow boars from neighbouring farmers. However, in the case of washing hands or safe reproductive practices, a substantial improvement in compliance wasn’t seen, which may simply indicate that a longer time frame is required to see changes.

However, this last practice on safe reproduction shows that despite efforts and focus on progressive improvements, there remain persistent

challenges beyond farmers’ control that can only be marginally improved without a more targeted structural support, such as availability of government-approved health breeding stock. Similarly, practices such as ‘no movement or sale of sick pigs’ faced Motivation barriers, particularly related to Reinforcement (farmers reported not feeling rewarded) (unlike for other practices, where this was frequently found to be an enabling factor) and negative Beliefs about Consequences (fear of financial loss). Addressing this likely requires intervention beyond individual farmer motivation, potentially through longer-term solutions like public-private-partnerships to fund compensation mechanisms that are implemented by the competent authority or insurance systems may be considered.

Sensitisation events were held to raise awareness so that practices were no longer seen as uncommon amongst farmers in the community, in addition to providing a space where farmers learn about behaviours of other farmers in their peer group and a sense of community is strengthened (33). It is well recognised that practices like the purchase of disease-free and healthy pigs are challenging, and to encourage adoption, partnerships with the private sector, for instance, the Tanzania Association of Pig Farmers (TAPIFA) could be explored during the scale up phase. TAPIFA is already responsible for sourcing pigs for its members and extending access of memberships to small-scale farmers may provide an opportunity or incentive to overcome this challenge. Approaches that take into consideration individuals’ preparedness for change as well as motivations for behaviours may provide a framework or catalyst for tangible change in biosecurity (15). Recognition of farmers that improve practices substantially can facilitate the identification of champions or ambassadors whose example can be used to foster changes among their peers.

Both farmers and public extension officers have transformed into agents of change in this pilot intervention through being empowered to contribute towards innovative solutions to improve biosecurity. In particular, LFOs have created an intent in farmers to change, adopt good practices and diffuse information while establishing



FIGURE 8

Examples of different types of handwashing stations implemented within participating farms. Both images also include signs with text in Kiswahili, which indicates to restrict entry of visitors onto the farm.

information-exchange relationships between themselves to share experiences and co-create solutions. In this way, LFOs are key to the success of such interventions and are likely to also function as scaling up agents on the ground in the next phase of the project.

4.2 Association between biosecurity, production and antimicrobial use

In terms of the impact on production, the study faced challenges to show a significant reduction in several parameters including pre-weaning and post-weaning mortality, as well as an impact on average daily gain after the intervention due to limited data and short timeframe of the study. However, the proportion of the herd treated with antimicrobials and the cost of antimicrobials also reduced by the end of the intervention (showing reductions of over 50% in both parameters). Given there was no statistically significant difference between baseline and endline, it is important to note that no inferential statements can be made about the effect of biosecurity on mortality rates or antimicrobial use and expenditure in this study and caution is recommended when drawing conclusions. This may be due to the study being underpowered to detect smaller effect sizes.

However, these findings do not indicate that there is an absence of an effect. It is likely that this is due to a type II error resulting in non-significance due to the study being underpowered, with a small sample size, which was a consequence of logistical challenges related to data collection when carrying out field studies in lower- and middle-income settings.

Few studies have been conducted to illustrate the association between biosecurity, production parameters and antimicrobial use in pig herds, and majority of these studies have been carried out in farrow-to-finisher production systems within high-income contexts

like the European Union (34–36). This means these findings are difficult to extrapolate to value chains in low-resource or lower- and middle-income settings where smallholder or small- to medium-scale farming systems predominate. It is essential to prioritise collaborations (for instance, through training LFOs to become enumerators) to improve the quantity and quality of data collected, thereby avoiding limitations in terms of data collected and inconclusive findings.

4.3 Sustainability

Sustainability remains a challenge for many community-level health interventions. Sustainability is defined as “the extent to which an intervention can deliver its intended benefits over an extended period after external support is terminated” (37). The approach used for this intervention was novel for development agencies in that no funding or resourcing was provided to directly support farmers to improve biosecurity on their farms, but instead, funding was only provided for capacity development through training (of LFOs as our scaling agents) and activities, such as sensitisation and recognition events. This was primarily to ensure farmers understood their individual responsibility to invest and improve biosecurity on their farms and to ensure sustainability beyond the pilot duration. This was achieved through using a specifically designed participatory, co-creation approach (22) where the local stakeholders are empowered to be the innovators of solutions.

Sustainability relies on the local government absorbing responsibility for implementation going forward, i.e., to continue at the local level and scale up, for instance, into surrounding local government administrations. The strong and steady support from LFOs doing voluntary farm visits as part of the ‘ad-hoc’ monitoring is a promising sign of commitment showing that the improvements are

not limited to the systematically monitored farms. In this regard, another positive sign is the fact that the local government in Sumbawanga MC has committed to integrate biosecurity audits into the regular work of LFOs after the pilot's completion, and regular budget allocation towards fuel allowance for LFOs to perform farm audits. This commitment came about after the data collected by LFOs during the pilot project were presented to the local government and it has been recognised that fuel allowance is necessary to sustain this level of extension service provision. This highlights the importance of working directly with and involving all stakeholders, including the local government. An alternative solution proposed to this challenge was remuneration for LFOs paid by the farmers. An example of the practical application of this solution was through organisation of farmers associations, where a proportion of member fees would be allocated to LFOs to ensure regularity of extension service provision, however, this was not implemented as part of the pilot intervention. Although this was suggested during the project design phase by farmers, FGDs conducted with LFOs revealed that public extension officers provide advisory services free-of-charge and monetary remuneration can only be provided for veterinary drug administration and procedures, rendering this solution unfeasible in the context of Tanzania, unless a cost-recovery policy reform is implemented.

It is important to note that a significant increase in compliance resulted following a sensitisation event, where farmers were recognised for their participation and awarded prizes in the form of biosecurity equipment (such as buckets, brushes, boots and overcoats) based on their compliance levels. The authors acknowledge that this raises the question of sustainability of the intervention as there is a risk that once incentives are discontinued, farmers will not continue implementing biosecurity on their farms. On the other hand, it is possible that farmers may have improved their compliance due to social pressure for recognition by their peers and local authorities; prioritised improvements from early in the pilot being completed, or genuine buy-in and understanding of potential benefits. In the future, alternative considerations for incentives may include social incentives or incentivising with prizes that are unrelated to biosecurity to avoid bias and promote sustainability or exploring partnerships with the private sector to sponsor such prizes in exchange of publicity.

4.4 Strengths and limitations

This study had several strengths and limitations. In particular, the representativeness of the sample of farms is a limitation since non-probability-based sampling was used, due to the list of farms initially provided from the last agricultural census being inaccurate and outdated. When the existing list of pig farmers was validated, many were not present anymore, which is likely related to the fast-changing nature of pig production systems in the selected area. The purposive sampling method may also contribute towards selection bias and impact findings and the sampled farmers should not be considered representative of all the pig farmers in the district. However, to minimise the risk of LFOs proposing farmers that would be more willing to participate, they were blinded from the pilot intervention details before being requested to share sampling frames of pig farms in their respective wards.

More generally, cross-sectional studies are conducted to evaluate the level of biosecurity while evaluation of measures to improve biosecurity are less frequently found in the literature. One exception to

this is the farmer field school initiatives (30) although their application in Tanzania is not well documented. Moreover, given the challenges and complexity of performing studies targeting small- and medium-scale farms (such as poor quality and availability of farm-level data and remote study locations) means that the existing evidence-base is limited and renders this study a valuable contribution in the authors' view. While some of the data collected at the farm-level was from records, good record-keeping was a new concept introduced to the majority of the farmers during this study, and as such, the data collected may suffer from recall bias or obsequiousness bias so findings should be interpreted with some caution. Considering that there are multiple components to the pilot possibly contributing towards increasing adoption of biosecurity measures (sensitisation, provision of incentives, training etc.), without adjusting for confounding, we cannot make inferences that the improvements in on-farm biosecurity observed were due to the checklist only. Observational studies suffer from difficulties in establishing causality, lack appropriate control groups and prospective follow-up periods. When considering conducting an interventional trial, factors such as withholding biosecurity-related extension services were considered ethically unjustifiable in this setting.

5 Conclusion

This study demonstrates the potential of a bottom-up approach and several innovative methods, including participatory approaches, blended learning concepts, local-level public-private partnerships and behavioural science to successfully improve biosecurity at farm level. Additionally, these methods can foster trust and strengthen partnerships between farmers and public and private LFOs, which is a crucial prerequisite for achieving meaningful impact on the ground. While the study suggests that improving biosecurity may contribute to reduced mortality, antimicrobial use and improved productivity on pig farms, further research is needed to investigate its long-term impacts, scalability, and sustainability in diverse farming contexts.

As a pilot study, our primary aim was however to test the practical feasibility of these methods, and the insights gained are valuable for other researchers in this field.

The government of Tanzania is currently in contact with FAO to prepare the scaling to neighbouring districts within Rukwa region. Beyond this current effort, we hope that this study inspires others to adopt more participatory approaches, which are applied to various settings and production systems. Strengthening record keeping and data collection is crucial to generate adequate evidence of the benefits resulting from improving biosecurity and can be used to provide motivation to farmers to change their behaviour and promote investments. The findings of this pilot intervention will also be shared to local and national stakeholders to present the case to formally adopt and progressively scale up implementation to other value chain nodes, livestock systems or geographic areas of Tanzania.

