DISEASE ECOLOGY: NOVEL CONCEPTS AND METHODS TO TRACK AND FORECAST DISEASE EMERGENCE, TRANSMISSION, SPREAD, AND ENDEMIZATION

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EDITORIAL
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Editorial: Disease Ecology: Novel Concepts and Methods to Track and Forecast Disease Emergence, Transmission, Spread, and Endemization

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Editorial on the Research Topic

Disease Ecology: Novel Concepts and Methods to Track and Forecast Disease Emergence, Transmission, Spread, and Endemization

Disease ecology focuses on host-pathogen systems in the context of their environment and evolution, analyzing how species interactions and abiotic components of the environment affect patterns and processes of infectious diseases (de Garine-Wichatitsky et al., 2021b; **Figure 1**). The discipline has emerged during the past two decades, and is particularly relevant for improving our understanding and management of diseases in complex wildlife-livestock-human interfaces across the globe. The need to collate the latest advances in disease ecology was identified and discussed by the guest-editors in 2019, and this Research Topic was launched with Frontiers in March 2020, just after the SARS-CoV-2 virus had emerged and spread, shaking entire societies and ecosystems and profoundly changing health perceptions and practices around the world. The wildlife origin of the SARS-CoV-2 virus has highlighted the relevance of inter-disciplinary approaches, integrating disease ecology with biomedical and social sciences in order to understand the complex intertwined ecological, genetic, socio-economic and cultural drivers of pathogen emergence from wildlife. In addition, the spread of COVID-19 pandemic, and the diversity and magnitude of its impacts, has dramatically demonstrated that national public health services cannot handle such crisis alone, calling for more integrated intersectoral and cross-scale One Health/Planetary Health collaborations (de Garine-Wichatitsky et al., 2020). In this Research Topic, we have collated nine articles that illustrate the diversity of approaches developed to anticipate and better manage diseases associated with wildlife.

Three papers in this volume focus specifically on diseases in wild host populations, adopting various approaches to identify threats and proactively reduce the risks to wildlife populations, and two papers illustrate the associated risks to human populations. Russel et al. review theoretical and empirical examples of how populations can persist in the face of emerging threats of disease.

FIGURE 1 | Monitoring ecological and epidemiological interactions between willdlife, livestock and humans: radiotracking African buffalo in the Great Limpopo Transfrontier Conservation Area, Zimbabwe (left: picture by Eve Miguel/Michel de Garine-Wichatitsky); swallow in urban environment, Thailand (top-right: picture by Micha Garine-Wichatitsky); dead cow carcass, Southeast lowveld Zimbabwe (middle-right: picture by Michel de Garine-Wichatitsky); farmer riding a water buffalo in rural Cambodia (bottom-right: picture by Michel de Garine-Wichatitsky).

Excitingly, the authors identify five principles of persistence spanning population, landscape, and species levels of organization. They illustrate each principal with case studies from a variety of taxa and show how demographic, evolutionary and geographic processes can help determine the ability of hosts to persist with disease. Ultimately, the authors provide a useful synthesis that, for example, can guide wildlife health practitioners and land managers alike to manage disease in wildlife populations. Belsare et al. bring out the value of incorporating analytical approaches from other disciplines, which is the heartbeat of disease ecology, to showcase how disease can be managed in a wildlife population. Obanda et al. focus on a zoonotic disease, anthrax, and expose some of the abiotic predictors modulating its endemization, and Choi et al. further show how host life history traits, such as migration, substantially increase infection rates by *Salmonella* in barn swallows, which has major implications for pathogen movement. The opinion paper by Campos and Lourenço-de-Morae unravels the adaptations and evolutions of diverse coronaviruses as they spill across human-animal hosts and the consequences of the emergence of novel infectious diseases.

Molecular approaches have been increasingly used to characterize and quantify multi-host disease transmission at wildlife-livestock-human interfaces, including the interactions with their microbionts. Tremendous technical progresses have been made in recent years in order to collect and analyse efficiently (Martínez-López et al., 2021) large datasets of genetic sequences, offering new insights regarding wild and domestic hosts and parasites interactions. In their paper, Choi et al. explore the effects of potential pathogens (i.e., *Salmonella*) on wildlife microbiomes (i.e., barn swallows *Hirundo rustica*). Utilizing 16S rRNA gene sequencing alongside standard culture techniques, they concluded that bacterial community composition and diversity differed between birds based on *Salmonella* status. This illustrates how the monitoring of pathogens in wild birds and investigating the ecology of host microbe-pathogen relationships may provide useful data for prediction and mitigation of disease spillover into domestic animals and humans. The findings from Omony et al. reveal presence of mutations in the predicted immune-dominant fusion (F) and hemagglutinin-neuraminidase (HN) genes of *Avian Avulavirus serotype-1* (AAvV-1) strains from waterfowl or poultry in Uganda, that may influence immunity in vaccinated and susceptible animals. Specifically focusing on COVID-19, Srivastava et al. highlight a significant association of the alternate allele (allele T on plus strand or allele A on minus strand) of variant rs2285666 with the lower COVID-19 infection as well as lower case-fatality rate among Indian populations. It is believed that the data generated from the study will serve as a basis for understanding the role of Angiotensin-converting enzyme 2 (ACE2) in COVID-19 susceptibility.

The interdisciplinary integration of ecological, bio-medical and social sciences into a single discipline of "disease socio-ecology" remains a major research frontier for improved management of wildlife-livestock-human interfaces (Caron et al., 2021; de Garine-Wichatitsky et al., 2021b). A key element for improving knowledge and innovation regarding health and the adoption of effective management is the use of participatory approaches to frame health issues in contextualized social-ecological systems and co-design interventions with relevant stakeholders (de Garine-Wichatitsky et al., 2021a). Mendes et al. provide an illustration of the interdependencies between epidemiological and economic factors, and the usefulness of an epidemiological-economic model for the management of a livestock disease. In many contexts, successful disease control depends on the voluntary uptake of costly control measures by individual actors in the population. Using a modeling approach, they showed that even if some actors implement appropriate control measures, others may free-ride on these actions and thus compromise the benefits of control strategies that rely on collective behavior. The mismatch between individual costs and collective benefits likely contributes to the continued endemicity of many diseases despite substantial investments in control. Another key challenge for improving future health management is how to improve the connections between research and knowledge production entities, with education and policy and decision makers. In their paper, Comizzoli et al. propose an approach to health interconnecting science, culture and education, derived from a One Health framework associating health for all life. Although the proposed framework is specific to the Smithsonian network, grounded on its conservation and education infrastructures (museums, biological collections/zoos, education and research centers…), the lessons learnt through the application of the interconnected framework should benefit other transdisciplinary health research and education initiatives.

In summary, the papers collated in this topic illustrate the diversity of methods and approaches that are currently implemented and combined to improve our understanding of the ecology of disease emergence at wildlife-livestock-human interfaces. More than ever, with the COVID-19 crisis added to the climate change and biodiversity crises, disease ecology must innovate and grow as a leading discipline aggregating multiple tools and disciplines to forecast, prevent and mitigate the next emergences (de Garine-Wichatitsky et al., 2020, 2021b; Caron et al., 2021).

AUTHOR CONTRIBUTIONS

MG-W, OL, NF-J, KV, and VO contributed to the initial ideas and edition of the Research Topic. MG-W drafted the first version of the manuscript. All authors contributed to revising it critically and approved the final version.

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Genetic Association of *ACE2* rs2285666 Polymorphism With COVID-19 Spatial Distribution in India

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Studies on host-pathogen interaction have identified human ACE2 as a host cell receptor responsible for mediating infection by coronavirus (COVID-19). Subsequent studies have shown striking difference of allele frequency among Europeans and Asians for a polymorphism rs2285666, present in *ACE2*. It has been revealed that the alternate allele (TT-plus strand or AA-minus strand) of rs2285666 elevate the expression level of this gene upto 50%, hence may play a significant role in SARS-CoV-2 susceptibility. Therefore, we have first looked the phylogenetic structure of rs2285666 derived haplotypes in worldwide populations and compared the spatial frequency of this particular allele with respect to the COVID-19 infection as well as case-fatality rate in India. For the first time, we ascertained a significant positive correlation for alternate allele (T or A) of rs2285666, with the lower infection as well as case-fatality rate among Indian populations. We trust that this information will be useful to understand the role of *ACE2* in COVID-19 susceptibility.

Keywords: *ACE2*, India, rs2285666, coronavirus, COVID-19, SARS-CoV-2

INTRODUCTION

The progressive and rapid spread of the novel coronavirus SARS-CoV-2, has created a worldwide wave of crisis by profoundly affecting the human health, and global economic stability. Recent researches have shown that the ACE2 (encoding Angiotensin-Converting Enzyme 2) is the main host cell receptor of spike glycoprotein responsible for the infection (Hoffmann et al., 2020; Lu et al., 2020; Zhou et al., 2020). It plays a crucial role for the entry of the virus into the cell to cause the final infection (Lu et al., 2020). ACE2 is a type I transmembrane metallocarboxypeptidase with homology to ACE, an enzyme long-known to be a key player in the Renin-Angiotensin system (RAS), and a target for the treatment of hypertension (Li et al., 2003; Shi et al., 2014). The secreted protein catalyzes the cleavage of the C-terminal dipeptide of Angiotensin I to produce Angiotensin 1–9 and Angiotensin II to Angiotensin 1–7.

The ACE2 is mainly expressed in vascular endothelial cells, the renal tubular epithelium, and in Leydig cells of the testes (Riordan, 2003; Kuba et al., 2010). PCR based analyses have revealed that

ACE2 is also expressed in the lung, kidney, gastrointestinal tract, and blood vessels, tissues (Harmer et al., 2002; Ksiazek et al., 2003; Jiang et al., 2014). More recent reports have suggested ACE2 expression in the mucosa of the oral cavity may grant easy access to the virus for a new susceptible host (Xu et al., 2020). This explains the high incidence of pneumonia and bronchitis in those with a severe SARS-CoV-2 infection (Zhou et al., 2020). The ACE2 regulates blood volume, systemic vascular resistance, and thus cardiovascular homeostasis. *ACE2* has previously been found to be associated with hypertension, stroke, dyslipidemia, cardiovascular diseases, and kidney diseases (Wang et al., 2014; Pan et al., 2018; Wu et al., 2018; Zhang et al., 2018). People on ACE inhibitors and ARBs (angiotensin II type I receptor blockers) produce more numbers of receptors, raising the question of increased susceptibility to the infection, as the SARS-CoV-2 attaches itself to the ACE receptors on heart and lung tissues (Mehra et al., 2020). The expression of ACE2 is also substantially increased in patients with type 1 or type 2 diabetes, who are treated with ACE inhibitors and ARBs (Fang et al., 2020).

Contemporary studies on the large number of samples have analyzed the genomic variations present among populations worldwide (Cao et al., 2020; Stawiski et al., 2020). It was unanimously shown that a polymorphism rs2285666 present in *ACE2*, varied significantly among European and Asians (Asselta et al., 2020; Cao et al., 2020). The expression experiments have suggested that the alternate allele (TT-plus strand or AA-minus strand) of rs2285666 elevated the expression of this gene upto 50%, hence may play a role in SARS-CoV-2 susceptibility (Wu et al., 2017; Asselta et al., 2020). Therefore, we studied the haplotype structure as well as association of this allele for COVID-19 susceptibility in India. In particular, we have analyzed the haplotypes downstream to rs2285666 among worldwide populations as well as compared the frequencies of this allele and number of cases and case-fatality rate in India for any existing association.

MATERIALS AND METHODS

The worldwide data for haplotype analysis was extracted from recent complete genome analysis (Pagani et al., 2016). NETWORK 5 (Bandelt et al., 1999) was used to construct the median joining network of haplotypes derived from rs2285666 polymorphism. All the SNV's observed downstream to rs2285666 have been listed in **Supplementary Table 1**. Illumina HumanHap 730K genotype chip has listed rs2285666 SNV in their panel,

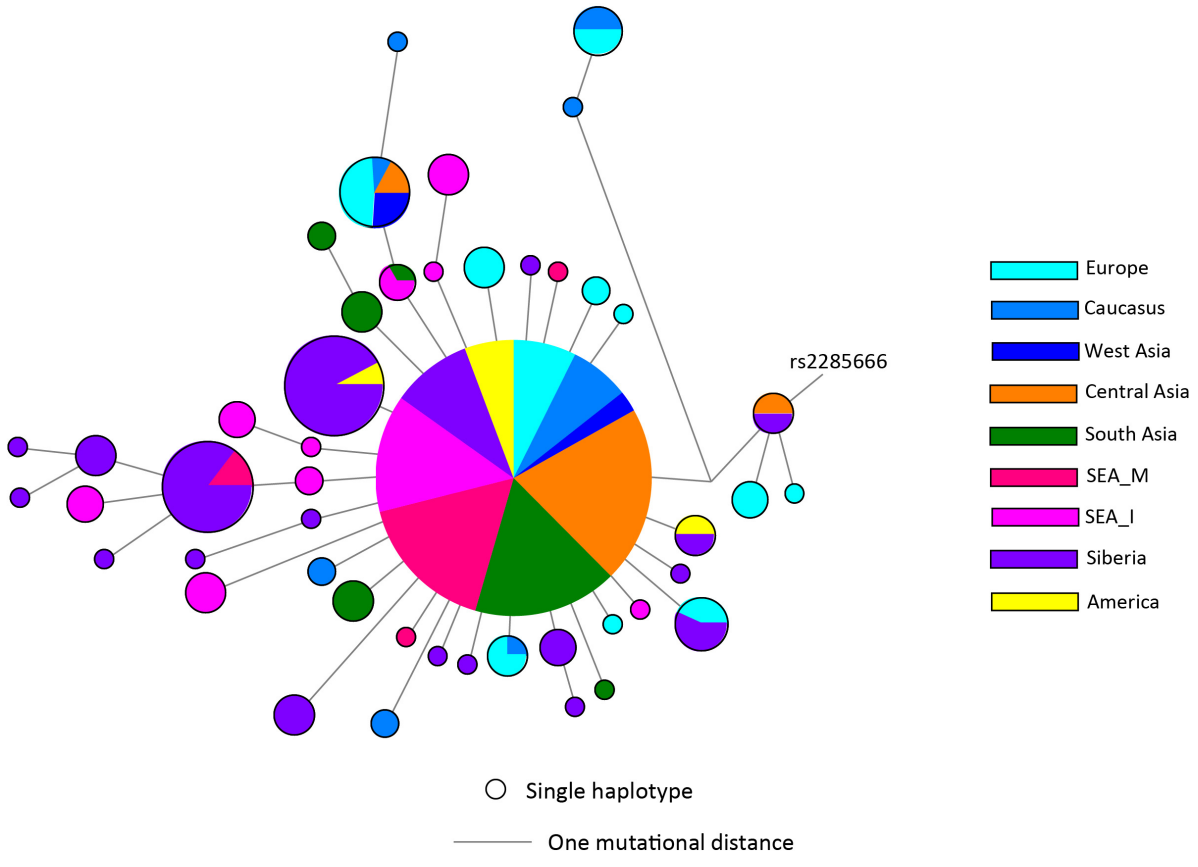

FIGURE 1 | The median joining (MJ) network analysis constructed from haplotypes downstream to variant rs2285666. The network was constructed manually from the analysis obtained from NETWORK 5 pogramme. SEA_M, Southeast Asian Mainland; SEA_I, Southeast Asian Island. The downstream polymorphisms of CNV rs2285666 have been shown in **Supplementary Table 1**.

therefore we looked the genotype data generated on this platform. The frequency data of SNV rs2285666, of various populations were extracted by using Plink 1.9 (Chang et al., 2015), from 1,000 genome project data phase 3 (1000 Genomes Project Consortiumet al., 2010), data published by Estonian Biocentre (Chaubey et al., 2017; Pathak et al., 2018; Tätte et al., 2019; Estonian Biocentre Public _Data, 2020) and our newly genotyped samples for various Indian states and the Bangladesh (**Supplementary Table 2**). The frequency maps were generated by https://www.datawrapper.de/. The regression plots were constructed by https://www.graphpad.com/quickcalcs/linear1/ and verified by the Microsoft excel regression calculations. We have also used Pearson's correlation coefficient test (Benesty et al., 2009) to validate our results. SPSS (ver 25) was used to estimate the Person correlation for two tailed significance test at 95% CI and 1,000 bootstrapping (2,000,000 seeds). The joint plots for all parameters were obtained from customized script of program R (ver 4) (R Core Team, 2012).