By prioritising locally tailored, feasible practices, farmers were empowered to adopt sustainable measures that may improve productivity and reduced morbidity and mortality. The findings highlight the potential of using participatory and bottom-up approaches, combined with sensitization and capacity-building, to address the unique challenges of biosecurity in low-resource settings. Despite limitations such as sample size and resource constraints, this

pilot intervention underscores the importance of integrating farmer-led initiatives with local government support. It lays the groundwork for scaling the intervention across other value chains and geographic locations as per the FAO-PMP-TAB framework.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Author contributions

AA: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Visualization, Writing – original draft. KM: Data curation, Investigation, Project administration, Writing – review & editing. HM: Project administration, Supervision, Writing – review & editing. DT: Conceptualization, Writing – review & editing. AB: Methodology, Writing – review & editing. UC: Conceptualization, Writing – review & editing. MM: Writing – review & editing. MH: Conceptualization, Data curation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The authors declare that no Gen AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2025.1567072/full#supplementary-material>

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Methods for assessing efficacy of cleaning and disinfection in livestock farms: a narrative review

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Cleaning and disinfection (C&D) procedures are essential components of farm biosecurity, aiming to reduce microbial load and eliminate the pathogenic microorganisms in livestock farms facilities. This review examines the various methods used to assess the effectiveness of both cleaning and disinfection, exploring their strengths, limitations, and optimal-use scenarios. For cleaning evaluation, common methods include basic visual inspections, ultraviolet (UV) fluorescence, adenosine triphosphate (ATP) bioluminescence, rapid protein tests (RPT), redox potential, and microbiological swabbing. However, visual inspections and UV fluorescence alone provide only qualitative insights. ATP offers quantitative data, though the accuracy can be influenced by the presence of detergents or disinfectants, requiring careful calibration. Additionally, ATP and RPT testing demands standardization to ensure consistent results. A new promising redox method is fast and more accurate, however still has limited field applicability. Microbiological methods, while highly accurate in detecting microbial contamination, are resource-intensive and therefore not in frequent use for routine evaluation of the cleaning procedures. For assessment of disinfection procedures microbiological tests such as colony-forming unit counts on agar plates, as well as the use of selective media for target microbes or hygiene indicator organisms are more appropriate than non-microbiological tests as they offer direct evidence of microbial elimination. However, these methods can be labor-intensive and time-consuming. Molecular methods can be powerful tools in detecting hard-to-culture organisms, however, are more expensive and require specialized equipment. Given these challenges, our study recommends a comprehensive C&D evaluation protocol, incorporating multiple methods tailored to the farm's specific biosecurity needs and epidemiological context. This integrated approach improves the reliability and efficiency of C&D monitoring, ensuring robust hygiene management in farm settings.

KEYWORDS

ATP, ACP, farm hygiene, efficacy of C&D, evaluation of cleaning, evaluation of disinfection, microbiological methods

1 Introduction

Hygiene monitoring of farm animal facilities encompasses the observation and evaluation of farm cleanliness. Through this, potential on-farm contamination sources can be identified, and likewise, possible lapses in the implementation of the cleaning and disinfection (C&D) procedures can be identified (1, 2). Inadequate hygiene practices can be associated with

various risks and negative outcomes, including disease outbreaks, compromised animal health and welfare, the spread of zoonotic pathogens, economic losses, contamination of animal products, increased antibiotic use, environmental pollution, and finally damage to the industry's reputation (3–5). Thus, the importance of hygiene management in livestock has been steadily increasing, particularly within the broader context of farm biosecurity (1, 2, 6).

From a biosecurity perspective, proper C&D procedures are important to reduce or eliminate organic and microbial load at the farm level to minimize the infection pressure (3, 7, 8). As was confirmed in previous studies the implementation of proper C&D measures in pig and poultry housing reduced pathogens like *Salmonella* spp. and *Campylobacter* spp. (9–13). Additionally, maintaining adequate hygiene on farms also mitigates the risk of colonization by antibiotic-resistant bacteria (1, 14). At the same time, it was emphasized that inadequate C&D procedures result in residual organic material, which can reduce the effectiveness of disinfectants (15) and create conditions favorable for microbial persistence, including the development of protective matrices that shield pathogens from inactivation (16). Considering that C&D incurs expenses related to working time, purchase of equipment, and consumables, improper C&D can result in a wasteful expenditure of resources (17). That is more concern, that even when C&D procedures are properly implemented, using the correct disinfectant, concentration, contact time, proper temperature, and applied to pre-cleaned surfaces, the elimination of all pathogens cannot be guaranteed (18). This is due to factors such as the formation of biofilms, the development of resistance to cleaning chemicals and/or disinfectants, or the presence of difficult-to-clean or disinfect locations (such as drains and lairage pens' cracks and holes) (19, 20). Therefore, assessing the efficacy of C&D procedures in livestock facilities is essential to confirm the successful elimination or inactivation of pathogens (21–23).

Generally, checking the efficacy of C&D procedures in animal housing facilities can be carried out as part of routine practices, periodically (scheduled audits), or as emergency response. However, the evaluation of C&D practices on animal farms is often not conducted at the recommended level due to time constraints, limited awareness of available evaluation methods, and insufficient understanding of hidden risks (24, 25). A similar tendency was identified in a study assessing the application of C&D evaluations procedures on pig farms across 10 European countries revealed that only 1% of farms regularly assess the effectiveness of their C&D measures (e.g., with hygienogram) (23). This finding can suggest that farmers, workers, and occasionally veterinarians may lack adequate awareness of proper methods for the evaluation of C&D procedures.

While there are different methods available for evaluation of C&D practices in human hospitals and food industries (17, 26–28), however in farm environments it is still complicated due to several key factors. These include the variability of farm settings, the abundance of organic matter, and the challenges in consistently applying and evaluating the C&D practices across different types of equipment, surfaces, and animal housing areas. Therefore, the aim of the present review is to analyze and evaluate the various methods used to assess the effectiveness of C&D practices/measures and to translate these findings into practical recommendations for animal health professionals /farmers.

2 Materials and methods

The literature search for this narrative review was conducted in two rounds, a first one from October 2023 to December 2023 and a second one from May 2024 to June 2024. For both, three primary databases, PubMed, Web of Science, and Scopus, were selected to ensure comprehensive coverage of relevant scientific publications. The search was based on a set of carefully chosen keywords related to the assessment of C&D procedures and farm hygiene, including terms such as: “evaluation of cleaning”; “evaluation of hygiene”; “monitoring of hygiene”; “hygiene monitoring on farms”; “hygiene control”; “hygiene in stables”; “hygiene of surfaces”; “evaluation of disinfection”; “efficacy of disinfection”; “effectiveness of disinfection”; “effectiveness of C&D”; “efficiency of C&D”; “cleaning and disinfection efficacy.”

A thorough evaluation of the selected papers was performed by two co-authors in accordance with the inclusion and exclusion criteria outlined in Table 1.

From all relevant publications, a comprehensive review was conducted to extract applicable data on current and potential methods for evaluating the effectiveness of C&D procedures. This systematic analysis yielded key insights into the various approaches/methods used to assess the efficacy of hygiene interventions across different types of farm facilities and production systems. Given the limited number of studies specifically focused on hygiene in animal housing, the review was further expanded to include information from related fields such as the food industry and human medicine as an example of the possibility to expand it to the farm environment. This broader scope provided a more comprehensive understanding of potential methodologies that could be adapted for varied farm conditions. The data extracted were synthesized and presented through a combination of visual representations and detailed tables to enhance clarity and understanding.

TABLE 1 The inclusion and exclusion criteria adopted in the literature review process.

	Inclusion criteria	Exclusion criteria
1	Peer-reviewed original research articles, book chapters, and standard guidelines	Manuscripts that were not peer-reviewed, commentary, and conference abstracts
2	Manuscripts written in English	Manuscripts not written in English
3	Studies focusing on pigs, poultry, and cattle farms	Studies focusing on pets, companion animals, wild animals and aquatic species
4	Studies including the effect of C&D on farm surfaces and equipment	Studies including the farm air and water systems

2.1 Data extraction and synthesis

The selected studies were classified into three main thematic sections based on the type of hygiene monitoring methods used on farms:

- (1) Non-microbiological assessment methods including studies focusing on visual, biochemical, and chemical assessment without direct microbial testing.
- (2) Microbiological assessment methods including studies with a focus on detection of targeted microbial contamination and/or assessment of hygiene indicator organisms.
- (3) Molecular methods including studies describing molecular techniques for the detection and identification of microorganisms.

For each of the identified methods, the strengths, limitations, and practical implications were listed and discussed.

3 Results

3.1 Overview of C&D procedures in farm facilities

The present review has identified distinct categories of methods used to evaluate the hygiene status in animal housing facilities, whether after cleaning, after disinfection or following the complete C&D process.

An effective C&D regime comprises seven essential steps: (1) Dry cleaning (to remove all organic material); (2) Soaking with a detergent (soaking of all surfaces preferably with detergent for appropriate contact time); (3) Pressure washing (high pressure cleaning with water to remove all dirt); (4) Drying (to avoid dilution of the disinfectant applied in the next step); (5) Disinfection (to achieve a further reduction or elimination of the concentration of the pathogens); (6) Final drying (drying of the stable to assure that animals afterwards cannot come into contact with the residues of used disinfectant); (7) Evaluation (testing of the efficiency of the procedure through sampling of the surface by using applicable methods). The final step (7) should involve testing of the efficiency of the procedure through sampling of the surface by using applicable methods such as visual inspection, ATP testing, or microbial swabbing, etc., to ensure the overall effectiveness of the entire process (8, 18, 29).

3.2 Non-microbiological assessment methods

3.2.1 Visual assessment

3.2.1.1 Basic visual inspection

Visual inspection is a common and conventional method for assessing hygiene in animal facilities, offering a quick evaluation of the cleaning effectiveness of stables, equipment, and materials, without requiring specialized tools (30). It can be applied both before and after

cleaning. Performing it after dry cleaning but before detergent cleaning or disinfection helps addressing gaps and may reduce residual organic material.

Description of the test. This approach is widely used, but only few studies described in detail the procedure. In a poultry farm study, the inspection was conducted for equipment and buildings after cleaning and before disinfection. Each building was divided into four sections identifying the specific control points for assessment based on the grid approach described by Rose et al. (31), and calculated cumulative scores as percentages, with 100% indicating perfect cleanliness (32). In pig farms, a three-point grading system was applied to evaluate visible soiling: 1 = satisfactory (no visible soiling), 2 = sufficient (minor soiling), and 3 = unsatisfactory (visible soiling) (24). Similarly, a poultry farm study adopted a three-tier scale: 0 = soiled, 1 = partially cleaned, and 2 = clean (20). However, as was highlighted by Heinemann (33), the lack of standardized definitions for “clean” and “dirty” makes such assessments subjective. Visual hygiene assessments of milking and feeding equipment in cattle farms have been employed in multiple studies using standardized scoring systems based on the presence of visible organic residues. A commonly adopted 4-point scale evaluates equipment surfaces that come into contact with colostrum or milk, where: score 1 denotes visibly clean equipment with no detectable fecal, milk, or colostrum residues; score 2 indicates minimal residual traces; score 3 reflects clearly visible contamination; and score 4 represents extensive contamination, often involving manure, milk, or colostrum deposits (34, 35). In some studies, visual scoring charts were used for evaluating items such as buckets and nipples (36), with additional modifications for esophageal and automatic milk feeders, taking into account factors like tube transparency to assess internal cleanliness. All assessments were performed by a single evaluator to ensure consistency (34). The goal should always be to achieve the better possible visual hygiene score immediately after the cleaning process and just before the equipment is used again (37).

Advantages. Visual inspection is a quick and simple method to do the checking, allowing evaluators (staff, auditors, and other stakeholders) to spot visible dirt or residue efficiently. If a surface fails this test, the use of more advanced methods like rapid tests, microbiological analyses, or even proceeding with disinfection is unnecessary (8, 18).

Limitations. The main limitations of basic visual inspection are its subjectivity, reliance on the inspector's perception, and external factors such as lighting and surface color (24). Only visible areas can be assessed, potentially missing hidden spots. For example, a poultry farm study found high adenosine triphosphate values on drinking cups, drain holes, and floor cracks after cleaning, which confirmed that these areas still contained notable amounts of organic material and/or bacteria after cleaning despite visual inspections showing cleanliness for the last two (20). Similar findings from studies on colostrum-feeding equipment in cattle farms revealed that nipples appearing clean upon visual inspection were found to be contaminated when assessed using luminometry, likely due to hard-to-see internal surfaces, especially in dark or narrow designs (34).

Overall reflection. Visual assessment is generally considered a poor indicator of cleanliness on farm (34–36). Although it is highly subjective (38) and only offers a qualitative assessment, making it difficult to track improvements, it remains a valuable and integral tool within a comprehensive C&D evaluation protocol (39, 40). To improve reliability, the visual inspection process on farms should be standardized by defining clear criteria (8, 31), training and calibrating inspectors regularly (37, 38), using detailed scoring systems and checklists (32), and incorporating digital imaging or AI tools (41) for more objective assessment. Emerging AI tools, such as computer vision and machine learning algorithms, can enhance the objectivity of farm visual inspections by automatically detecting hygiene deficiencies and standardizing image-based evaluations.

3.2.1.2 Ultraviolet (UV) fluorescence

UV fluorescent markers, also known as UV tracers or UV fluorescent dyes, provide a visual indication of areas in animal houses that are not properly cleaned (42).

Description of the test. UV fluorescent markers emit visible fluorescence under UV light and are easily removed by wet mopping. In animal housing facilities, these dye-based markers are typically applied to surfaces prior to cleaning, and the cleaning process is considered effective if more than 90% of the marker is visibly removed. In studies of veterinary and hospital cleanliness, outcomes are categorized as “clean” (mark faded) or “dirty” (mark persists) (Figure 1) (42, 43). UV markers are also used to assess hand hygiene in hospitals using UV light boxes (44), with a 4-point scale from “very dirty” to “very clean” (45).

An American study on pig farms highlighted the broad application of specialized fluorescent gels and powders, such as “Glo Germ”, which can simulate germs or contaminants visible under UV light. These tools have demonstrated effectiveness as educational aids, improving biosecurity practices across various contexts. They are particularly valuable for training individuals in identifying contamination risks, serving as practical and engaging tools in swine facilities and other agricultural settings (46).

In addition, a study conducted in the USA applied deep learning algorithms, Xception and DeepLabv3+, to analyze images of surfaces

and equipment in the food industry. The models accurately distinguished between contaminated and clean surfaces with 98.78% accuracy (41). This approach holds potential for future application in farm facilities.

Advantages. UV fluorescent markers are relatively easy to use and have low costs related to them (47). They do not require specialized equipment or complex procedures, making them accessible and practical for routine assessments. UV fluorescent markers are non-destructive to surfaces and most of them are non-toxic, posing minimal risk to humans or animals (41).

Limitations. Major limitations are additional work/time for marking surfaces prior to cleaning procedures and that this methodology requires a UV light source (42). Its effectiveness depends on the observer’s ability to detect fluorescence, which can be influenced by lighting conditions and improper use of UV light. Additionally, it does not provide microbial contamination data.

Overall reflection. UV fluorescence can be useful for assessing the efficacy of cleaning procedures. They are especially used as a training and educational tool to raise awareness and promote better cleaning as well as hand hygiene practices (44, 45). Works best when subjectivity is minimised using pre-printed scoring templates (such as % of marker removed) or pictures.

3.2.1.3 Adhesive tape evaluation

Adhesive tape sampling methods have been used in clinical, environmental, and food microbiology since the early 1950s (48). This sampling method can be used for porous surfaces or uneven areas that may be difficult to inspect visually directly or to sample using swabs or agar contact plates (49).

Description of the test. The test methodology involves pressing adhesive tape onto a surface to collect residual particles, both organic and inorganic, which are subsequently visually inspected or microbiologically cultured to evaluate C&D efficiency (48). Advanced techniques like spectroscopy or microscopy may be used for further analysis, helping to identify specific particles or microorganisms.

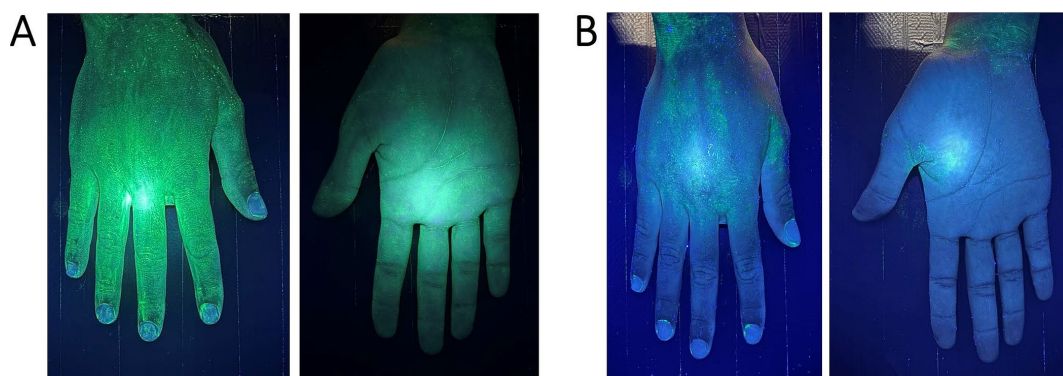


FIGURE 1

Hand examination under UV light in a box: (A)—pre-cleaning view (after applying UV gel the fluorescent light marks the whole hand) and (B)—post-cleaning view (fluorescent spots mark the areas that are not properly cleaned).

Advantages. The method is simple, cost-effective, and requires minimal equipment, making it suitable for routine use. It is versatile, easily covering irregular surfaces and hard-to-reach areas. The collected samples are manageable, transportable, and non-destructive (50). This method furnishes a qualitative appraisal of the presence of visible residues, aiding in the identification of areas necessitating further attention.

Limitations. The use of adhesive tape sampling is limited to collecting contaminants from small surface areas and does not capture those embedded in deeper layers of rough surfaces (e.g., concrete). Its effectiveness is influenced by factors such as the sampling technique, applied pressure, and the type of tape used. Additionally, it may miss larger or non-adherent contaminants, leading to potential inconsistencies in results. If only visual analysis is done, interpretations can be subjective.

Overall reflection. The technique is simple and requires minimal equipment, making it an accessible option for routine monitoring of small surface areas in animal farm environments. It is primarily suited for detecting visible residues rather than accurately quantifying the microbial load on surfaces.

3.2.2 Biochemical assessment methods

3.2.2.1 Adenosine triphosphate (ATP) bioluminescence technology

ATP analysis is a quantitative method that can be used for monitoring hygiene after C&D (8, 20). It is typically applied to high-touch surfaces and critical control points, focusing on areas prone to contamination.

Traditionally, this technique is more commonly implemented in human medicine and the food industry to assess potential contamination points as well as C&D regimen. However, recent studies have demonstrated that this method has gained popularity and is increasingly utilized to assess hygiene effectiveness in veterinary medicine, especially for the evaluation of specific surfaces in scientific studies (38, 39, 51). For example, in dairy

farms, studies highlighted the potential of ATP as an on-farm tool for evaluating the hygiene of rubber liners (35), for cleanliness of equipment used to collect and feed colostrum (36, 52), for assessing the cleanliness of feeding equipment in pre-weaning calves (34), and for cleanliness of milking equipment (53). In the poultry industry, ATP bioluminescence was used to assess cleanliness in broiler houses from 12 different sample points (such as floor, wall, drinking cup, feed hopper, loose material, etc.) (20), in battery cages and on-floor layer houses (32), in carcass processing environments to identify critical control points and equipment surfaces (54). In pig farming, ATP assay was used for testing floor corners, floor centers, and feeding troughs in an empty pig farrowing unit before and after standard cleaning procedures (55).

Description of the test. ATP analysis detects biological residues, including cells from plants, animals, and microorganisms, as ATP is the universal energy source in living cells (17). As the dirt that is left in farm animal housing is often a mixture of feces, urine, leftover feed, animal cells, and bacteria, the ATP levels provide an indication of surface cleanliness (33).