RESULTS AND DISCUSSION

Studies have shown that angiotensin-converting enzyme 2 (ACE2) acts as an entry receptor for coronavirus (Li et al., 2003). The interaction between SARS-S and ACE2 has already been expounded at molecular level in detail suggesting ACE2 as key determinant of SARS-CoV transmission (Li F. et al., 2005; Li W. et al., 2005). It has been revealed that human recombinant soluble ACE2 (hrsACE2) inhibits growth of SARS-CoV-2 and interrupts early stages of infections (Monteil et al., 2020). The variable susceptibility to the SARS-CoV-2 infection may be associated with the certain genomic variants within *ACE2*, that modulate its function or expression.

Among all the common exonic variants, some very recent studies done on *ACE2* variants reported population-based frequency differences for a single nucleotide variant (SNV) rs2285666 (also called G8790A) (Asselta et al., 2020; Cao et al., 2020; Strafella et al., 2020). This variant of *ACE2* was significantly different for Europeans (0.2), than the East Asians (0.55) (Asselta et al., 2020; Cao et al., 2020). Our analysis on Indian population has revealed mean frequency ∼0.6 of this allele (Srivastava et al., 2020). Moreover, our haplotype analysis for this gene revealed excessive sharing of the frequent South Asian haplotypes with East Eurasian populations, rather than West Eurasian populations (Srivastava et al., 2020). Similar to the East Asians, we have also noted that the frequency of this allele is significantly higher (two tailed $p < 0.0001$) among Indian populations in comparison with either of European, American, or African.

Since rs2285666 has already been proven to be a potential risk factor for hypertension, type 2 diabetes, and coronary artery disease (Chaoxin et al., 2013; Asselta et al., 2020), therefore, may possibly be a predisposing factor associated

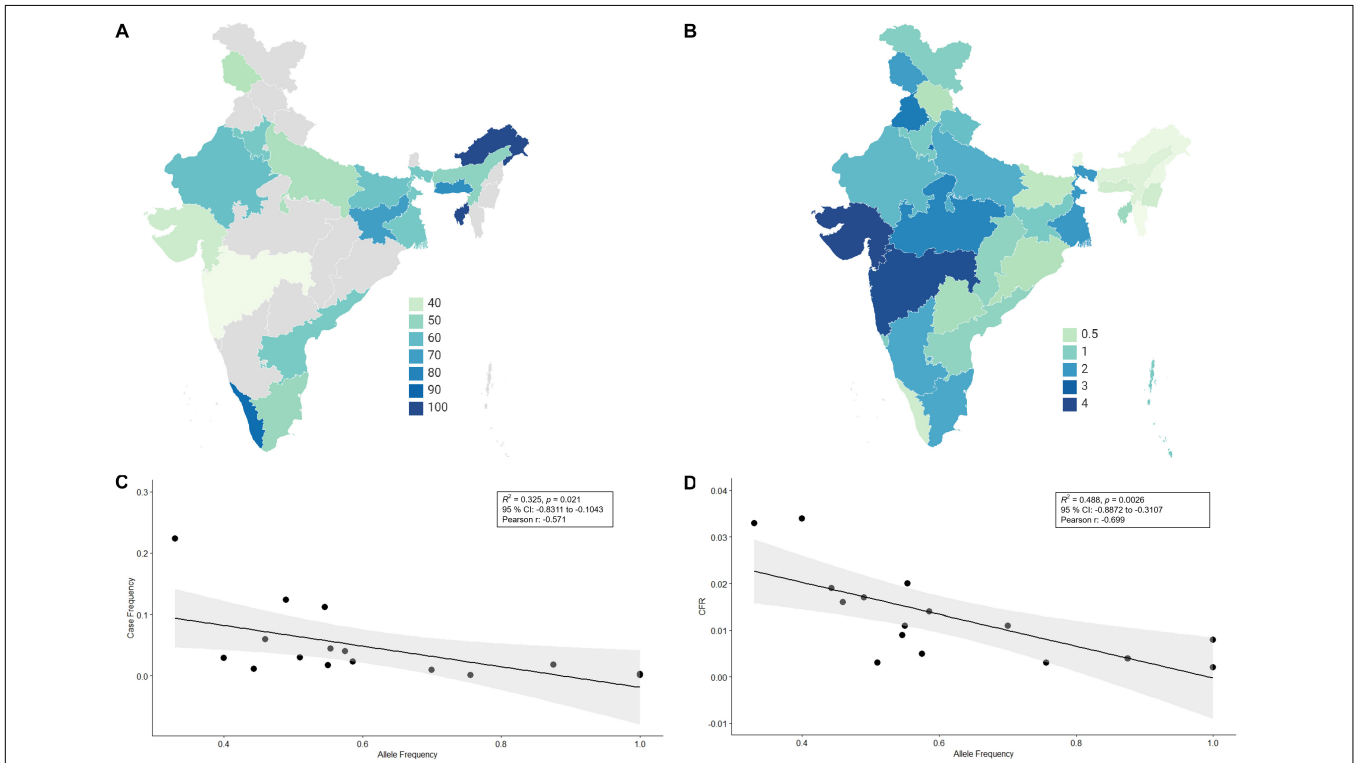

FIGURE 2 | (A) The frequency (%) of allele rs2258666 among Indian populations. The gray colored areas in the map show the absence of data. **(B)** The statewise frequency (%) of case-fatality rate (CFR) (updated till 23rd August 2020). **(C)** The regression analyses showing the goodness of fit and Pearson correlation coefficient for the allele frequency and frequency of cases as well as **(D)** allele frequency vs. CFR (case-fatality rate) among various states of India (updated till 23rd August 2020).

with the comorbidities observed in COVID-19 patients. Variant rs2285666 is located at the beginning of the intron 3, theoretically affecting gene expression with alternative splicing mechanisms (Li, 2012; Yang et al., 2015). A study also reported for the association of three rs2285666 genotypes with ACE2 protein level measured in serum by ELISA, with the A/A genotype having an expression level almost 50% higher than the G/G genotype (Li, 2012). More recently, it is shown that the substitution of G with A is predicted to increase the strength of the splice site of about 9.2%, resulting higher expression of ACE2 protein (Asselta et al., 2020). It has been also noted that patients characterized by higher ACE1 activity (a protein similar to ACE2) in conjunction with reduced ACE2 activity (i.e., CC/GG females or hemizygous C/G-males for rs2285666) account for increased susceptibility to hypertension, mainly in association with classical cardiovascular risk factors such as old age, dyslipidemia, and diabetes (Pinheiro et al., 2019; Ghafouri-Fard et al., 2020). Thus, it is clear that decreased ACE2 level contributes to severe consequences of SARS-CoV-2 infection (Samavati and Uhal, 2020; Verdecchia et al., 2020).

There has not been any study so far on this SNV among Indian Populations. Therefore, we first looked the haplotype sharing, derived after variant rs2285666 among worldwide populations (**Figure 1**). Most of the haplotypes downstream to variant rs2285666 were belonging to South Asian, Central Asian, and East Eurasian populations. The starlike structure of rs2285666 derived haplotype indicate a case of positive selection among Asian populations, which needs further exploration (**Figure 1** and **Supplementary Table 1**). Subsequently, our spatial analysis showed that in India, frequency of alternate allele of this SNV (rs2285666) varied between 33% and 100% (**Figure 2A** and **Supplementary Table 2**). The frequency gradient (lower to higher) is observed from Northwestern and Western region to Northeastern part of the subcontinent. In order to understand the correlation of allelic frequency with respect to the frequency of cases among Indian populations (**Figure 2B**), we performed linear regression and Pearson's correlation coefficient analyses for variant rs2285666 and frequency of cases as well as case-fatality rate (CFR) (**Figures 2C,D** and **Table 1**). The regression analysis showed a significant correlation between allele frequency and number of cases ($p < 0.05$) (**Figure 2C** and **Table 1**). More number of cases are observed where frequency of this allele is lower and vice versa. The goodness of fit (R^2) explained 34.6% of the variation. This suggests that the effect size of this allele for Indian populations is large. Since this is an ongoing pandemic and the number of infected people changes with time, we tested this result by adding the latest number of cases (August 2020) as well as the CFR (**Figure 2** and **Table 1**). The latest data is also consistent with the older observation. We didn't find any significant difference between both of the results. Moreover, the CFR data showed stronger association with the allele frequency of rs2285666 (**Table 1**). Further, in order to confirm our findings, we have also performed the Pearson's correlation coefficient test. The genetic variation (frequency of rs2285666) and number of cases are negatively correlated with $r = -0.571$, $p = 0.05$ (August 2020), as well as frequency of rs2285666 and CFR are also negatively correlated with $r = -0.699$, $p = 0.005$ (**Figures 2C,D** and **Table 1**). The Pearson correlation analyses supported previous observations by showing a strong negative correlation of rs2285666 (allele T/A) with the number of cases and case-fatality ratio.

TABLE 1 | Outcome of the various tests performed for statistical significance.

Observation	Linear regression		Pearson's correlation	
	R square	*p*-value	r	*p*-value
May 2020_Cases	0.346	0.0165	−0.588	0.0167
August 2020_Cases	0.325	0.021	−0.571	0.021
August 2020_CFR	0.488	0.002	−0.699	0.002

Thus, for the first time, we showed a strong correlation of alternate allele (allele T on plus strand or allele A on minus strand) of variant rs2285666 with the lower infection rate as well as lower CFR among Indian populations. Although whole genome sequencing of a considerably large sample of cases and control individuals in India need to be performed to secure a robust genetic information on susceptibility for the disease, we here establish a possibility of the SNV (rs2285666) being associated with a protective role against COVID-19.

We caution that this is just one of the factors affecting the transmission, however there are several other elements (e.g., variation of rs2285666 among diverse ethnic groups of a state, sex of a person, comorbidity, virus strain, temperature, humidity, population density, social distancing, lockdown, etc.), which can perturb the infection rate and CFR substantially. If more of genetic factors or polymorphisms are recognized that may have played a significant impact on the variability of SARS-CoV-2 course, it would be worthwhile to design a cheap and accurate DNA based test for coronavirus susceptibility.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GC concived and designed this study. AS, AB, DD, RP, VS, NK, NS, PS, PD, AP, PG, NR, and GS collected the data for allele and COVID-19. AS, AB, PS, PD, AP, and GC analyzed the data. AS, AB, DD, PS, and GC wrote the manuscript from the inputs of other co-authors. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.564741/full#supplementary-material

Supplementary Table 1 | The details of SNV's downstream to the rs2285666 polymorphism. This data is obtained from the Network analysis.

Supplementary Table 2 | The summary of data used in present analyses.

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Principles and Mechanisms of Wildlife Population Persistence in the Face of Disease

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Emerging infectious diseases can result in species declines and hamper recovery efforts for at-risk populations. Generalizing considerations for reducing the risk of pathogen introduction and mitigating the effects of disease remains challenging and inhibits our ability to provide guidance for species recovery planning. Given the growing rates of emerging pathogens globally, we identify key principles and mechanisms for maintaining sustainable populations in the face of emerging diseases (including minimizing the risk of pathogen introductions and their future effects on hosts). Our synthesis serves as a reference for minimizing the risk of future disease outbreaks, mitigating the deleterious effects of future disease outbreaks on species extinction risk, and a review of the theoretical and/or empirical examples supporting these considerations.

Keywords: adaptive capacity, disease risk, emerging infectious disease, extinction risk, refugia, wildlife populations, spillover

INTRODUCTION

Historically, the role of disease in wildlife conservation was underestimated; possibly due to the difficulty of attributing causal mechanisms to population declines (Plowright et al., 2008; Hefley et al., 2017) and the inability to detect dead or dying animals (Skerratt et al., 2007). However, theoretical and empirical studies demonstrate that disease increases species extinction risk through demographic stochasticity, Allee effects, or other mechanisms (De Castro and Bolker, 2005). Infectious disease has contributed to multiple species declines and limited the ability of species to maintain sustainable populations without human intervention (Deem et al., 2001; Smith et al., 2006; Canessa et al., 2018). Despite this, developing general guidelines for managing wildlife populations in the face of emerging diseases remains challenging.

Disease outbreaks can cause rapid and catastrophic declines in wildlife populations. White-nose syndrome, for example, has resulted in the decline of many cave-hibernating North American bat species (Blehert et al., 2009), and has contributed to the listing of the Northern long-eared bat (*Myotis septentrionalis*) as Threatened (United States Fish and Wildlife Service (USFWS), 2015). Likewise, two chytrid fungi (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) have

contributed to global amphibian declines (Scheele et al., 2019), and macroparasites have decimated honeybee populations (Brosi et al., 2017), endangering human food system sustainability. Because species do not occur in isolation, these disease-induced declines can cause top-down and bottom-up consequences in ecosystems.

Ecosystem-level changes caused by disease affect predators and prey. For example, in Australia, Devil Facial Tumor Disease (DFTD) has caused the severe decline of an apex predator (*Sarcophilus harrisii*, the Tasmanian Devil) and resulted in changes to the mammalian community (Hollings et al., 2016). The loss or decline of a keystone species can also contribute to ecosystem changes via trophic cascades, as is hypothesized in the case of Sea Star Wasting disease and the decline of kelp forests in the Pacific Northwest (Schultz et al., 2016; Rogers-Bennett and Catton, 2019), and snake declines driven by chytridiomycosis-induced amphibian losses (e.g., Whiles et al., 2006; Zipkin et al., 2020). Although these types of examples are scarce in the literature, they may be more widespread than currently recognized.

In addition, disease may exacerbate the effects of other stressors. Other stressors such as habitat fragmentation and climate change may prime populations to be more vulnerable to disease. For example, populations that are declining due to habitat fragmentation and loss, or climate change may be more vulnerable to the effects of disease (McCallum and Dobson, 2002; Gallana et al., 2013). Under these scenarios, disease can reduce the effectiveness of conservation actions, such as translocations (Cunningham, 1996), if translocated animals bring novel pathogens with them, or corridor creation (Hess, 1994), which can also facilitate disease spread. Therefore, consideration of how actions affect both pathogen spread and population perseverance is important for conservation practices.

Given the growing frequency of pathogen emergence (Daszak et al., 2000; Dobson and Foufopoulos, 2001; Fisher et al., 2012), conservation and management agencies are facing new challenges in managing for sustainable populations under the threat of epizootics. The concept of resiliency, defined as the population's ability to withstand stressors (such as environmental fluctuations, habitat loss, or disease), has recently been suggested in the literature as a means for improving wildlife population management in general (Hanisch et al., 2012; Stephens, 2014; Cassirer et al., 2018). Correspondingly, there is a need to identify principles and mechanisms to manage populations to improve resiliency to emerging infectious diseases, including characteristics of populations that are amenable to management to reduce disease risk directly (i.e., population size, genetic diversity), and those that are difficult to manage but can be used to assess disease risk to a species (i.e., species life history).

Here we present a synthesis that serves as a reference for wildlife managers on how to (i) assess the vulnerability of species to epizootics, (ii) minimize the potential for disease to result in species declines, and (iii) increase the likelihood that species recover following outbreaks. We focus on the principles behind the management of wildlife hosts in the face of emerging diseases rather than pathogen characteristics that can affect the ability of hosts to persist with disease. In doing so, we aim to provide managers with a set of principles and mechanisms focused on wildlife population persistence in the face of disease.

POPULATION-LEVEL PRINCIPLES

Principle 1. Maintain Large Population Size and Demographic Rates

In general, population size is correlated with species extinction risk (e.g., Diamond et al., 1987; Berger, 1990; Foufopoulos and Ives, 1999; O'Grady et al., 2004). When epizootics occur, the robust demographic structure, high abundance, and reproductive capacity associated with large population sizes buffer species from disease-induced extinction (**Table 1**).