ATP analysis operates on the principle of introducing a solution containing a lysis reagent, luciferin substrate, and luciferase enzyme to a swab sample. The lysis reagent facilitates the release of ATP from all living cells. When ATP is released, it is utilized by the luciferase enzyme to convert the luciferin substrate, resulting in a bioluminescent reaction that produces light. The intensity of this light correlates with ATP levels (8). The light is measured in relative light units (RLUs), where higher RLUs indicate greater contamination (Figure 2). ATP levels vary by cell type (e.g., yeast, bacteria) and growth phase, but regulatory mechanisms maintain a consistent ATP pool (17). Thus, routine cleaning assessments should be compared with baseline data of acceptable cleanliness values. ATP bioluminescence thresholds for hygiene assessment vary considerably across studies, depending on animal species, facility type, and the specific surface or equipment being tested. For instance, Lindell et al. (35) reported ATP cutoffs for milking equipment ranging from ≤ 150 relative light units (RLU) to indicate cleanliness, to ≥ 300 RLU as indicative of contamination. In

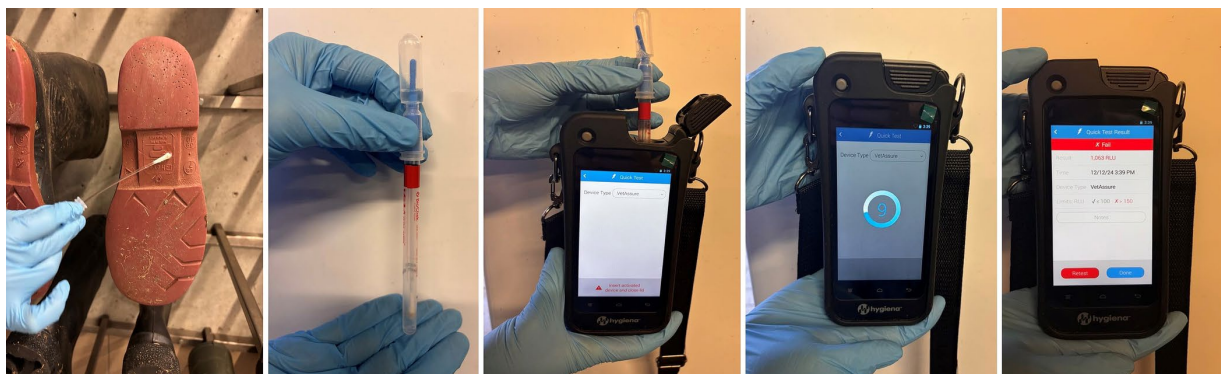


FIGURE 2

ATP procedures: measuring surface contamination levels (swabbing the boots after cleaning; mixing with reactive solution; placing the vial into ATP testing device; waiting for results 10 s; receiving the results).

the context of colostrum feeding equipment, Buczinski et al. (52) proposed a general threshold of <1,000 RLU for clean devices, and $\geq 1,000$ RLU for contaminated ones. Similarly, Van Driessche et al. (38) classified ATP readings as follows: ≤ 500 RLU (clean), 501–1,000 RLU (alert), and $> 1,000$ RLU (fail). A wider range was observed by Vilar et al. (53), who reported thresholds from <152 to <1,182 RLU, depending on sampling sites within cattle operations. In poultry barns, Mateus-Vargas et al. (56) considered values ≤ 150 RLU as indicative of effective cleaning, with readings above this level reflecting insufficient hygiene. For pig fattening units, Heinemann et al. (33) identified > 500 RLU as a threshold indicating inadequate cleanliness of pen surfaces. These variations underscore the importance of establishing facility-specific pass/fail benchmarks when using ATP monitoring as a routine hygiene assessment tool, as recommended by Heinemann et al. (24).

Advantages. ATP bioluminescence tests swiftly detect organic residues, providing quantitative results within a short time (around 5–10 s) without the need for microbial cultivation. This allows for immediate feedback on surface cleanliness, enabling real-time adjustments and interventions.

Limitations. A major limitation is the absence of specific standards for the RLUs to define cleanliness (57). Each ATP device manufacturer employs its own RLU scale, based on their specific ATP luminescence curve, making all measurements and RLU values relative to that particular system. Furthermore, users should understand that the ATP value does not relate directly to the microbial load of surfaces, even when only bacteria are measured. Similarly, non-microbial sources of ATP, such as plant material, could lead to an overestimation of microbial contamination (33). Therefore, if ATP values are very high, additional microbial testing is recommended for verification. Although the ATP assay can lead to a false positive result, the cost of an additional cleaning is typically lower than the potential expenses associated with an infectious disease outbreak, such as PEDV (Porcine Epidemic Diarrhea Virus) or PRRSV (Porcine Reproductive and Respiratory Syndrome Virus) (39, 58).

Another limitation of the methodology is that residual detergents and disinfectants may interfere with the ATP bioluminescence reaction and alter RLU values leading to inaccurate results (27, 59, 60). As was observed in previous studies, among the nine chemicals that were tested for impact on ATP, quaternary ammonium was the only one that increased $\log_{10}$ RLU measurements. In contrast, hydrogen peroxide and peroxyacetic acid sanitizer caused larger $\log_{10}$ RLU reductions in ATP measurements from organic sources (chicken exudate) compared to pure ATP sources (61). For this reason, it is advisable to use commercial bioluminescence detection kits that include neutralizers to mitigate the effects of detergents and sanitizers (61) or leave a drying period of a minimum of 12 h after disinfection of a surface before using ATP to evaluate cleanliness. In addition, it's important to note that ATP systems are also unable to detect spores, viruses, or prions, as they do not contain ATP (61). Moreover, when samples are collected from areas that are not visibly clean, the contamination load of the swab may be too high, preventing an accurate ATP measurement, which could result in false negative results. Thus, it is recommended to

use this technique exclusively on visually clean surfaces, as advised by the manufacturers (52). A similar statement was confirmed in a study in pig barns which found a strong correlation ($r = 0.698$, $p < 0.001$) between ATP and total aerobic bacteria (TAB) levels after cleaning. However, the correlation was weakened in the presence of fecal bacteria, emphasizing the need for thorough cleaning and visual inspection before testing (55).

In comparison to the food industry, where ATP testing is already standardized, its application for veterinary purposes still requires further standardization. Until this is achieved, each manufacturer should, at the very least, provide its own upper and lower RLU limits for application within the veterinary context (55). To accurately interpret the hygiene status while using ATP testing on a regular basis, the user must set a pass or fail benchmark as was suggested by Heinemann et al. (24).

Overall reflection. Despite its limitations, ATP bioluminescence is increasingly popular (especially in the frame of scientific projects) for environmental cleanliness monitoring in farm facilities serving as a technique suitable for real-time assessment of surfaces by focusing on dirt absence, not microbial count where cleanliness, but not sterility, is required (20, 35, 57). However, opinions on the effectiveness of ATP testing vary. Some argue that ATP testing should not replace quantitative methods for determining microbial load and should, therefore, be supplemented with microbiological methods (35, 57, 58, 62–64). On the contrary, other researchers have confirmed that ATP testing can be a cost-effective alternative to microbiological methods along with visual inspection (55) and alone (24).

However, based on the analysis of the different studies there are important prerequisites: in case of use after cleaning, the ATP testing should be used only on visually clean surfaces, and in case of using ATP testing after disinfection, it can only be applied when surfaces are completely dry, in both cases in order to avoid false results. A future standard approach requires identifying a benchmark and establishing a cut-off value to alert farmers when extra cleaning is needed (65).

3.2.2.2 A3 system (ATP+ADP+AMP)

The A3 test quantifies the total adenylate content, ATP, ADP (adenosine diphosphate), and AMP (adenosine monophosphate), collectively on surfaces to assess cleanliness. Therefore, measuring total adenylates (ATP + ADP + AMP, known as A3) may provide a more reliable indicator of residual contamination that can lead to biofilm formation and other contamination (65).

Description of the test. In this method, samples collected from surfaces are processed to convert all adenylates into ATP using enzymatic reactions. The resulting ATP is then measured using a standard bioluminescence reaction catalyzed by the luciferase enzyme. Because the test captures all forms of adenylates rather than ATP alone, it provides a more accurate measure of residual organic matter, including degraded biological material, on cleaned surfaces.

Advantages. By measuring all three adenylates (ATP, ADP, AMP), the test captures residual organic matter even when ATP has partially

degraded, increasing detection accuracy. A3 levels correlate with residues that promote biofilm formation, enabling earlier identification of sanitation issues. Like ATP bioluminescence, A3 testing can be performed quickly with portable luminometers (66).

Limitations. A3 testing equipment and reagents are less common and can be more expensive than standard ATP tests. The inclusion of ADP and AMP can complicate result interpretation, requiring calibration and training. Some cleaning agents or sample matrices may affect adenylate stability or detection, causing false readings (67).

Overall reflection. The A3 test offers a more comprehensive and reliable alternative to traditional ATP testing by accounting for ATP degradation products, improving cleanliness assessments in environments where heat or chemicals degrade ATP. Finally, this approach allows detection of degraded organic residues that may not contain intact ATP but still pose hygiene risks, providing a more reliable indicator of surface contamination. Although promising, its broader adoption depends on further validation, cost reduction, and user training to interpret results accurately. Integrating A3 testing with other hygiene monitoring methods can enhance overall sanitation control.

3.2.2.3 Luciferase-based methods

All ATP bioluminescence tests use luciferase, but not all luciferase-based tests are ATP tests. Some are designed to find live pathogens. These tests are more specific than general ATP tests and can confirm if harmful microbes (such as *Salmonella*, *Listeria*, or *E. coli*) are still present after C&D procedures.

Description of the test. Luciferase-based microbe detection combines the bioluminescent reaction of the luciferase enzyme with biological targeting mechanisms, such as bacteriophage-based systems or pathogen-specific genetic probes. Phage-based tests use luciferase as a biosensor to detect specific microbe, often via genetically modified bacteriophages or molecular probes that only activate the luciferase signal when a viable target pathogen is present (68, 69). Genetic probe-based methods use nucleic acid amplification coupled with luciferase to quantify specific bacterial DNA (70). These approaches ensure that luminescence occurs exclusively in response to viable and correctly identified microbe, enhancing both specificity and reliability.

Advantages. Offers high specificity, as it targets viable pathogens rather than general organic material, allowing for accurate confirmation of disinfection effectiveness. The test delivers results within hours, significantly faster than traditional culture methods, and is sensitive enough to detect low pathogen levels. It is also non-destructive, requiring minimal sample preparation, and the intensity of luminescence correlates quantitatively with pathogen concentration (68, 71).

Limitations. More complex, time consuming and expensive than ATP tests. Require specific reagents and instruments, require skilled lab personnel to carry them out, typically suited for lab or semi-lab settings, which limit field applicability. The test generally targets

specific pathogens, so it is not suitable for broad-spectrum screening. Additionally, sample matrices can sometimes interfere with the luciferase reaction, causing false results. Proper sample collection and handling, as well as operator training, are critical to ensure accuracy (68, 71).

Overall reflection. Luciferase-based pathogen detection provides a valuable balance between rapid turnaround and specificity for viable pathogens, making it a useful tool for verifying the effectiveness of C&D protocols on farms. However, it is best employed as part of an integrated hygiene monitoring strategy that also includes complementary methods such as ATP bioluminescence and microbiological cultures to ensure comprehensive pathogen detection and control (68).

3.2.2.4 Rapid protein tests (RPT)

RPTs are widely used across multiple industries, including the food industry, healthcare, and environmental monitoring. On farms, rapid protein tests can be applied to assess the cleanliness and hygiene of equipment, surfaces, and animal housing facilities. By detecting protein residues, these tests can reveal the presence of organic contaminants, such as manure, feed residues, or milk, which may potentially carry harmful microorganisms (24).

Description of the test. Rapid protein tests detect protein residues through chemical reactions that cause a color change, typically within 1–15 min. Using swabs, test strips, or pads, samples are mixed with a reagent to induce a color shift, which indicates the presence and extent of protein contamination. The color change is often assessed using a predefined scale, such as the 5-point scale used in a German study to evaluate the intensity of the shift from green to violet after 15 min (24). The semi-quantitative scoring system typically ranges from 1 (no detectable color change) to 5 (intense color change), with scores exceeding 2 or 3 generally interpreted as indicative of insufficient cleaning or inadequate hygiene.

Advantages. RPTs allow for the immediate semi-quantitative assessment of surface cleanliness. According to Heinemann et al. (24), rapid protein tests are highly inviting for on-farm monitoring due to their short duration, in contrast to microbiological techniques. While the interpretation of color changes could potentially be subjective, this can be supplemented by inexpensive tools to measure and/or record results when needed (24).

Limitations. These tests do not detect non-organic substances, chemical or residues (24, 33, 53, 72). Their sensitivity can vary across surfaces and test brands, leading to inconsistent results that require validation and standardization. Additionally, detergent or disinfectant residues may interfere with test outcomes, causing false positives or negatives. To address this, low-residue disinfectants or post-application residue removal methods can be used, although the term “low residue” does not guarantee zero residues, and levels are typically defined as <25 ppm (24).

Overall reflection. Results of rapid protein tests are often qualitative (pass/fail) or semi-quantitative, providing less precise information. When comparing ATP and RPT, ATP tests more accurately reflect

subtle differences, whereas rapid protein tests only allow for the visual recognition of coarse color graduations (24).

3.2.3 Chemical assessment method

3.2.3.1 Redox potential measurement

The redox potential method is one of the complex indicators of the physiological state of microbial cultures and its measurement could be a useful tool for the qualitative and quantitative determination of microbial contamination (73).

Description of the test. The redox potential method relies on oxidation–reduction reactions in biological systems, driven by microbial activity. During microbial growth, biological oxidation leads to oxygen depletion and the production of reducing compounds, causing a measurable decrease in the redox potential (Eh) of the medium (74). This change, governed by the Nernst equation, serves as an indicator of microbial activity and contamination levels (73). Researchers have validated this approach for coliform bacteria, finding a strict linear correlation between time-to-detection and the log of the initial CFU count (73, 75). Where the ‘time-to-detection’ is the interval between inoculating the sample into the redox-sensitive medium and the moment when the measured redox potential (Eh) drops to the chosen cutoff, indicating significant microbial oxidation and reduction activity.

Advantages. Redox potential measurement can detect microbial activity within 16 h, faster than the minimum 24-h incubation required for the reference plate culture method. This technique is efficient and can be tailored to specific bacterial strains by using selective media, as each bacterial strain exhibits a unique kinetic pattern in terms of redox potential change. This makes the method not only faster but also adaptable to different types of microbial contamination (75). Recent studies have led to the development of mobile devices for measuring microbiological activity on farms (still in progress), utilizing the redox potential measurement technique, opening new ways to its use by the sector. Also, mathematical models have been successfully used to describe the specific shape of redox potential curves for several bacteria optimizing the readings and leading to higher accuracy when classifying bacterial species (75).

Limitations. The redox potential is a complex indicator influenced by various factors, requiring careful interpretation. Automation is possible but demands advanced redox electrodes and measurement systems. Despite its potential, the method has historically seen limited application (73, 75, 76). Due to the non-availability of devices for field conditions (available devices are only non-portable), this method needs more improvement.

Overall reflection. This method can be a cost-effective alternative for monitoring microbial contamination. Its adaptability and minimal environmental control requirements make it a valuable tool, though broader adoption and technological improvements are necessary to fully leverage its capabilities (73, 75). Therefore, integrating redox potential monitoring, based on microbial metabolic activity reducing oxidation–reduction potential, can provide faster, indirect microbial detection and offers potential for field-adapted C&D evaluation.

3.3 Microbiological assessment methods

Microbiological assessment is critical for detecting and measuring microorganisms on surfaces, encompassing a wide range of microorganism types (55). Unlike non-microbiological testing, microbiological ones may provide quantitative data on contamination levels through viable colony counts (77) offering insight into the efficacy of C&D (78).

Culturing techniques form the cornerstone of microbiological assessments, enabling the growth and enumeration of microorganisms from collected samples. The mesophilic aerobic total viable count (TVC) is a widely used parameter for assessing surface cleanliness, reflecting the presence of a total number of aerobic and facultative anaerobic microorganisms. Additionally, indicator microbes like *Enterobacteriaceae*, *E. coli*, and total coliforms are used to assess faecal contamination, aiding in identifying hygiene lapses (20, 22, 33). Specific pathogens such as *Salmonella* spp., *Campylobacter* spp., and methicillin-resistant *Staphylococcus aureus* (MRSA) are also commonly targeted in assessments (18, 33, 79).

Microbiological assessment involves collecting microorganisms from surfaces using various techniques, including swabbing and agar contact plates (ACP) (17). A brief description of these two commonly used techniques is provided below.

3.3.1 Swabbing

Swabbing is a versatile sampling technique that involves wiping targeted surfaces with swabs made of materials like cotton, rayon, or nylon, which are then processed for microbiological analysis.

Description of technique. For direct swabbing, a sterile frame marks the sample area, and a dry or pre-moistened swab is wiped horizontally and vertically with rotation. The swab is then placed in a transport media for microbiological analysis (Figure 3).

This technique is highly effective for sampling irregular or hard-to-reach areas, such as artificial teats or inside equipment (53). Swabbing is commonly used to sample the animal farms from the floor, wall, drinkers, and feeders (Figure 4). The boot sock or boot swab sample method is recommended to examine *Salmonella* spp. occurrence in poultry houses (CR (EU) No 200/2010) (24). This technique also involves pulling sterile disposable hairnets or cotton covers over disinfected shoes (“sock” samples) and walking a defined number of steps through the barn. This method was previously used to detect *Campylobacter* spp., MRSA, total aerobic bacteria, and fecal indicator bacteria such as Enterococci and *Enterobacteriaceae* (33, 79). In the study by Mateus-Vargas et al. (56), boot swab samples collected by walking over poultry barn floors were used to evaluate C&D efficacy through log₁₀ reductions in total aerobic counts (TAC). The results demonstrated a 3 log₁₀ reduction in TAC following cleaning and a reduction of less than 2.5 log₁₀ after disinfection. Similarly Luyckx et al. (20, 22), reported a decrease in total aerobic flora from 7.7 ± 1.4 to 4.2 ± 1.6 log CFU/625 cm² after disinfection using swab-based culture methods in poultry barns. Although no definitive threshold was proposed, lower bacterial counts were consistently associated with improved hygiene status. In cattle farm settings, Lindell et al. (35) employed swab sampling followed by culture for TAC assessment, where

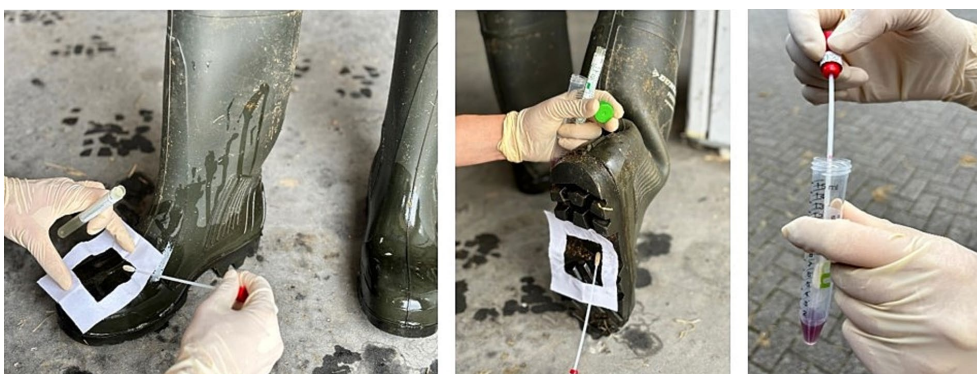


FIGURE 3

Boot swab sampling process: a swab is wiped horizontally and vertically with rotation over a marked area and placed in a transport media for further assessment.