Abundance

Both stochastic and deterministic extinction risk is correlated with population abundance (Payne and Finnegan, 2007). Small population sizes have been associated with extinction risk from disease for several species, including black-footed ferrets (*Mustela nigripes*), bighorn sheep (*Ovis canadensis*), golden (*Incilius periglenes*) and boreal toads (*Anaxyrus boreas*), and other species (**Table 2**) that either historically lived in small populations, or in populations that have been reduced in size by stressors other than disease. For each of these species, disease resulting in direct mortality of individuals increased extinction risk. For example, Page (2013) identified small population sizes as a characteristic of disease-induced extinctions, and described how habitat fragmentation and loss resulted in small population sizes for Allegheny woodrats (*Neotoma magister*) that combined with the parasite, *Baylisascaris procyonis,* to reduce population viability. In addition, small population sizes contribute to reductions in other characteristics of resilient populations, such as genetic diversity (for example resulting in Allee effects; Allee and Bowen, 1932), that are described in more detail below. In contrast, large population size may increase disease transmission. For example, rapid spread of white-nose syndrome (Frick et al., 2016) may have been enhanced by large geographically distributed populations of North American bats. Similarly, populations that are artificially dense (such as elk populations at feeding stations in Wyoming or game farms) may lead to increased disease transmission risk (Cotterill et al., 2018).

Demographic Structure

The age structure of a population can contribute to its ability to persist in the face of an emerging disease when disease susceptibility varies by life stage. For example, the larval stages of amphibians may be less susceptible to chytridiomycosis, but more susceptible to ranavirus (Haislip et al., 2011; Bradley et al., 2019), and phocine distemper in European harbor seals has distinct age-related mortality rates (Härkönen et al., 2007). Maintaining diversity in a species age structure may limit the effects of disease on the population by limiting the opportunity for a pathogen to affect a susceptible age class and allowing for the population to subsequently rebound. If the life stages that are susceptible to disease are critical for population growth (i.e., reproductive stages), and other stressors

TABLE 1 | Summary of principles and mechanisms that minimize species extinction risk following an epizootic, increase the likelihood of recovery from an epizootic, and mitigate the effects of future epizootics.

Principle	Scale	Mechanism	Inherent (I)/ amenable to management (A)	Minimize risk of catastrophic disease	Increase the likelihood of recovery from an epizootic
Principle 1. Maintain large population size	Population				
		Abundance	A	Y	Y
		Demographic structure	A	?	Y
		Reproductive capacity	I	N	Y
Principle 2. Minimize contacts with domestic, invasive, or novel species	Population				
		Biotic reservoirs	A	Y	Y
Principle 3. Maintain multiple connected populations	Landscape				
		Demographic or genetic rescue	A	?	Y
Principle 4. Maintain geographic distribution	Landscape				
		Epizootic asynchrony	A	Y	Y
		Environmental refugia	A	Y	Y
Principle 5. Maintain adaptive capacity	Species				
		Phenotypic plasticity	I	Y	Y
		Evolutionary rescue	A	?	Y
		Life history traits	I	N	Y

Letters in the final two columns (Y and N) indicate whether principles or mechanisms can be actively managed (Y) or not (N).

act synergistically with the effects of disease (Schisler et al., 2000), then reducing the effect of other stressors may enhance population stability. This would reduce the overall mortality of the susceptible class and ensure that some survive to reproduce. Alternatively, population reduction efforts could focus on age or stage classes that are responsible for the greatest risk of disease transmission.

Demographic Rates That Promote Population Growth

The reproductive capacity of a population, or the ability of a population to increase reproductive capacity when population levels are low (i.e., compensatory births or recruitment), can determine the rate at which a population can recover to its original size after a disturbance (Holling, 1973). For example, cave-hibernating *Myotis* spp. have a reproductive rate of one pup per female per season, which can result in an extended recovery period from white-nose syndrome, even if the effects of the disease are abated (Russell et al., 2015). Density-dependent and compensatory mechanisms can also promote recovery post-outbreak. Populations that exhibit negative density-dependence (i.e., reduced mortality or increased recruitment at lower densities), will rebound faster than populations that require a minimum threshold number of individuals for recruitment to occur (an ecological mechanism leading to rescue; McDonald et al., 2016). Disease outbreaks that do not cause direct mortality may also affect populations by reducing reproductive rates (Breed et al., 2009), which can slow a species recovery following a disturbance and make populations more vulnerable to stochastic extinction. Ensuring demographic rates are maintained such that population growth rates are equal to or above 1.0 will improve the ability of the species to withstand an epizootic. Managing populations for growth rates greater than one will provide a buffer against disease-induced extinction by ensuring that populations have enough individuals to withstand an increase in mortality rates.

Principle 2. Minimize Contacts Between Domestic, Invasive, and Novel Species

When populations interact with novel species, a pathogen can "spillover" from one host species to another. For example, the recent coronavirus outbreak was likely the result of a spillover event of a pathogen from wildlife to humans (Rodriguez-Morales et al., 2020). When the introduction and maintenance of disease in a population results from the interaction with alternative competent hosts, the resilience of populations to disease may be threatened (Power and Mitchell, 2004).

Biotic Reservoirs

Pathogens carried by species that are less prone to disease (i.e., reservoir species) can maintain the pathogen in wildlife populations through continuous reintroductions from the reservoir. This disease "spillover" might take place even when the wildlife population has declined to a population size where we would expect epizootic fadeout (McCallum and Dobson, 2002; Lloyd-Smith et al., 2005). There are several examples of damaging spillover events from biotic reservoirs, especially between domestic and wildlife hosts. For example, orange-bellied parrots (*Neophema chrysogaster*; Peters et al., 2014), Ethiopian wolves (*Canis simensis*; Haydon et al., 2002; Randall et al., 2006), red squirrels (*Sciurus vulgaris;* Rushton et al., 2006), and African wild dogs (*Lycaon pictus*; Woodroffe et al., 2012) have all suffered population declines attributed to the interaction between wild and domestic animal populations that resulted in the introduction of novel pathogens to wild populations. Human-assisted movements of wildlife can result in spillover events by introducing novel species into native

TABLE 2 | List of empirical examples where organisms have been negatively affected by disease.

Species	Castro's designation	Explanation	Citations
Golden toad (*Bufo periglenes*)	Probable extinction	*Batrachochytrium dendrobatidis* likely contributed to the demise of the small isolated populations	Pounds et al., 1997
>500 species of amphibians declines	>90 proposed extinct in the wild	*B. dendrobatidis* driven disease declines	Scheele et al., 2019
Black-footed ferret (*Mustela nigripes*)	Extinction in the wild	Spillover (canine distemper), introduced pathogen (plague), territorial and live in small isolated populations. Each ferret family needs a lot of territory. Primary impediment to recovery is plague killing their prey items	Thorne and Williams, 1988
Mednyi arctic fox (*Alopex lagopus semenovi*)	Probable extinction	Spillover (mange) likely caused a population bottleneck that contributes to slow recovery. Population switched diets after going through the bottleneck. Mercury levels may keep populations small	Goltsman et al., 1996; Bocharova et al., 2013
African wild dog (*Lycaon pictus*)	Population crash	Spillover (rabies), domestic dogs, and other predators keep populations small	Woodroffe et al., 2012; Canning et al., 2019
Boreal toad (*Bufo boreas*)	Population crash	Bd created small isolated populations. Reintroductions are preserving the species (Gerber et al., 2018)	Muths et al., 2003
Spanish ibex (*Capra pyrenaica hispanica*)	Population crash	Spillover, (Castro et al., 2016) animals that are healthier can resist disease	León-Vizcaíno et al., 1999
Bighorn sheep (*Ovis canadensis*)	Possibly population crash and extinction	Spillover	Gross et al., 2000
Wolves on Isle Royale (*Canis lupus*)	Increased susceptibility to disease	Spillover of parvovirus lead to small population and inbreeding which caused extinction.	Peterson et al., 1998
Cheetahs (*Acinonyx jubatus*)	Increased susceptibility to disease	Low representativeness may make population more susceptible to disease though habitat loss is main threat.	O'Brien et al., 1985
Koala/Chlamydia*	Possible population crash (model)	Non-density dependent transmission, connectivity is contributing to population loss, possible spillover	Augustine, 1998; Polkinghorne et al., 2019
Rabbit/Rabbit haemorrhagic disease*	Possible population crash (model)	Inhomogeneous mixing, spillover, rabbits survive because they are populous and geographically distributed.	Abrantes et al., 2012
Iiwi (*Vestiaria coccinea*), Amakihi (*Hemignathus virens*)	Population crash	Biotic reservoir, geographic representativeness, genetic representativeness may allow the development of resistance	Atkinson et al., 1995, 2000
White-tailed deer (*Odocoileus virginianus*)/moose (*Alces alces*)	Population reduction	Spillover from WTD to moose, meningeal worm, controversial as to the cause of moose decline.	Schmitz and Nudds, 1994; Vanderwaal et al., 2014
Red squirrel (*Sciurus vulgaris*)	Population reduction	Spillover, affects population resilience, management of invasive host populations (gray squirrels) may help	Rushton et al., 2000
Ethiopian wolf (*Canis simensis*)	Population reduction	Spillover (Rabies and distemper)	Haydon et al., 2002
Cave-hibernating bats*	Population reduction	Novel pathogen introduction (spillover)	Frick et al., 2010
Ungulates*	Decreased individual survival rates	Chronic wasting disease spillover from game farms, large distributed populations help preserve the species	Miller et al., 2008
Allegheny woodrats (*Neotoma magister*)*	Disease as an impediment to recovery	Parasite that along with other stressors affects populations that are fragmented and small	Page, 2013

*This table has been modified from De Castro and Bolker, 2005 and focuses on terrestrial vertebrates. We added a few more examples that have occurred since the original publication, and updated some of the designations to represent current literature. *Added to table.*

communities. For example, the pet trade (Karesh et al., 2005) can result in releases of non-native species into the wild, which can introduce new diseases into previously naïve wildlife populations. Movements of disease vectors, such as mosquitoes (Warner, 1968), or pathogens (such as fungal spores or prions) through the redistribution of abiotic reservoirs, such as contaminated soil and water (Almberg et al., 2011), can also result in novel introductions leading to spillover. For diseases for which alternative hosts play a large role in the maintenance of epizootics, reducing the potential pathogen spillover by limiting introductions of non-native species and reducing interactions with alternative hosts can help reduce extinction risk from disease. Creating barriers to movement, changing seasonal grazing patterns, restricting the pet and exotic animal trade, and reducing the movements of contaminated reservoirs are management actions that can be taken to reduce spillover risk.

LANDSCAPE-LEVEL PRINCIPLES

Principle 3. Maintain Multiple Connected Populations

A large theoretical literature regarding the effects of population connectivity on species persistence in the face of disease investigates trade-offs between population connectivity and disease risk (Hess, 1994, 1996; Gog et al., 2002; McCallum and Dobson, 2002; Reed, 2004; Cross et al., 2005; Jesse et al., 2008). Well-connected populations may be more likely to succumb to a disease outbreak as animal movements

spread pathogens between populations (Hess, 1996); however, isolated populations may go extinct without the benefits of immigration from neighboring populations (i.e., demographic rescue, Gotelli, 1991).

Demographic or Genetic Rescue

The optimal degree of population connectivity depends on a variety of factors, including the pathogen transmission dynamics (McCallum and Dobson, 2002), and the rate of animal movements relative to the infectious and recovery periods (Hess, 1996). For pathogens that have an environmental transmission route (such as fungal pathogens including *Pseudogymnoascus destructans* and amphibian chytrids), a lack of host population connectivity may not be a barrier to spread, but rather present an impediment to the species' ability to withstand an epizootic because subpopulations cannot benefit numerically from immigration of new individuals or genetically via the introduction of protective alleles.

Fragmented and small populations have been identified as key determinants of extinction risk from disease (De Castro and Bolker, 2005; Woodroffe et al., 2012, **Table 1**), and increase vulnerability to stochastic events, such as spillover of disease from other populations (see Gog et al., 2002, **Table 1**), extreme weather, and habitat destruction (De Castro and Bolker, 2005). For example, bighorn sheep (*O. canadensis*) suffered severe population declines post-European settlement (Monson and Sumner, 1985), and now persist in small relatively isolated herds. Respiratory disease is an impediment to recovery for those isolated herds unable to recruit from neighboring populations (Wehausen et al., 2011; Cassirer et al., 2018).

Dispersal-limited species, such as amphibians, may rely on the ability to change behaviors in order to respond to catastrophic events such as disease (Cushman, 2006; Ruiz-Aravena et al., 2014), but populations that consist of individuals that are naturally (e.g., sessile organisms) or artificially (e.g., due to land-use change) restricted in movement may face greater obstacles in recovering from a pathogen introduction. Further, restricted dispersal ability may subject these species to environmental stressors that are synergistic with disease. For example, coral disease outbreaks are likely exacerbated by warming ocean temperatures, and with limited dispersal ability, coral are unable to relocate in more suitable environments (Sokolow, 2009; Altizer et al., 2013). For some species, climatic or land-use changes have disrupted migration routes, leading to an increase in exposure to pathogens and pests. Increased parasite burden has been documented in monarch butterflies (*Danaus plexippus*) that forgo migration due to milder winters (Altizer et al., 2011). Finally, there is evidence that some species have evolved movement behaviors to reduce disease risk, as in the case of post-calving reindeer (Rangifer tarandus) migration, where herds that migrate greater distances have decreased warble Hypoderma spp. fly abundance (Folstad et al., 1991). When movement is limited, this strategy for disease avoidance becomes compromised. Wildlife managers are tasked with balancing the trade-offs between managing populations and landscapes to promote host population connectivity and gene flow, while reducing dispersal among populations to reduce the risk of pathogen movement among populations (Cassirer et al., 2018). Increasing substructure of recovering populations (i.e., creating more populations with varying levels of connectivity between them), as well as increasing the population size, may be a solution to this dichotomy.

Principle 4: Maintain Broad Geographic Distributions

Geographically distributed self-sustaining populations provide several advantages for maintaining viable populations of a species in the face of disease (Bascompte et al., 2002; **Table 1**). Two such advantages include epizootic asynchrony and environmental refugia, which may act alone or in synergy.

Epizootic Asynchrony

Studies have shown that populations that are closer together or otherwise connected tend to be more synchronous in their dynamics and response to disturbances (Ranta et al., 1997); synchrony in population dynamics can lead to global extinctions (Heino et al., 1997), and this is true for disease outbreaks. In addition, more widely distributed populations tend to experience a wider variety of environmental conditions and have uncorrelated population dynamics that allow a species to persist in the face of stressors (Allen et al., 1993). For example, Duke-Sylvester et al. (2011) demonstrated how rabies in raccoon Procyon lotor populations tended to be synchronized across large geographic areas that lack strong seasonal pulses, whereas in areas with strong seasonal changes in environmental conditions (northern latitudes) outbreak dynamics appeared to be asynchronous. This indicates that geographic areas with strong seasonality (i.e., large changes in environmental conditions between seasons) may represent refugia from disease outbreaks. Finally, increasing asynchrony in disease dynamics (i.e., temporal differences in when outbreaks occur) allows for ecological mechanisms, such as the rescue effect, to occur. For example, prairie dog Cynomys spp. colonies subject to frequent plague outbreaks can persist due to the asynchronous nature of plague outbreaks among populations that allow for re-colonization of extirpated populations (Stapp et al., 2004).

Environmental Refugia

Large numbers of geographically distributed populations are also more likely to occur across gradients of environmental conditions, providing some insurance against a pathogen with a limited environmental niche. Such refugia, stemming from within and among-patch environmental heterogeneity, results in the conditions necessary for pathogen to infect hosts, grow, and spread. For example, in Hawaii, thermal constraints on the distribution of avian malaria (*Plasmodium relictum*) allow Hawaiian honeycreepers (*Drepanidinae*) to persist at high elevations (LaPointe et al., 2010), while in Australia, high elevation dry forest may create refugia for frogs from *B. dendrobatidis* (Puschendorf et al., 2011). *Litoria lorica* was thought to have gone extinct from their higher elevations during chytridiomycosis outbreaks in the 1980s and early 1990s, but in 2008, a previously unknown population was discovered in a high-elevation dry sclerophyll forest (Puschendorf et al., 2011).