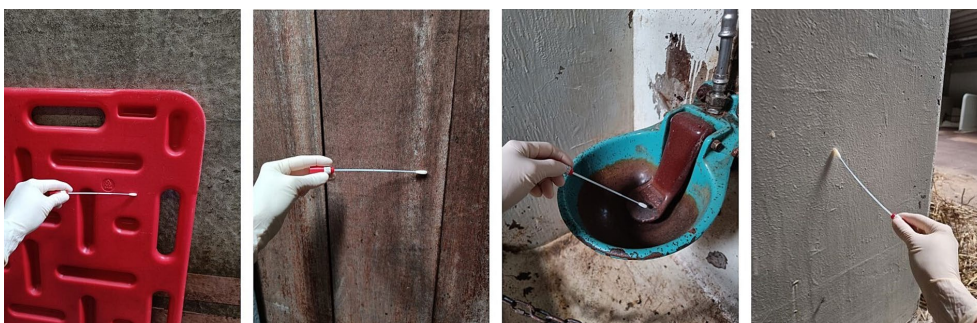


FIGURE 4

Swabbing of equipment, drink pillars, and walls in farm environments.

post-cleaning levels below 2–3 log CFU/cm² were generally considered indicative of acceptable surface cleanliness.

Advantages. Swabs can be further processed to quantify total microbial contamination, detect specific pathogens, or identify indicator organisms. The type of swab and the choice of moistening and transport solution can significantly impact the recovery of microorganisms, with studies showing nylon flocked swabs with non-growth-enhancing moistening solutions as particularly effective for wet surfaces (28). In cases where swabs are taken after disinfection, the use of a neutralising transport medium ensures that residual disinfectants in the sample are inactivated, enabling an accurate bacterial count during processing. A key advantage of swabs is the ability to dilute the transport solution, which helps prevent microbial overgrowth, an option not available with ACP. Additionally, unlike ACPs, swabs allow the same sample to be plated onto multiple types of agar, enabling the detection and enumeration of different bacterial species from the exact same swabbed surface.

Limitations. Swabs as a sampling method have some limitations, including being labor-intensive and highly susceptible to variability in sampling technique. The process is time-consuming, detects only

culturable bacteria, and requires access to laboratory facilities. Additionally, there is no universally accepted cut-off for interpretation, and results typically take 2–3 days to obtain (8, 17, 56).

Overall reflection. Swabs are useful for certain surfaces that are challenging to sample and provide evidence-based feedback on the effectiveness of C&D protocols.

3.3.2 Agar contact plating (ACP) method

ACP is another essential tool for microbial sampling, particularly for smooth, dry surfaces. In agricultural settings, such samples are frequently taken as part of evaluations after production cycles to evaluate the effectiveness of C&D protocols. Additionally, regulatory bodies may implement ACP testing if specific pathogens are suspected on a farm.

Description of the methodology. The choice of culture medium depends on the targeted bacterial species (33). ACP involves pressing (for several seconds) convex agar plates onto surfaces to capture microbial contaminants (Figure 5).

Further, these plates are incubated to allow bacterial colonies to grow, which are then counted to estimate microbial contamination levels. ACP results are typically expressed in CFU (Colony-Forming Units) or TAC (Total Aerobic Count), and these metrics are



FIGURE 5
Sampling stables, walls, and floors by using agar contact plating.

commonly referred to as a hygienogram. The CFU count reflects viable, colony-forming microorganisms, indicating microbial activity, while TAC measures all aerobic microorganisms present on a non-selective agar plate. The low CFU/TAC indicating the good hygiene and high CFU/TAC pointing to inadequate cleaning or contamination. The application of ACP for hygiene monitoring has been described in previous studies, where results are translated into a hygienogram scoring system that assigns values from 0 (very good disinfection) to 5 (very poor disinfection) based on colony counts (20). According to Mateus-Vargas et al. (56), this scoring system allows for the categorization of C&D outcomes as: “good” (score ≤ 1.5), “satisfactory” (score 1.6–2.9), and “poor” (score ≥ 3.0). In practical applications, Huneau-Salaün et al. (32) reported that, for poultry farms, total aerobic counts below 200 CFU/25 cm² are considered acceptable, while higher counts reflect microbial overgrowth and inadequate hygiene. On pig farms, the upper threshold for acceptable contamination has been set at 33.3 CFU/cm² (33).

Advantages. ACP provides standardized sampling of smooth surfaces with minimal processing and allows for direct colony enumeration, making them particularly suitable for use in hygienograms (20, 33). Compared to swab sampling, ACP offers a simpler and less labor-intensive alternative by enabling direct contact with surfaces, thereby eliminating the need for additional tools such as transport media or extra steps like enrichment and plating. Typically, ACPs use basal agar media, which support the growth of a broad spectrum of microorganisms. However, the agar plates can be customized by using selective media to target specific bacterial types. ACP is particularly advantageous for providing quantitative data about the microbiological load and tracking contamination trends.

Limitations. ACPs are limited to flat, smooth surfaces and cannot be used effectively on curved, uneven, or porous surfaces. They may also underestimate contamination from biofilm-embedded microorganisms and are prone to colony overgrowth if the sampled surface is heavily contaminated or not properly cleaned beforehand (8, 24). Like other microbiological assays, ACP demands an incubation period, ranging from hours to days, depending on species and conditions. This might not suit real-time result needs. Also, the interpretation and counting of the colonies on ACP might be subjected to observer variation due to variability in colony size, colour, and morphology (24).

Overall reflection. Agar contact plates are a practical sampling method for evaluating C&D in animal farms, making them a reliable method for surface contamination checks.

3.3.3 Targeting specific pathogens

Both swabs and ACP can be applied for selective plating techniques, which enable the identification and quantification of specific microorganisms (80). Selective media, such as Eosin Methylene Blue agar for *E. coli*, Xylose Lysine Deoxycholate agar for *Salmonella* spp., and Campylobacter Blood-Free Selective Medium for *Campylobacter* spp., are commonly used for targeting pathogens of interest (33, 81). These selective media are particularly valuable during outbreaks or persistent health issues, as they allow for precise pathogen detection and quantification (82, 83). Microbiological plating focuses only on viable cells, allowing selective plating techniques to provide a more accurate assessment of disinfection efficacy by confirming the survival or elimination of targeted pathogens (Figure 6).

However, while selective plating is effective in identifying specific bacteria, it may not capture the full spectrum of microbial diversity present on surfaces, potentially overlooking non-target organisms (84). Nevertheless, these approaches remain essential for evidence-based evaluation of C&D protocols or during investigation of the disease outbreaks due to suspected pathogen(s).

The use of indicator organisms such as *Enterobacteriaceae*, *E. coli*, and *Enterococcus* spp. provides valuable information about the overall hygiene status of a facility (17, 20). In general, criteria for hygiene indicators include being more abundant than the pathogen to enhance detection (85), having a survival rate similar to or greater than the pathogen in the environment (24, 33) and being easily detectable with reliable, faster, and safer methods than those used for the pathogen (86). For example, *E. coli* serves as an indicator of fecal contamination and correlates with the likelihood of detecting other enteric pathogens. Past studies have shown that *E. coli* can be a suitable index organism for detecting the possible presence of *Salmonella* spp. (85). Further Luyckx et al. (20), observed that *Enterococcus* spp., due to their resilience, persistence on surfaces, and higher probability of recovering, may be even more effective indicators of cleaning efficacy compared to *E. coli*. The choice of hygiene indicators depends on the farm's specific needs, the type of animals, and potential pathogens of concern. Since current hygiene indicators primarily focus on fecal contamination; the ideal goal is to develop hygiene indicators specifically designed to address biosecurity lapses in different farm areas.

3.4 Molecular assessment methods

Molecular methods are particularly advantageous for their potential to identify microbial species strains, and even discrete subtypes, including pathogenic variants, with enhanced sensitivity and specificity (33). Molecular tools like traditional polymerase chain reaction (PCR) and real-time PCR, along with advanced

next-generation sequencing (NGS) techniques, are highly effective in characterizing pathogens down to the genetic level. Typically, these techniques (NGS) are applied either to trace back outbreaks by identifying the pathogen and its possible source through phylogenetic analysis or for experimental or research purposes.

Description of the methodology. These methods require genetic material (Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)), which can be extracted from samples collected on animal farms using commercially available kits (87). The repertoire of techniques includes PCR, quantitative polymerase chain reaction (qPCR), reverse transcriptase PCR (RT-PCR), nucleic acid sequence-based amplification, as well as NGS techniques such as 16S rRNA gene sequencing and metagenomics (88).

Advantages. Molecular methods are considered as highly sensitive and specific, capable of detecting even very small quantities of genetic material to ensure accurate identification (89). These methods provide rapid results, which is especially valuable in outbreak investigations and in situations where culturing is difficult or impractical. NGS techniques offer comprehensive analysis, revealing detailed information about the genetic makeup of pathogens, including potential resistance genes and virulence factors. Their versatility allows application to a wide range of samples, including environmental, animal, and food samples, making them highly adaptable for various research and diagnostic purposes. Real-time PCR, in particular, facilitates the quantification of pathogen load, crucial for assessing infection severity and the effectiveness of C&D measures. The details on NGS techniques are beyond the scope of this review; however, readers are encouraged to refer to applied aspects of NGS for further information (90, 91).

Limitations. These methods can be costly, requiring expensive and specialized instrumentation and reagents, and trained personnel to perform and interpret the results (17). The complexity of these techniques can lead to technical errors and contamination.

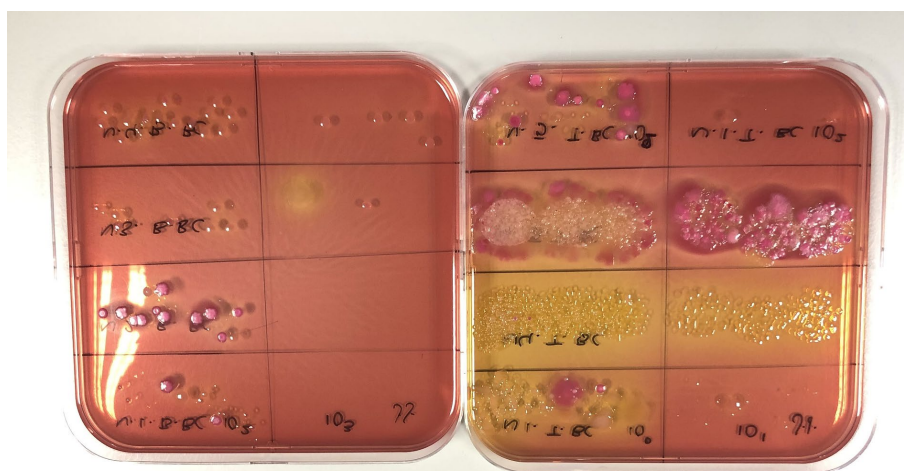


FIGURE 6

Use of selective MacConkey agar for detecting Gram-negative bacteria and differentiating based on lactose metabolism (pink colonies are lactose fermenting, while non-pink colonies are non-lactose fermenting).