Investigators hypothesized that the lack of canopy cover allowed the rocks on which frogs perched to warm up, thereby slowing growth and reproduction of the pathogen on the hosts.

SPECIES-LEVEL PRINCIPLES

Principle 5. Maintain Species' Adaptive Capacity

Adaptive capacity is multi-faceted and defined as "the capacity of a species to cope with and persist under new conditions" (Dawson et al., 2011). Phenotypic plasticity, genetic diversity, dispersal ability (discussed above), and life-history traits are components that contribute to a species' fundamental adaptive capacity (Nicotra et al., 2015; Beever et al., 2016). These characteristics, which vary among species, combine with extrinsic factors, such as habitat or community composition, to create variability in potential responses to stressors like changes in temperature, precipitation, and food availability (i.e., realized adaptive capacity, Beever et al., 2016). Within this framework, disease can be thought of as an additional stressor, and the ability of a species to respond effectively is influenced by both their intrinsic adaptive capacity and the extrinsic factors (such as environmental conditions, population size, and connectivity) that allow that capacity to be realized (Vander Wal et al., 2014).

Phenotypic Plasticity

Plasticity in life history traits may reduce time spent in a susceptible stage or result in early maturation and reproduction (Jones et al., 2008; Scheele et al., 2017), and this may be beneficial for increasing an individual's probability of surviving disease outbreaks. For example, after DFTD decimated populations of Tasmanian Devils > 2 years of age, the reproductive age of Tasmanian Devils decreased (Jones et al., 2008). This reduction in age of reproduction potentially increased the ability of this species to persist in the face of disease. Plasticity in behavioral traits may also provide a mechanism of persistence. For example, the effects of fungal diseases, such as snake fungal disease and white-nose syndrome, may be mitigated by behavioral changes related to thermal refugia, such as basking (Lorch et al., 2015), or finding colder microclimates within hibernacula (Boyles and Willis, 2010; Langwig et al., 2012). For amphibian populations, these shifts to warmer thermal refugia can result in spatial distributions shifts, such as the case with *L. lorica* (Puschendorf et al., 2011). Additionally, in the case of the amphibian *B. dendrobatidis* (Woodhams et al., 2003; Richards-Zawacki, 2010) and ranavirus (Sauer et al., 2019), amphibians have been documented as inducing a "behavioral fever" to elevate body temperature and cure infections. Townsend et al. (2020) summarizes several examples (including human) of changing behaviors from social-distancing of diseased individuals or groups (or individuals that are perceived to be diseased) to increased sociality to dilute the potential infection rates.

Evolutionary Rescue

Evolutionary rescue is a heritable mechanism of recovery from reduced demographic rates caused by disease (Carlson et al., 2014). This genetic adaptation occurs via multiple possible pathways (i.e., natural selection, genetic drift, bottleneck, and mutations). Genetic diversity provides some protection from the effects of disease outbreaks because individuals are more likely to have the genetic basis to evolve disease resistance (Spielman et al., 2004, see Blanchong et al., 2016 for a review). For example, in Australia, France, and the United Kingdom, rabbit species rapidly evolved resistance to disease effects in response to the introduction of the myxoma virus for purposes of biocontrol (Alves et al., 2019). Similarly, in the case of Tasmanian Devils, some small populations do appear to be developing resistance and co-existing with DFTD (Epstein et al., 2016; Margres et al., 2018a,b). However, for other species, resistance resulting in population persistence has not evolved due to fitness consequences of resistant genes (Monello et al., 2017), pathogen co-evolution (Chen and Holmes, 2006), or other factors, such as loss of connectivity in populations that may prevent or slow natural selection processes that would result in resistance to infection (Russell et al., 2019). The potential to adapt may be enhanced if factors affecting resistance are linked to other alleles that are selected for by other drivers (e.g., abiotic conditions, Wolinska and King, 2009).

A lack of genetic diversity may lead to a population being more vulnerable to disease. For example, for DFTD in Tasmanian Devils (Siddle et al., 2007; Morris et al., 2012), coronavirus in cheetahs (*Acinonyx jubatus*; O'Brien et al., 1985), and virulent canine distemper in black-footed ferrets (Thorne and Williams, 1988) low genetic diversity is a contributing factor in the ability of the disease to have devastating population-level consequences. Populations that are genetically poor may require an influx of individuals either through increased connectivity or translocations (Weeks et al., 2011) to improve the adaptive potential of the population (Hoffmann et al., 2015). Connected populations will also ensure that random genetic drift and/or geographic isolation does not occur and result in a loss of alleles (Franklin and Frankam, 1998). Understanding and maintaining the landscape-level distribution of genetic diversity for a species while maintaining locally adapted populations is also important (Hoffmann et al., 2015). Recently, researchers have noted the potential pitfalls of outbreeding depression. For example, when individuals are relocated from one area to another or released from a captive breeding program to enhance the local genetic diversity, then the new individuals may dilute the locally adapted gene pool (Hohenlohe et al., 2019).

Life-History Traits

Characteristics that define species' life history or ecological niche may also affect their ability to recover from the effects of epizootics. Specialist species that are narrowly dependent on one or a few other species – particularly for dietary or habitat needs – may face severe indirect effects from threats to that source. For example, black-footed ferrets are heavily dependent on prairie dogs as prey, and epizootics of plague that threaten prairie dog populations or result in local extirpations have complicated recovery efforts for the imperiled ferrets (Antolin et al., 2002). Similarly, Monterroso et al. (2016) documented declines in

Iberian lynx (*Lynx pardinus*) and Spanish Imperial eagle (*Aquila adalberti*) populations after an outbreak of rabbit haemorrhagic disease virus (RHDV2) caused severe declines in European rabbit (*Oryctolagus cuniculus*) populations. Recent outbreaks of RHDV2 in North America may result in similar effects to predator species, such as Canada lynx (*Lynx canadensis*; Stokstad, 2020). Trade-offs may exist in life-history traits related to disease. In common eiders (*Somateria mollissima*), for example, larger clutch size has been shown to correlate with higher rates of death from avian cholera (*Pasteurella multocida*, Descamps et al., 2009). One study found that bighorn sheep ewes that had their first lamb at the age of two were more likely to die from a pneumonia epizootic than ewes that had their first lamb after the age of two (Festa-Bianchet, 1989). One study of 13 different amphibian species found that "fast lived" species (i.e., those that developed quickly and had shorter metamorph stages) showed higher levels of parasite load and pathology (Johnson et al., 2012). These examples demonstrate trade-offs of early reproduction versus pathogen susceptibility. In addition to life history characteristics, seasonal dynamics, such as seasonal breeding and social aggregation, have also been documented to play a role in the transmission dynamics of some wildlife diseases. For example, *Mycoplasma gallisepticum* in house finches (*Haemorhous mexicanus*) transmission is exacerbated through an influx of new susceptible individuals into the population, and aggregation around feeding sources during winter months (Hosseini et al., 2004). Finally, while it seems unlikely that these life-history characteristics will lead to devastating population losses in most species, due to existing compensatory mechanisms, understanding a species' breadth of life history traits and the effects of those traits on vulnerability to disease outbreaks can help inform an overall assessment of risk for different species.

CONCLUSION

Here, we outlined the principles and mechanisms that may help understand how to minimize species extinction risk following an epizootic, increase the ability of a species to persist in the face of disease outbreaks, and mitigate the effects of future epizootics. However, population, ecosystem, and disease dynamics are complex and may interact in ways that contradict these principles. For example, the ability of an individual to disperse is a mechanism for species persistence, but also enhances the ability of pathogens to spread among populations. Likewise, the maintenance of large populations contributes to genetic diversity, phenotypic, and behavioral plasticity, though large populations may also be associated with high densities that place populations more at risk for catastrophic disease effects. Further, the connectivity of host populations, as well as the mechanisms of pathogen transmission (i.e., direct contact and environmental reservoirs), contribute in complex and interacting ways to the ability of a species to adapt to disease (McKnight et al., 2017). However, without genetic variability and spatially distributed populations pre-outbreak the possibilities for adaptation are greatly reduced – so the general principles may still apply without a need to understand the detailed mechanisms underlying population and disease dynamics.

Though disease may rarely be the ultimate cause of species extinction, disease can have substantial effects on the recovery efforts for endangered or threatened species. Infectious diseases can emerge rapidly leaving only a short window of time within which an effective response may be implemented (Canessa et al., 2018; Mysterud and Rolendson, 2018). Controlling disease once it has entered a population is extremely difficult (Gerber et al., 2018; Martel et al., 2020), but proactive management actions are rarely implemented prior to disease detection, often due to the uncertainty in predicting effects (Russell et al., 2017), a lack of identified and tested management actions (Bernard and Grant, 2019), or a lack of clear decision context, among other challenges (Grant et al., 2017). Therefore, incorporating proactive management of a species into planning documents (e.g., species status assessments) to increase the probability of species persistence in the face of future disease outbreaks is of paramount importance (Deem et al., 2001).

The principles that we outline here are broad guidelines, specific considerations regarding how to minimize the effects of a novel disease on a population depends on multiple, interacting factors of the host, the pathogen, and the abiotic habitat conditions within and among habitat patches occupied by a species. We also note that the success of these strategies can be sensitive to the degree of pathogen evolution, which can lead to changes in virulence (Boots et al., 2004; Berger et al., 2005), jumps between species (Gabriel et al., 2005; Woolhouse et al., 2005; McCarthy et al., 2007), and loss of vaccine effectiveness (Gandon et al., 2003). The evolution of pathogens can have substantial effects on the ability of species to persist, as hosts and species engage in an evolutionary arms race. However, there are currently few recommendations regarding the management of wildlife populations in the face of pathogen evolution. The integration of pathogen evolution and wildlife management is an expanding area of research (Vander Wal et al., 2014). While better predictions may be made to inform specific management strategies, as a first step we outline the principles and mechanisms for wildlife population persistence in the face of novel and emerging infectious disease. The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

AUTHOR CONTRIBUTIONS

JS, EG, and RR came up with the idea. EG and RR drafted the manuscript. EG, GD, and RR revised the manuscript. All authors contributed to revisions of the final version.

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frontiers in Ecology and Evolution

OPINION
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Ecological Fever: The Evolutionary History of Coronavirus in Human-Wildlife Relationships

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THE OVERLOOKED WILDLIFE SPILLOVER IN HUMAN-DOMINATED ECOSYSTEMS

The rapid dissemination of severe acute respiratory syndrome coronavirus 2 (SARS-CoV−2) has opened up an environmental dilemma—investigating the relationship between the evolutionary history of coronaviruses (CoVs) and the zoonotic spillover in humans to avoid new rapidly evolving pathogens. To guide politicians in health policy decision-making, scientists have an urgent need to explore how cross-species virus transmission can help prevent pandemics (Zhou et al., 2020). The emergence of new epidemic diseases varies among different taxonomic groups, and the human-made change in natural environments causes eco-evolutionary consequences. Therefore, the alteration of this natural role caused by human pressures on wild species, we label as "ecological fever" —a new One Health perspective from ecology to society. Following the new phylogenies of coronavirus proposed by Gorbalenya et al. (2020) and Zhang et al. (2020), we explore the adaptive evolution of coronaviruses across mammal species and its importance for wildlife conservation. Here, we show reconstructed ancestral states of coronaviruses under maximum-likelihood estimations across an entire class of host organisms (i.e., Mammalia). In this opinion paper, we explore the evolution and cross-species transmission of coronaviruses and highlight the need to preserve natural habitats of wildlife in order to prevent future pandemics.

PHYLOGENETIC RELATIONSHIPS

We reconstruct a full trait-based evolution for a range of coronaviruses (CoVs) through the following steps: (1) According to the new phylogenies of CoVs proposed by Gorbalenya et al. (2020) and Zhang et al. (2020), we build a maximum clade credibility tree based on likelihood ratio tests for the genome tree of 21 CoVs (Bat Hp-BetaCoV, SARSr-CoV BtKY72, SARS-CoV, SARS-CoV PC4–227, Pangolin-CoV, SARSr-CoV RaTG13, SARS-CoV−2, Ro-BatCoV GCCDC1, Ro-BatCoV HKU9, Ei-BatCoV C704, EriCoV, MERS-CoV, Ty-BatCoV HKU4, Pi-BatCoV HKU5, MrufCoV 2JL14, HCoV OC43, ChRCoV HKU24, HCoV HKU1, MHV, HCoV 229E and HCoV NL63); (2) Using the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), we assess all mammalian hosts ($n = 44$ species; see **Supplementary Table 1**) of the 21 CoVs; (3) We create a data matrix to compare the pairwise distribution of the CoVs and the mammalian orders (i.e., Artiodactyla, Carnivora, Chiroptera, Eulipotyphla, Lagomorpha, Pholidota, Primates, and Rodentia), providing ancestral character states under stochastic character mapping analysis—SIMMAP (Bollback, 2006) with 1,000 simulations, accounting for phylogenetic uncertainty; (4)

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Using the full time-calibrated phylogeny of Mammalia proposed by Upham et al. (2019), we evaluate the proportional phylogenetic relationships of the 21 CoVs across their mammalian hosts. To test phylogenetic signals, we calculate D-statistic for discrete attributes to show the degree of phylogenetic clustering under Brownian expectations, as defined by Fritz and Purvis (2010). We perform all the phylogenetic analysis and the null simulations in R software (R Development Core Team, 2019), using the packages "caper" (Orme et al., 2018) and "phytools" (Revell, 2012); (5) To allow further comparisons between the CoVs evolution and the mammalian phylogeny, we investigate the evolutionary adaptation of coronaviruses under the Phylogenetic or Brownian trait evolution in adaptive radiations of mammals.