Additionally, these methods typically require high-quality genetic material, and the presence of inhibitors in the samples can interfere with the accuracy of the analysis. The extensive data generated by NGS can be challenging to analyze and interpret, requiring sophisticated bioinformatic tools and expertise.

However, a major limitation of this methodology, particularly the qPCR approach, is its inability to differentiate between nucleic acids from viable and non-viable organisms. This can lead to an overestimation of microbial contamination or infection risk, especially in post-disinfection assessments where non-viable cells may still be present (92). In a laboratory-based study by Buttner et al. (93), a comparative assessment was performed between traditional cultivation methods and qPCR across various surface substrates using a single target microorganism. The results demonstrated that cultivation methods detected only a small number of viable cells, whereas qPCR produced considerably higher measurements. Therefore, careful interpretation of threshold cycle values is needed to accurately interpret the relevance of qPCR results (24). Emerging techniques like the propidium monoazide qPCR method (PMA-qPCR) aim to address this, but routine on-farm application remains limited by cost and complexity (94).

Overall reflection. Molecular methods can serve as powerful tools for evaluating the quality of the C&D process, especially in detecting hard-to-culture or fastidious organisms that might evade detection through traditional methods. Molecular methods, particularly PCR and qPCR, are increasingly used in farm settings for detecting pathogens such as *Coxiella burnetii* and *Mycobacterium avium* subsp. *paratuberculosis* in environmental swabs or dust samples (95, 96). Furthermore, they can identify pathogens on a strain or even subtype level and offer significantly faster turnaround times compared to culture-based approaches, making them applicable for real-time monitoring of farm hygiene. However, due to the cost and effort involved, these methods are more practical for addressing persistent issues rather than for routine use.

4 Comparison of methods

Given the importance of hygiene measures within animal production systems, an effective method to evaluate hygiene should serve as more than just an indicator of the success of C&D procedures. It should also serve as a stringent control measure with proper identification of critical control points, validating the proper execution of every step of the process (56). An ideal assessment method for the evaluation of C&D should possess several critical attributes, including high sensitivity and specificity in detecting microorganisms, user-friendliness, rapidity, cost-effectiveness, consistent performance on both wet and dry surfaces, suitability for curved or rough surfaces, non-destructiveness, non-toxicity, resilience to chemical residues, strong repeatability and reproducibility. It should also provide recordable, and tamper-proof results, deliver objective and quantitative data, be suitable for real-time monitoring, and allow for trend analysis (22, 33, 60).

However, based on the analyzed studies in this review, there is no single method meeting all the above-mentioned criteria, with each

method having both advantages and disadvantages concerning its use in evaluating C&D in a farm context.

Table 2 highlights the comparison of various methods for assessing the effectiveness of C&D procedures. In general, the convenient and most widespread method for assessments of C&D efficacy on farms is visual inspection, but visual inspection alone is considered as an unreliable indicator of cleaning effectiveness and is insufficient for accurately assessing the hygiene status (20, 97). Similarly, other kinds of visual examination such as UV fluorescence are subjective methods that are more applicable to the training of farmers. The ATP measurements can provide a more objective (quantitative) identification for use in critical or difficult-to-clean sampling points in comparison with the visual examination (20, 22). Additionally, total adenylate content, which consists of ATP, ADP, and AMP, can be used as a more reliable indicator of residual contamination in different conditions. At the same time, ATP bioluminescence measures overall cleanliness by detecting ATP in all living cells and organic matter, but it cannot distinguish harmful pathogens. In contrast, luciferase-based tests target specific viable pathogens with higher accuracy but are costlier and need specialized equipment, limiting their use in routine field inspections. The redox potential measurement technique to assess microbiological activity, which does not depend on surfaces, can be innovative applications for the agricultural sector. Yet it requires further technological development (75). One feasible approach could involve combining visual inspections with rapid tests to mitigate the added expenses associated with microbiological examinations (24).

However, a combination of visual examination and microbiological tests is more relevant in the case of a longer-term study/evaluation. For example, in pig production, according to Heinemann et al. (24), hygiene has already been proposed as a critical control point for on-farm assessment with daily visual inspections and additional monitoring of C&D procedures. One potential suggestion was to implement hygienogram scores similar to those already established in poultry farming, by investigating the TVC using agar plates to enhance the regular assessment of C&D practices (11). Introducing a system akin to hygienogram scores in piggery farm management could potentially enhance cleanliness but requires further development, particularly since the highest bacterial loads were detected at sampling points where ACP are not suitable for use (20, 98). Furthermore, microbiological tests could be used to target suspected pathogens in cases of persistent health issues and severe illnesses. The molecular methods can be applied during outbreak investigation, especially when the causative agent is unknown or to carry out evidence-based trace backing of contamination source(s). Moreover, the higher cost and complexity of molecular assays compared to traditional culture methods may restrict their routine application in livestock facilities.

It's also critical to comprehend how each technique contributes to the detection of biofilms, which significantly reduce the effectiveness of C&D by shielding microorganisms from disinfectants and contributing to the development of antimicrobial resistance (15). Standard visual and ATP-based assessments fail to detect biofilm-embedded microbes. Culture-based methods may also underestimate contamination from biofilm-embedded microorganisms, and PCR may detect DNA from dead biofilm cells, complicating interpretation (93). Chemical methods testing and emerging biosensors (e.g., redox)

TABLE 2 Comparison of methods for evaluating the efficacy of cleaning and disinfection (C&D) procedures.

No	Method	Price	Speed	Ease of use	Reliability of results	Sensitivity	Quantitative	Applicable for evaluation of C / D / C&D	Key applications in context with assessing C&D at the farm level	Surface applicability	Study references
Visual assessment											
1	Visual inspection	Lowest	+++	+++	–	–	NA	Cleaning	Simple and immediate; provides a basic assessment of cleanliness but is subjective and may miss microscopic contaminants.	For any surfaces and equipment available for visual assessment (easy to see).	(24, 32, 33, 38, 40)
2	Adhesive tape sampling	Lowest	+++	+++	–	–	–	Cleaning	Primarily applicable for detecting visible residues. of more substantial debris, and other discernible contaminants.	For any surfaces, especially porous or uneven areas that may be difficult to sample using swabs or agar contact plates.	(17, 48–50).
3	UV fluorescent markers	Low	++	++	+	+	–	Cleaning	Highlights residual contamination invisible to the naked eye; useful for training and immediate feedback but requires a UV light source.	For any areas that potentially can be missed during the cleaning process (easy to access).	(41–45, 47)
Biochemical assessment											
4	Adenosine triphosphate (ATP) bioluminescence testing	Low	+++	++	+	++	++	C&D	Measures organic matter, including living and dead cells, via ATP presence in more stable conditions; rapid results and useful for overall cleanliness but not specific to pathogens, not standardized.	For specific areas of concern, high-touch surfaces, or critical control points to focus on areas that are more prone to contamination (in difficult places).	(8, 17, 20, 27, 32–36, 38, 39, 52–55, 58, 60, 61, 66, 111)

(Continued)

TABLE 2 (Continued)

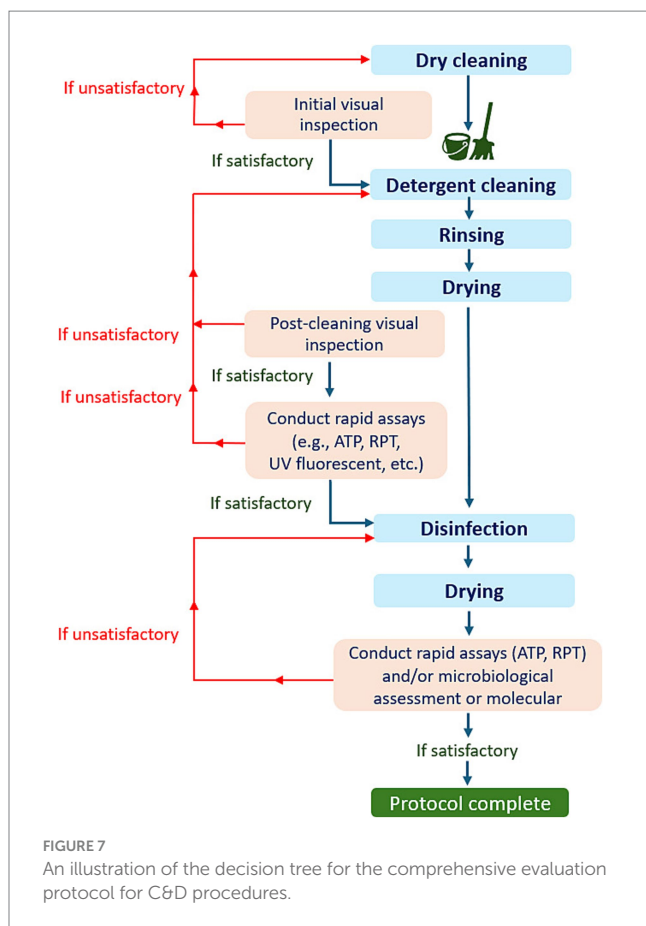
No	Method	Price	Speed	Ease of use	Reliability of results	Sensitivity	Quantitative	Applicable for evaluation of C / D / C&D	Key applications in context with assessing C&D at the farm level	Surface applicability	Study references
5	A3 system (ATP+ADP+AMP)	Medium	+++	+	+	++	++	C&D	Measures organic matter, including living and dead cells in environments where heat or chemicals can degrade a molecule of ATP	Useful for specific areas of concern, such as high-touch surfaces or critical control points that are more prone to contamination, especially in difficult-to-clean areas.	Bakke (65), Bakke and Suzuki (66)
6	Luciferase-based methods	High	++	+	+++	+++	++	C&D	Detect viable microbes	Recommended for high-risk zones and post-disinfection verification in food or veterinary environments	(68–71)
7	Rapid protein tests (RPT)	Medium	++	++	++	++	+	Cleaning	Detects protein residues as an indicator of organic contamination; quick and easy to use, giving immediate results but not specific to microbial contamination.	For particular areas of concern, high-risk surfaces, or critical control points.	(24, 33, 55, 72)
Chemical assessment											
8	Redox potential method	Medium	++	–	++	+++	++	Cleaning	Measuring microbiological activity on farms, utilizing the redox potential measurement technique	For various surfaces	(73, 75)