COEVOLUTION CORONAVIRUS IN MAMMAL HOSTS

Given the strong transmission patterns of the coronavirus infection in mammals, their potential zoonotic transfers rely on the number of phylogenetically distinct hosts. Searching for coronaviruses' phylogenetic signals in infected species, we find differences in species-level host relationships within the full phylogeny of Mammalia proposed by Upham et al. (2019). Using phylogenetic inferences based on ecological trait evolution, we show that 44% of the coronaviruses have zoonotic origins based on a non-random phylogenetic structure, and 56% have their origins expected by Brownian phylogenetic structure under random processes (**Figure 1**). Mapping the full-length

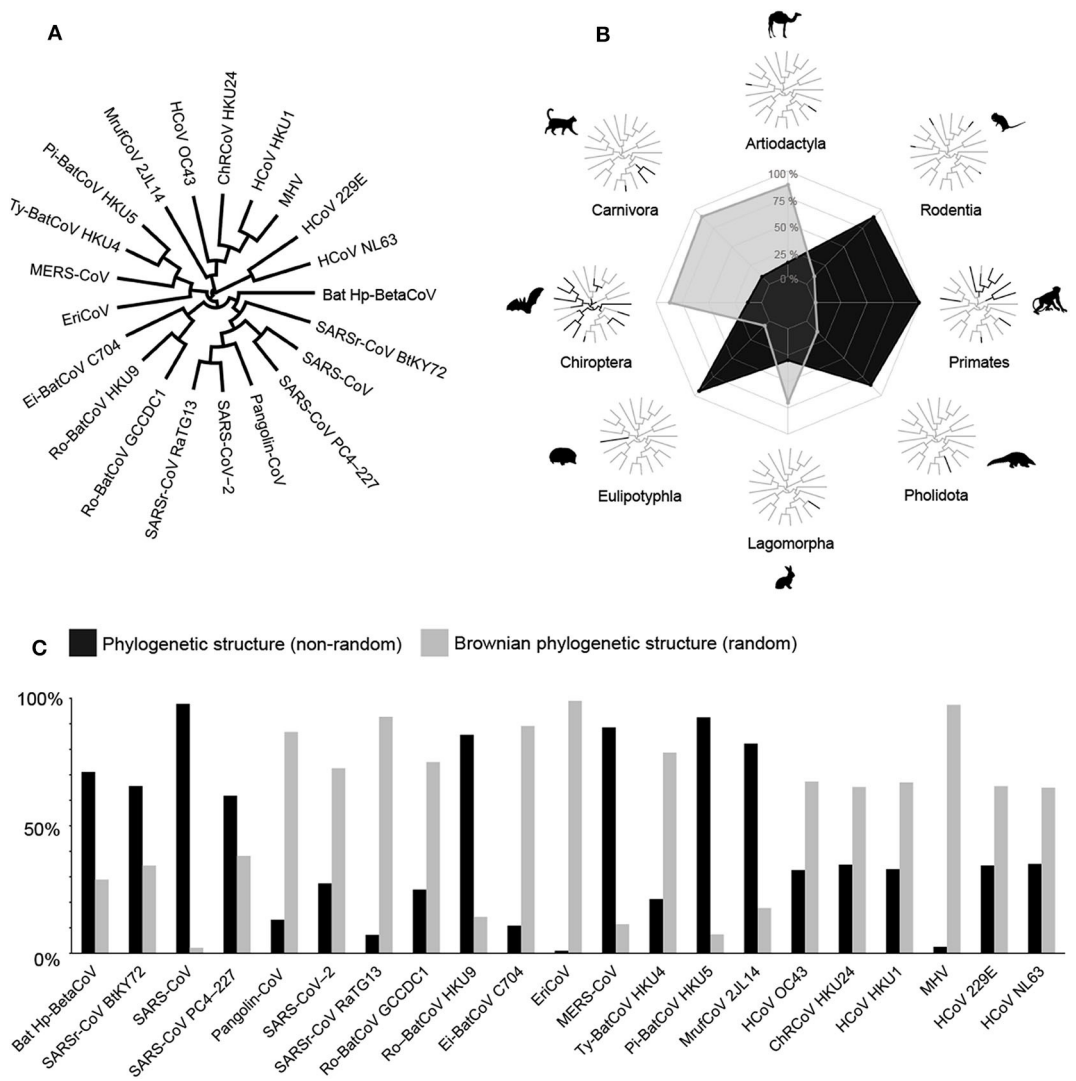

FIGURE 1 | Phylogenetic relationships between coronaviruses and their mammalian hosts. **(A)** Full genome tree of 21 CoVs. **(B)** Maximum-likelihood ancestral state reconstructions for eight mammalian host orders by stochastic character mapping (SIMMAP). Radar chart shows the variation (%) between Phylogenetic and Brownian structure evolution of CoVs in mammalian orders. **(C)** Proportional differences of Phylogenetic and Brownian structure variation (%) in the evolutionary history of CoVs on the time-calibrated phylogeny of Mammalia.

genomes of coronavirus across mammals, we find that they are evolving to adapt to specific mammalian taxa as well as a random evolutionary pattern, suggesting easy transmission across different mammalian species.

We show that the coronavirus radiation into the orders Chiroptera, Lagomorpha, Carnivora, and Artiodactyla have a Brownian phylogenetic structure, with a random origin within Mammalia. This evolutionary pattern can be explained by the high plasticity of the coronavirus to infect species from these mammalian orders. In this way, they are able to mutate and evolve in new types of hosts, which then become reservoirs of infection (Andersen et al., 2020). Within all mammalian orders, Chiroptera has the highest diversity of coronavirus with a worldwide distribution, occurring in all continents except Antarctica (Calisher et al., 2006). From an ecological perspective, bat-related coronavirus relies on their higher population sizes in the breeding seasons, which increase fecal-oral transmissions and highlight the necessity to keep them protected from human interference in natural habitats (Drexler et al., 2014). In bats, multiple viruses can coexist asymptomatically (Calisher et al., 2006), while in other mammal species, a single virus can cause numerous health problems (Zhou et al., 2020). In the case of the SARS-CoV, we find a Brownian phylogenetic structure for Chiroptera and a phylogenetic structure among the other mammal groups infected as hosts. In Carnivora, our estimates show the SARS-CoV PC4–227 has a phylogenetic structure, with a single host species represented by the common palm civet (*Paradoxurus hermaphroditus*), suggesting a low capacity to infect other hosts. Recent research on the potential origin of SARS-CoV−2 shows its closely phylogenetic relatedness to Pangolin-CoV and bat SARSr-CoV RaTG13 (Andersen et al., 2020). However, it is already known that SARS-CoV−2 does not originate directly from pangolins (*Manis javanica*)—indeed infected with Pangolin-CoV (Andersen et al., 2020). Our results suggest that the SARS-CoV−2 has a Brownian phylogenetic structure, and cross-species transmission from an unknown animal species could be acting as an intermediate host among bats, pangolins, and humans (Zhang et al., 2020).

EVOLUTIONARY PATHWAYS OF SHARED PATHOGENS

On the intermediate host species-relationships, some researchers show that the SARS-CoV initially jumped from bats-to-civets-to-humans (Song et al., 2005). In contrast, the MERS-CoV jumped from bats-to-camels-to-humans (Müller et al., 2014). Moreover, some bat coronavirus shows coevolution with their specific host genera (Drexler et al., 2014). Besides being considered as natural and important coronaviruses reservoirs (Zhou et al., 2020), bats have a key functional role in providing ecosystem services to improve human well-being, such as biological control and pollination (Kunz et al., 2011). Therefore, there is an urgent need to conserve bat species in their natural habitat for keeping humans safe from infectious diseases. Although humans are highly susceptible to infection by SARS-CoV related viruses (Zhou et al., 2020), coronaviruses can co-exist harmoniously in wild environments with bats (Calisher et al., 2006), which are essential to preserve crucial relationships between biodiversity and ecosystem functioning for improved public health outcomes (Kunz et al., 2011).

IMPLICATIONS FOR CONSERVATION AND FUTURE DIRECTIONS

Accelerated modern human-induced changes in natural environments can have many consequences beyond infectious diseases, species extinction and climate-health issues. Our findings support conservation studies aimed at exploring the eco-evolutionary consequences of environmental change in managing public health. The coronavirus crisis results from the human disregard for biodiversity in their natural habitat and the wildlife trade spillover to spreading diseases. The substantial increase in deforestation of species-rich regions (e.g., Amazon forest and Southeast Asian forests) may be the beginning of a new pandemic. Opportunities for prevention may emerge from ecology, evolution, and conservation science, which can play a key role in defining links between people and nature, having several implications for human well-being in the short-run. For post-pandemic projections, future studies should focus on outbreak prevention through ecological and evolutionary knowledge instead of disease-related health efforts. Using trait-based science across the Tree of Life for mammals, we find evidence on the evolutionary role of coronavirus in human-wildlife relationships.

DATA AVAILABILITY STATEMENT

The raw data used in this paper are available in **Supplementary Table 1**.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.575286/full#supplementary-material

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BRIEF RESEARCH REPORT
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Socially vs. Privately Optimal Control of Livestock Diseases: A Case for Integration of Epidemiology and Economics

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This paper aims to illustrate the interdependencies between key epidemiological and economic factors that influence the control of many livestock infectious diseases. The factors considered here are (i) farmer heterogeneity (i.e., differences in how farmers respond to a perceived disease risk), (ii) off-farm effects of farmers' actions to control a disease (i.e., costs and benefits borne by agents that are external to the farm), and (iii) misalignment between privately and socially optimal control efforts (i.e., privately optimal behavior not conducive to a socially optimal outcome). Endemic chronic diseases cause a wide range of adverse social and economic impacts, particularly in low-income countries. The actions taken by farmers to control livestock diseases minimize some of these impacts, and heterogeneity in those actions leads to variation in prevalence at the farm level. While some farmers respond to perceived disease risks, others free-ride on the actions of these individuals, thereby compromising the potential benefits of collective, coordinated behavior. When evaluating a plausible range of disease cost to price of control ratios and assuming that farmers choose their privately optimal control effort, we demonstrate that achievement of a socially optimal disease control target is unlikely, occurring in <25% of all price-cost combinations. To achieve a socially optimal disease control outcome (reliant on farmers' voluntary actions), control policies must consider farmer heterogeneity, off-farm effects, and the predicted uptake of control measures under the assumption of optimized behavior.

Keywords: livestock, infection, disease control, model, epidemiology, economics

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INTRODUCTION

Livestock production is vital to economic development in many low-income countries, contributing to poverty alleviation, investment in children's education, and food security (1, 2). Infectious diseases jeopardize these societal functions, yet their control has become increasingly reliant upon privately funded actions (3–8). This "voluntary approach" to disease control distributes costs and responsibility between private agents (farmers that invest in control measures) and the government (animal health authorities that promote and often regulate farmers' actions) (9). The problem of the voluntary approach is that self-interested behavior can fail to produce a socially optimal level of disease control (i.e., the level of disease control that maximizes the sum of all

agents' payoffs in society, the social optimum) (8, 10). The market thus "fails" to achieve the social optimum.

One reason for such "market failure" in disease control is the presence of externalities: costs and benefits borne by agents who are not parties to a market transaction (8). Externalities can be positive (e.g., a farmer enjoys a lower risk of disease incursion into his herd when neighboring farmers vaccinate their animals) or negative (e.g., a farmer has a higher risk of disease incursion into his herd when other farmers sell sick animals into a livestock market). One farmer's investment in animal health can thus influence other farmers' levels of disease risk. The way individual farmers perceive and act upon their level of risk may fail to produce a social optimum: some farmers free-ride on the control efforts of others, and the potential benefits of one farmer's actions can be limited by others' inaction (9, 11). Achieving the socially optimal level of disease control often requires governments to intervene (e.g., through financial incentives and regulation) in a way that accounts for the nature of farmers' decision-making processes and the interdependencies between farmers' choices and disease dynamics (3, 8, 9, 12–19).

The way farmers perceive and respond to disease risks is not only affected by disease spread but also impacts upon it. There are feedback processes between how a disease spreads and the responses of farmers to changing risks (20–22). When the inter-dependent dynamics of economic, behavioral, and epidemiological factors are not understood or well-represented in models of disease transmission, then policies aimed at promoting farmers' action may have perverse effects (23–26).

Our objective in this paper is to illustrate interdependencies between key epidemiological and economic factors that are not consistently taken into account in models of livestock diseases: (i) farmer heterogeneity (i.e., differences between farmers in how they respond to perceived risks); (ii) associated externalities; and (iii) misalignment between privately and socially optimal control efforts. We demonstrate the impact of farmer heterogeneity on disease prevalence over time. We quantify the externalities associated with farmer heterogeneity and show how the outcome of any individual farmer's control effort depends on the behavior of others. Finally, we examine a farmer's decision in trading-off *ex ante* disease prevention expenditure and *ex post* treatment expenditure and output loss, to estimate the probability that adoption of privately optimal behavior will achieve a socially optimal disease control target. The consequences of a socially sub-optimal control level depend on, among other factors, the population at risk. Here, for simplicity and to illustrate the general case, groups of farmers represent society.

METHODS

We explore the relationships between epidemiological and economic factors using a deterministic compartmental model (27). The model assumes a chronic infectious disease of livestock with a low basic reproduction number ($R_0 = 1.11$), a livestock species with an average lifespan of five time units, and a constant herd size over time (250 animals on farm). We apply the model in three scenarios that explore farmer heterogeneity, quantify externalities, and examine differences between privately and socially optimal control efforts. In all scenarios, the animal-level prevalence in each farm starts at 10% (the endemic equilibrium, i.e., when prevalence remains steady and the effective reproductive number, R_e, is 1.00). We simulate the time required to achieve a prevalence of <2% following a hypothetical awareness-raising event (e.g., information provision at time zero) and a time horizon of 25 time units. This target prevalence of <2% represents an economic optimum from society's perspective (i.e., the socially optimal level of disease control). A 2% threshold can also be considered a prevalence level below which costly government-led strategies aimed at eliminating the disease (e.g., test-and-slaughter) are required (28). In our simulations, for simplicity and without loss of generality, we assume infected animals show chronic symptoms and lose productivity, but there is no disease-associated mortality or infertility. Individuals can be either susceptible or infected; once infected, they remain infected and infectious for the remainder of their lives. Complete recovery from the disease is not possible.

The model set-up is as simple as possible for demonstration purposes. The basic features are (i) two states (susceptible—*S*; and infected—*I*), (ii) continuous time steps, (iii) equal birth and death rates ($\alpha = \mu = 0.20$), and (iv) an initial transmission rate (β) of approximately 8.89×10^{-4}, reflecting the endemic equilibrium. The model can be run with one or more farms, and disease transmission can occur within farm only or both within and between farms. The proportion of the initial overall β attributable to between-farm transmission (q) is 0.0 when there is no transmission between farms, and 0.1 when between-farm transmission is included. The amount of control effort applied on farm is represented by a proportionate reducer on β (r_i^{β}). The model's differential equations are as follows:

$$\frac{dS_i}{dt} = \alpha (S_i + I_i) - (1 - q)\left(1 - r_i^{\beta}\right)\beta S_i I_i - q\beta S_i \sum_{\substack{j=1 \\ j \neq i}}^{n} I_j - \mu S_i \quad (1)$$

$$\frac{dI_i}{dt} = (1 - q)\left(1 - r_i^{\beta}\right)\beta S_i I_i + q\beta S_i \sum_{\substack{j=1 \\ j \neq i}}^{n} I_j - \mu I_i \quad (2)$$

The differential equations were solved in *R* (29) (version 3.6.2) with the package *deSolve* (30) (version 1.27.1). All plots were generated using the *ggplot2* package (version 3.3.0) (31).

We demonstrate the effect of farmer heterogeneity on the predicted prevalence of disease over time by simulating transmission-reducing measures in three farms following a hypothetical awareness-raising event. As farmers continually assess the benefits and costs of their actions over time, the amount of control effort in each farm is set to be a function of disease prevalence (p_i). We assume that (i) disease transmission occurs within each farm only, and (ii) as the prevalence (and disease impact) at the farm declines, the control effort applied (r_i^{β}) also

falls (32), as follows:

$$r_i^{\beta} = \frac{1}{1 + e^{-100(p_i - \gamma_i)}} \quad (3)$$

where γ_i represents the farmer responsiveness. γ was set at 0.025, 0.100, and 0.175 for the farmer that is "highly responsive," "moderately responsive," and "slightly responsive," respectively. Note that, as the prevalence decreases in any farm due to the control effort applied, the farmer responsiveness does not change—it is a characteristic of each individual farmer.

We then estimate the off-farm effects (externalities) of livestock disease control in two of the above farms: one in which the farmer is highly responsive and the other in which the farmer is slightly responsive. Firstly, we quantify the *ex post* cost of disease over time at the farm level by assigning a monetary value (δ) to the cost of each infected animal per time step. We assume that the *ex post* cost of the disease varies linearly with prevalence (i.e., the cost per time step is the product of the number of infected animals and the cost per infected animal). We then compare the *ex post* cost of disease over time in each farm when the transmission rate between farms (bβ) is 0% (q is 0.0) and 10% (q is 0.1) of the initial within-farm transmission rate (wβ). Actions taken by each farmer to reduce transmission in their farms only reduce the wβ—and do not influence bβ, which is kept constant over time. By holding the bβ constant over time and at a common value for the two farms, we demonstrate the off-farm effects of on-farm actions only—this assumes that (i) the contact rate between farms is kept constant, and (ii) the force of infection is proportional to the number of infected animals on each farm. Variation in farm number and density is unlikely to affect the qualitative inferences from this simulation.

We define "cost of disease" as the sum of disease-related output loss (production potential that is not realized over time) and expenditure on treatment of infected animals, plus the cost of control. Treating infected animals does not affect their infectiousness. Cost of control refers to money spent on reducing the wβ to prevent new cases, for instance, through improvements in hygiene and biosecurity. The cost of disease can thus be split into *ex ante* cost (cost of control) and *ex post* cost (disease-related output loss and treatment-related expenditure).