(Continued)

TABLE 2 (Continued)

No	Method	Price	Speed	Ease of use	Reliability of results	Sensitivity	Quantitative	Applicable for evaluation of C / D / C&D	Key applications in context with assessing C&D at the farm level	Surface applicability	Study references
Microbiological assessment											
9	Agar contact plates (ACP)	High	–	+	+++	++	+++	More applicable after disinfection, for C&D	Allows for the growth and identification of surface microbes; useful for detecting viable organisms but takes time for colonies to grow (24–48 h)	For smooth surfaces in different settings such as floors, walls, feed hoppers	(11, 17, 20, 33, 77, 78, 85, 86, 98)
10	Swabs samples for microbiological examination	High	–	++	+++	++	+++	More applicable after cleaning, for C&D	Collects samples from surfaces for subsequent culture analysis	For various surfaces, useful for challenging areas, like inside artificial teats or pipes	(28, 33, 53, 56, 79)
Molecular methods											
11	PCR based assays	Highest	+	+	+++	+++	+++	Disinfection	Detects and quantifies specific microbial DNA; highly sensitive and specific, useful for detecting low levels of pathogens but requires lab equipment and expertise.	For various surfaces	(87–91, 93)
12	16 s RNA gene sequencing	Highest	+	+	+++	+++	+++	Disinfection	Identifies and quantifies bacterial populations; provides detailed microbial community profiles but requires extensive data analysis and is more time-consuming.	For various surfaces	(87–91, 93)
13	Metagenomics and next generation sequencing	Highest	+	+	+++	+++	+++	Disinfection	Offers a comprehensive analysis of all microbial DNA in a sample; highly detailed and informative about microbial communities and resistance genes but is complex and costly.	For various surfaces	(87–91, 93)

The table uses a color-coded system to visually represent the performance and applicability of various methods used to evaluate the efficacy of cleaning and disinfection. Shades of green indicate positive performance, with dark green corresponding to the highest rating (+++), medium green to good (++), and light green to moderate or fair performance (+). These shades are applied to parameters such as speed, ease of use, reliability, and sensitivity. Red cells represent poor or low performance, typically marked with a minus sign (–), and are commonly associated with limitations in reliability and sensitivity for visual methods.



provide improved sensitivity for early biofilm detection (65, 75). A multipronged strategy including mechanical removal, biofilm-active disinfectants, and enhanced monitoring is essential for robust biofilm control. However, the detailed discussion on biofilm detection and control strategies in livestock farming environments is beyond the scope of this review. Readers seeking in-depth insights are encouraged to consult recent comprehensive reviews focused specifically on biofilm management in agricultural and veterinary contexts (99–101).

Generally, it is important to select appropriate sampling methods based on the specific objectives and requirements of the assessment (29). The selection of testing methodologies should be an evidence-based process, aligning with key variables such as potential surface contamination, the specific hazards targeted by the C&D regimen, and the required level of cleanliness specific to each surface. Moreover, factors like when, where, and how to sample should be considered. Consequently, these considerations must guide decisions regarding the timing and methodology of sampling (29). Subjectivity and variation in individual perception may affect the precision of outcome. If a single individual is responsible for both the cleaning process and the visual inspection of farm facilities, there exists the possibility of overlooked areas during cleaning being similarly disregarded during the inspection (38, 55). Similarly, interpreting monitoring results is hindered by the absence of universally accepted guidelines defining when a surface is adequately cleaned (51).

Our analysis indicates that there are currently a limited number of studies describing the various techniques for evaluating hygiene in farm facilities and comparing their sensitivity and specificity under

field conditions. Furthermore, research on the impact of different materials surfaces (wood, plastic, metal, concrete, etc.) on hygiene outcomes is limited, highlighting the need for further investigation in this area.

Finally, the evaluation of hygiene effectiveness in diverse settings, particularly in the context of livestock farming, presents a complex challenge. To address this challenge effectively, it is imperative to integrate various assessment methods within a comprehensive protocol (Figure 7). This multi-step approach ensures that the C&D process is thoroughly assessed at each stage to maintain appropriate standards of hygiene. It is an ideal scenario which will probably not always be feasible under field condition.

As shown in Figure 7, the proper evaluation protocol should involve the following key steps:

- Initial visual inspection:* A thorough visual examination should be conducted before cleaning begins.
- Post-cleaning visual inspection:* After the cleaning and drying process, another visual inspection can be performed.
- Decision point:* If notable organic residues or missed areas are detected during the visual examination, the detergent cleaning process should be repeated. If not, can proceed with rapid tests (such as ATP, or RPT).
- Post-test evaluation:* If the results from rapid tests fail to meet established benchmarks, detergent cleaning should be repeated. If the results are satisfactory, proceed with disinfection.
- Post-disinfection testing:* After disinfection and drying, samples should be tested using rapid tests, microbiological methods, or a combination of both. In the case of an outbreak, molecular testing can also be employed.
- Final decision:* If the results of the applied tests fall below acceptable standards, the disinfection must be repeated.

Finally, to enhance hygiene management, it is highly beneficial to develop a farm-specific C&D protocol in collaboration with a supervising veterinarian, tailored to the farm's needs, health status, and current epidemic conditions, as suggested by Heinemann et al. (24). This protocol would involve task verification similar to self-monitoring controls in the food industry. Regular in-house training sessions, possibly led by a specialized consultant and conducted periodically, could improve procedural efficiency and prevent lapses. Effective C&D also depend on the qualifications and understanding of risks by the personnel involved (13, 102, 103). Engaging professional cleaning contractors is advantageous, a practice commonly adopted in poultry production. Past studies have demonstrated that the efficacy of C&D performed by professional cleaning firms surpasses that conducted by in-house farm staff (11, 24). Therefore, it is essential to enhance awareness of the critical importance of hygiene in livestock production.

Furthermore, improving the evaluation approach and developing a comprehensive protocol should involve the integration of cutting-edge technologies, along with a forward-looking discussion on future advancements and trends in the field. Emerging technologies such as nanomaterial-based antimicrobials, photodynamic treatments, and

pulsed light disinfection offer promising future alternatives to conventional disinfectants, particularly for targeting biofilm-associated pathogens (104–106). Additionally, intelligent sensors using electrochemical or optical biosensing, often integrated with artificial intelligence (AI) and Internet of Things (IoT) platforms, are being developed for real-time hygiene monitoring in food and farm environments (107, 108). These tools can detect residual ATP, pathogens, or environmental markers with high precision, enabling proactive sanitation management (41, 109). The integration of diverse technologies, such as biotechnology, physical technologies, and information technology, offers promising opportunities to enhance the evaluation of C&D effectiveness (92, 110, 111). Biotechnology enables the development of sensitive diagnostic tools and biosensors for rapid pathogen detection, while physical technologies can provide objective measurements of hygiene levels. Information technology, including AI, machine learning, and IoT systems, can further support real-time data collection, automated analysis, and decision-making. By combining these approaches, a more comprehensive, efficient, and data-driven framework for hygiene assessment can be established. This multidisciplinary integration not only improves accuracy and response time but also promotes the development of smart, scalable systems tailored for modern farm environments.

5 Limitations

This review has some limitations. First, it is a narrative review, while based on a systematic literature search, no quantitative synthesis or meta-analytic statistics were performed. Instead, the review offers a qualitative and comparative evaluation of methods used to assess C&D efficacy in reducing pathogen presence. Second, the scope was deliberately limited to farm livestock housing. Studies related to companion animals, laboratory animals, or other facility types were excluded to maintain a focused and practically relevant discussion. Finally, the review does not cover all existing C&D practices. Rather, it emphasizes the field applicability and comparative performance of selected field applicable evaluation methods, including biochemical, chemical, cultural, and molecular techniques. While this targeted approach enhances practical relevance, it may exclude broader methodological perspectives.

6 Conclusion

Assessing hygiene and microbial contamination in farm environments necessitates an evidence-based approach that leverages the strengths of multiple assessment methods. While no single method is flawless, the judicious combination of various techniques within a comprehensive C&D evaluation protocol provides a more holistic and accurate understanding of hygiene conditions. This integrated approach enables more precise monitoring and control of microbial contamination, ultimately enhancing the effectiveness of hygiene practices. The present study recommends choosing the appropriate combination of methods based on the specific needs of the farm, the frequency of evaluation, and the epidemiological status of the farm, district, and region. This strategic selection is crucial not only for improving hygiene

management but also for ensuring the overall well-being of the animals and improving animal health. Also, periodic sensitization and training of the farm staff can improve the compliance and procedural efficiency. Future research should focus on developing comprehensive evaluation models for assessing C&D effectiveness, optimizing sampling strategies to improve accuracy and efficiency, and advancing automated and intelligent detection technologies. The integration of AI-driven systems, real-time biosensors, and IoT-based tools holds strong potential to enhance the reliability and responsiveness of hygiene monitoring in farm settings.

Author contributions

IM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. EB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Validation, Writing – review & editing. PD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. LK: Formal analysis, Investigation, Validation, Writing – review & editing. JJ: Investigation, Validation, Visualization, Writing – review & editing. LC: Investigation, Validation, Visualization, Writing – review & editing. IC: Investigation, Supervision, Validation, Writing – review & editing. JD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

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