We demonstrate the market failure in livestock disease control by comparing the private net profit of farmer i (Π_i) with the social net profit (Π_s), the sum of the private net profits of n farmers ($\Pi_s = \sum_{i=1}^{n} \Pi_i$). Π_i denotes the private net profit of farmer i for the whole simulation period of 25 time units ($\Pi_i = \sum_{t=1}^{25} \Pi_{it}$). The example has only two farmers for simplicity, so $\Pi_s = \Pi_R + \Pi_r$; the social net profit is the net profit of the highly responsive farmer (R) plus the net profit of the slightly responsive farmer (r). Considering that the benefit (b_i, the number of animal infections averted) enjoyed by the highly responsive farmer as a result of disease control effort is a function of control effort applied in their farm (r_R^{β}) as well as in the other farm (r_r^{β}) (i.e., $b_R = f\left(r_R^{\beta}, r_r^{\beta}\right)$), the net profit Π_{Rt} (in time step t) is given by $\delta b_R - \theta r_R^{\beta}$, where δ is the *ex post* cost per infected animal (treatment expenditure and output loss), and θ is the unit price of control action (the price of reducing the transmission rate by 1%). For this simulation, we assume that δ and θ are fixed at 50 and 0.10 monetary units, respectively.

We examine the effect of optimizing behavior on disease prevalence by assuming that the farmer reduces the wβ so that the total cost of the disease (the sum of *ex ante* and *ex post* costs) at each time step is minimized (32). This process is repeated iteratively for each level of the unit price of control (θ, ranging from 0.00 to 1.25 monetary units per time step) and *ex post* cost per infected animal (δ, ranging from 10 to 85 monetary units per time step). We assume (conservatively) that (i) the farmer has perfect information of both the *ex ante* and *ex post* costs of disease, and (ii) their behavior is no longer determined by a pre-specified logistic function. Instead, at each time step, the farmer reviews and optimizes their control effort given the prevalence. The short planning horizon (one time step, equivalent to one-fifth of the animals' average lifespan) reflects the generally high time-preference rates in low-income countries (33).

The qualitative inferences drawn from the model outputs are observed under a range of alternative scenarios and assumed parameter values. Readers can explore different scenarios and assumptions in the interactive web application, which is available here: http://boydorr.gla.ac.uk/eemodel/epiecon. This application was developed with *shiny* (34) (version 1.4.0) and *shinydashboard* (35) (version 0.7.1) packages in R (29). The R script for all the simulations is available at: https://doi.org/10.5281/zenodo.4108335.

RESULTS

The Impact of Farmer Heterogeneity

The prevalence of the disease is estimated over time within three model farms managed by three different "types" of farmers who vary in their responsiveness to an awareness-raising event. The responsiveness level of each farmer type is given by a behavioral parameter (γ_i). The model uses this parameter in a logistic function that determines the reduction in transmission rate caused by each farmer's different actions at any given level of prevalence (**Figure 1A**). For instance, at the initial prevalence of 10%, (i) the highly responsive farmer reduces transmission by 100% (red solid line), (ii) the moderately responsive farmer reduces transmission by 50% (green dotted line), and (iii) the slightly responsive farmer does not reduce transmission at all (blue dashed line). The impacts of these three levels of responsiveness on disease prevalence over time are shown in **Figure 1B**. The heterogeneity in farmer responsiveness has an impact on the level of control effort applied and thus on the reduction in prevalence achieved. Among the three farmer types, only the highly responsive type achieves the target prevalence (i.e., <2%) within 25 time units.

Externalities: The Off-Farm Effects of Farmers' Control Actions

Now we model disease prevalence in two neighboring farms (managed by highly and slightly responsive farmers, respectively), allowing for between-farm transmission, to explore the off-farm effects of farmers' control actions upon within-farm and societal costs of disease, represented by the sum of costs over the two neighboring farms.

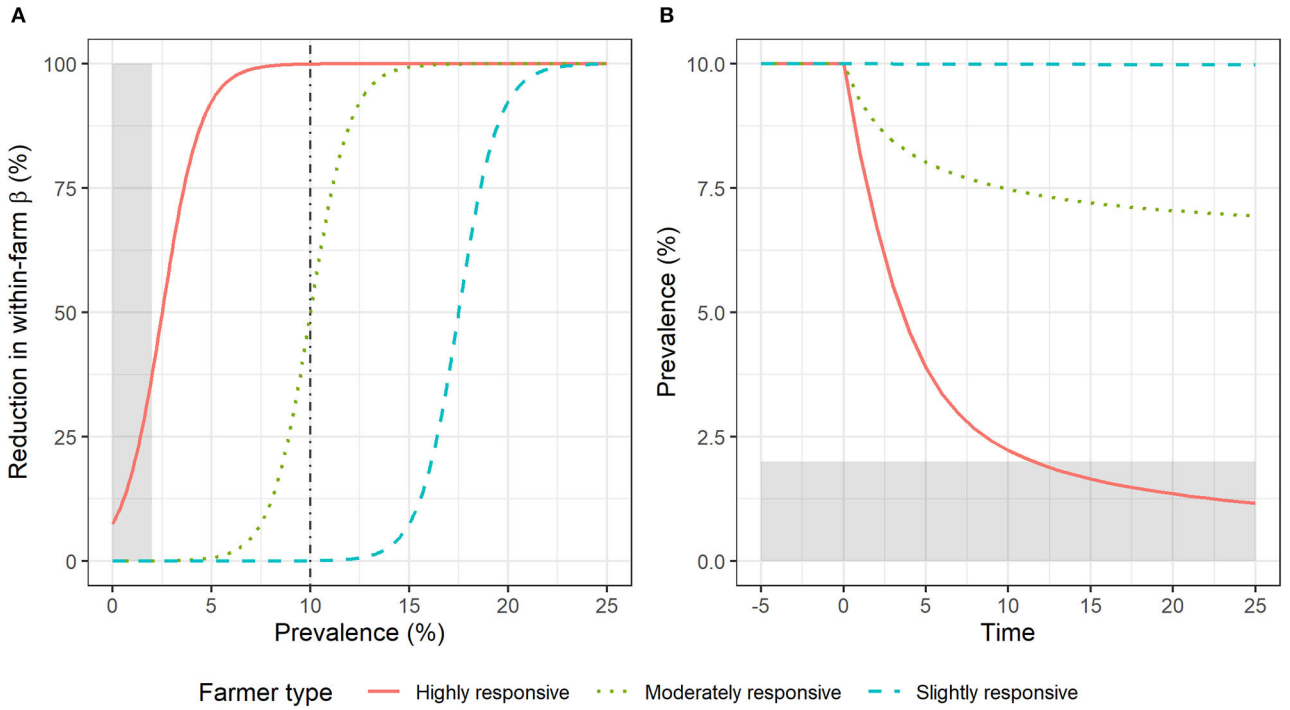

FIGURE 1 | Effect of farmer type on disease prevalence following a common starting prevalence and a hypothetical awareness-raising event (at time zero). **(A)** Control effort applied (percentage reduction in transmission rate—β—within the farm in relation to the initial β in endemic equilibrium) as a function of disease prevalence for three different farmer types. The vertical dash-dotted line indicates initial prevalence. **(B)** Disease prevalence over time within the three farms. In both panels, the gray shaded area indicates the target prevalence level from society's perspective (<2%).

For each farm, we estimate the *ex post* cost of disease over time following an awareness-raising event. **Figure 2** shows the estimated *ex post* cost in two farms with and without between-farm transmission. With no off-farm effects (i.e., $b\beta = 0$, and thus no effects of neighbor's actions on costs), a highly responsive farmer (solid red line) reduces prevalence and *ex post* cost. In contrast, for a slightly responsive farmer (dotted blue line), the prevalence and the *ex post* cost are unchanged over time. When the off-farm effects or externalities are included (i.e., $b\beta > 0$, transmission between farms is possible), the highly responsive farmer sees a reduced benefit of their actions, and the slightly responsive farmer benefits from their neighbor's actions (compared to the equivalent costs when $b\beta = 0$). The extent by which the first farmer (highly responsive) reduces the prevalence of disease (and its associated cost) is limited by the force of infection originating from the second farm, where the farmer is only slightly responsive. Also, despite the lack of control action, the prevalence and *ex post* cost of disease at the second farm drops over time as a result of a decreasing force of infection originating from the first farm, where the prevalence is decreasing due to the control measures applied. Using a discount rate of 5%[1], the extent of market failure[2] is the difference between the Π^s (849 monetary units) and Π^R (695 monetary units). This difference is due to the benefits enjoyed by the slightly responsive farmer, who does not incur any costs of control.

Privately Optimal Behavior and Reductions in Prevalence

Here we model disease prevalence (with a single farm and $b\beta = 0$) to establish if privately optimal actions of a farmer will achieve the socially optimal target prevalence (i.e., <2%). The prevalence over time assuming privately optimal behavior for different unit prices of control (from "free" to "high") is shown in **Figure 3**. Following an awareness-raising event (at time zero) and assuming a fixed *ex post* cost per infected animal ($\delta = 50$ monetary units), the extent by which the within-farm prevalence is reduced varies inversely with the unit price of control (θ). Achieving the socially optimal target prevalence is possible if the unit price of control is very low. Disease elimination (prevalence below 0.4% or <1 infected animal in a herd of 250; note that the predicted prevalence is strictly continuous) only occurs when the unit price of control is equal or <0.05 monetary units (first line above "free" in **Figure 3**). When the unit price of control is above 0.05 monetary units, the farmer's optimal control effort either reduces the prevalence until it reaches a new endemic equilibrium or does not reduce it at all (shown as the uppermost flat line in **Figure 3**; the prevalence remains at the initial endemic equilibrium level). This model of optimizing behavior shows that self-interested farmers, who optimize their

[1]The discount rate accounts for the decreasing value of benefits and costs over time in "real terms," as viewed from the present.
[2]More details available here: http://boydorr.gla.ac.uk/eemodel/epiecon.

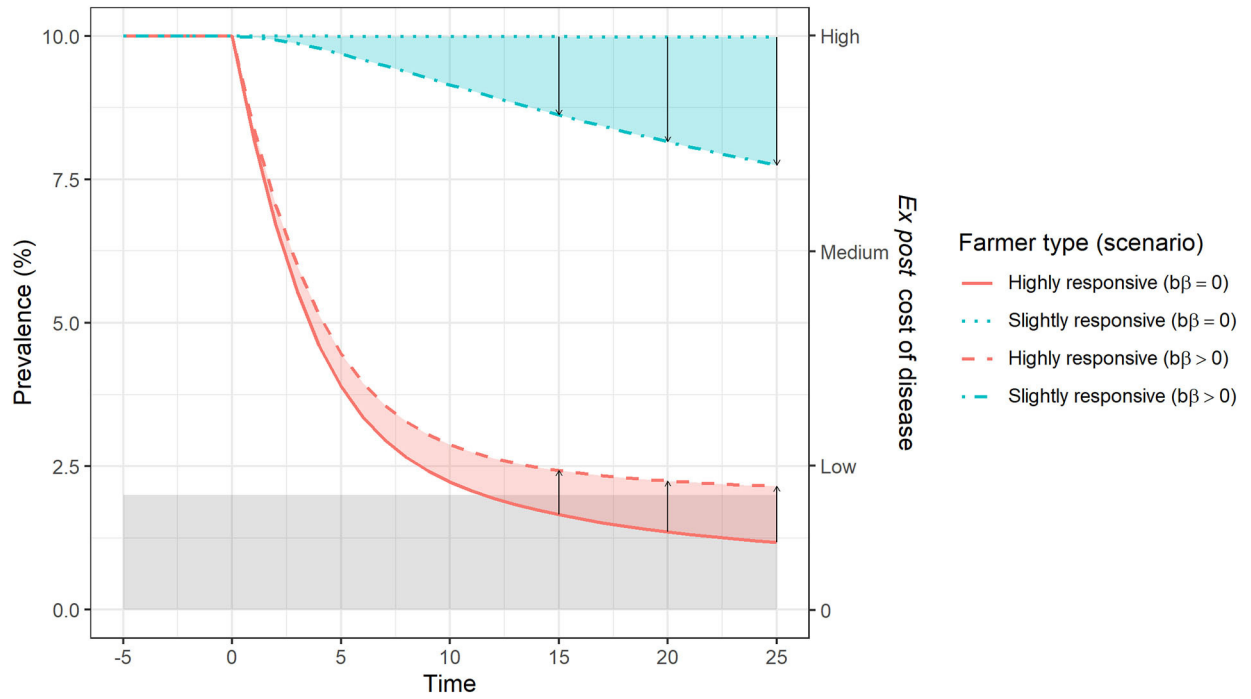

FIGURE 2 | Externalities occurring as a result of different effort to achieve disease control in two neighboring farms. Arrows indicate a simulation scenario shift, from absence of transmission between farms ($b\beta = 0$) to a situation in which between-farm transmission is possible ($b\beta > 0$). Assuming an *ex post* cost per infected animal per time step (δ) of 50 monetary units, the labels "Low," "Medium," and "High" on the right vertical axis correspond to 313, 781, and 1,250 monetary units. Blue and red shaded areas correspond to the positive and negative externalities, respectively. The gray shaded area indicates the target prevalence level from society's perspective (<2%).

private investment in disease control, as assumed here, may not achieve the target prevalence from society's perspective in the absence of an intervention by, for example, the government.

In our simulations, under the assumption of optimizing farmer behavior, as prevalence drops, the marginal cost of control actions (i.e., the cost of reducing the prevalence by an additional unit, 1%), increases sharply. It is more expensive to reduce the prevalence from 3 to 2% than from 10 to 9% because the number of cases prevented per time step by reducing the transmission rate depends on the number of currently infected individuals. If the number of currently infected individuals is high (e.g., 10% prevalence), the prevention of new cases yields a quick reduction in prevalence. If the number of currently infected individuals is low (e.g., 3% prevalence), further reductions of the prevalence rely to a greater extent on the elimination of currently infected individuals. Under the assumption of optimizing behavior, as the marginal costs of control increase, the farmer's investment in control therefore gradually declines until an equilibrium is reached in both epidemiological and economic terms (shown as the plateau for each line in **Figure 3**). This equilibrium prevalence level is reached when the *ex ante* expenditure in one time step equals the averted *ex post* cost of disease.

The achievement of a socially optimal outcome prevalence depends on the specific values of the unit price of control and cost per infected animal selected. The results shown in **Figure 3** considered a fixed *ex post* cost of one infected animal (δ = 50 monetary units). However, for the same disease and animal species, this value could in practice vary with the price of livestock, cost of production inputs, type of production system, etc. **Figure 4** shows the predicted prevalence after 25 time units for a range of values of price per control unit and a range of values of *ex post* cost per infected animal. When we allow the cost per infected animal to vary in plausible ranges in relation to the price of control unit (e.g., eliminating disease transmission by 100% would cost a maximum of 125 monetary units per time step, which is <1.5 times the highest *ex post* cost of one infected animal, i.e., 85 monetary units), the previously observed pattern holds: farmers who optimize their control effort may not achieve the target prevalence from society's perspective.

DISCUSSION

Analytical frameworks introducing human behavioral dynamics in the field of livestock infectious disease modeling have emerged in the last two decades. From the pioneering works of Bicknell et al. (24) and McInerney et al. (32) to the most recent individual-based, network-based, and game-theoretic models (9, 36–38), many significant contributions have improved our understanding of the interplay between human behavior and livestock disease spread (and the likelihood of disease control). However, this study is unique and contributes to that body of knowledge for two reasons. Firstly, it provides a simple but robust

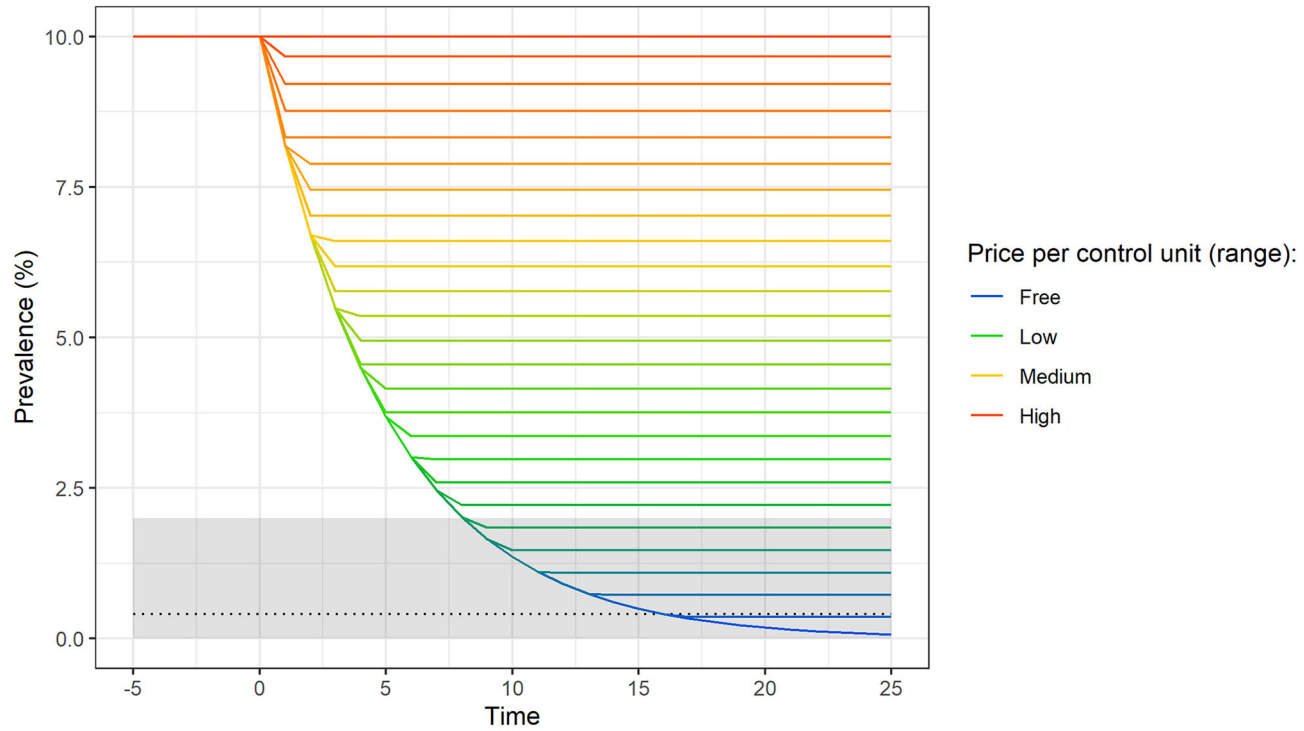

FIGURE 3 | Disease prevalence over time, assuming privately optimal behavior in a single farm for a range of prices per control unit. This simulation assumes a cost of one infected animal per time step (δ) of 50 monetary units. The price of reducing the transmission rate by 1% (θ) ranges from "Free" (0.00 monetary units) to "High" (1.25 monetary units). "Low" and "Medium" correspond to 0.40 and 0.85 monetary units, respectively. Colored lines represent increases by 0.05 monetary units. The gray shaded area indicates the target prevalence level from society's perspective (<2%). The black dotted horizontal line indicates a prevalence consistent with disease elimination (0.4%; <1 infected animal in a herd of 250 animals).

framework for exploring key factors in the control livestock diseases: farmer heterogeneity, externalities, and private-social misalignment of control optima. Secondly, we apply this framework to the control of endemic chronic diseases through privately funded actions in a low-resource setting.

Despite the growing literature on livestock infectious disease modeling, very little attention has been given to the assumption of homogeneous human behavior, the validity of which we evaluate here. Models of livestock disease typically assume that the farmers' perception of risk and the way they respond to that level of perceived risk remain unchanged over time (22). In addition, many epidemiological models entirely ignore the effects of changing prevalence on privately optimal spending on disease control (22, 23). However, several empirical studies have shown that a farmer's likelihood to take control actions is the result of a dynamic and complex interplay between epidemiological, economic, environmental, cultural, and social factors (39–43). Models of disease transmission that ignore such heterogeneity are likely to be misleading, resulting in reduced value for informing control efforts. Like many decision-makers, farmers make decisions based on conscious and unconscious, cognitive and affective shortcuts or rules of thumb (42, 44, 45). We model the implications of this type of individual decision-making process by simulating the effects on prevalence and costs of different levels of farmer responsiveness. These simulations quantify the consequences of heterogeneous behavior, the occurrence of externalities, and the consequent disparities between privately and socially optimal control efforts.

The simulations of our theoretical scenario of optimizing behavior assumed perfect information and rational behavior by the individual farmer. In this scenario, the farmer was able to accurately choose the optimal control effort at any point in time by trading-off the marginal benefits of reducing the prevalence against the marginal costs of their actions. These models assume a conservative range of values for the unit price of control action (from 0.00 to 1.25 monetary units to reduce the transmission rate by 1% per time step) and cost of one infected animal (from 10 to 85 monetary units per time step). For example, without administration and overhead charges, the annual cost of vaccinating a herd of 250 cattle for brucellosis in India (167.50 USD; 0.67 USD times 250) would be over twice as much the average loss caused by brucellosis per infected animal (73.44 USD) (46). The values that we used are considered appropriate and conservative for this evaluation because the highest possible cost of eliminating disease transmission completely would be equivalent to <1.5 times the highest *ex post* cost of one infected animal per time step. Despite these conservative assumptions, the societal disease control target was only achieved for high disease cost to price of control ratios (<25% of all price-cost combinations; **Figure 4**). Given these findings, we can infer that

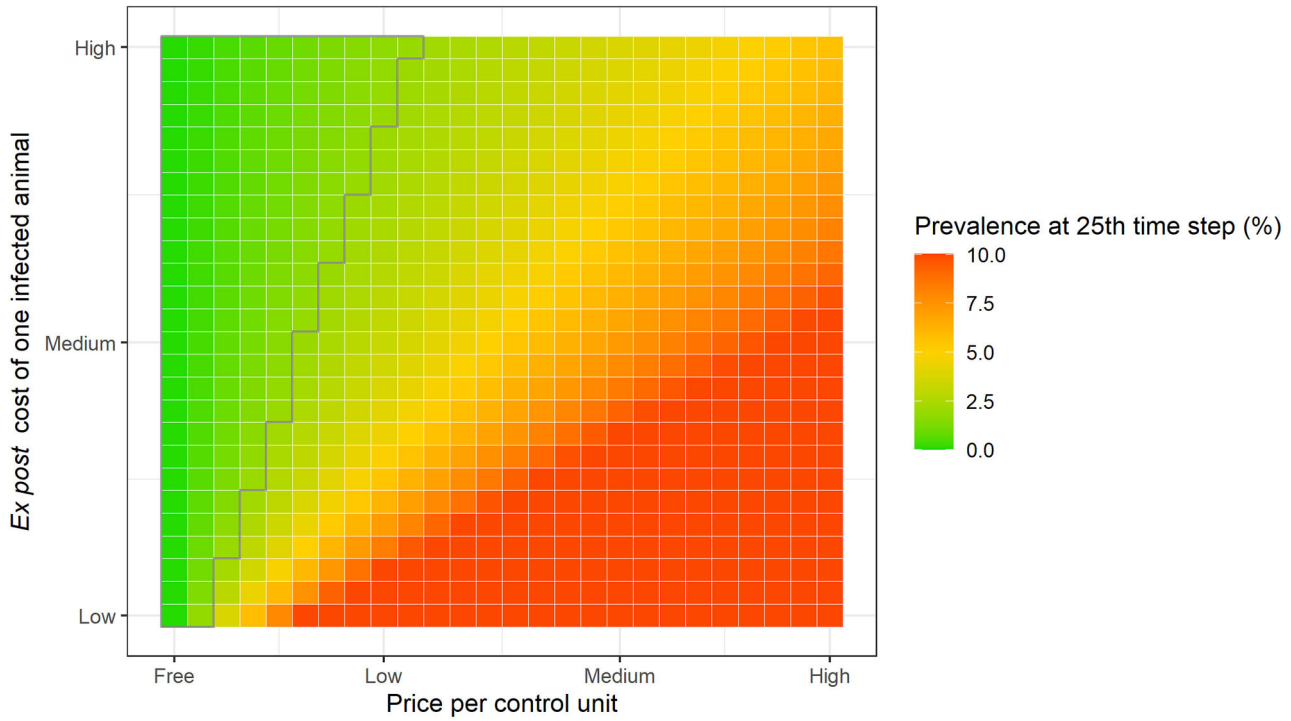

FIGURE 4 | Predicted prevalence at 25th time step for different values of price per control unit ("Free" = 0.00; "Low" = 0.40; "Medium" = 0.85; and, "High" = 1.25 monetary units) and *ex post* cost per infected animal ("Low" = 10; "Medium" = 46; and, "High" = 85 monetary units), assuming privately optimal behavior in a single farm. The polygon with gray border indicates the target prevalence level from society's perspective (<2%).

effective disease control through voluntary actions is unlikely to be privately optimal in many real-world cases. Additionally, as prevalence decreases, there is a sharp increase in the marginal costs of control action, which leads to a fall in optimal control effort. This result is consistent with the economic principle of diminishing returns and has also been reported by other theoretical (32) and empirical studies, namely those that analyzed control actions against tuberculosis (24, 47) and brucellosis (48, 49) in cattle.

Our model illustrates the misalignment of private and social optima for livestock disease control through a basic trade-off between the benefits of reductions in herd prevalence and the costs of transmission-reducing actions. Essentially, farmers "buy reductions in disease transmission," which is a simple and logical representation of many disease control actions. However, this approach is likely to underestimate the gap between private and social optima in many cases: the model does not capture many of the benefits of farmers' actions such as (i) the control of multiple pathogens through improved biosecurity, and (ii) the human health benefits in the cases of zoonoses. For instance, brucellosis is a bacterial zoonosis that causes abortion in livestock and debilitating symptoms (e.g., fever, joint pain, myalgia) in humans (50, 51). Given this burden on human health, in addition to animal health, voluntary actions taken by farmers to control the disease in their livestock (e.g., through vaccination) generate positive externalities, as society benefits from farmers' actions whilst not bearing the costs of these actions. This is a market failure that requires coordinated action to achieve socially optimal outcomes in terms of both human and animal health and productivity (48). Hence, the benefits enjoyed and the costs born by both public and private sectors must be considered throughout the planning phase of control interventions, which can include, among others, the provision of subsidized goods and services (e.g., vaccination), creating and enforcing regulations (e.g., movement restrictions) and setting compensation schemes (e.g., for culled seropositive animals) (8, 48).

To enable private farmers' actions to achieve a socially optimal disease control target, policy design and development must consider the heterogeneity of farmer behavior, the associated off-farm effects or externalities, and the predicted uptake of control measures under optimized farmer behavior. Failure to acknowledge these factors may result in potentially misleading predictions about disease transmission and the associated economic costs. This, in turn, perpetuates conditions in which private investments by farmers are inadequate to achieve disease control from society's perspective. For multi-host diseases that can impact multiple sectors (e.g., multi-host livestock diseases and zoonoses), inadequate disease control can have profound repercussions on international trade and, indeed, threaten human health.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ÂM, DH, EM, NH, and JH contributed to conception and design of the study and writing of the manuscript. ÂM and DH wrote the code in R. ÂM developed the interactive web application. All authors contributed to the article and approved the submitted version.

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Getting in Front of Chronic Wasting Disease: Model-Informed Proactive Approach for Managing an Emerging Wildlife Disease

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Continuing geographic spread of chronic wasting disease (CWD) poses a serious threat to the sustainable future of cervids and hunting in North America. Moreover, CWD has been detected in captive cervids in South Korea and, in recent years, in free-ranging reindeer in Europe (Norway). Management of this disease is limited by logistical, financial, and sociopolitical considerations, and current strategies primarily focus on reducing host densities through hunter harvest and targeted culling. The success of such strategies in mitigating the spread and prevalence of CWD only upon detection is questionable. Here, we propose a proactive approach that emphasizes pre-emptive management through purposeful integration of virtual experiments (simulating alternate interventions as model scenarios) with the aim of evaluating their effectiveness. Here, we have used a published agent-based model that links white-tailed deer demography and behavior with CWD transmission dynamics to first derive a CWD outbreak trajectory and then use the trajectory to highlight issues associated with different phases of the CWD outbreak (pre-establishment/transition/endemic). Specifically, we highlight the practical constraints on surveillance in the pre-establishment phase and recommend that agencies use a realistic detection threshold for their CWD surveillance programs. We further demonstrate that many disease introductions are "dead ends" not leading to a full epidemic due to high stochasticity and harvesting in the pre-establishment phase of CWD. Model evaluated pre-emptive (pre-detection) harvest strategies could increase the resilience of the deer population to CWD spread and establishment. We conclude it is important to adaptively position CWD management ahead of, rather than behind, the CWD front.

Keywords: agent-based models, chronic wasting disease, pre-emptive strategies, surveillance, white-tailed deer

INTRODUCTION

Chronic wasting disease (CWD) is an emerging prion disease of North American cervid populations (including white-tailed deer *Odocoileus virginianus*, mule deer *Odocoileus hemionus*, and elk *Cervus canadensis*) that has been detected in free-ranging and captive cervids in 26 U.S. states and three Canadian provinces as well as in free-ranging reindeer *Rangifer tarandus* in Norway and in captive cervids in South Korea. The seemingly inexorable spread of CWD presents both short- and long-term threat to free-ranging cervids and to wildlife conservation in general. Field data show that CWD can markedly reduce cervid populations (1, 2); while modeling studies suggest that CWD may even cause extirpation of local cervid populations (3). As well, CWD-driven declines in hunting license revenue, and the unsustainable cost of existing CWD surveillance and management programs have obvious economic implications (4, 5). Deer and deer hunting underpin the economic well-being of North American conservation as deer hunting is an important source of revenue for wildlife related work and added conservation value through the protection and management of private lands that benefit other wildlife (6, 7).

The management of wildlife diseases in general and CWD in particular is constrained by limited scientific knowledge about transmission dynamics (8). Moreover, uncertainties about the current status of infection in many populations and the likelihood of success of different management actions are major challenges. These challenges are compounded by the controversial nature of deer management and popular and political opposition to disease management interventions (9, 10). Regardless, affected publics and politicians expect and demand that wildlife agencies respond promptly and effectively to disease threats. An effective approach to addressing uncertainties while dealing with complex systems is through the integration of formal models with management and related policy decision making (11). Modeling presents an economical and time sensitive alternative to direct field tests that are costly, require years to complete and are difficult to sustain. Such virtual explorations can enhance scientific understanding of complex systems and can be developed specifically for evaluating the utility of various interventions and regulatory packages.

There are many different ways to model CWD dynamics depending on the objectives (12). Here, we build our argument using a published agent-based modeling framework, *Ov*CWD, that simulates the complex white-tailed deer-CWD system (13, 14). An important feature of this agent-based modeling framework is the ability to simulate age–sex-specific scenarios and interventions, as relevant individual host characteristics (age, sex, and group membership) and behaviors (dispersal, grouping behavior) have been incorporated in the constituent model programs. We illustrate how this model-based approach can be used to better understand phase-specific issues associated with CWD outbreaks. In this perspective, we argue that CWD management approaches should be phase specific (pre-establishment/transition/endemic) and should specifically take into consideration the need for pre-emptive, pre-detection strategies due to imperfect detection in the pre-establishment phase. A unique feature of CWD is the slow epidemic growth in the initial stage, and stochasticity plays an important role in determining whether the infection gets established in the deer population. Moreover, harvest strategies can influence the probability of CWD persistence. We conclude that a way forward should include the development of defensible pre-emptive harvest strategies that are locally relevant, sustainable and cost-effective, and prevent the establishment of CWD.

UNDERSTANDING CWD DYNAMICS

We derived CWD outbreak trajectories to provide a context for designing more efficient and sustainable CWD surveillance and management strategies. Model parameterization and implementation are detailed elsewhere (13). Here, we briefly describe the modeling process to derive outbreak trajectories. CWD was introduced in the model deer population (pre-harvest deer abundance ∼52,800, representing a midwestern county landscape of ∼721 square miles) by a dispersing yearling during the first year of the model run. The subsequent spread of CWD in the model deer population was documented over a 25 year period for each model simulation. Model output data from 100 iterations were summarized to generate a statistical portrait of CWD prevalence for each year of the model run (**Figure 1**; the dashed blue line represents CWD outbreak trajectory). CWD prevalence remains low (below 1%) for at least a decade after introduction, and this pattern is in agreement with field and modeling data from other studies in North America (15–17). Similar CWD outbreak patterns have been documented in white-tailed deer populations from Wisconsin, Pennsylvania and West Virginia. Such low prevalence rates after introduction have important implications for surveillance and management of CWD.

The Issue of Sampling and Imperfect CWD Detection

In the early phase of the outbreak (∼10–15 years after introduction into a natural population), detection of CWD using hunter-harvested deer (non-probabilistic sampling) is difficult, if not practically impossible, because the overall prevalence remains very low and cases are clustered, not randomly distributed (18–20). Nevertheless, at present, sample size calculations are usually undertaken without accounting for the clustering of cases and the non-probabilistic nature of hunter-harvest. As a result, the detection probability (or the confidence of detecting CWD) is likely overestimated (14, 21), and therefore, inferences about the presence or absence of CWD in the targeted population during the pre-establishment phase are unreliable (22, 23). Most often, by the time active surveillance detects CWD in a population, the disease is already well-established and difficult to eliminate (16, 24).

To highlight phase-specific sampling issues, we used the surveillance model (14) to calculate sample size targets for high detection probability (95%) over the course of the CWD outbreak simulating (a) current standard assumptions (randomly distributed CWD cases, random sampling) and (b) realistic

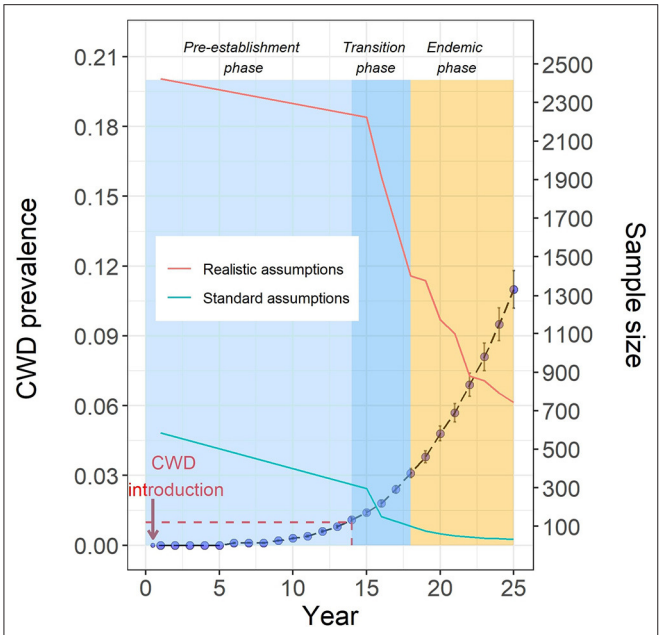

FIGURE 1 | Blue solid circles represent the mean CWD prevalence (±SE) for iterations with active CWD cases. The model-derived CWD trajectory is divided in three phases: pre-establishment, transition, and endemic. Model-derived sample size requirements for CWD detection (turquoise line = standard assumptions, red line = realistic assumptions) are plotted along with the CWD outbreak trajectory. The y-axis on the right side is for sample size. For example, when the true prevalence of CWD in the model deer population is ∼1% (year 14), the sample size needed for a 95% detection probability is 300, assuming a random distribution of CWD cases in the population and random sampling method (standard assumptions). For the same prevalence, but assuming a clustered distribution of CWD cases and non-random sampling (realistic assumptions), the sample size necessary for a 95% detection probability is ∼2,200. Red dashed line highlights the pre-establishment phase features: prevalence below 1% for ∼14 years post-introduction.

assumptions (clustered CWD distribution and non-random sampling). Model-derived sample size requirements are plotted along with the CWD outbreak trajectory (**Figure 1**). Based on the model-derived insights about CWD spread dynamics and sampling requirements, we define three phases of CWD outbreak: (a) *pre-establishment* phase is the early stage of the outbreak characterized by low CWD prevalence (below 1%) and sample size requirements that are either difficult to achieve and unsustainable (using realistic assumptions) or unreliable (detection probability overestimated due to unrealistic assumptions); (b) *transition* phase follows the pre-establishment phase, characterized by an increasing prevalence (above 1%, but <3%) and a considerable decrease in the sample size requirement even with realistic assumptions; and (c) *endemic* phase characterized by rapidly increasing CWD prevalence and a corresponding decrease in sample size requirement for CWD detection using realistic assumptions. Thus, surveillance strategies at each phase necessitates different sample sizes to confidently detect the existence of CWD. Among other factors, environmental contamination likely plays an increasingly important role in CWD transmission in the endemic phase, further complicating disease management (3).

Here, we show that model-derived sample size requirements for confidently detecting CWD in the pre-establishment phase are considerably large if realistic assumptions are used (**Figure 1**). Moreover, the surveillance model as presented here assumes 100% test sensitivity. But the probability of obtaining a positive result when testing an infected individual (test sensitivity) is <100% which is of special relevance for CWD because test sensitivity is typically lower in the early infection stages of the disease (25). Therefore, the actual samples required for confidently detecting CWD will be likely larger than the model-derived sample size.

Other model approaches, such as Bayesian weighted surveillance (26) and risk-based scenario tree modeling (27, 28) have been proposed for the early detection of CWD and other emerging diseases. Utilizing targeted sampling of high–risk individuals may be more cost-effective and reduce the sample size needed to detect disease when compared to random or convenience sampling. However, regardless of the approach to determine sample sizes, CWD surveillance data will be biased if clustering of cases in the pre-establishment phase and very low prevalence are not accounted for. A modeling tool that incorporates spatial clustering of cases (like *Ov*CWD) will be particularly useful for determining realistic sample size requirements for confidently detecting CWD in the pre-establishment phase. Better still, such a tool can be used to set a realistic detection threshold to economize surveillance efforts for efficient management of CWD.

Pre-establishment Phase CWD Dynamics and Pre-emptive Harvest Strategies

CWD is difficult to detect in the early phase of the outbreak when prevalence is low, and difficult to eliminate in the later stage of the outbreak when CWD is established. Preventing widespread establishment of CWD in regional populations is the key to avoiding long-term population health and economic impacts caused by CWD. The early-stage CWD dynamics in the model deer population underscores an important feature that sets the context for pre-emptive management of CWD: every introduction event does not necessarily result in persistent CWD transmission in the population, and it is possible that multiple introduction events occur before CWD is eventually established. The probability that a CWD introduction event results in persistent CWD transmission in the population is underpinned by two stochastic processes occurring at the individual level: actual transmission of infection between an infected and susceptible individual and an infected individual surviving harvest mortality. *Ov*CWD explicitly simulates these stochastic processes, and therefore can be used to derive "CWD persistence probability" [calculated as the proportion of iterations that have active CWD transmission in year 10 post-introduction; this was referred to as CWD Outbreak probability by (13)]. For example, for the scenario described in this paper, CWD transmission persisted in 41 out of 100 iterations while CWD transmission was extinguished before year 10 in 59

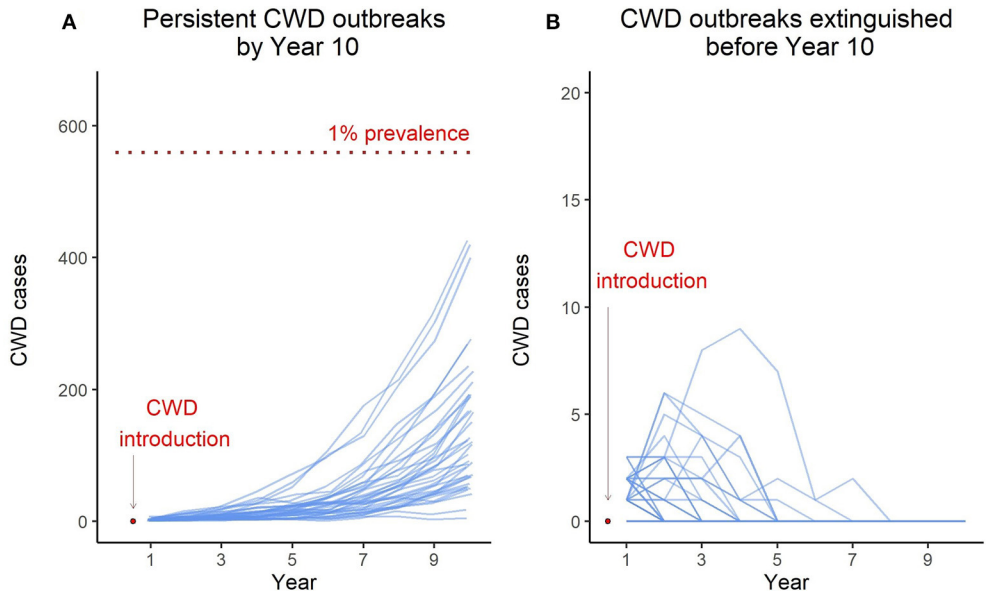

FIGURE 2 | Blue lines in the left panel **(A)** represent model iterations where the CWD transmission persisted for 10 years. The red dotted line indicates 1% prevalence level. Blue lines in the right panel **(B)** represent model iterations where CWD was extinguished from the population before year 10. Note the y-axis scale for the right panel, the maximum number of cases in non-persistent outbreaks remained under 10.

iterations (**Figure 2**). Therefore, the CWD persistence probability for the model deer population under the current harvest regime is 0.41. This metric can be used to compare and contrast the efficacy of alternate management actions implemented in the early phase of CWD outbreak.

For a given region of interest, CWD transmission (and therefore CWD persistence probability) is influenced by multiple factors, including: habitat characteristics, host community (species-specific differences in transmission and susceptibility), genetic structure (e.g., PRNP variation), demographics, behavior (dispersal, migration, aggregation, etc.), population connectivity related to natural and man-made barriers, and, of course, harvest (19, 20, 29–34). Some of these features are difficult to manipulate on meaningful spatial and temporal scales whereas others, especially demographics, can be manipulated by altering harvest strategies so as to decrease the CWD persistence probability. Implementing such harvest strategies before CWD is detected in a region (therefore, pre-emptive strategies), with the objective of increasing the resilience of the deer population to the spread and persistence of CWD, could be an important step forward in the current fight against CWD.

DISCUSSION

The management of CWD shares challenges common for many wildlife diseases. A common limitation is the lack of a firm theoretic basis when managing wildlife diseases (35). We propose the use of *Ov*CWD as one possible solution in the case of harvest management of CWD. Phase-specific management actions have been advocated for wildlife diseases in general (36), and for CWD, is one clear recommendation arising both from our modeling and from earlier literature surveys (8). Yet, implementing actions sufficiently early is made difficult by the uncertainty about the current status of infection in areas where wildlife disease has not been detected (11). In the case of CWD, this uncertainty stems from logistical constraints on wildlife agencies' ability to match the sample size targets required for detection of CWD in the early phase of the outbreak. For this reason, a blanket surveillance strategy, without considering the phase of the outbreak, may be inherently appealing and politically popular but is inefficient and unsustainable. Furthermore, non-uniform sampling effort across regions complicates the assessment of CWD status in regional deer populations. What is needed is the ability to rapidly assess the status of CWD in areas where it has not been detected yet.

We have already illustrated the use of our model-based framework for guiding collection and analysis of surveillance data that relies on harvest-based sampling (14). It is nearly impossible to confidently confirm the absence of CWD in a population or to confidently detect CWD in the early phase of the outbreak. In our view, surveillance strategies for areas with uncertain CWD status should acknowledge inherent logistical and practical limitations and use a realistic disease detection threshold. As illustrated in this paper, large number of samples are required for confident detection of CWD in the *pre-establishment phase* compared to the *transition phase* (**Figure 1**). Therefore, instead of a difficult to achieve and unsustainable surveillance target, agencies should consider setting the detection threshold to coincide with the *transition phase* of the CWD outbreak (prevalence above 1%, but <3%). Moreover, the

effect of non-random sampling is scale-dependent, and using smaller, ecologically based sampling units might reduce bias in probability of detection from non-random sampling and disease clustering (37).

From veterinary epidemiology, it is known that pre-emptive culling is required for diseases with latent stages or for those with initial low prevalence building up environmental reservoirs (38). Therefore, if CWD remains undetected with the detection threshold set for the transition phase, agencies should consider implementing pre-emptive harvest strategies tailored specifically for the target population. First, locally relevant harvest strategies that could be reasonably applied should be identified. These strategies will mostly involve manipulation of age and sex specific harvest rates, numbers removed, and spatial extent of affected area. The efficacy of such pre-emptive harvest strategies can then be evaluated using a simulation modeling framework like *Ov*CWD by deriving CWD persistence probabilities (13). Lower persistence probabilities can be interpreted as higher resiliency in the deer population to the spread and establishment of CWD. Additionally, CWD outbreak trajectories (and outbreak sizes) can be compared among different scenarios to assess their efficacy in limiting the spread of CWD. Wildlife agencies are likely to encounter resistance for pre-emptive strategies from stakeholders. Model-based simulations and virtual experiments can be used to communicate nuanced and complex management issues to stakeholders. Furthermore, user-friendly apps (Shiny apps, dashboard; e.g., https://rpubs.com/anyadoc/OvCWD_APR) can be developed to effectively illustrate these outcomes and ideas. Despite the publics' resistance that could occur with pre-emptive management strategies, such actions could help avoid much more invasive procedures such as population eradication.

Our modeling work highlights the importance of pre-emptive harvest strategies before CWD detection. By implementing model-evaluated harvest strategies pre-emptively, before CWD is detected in the population, wildlife agencies can improve their chances of reducing spread of CWD. Furthermore, management strategies implemented in the early stage of the outbreak during pre-introduction or pre-establishment will be relatively sustainable and logistically doable. Such pre-emptive strategies would be more palatable to the North American public than eradicating entire populations [e.g., (39)].

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://github.com/anyadoc/proactiveCWDmgmt. Model codes are openly available for download here: MIOvPOP (Version 1.1.0) https://doi.org/10.25937/kv07-3e08; MIOvCWD (Version 1.0.0) https://doi.org/10.25937/6qeq-1c13; MIOvPOPsurveillance (Version 1.0.0) https://doi.org/10.25937/fdke-rp28.

AUTHOR CONTRIBUTIONS

AB: conceptualization, model simulation and model output analysis, writing—original draft, writing-review and editing, prepared, and reviewed the manuscript. JJM, JRM, JS, HV, and AM: conceptualization and writing-review and editing. All authors contributed to the article and approved the submitted version.

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PERSPECTIVE
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The Interconnected Health Initiative: A Smithsonian Framework to Extend One Health Research and Education

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To better tackle diseases and sustain healthy ecosystems, One Health programs must efficiently bridge health in humans, domestic/livestock species, wild animals and plants, agriculture/aquaculture, and the environment. The Smithsonian Institution proposes to address this by considering 'health' in a broad sense – the absence of undue pathogens and unnecessary stress for any organisms as well as access to good living conditions in functional environments. Considering the interconnectedness of all life forms, the Smithsonian plans to create a framework that will integrate cultural, social, and educational components into health research on humans, animals, plants, or ecosystems. The objectives of this perspective article are to (1) propose an innovative framework to support an interconnected/integrated approach to health and (2) provide examples fostering impactful collaborations on One Health research and education. Based on the core strengths of the Smithsonian (multidisciplinary research, outreach and education programs, libraries/archives, and collections) and central institutional support, this framework has the potential to extend existing health-related projects, address new needs and situations (e.g., response to pandemics), provide invaluable resources to inform policy and decision makers, and educate all audiences globally.

Keywords: One Health, disease, education, research, interdisciplinary, multidisciplinary, museum, collections

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INTRODUCTION

Definitions of One Health and Examples of Current Programs

Historically, the concept of One Health has primarily focused on the spread of pathogens from animals to humans (zoonoses). However, the quality of the environment has major impacts on human health either directly through zoonotic disease transmissions or indirectly through management of natural resources (1). The World Health Organization (WHO) currently defines One Health as '*an approach to designing and implementing programs, policies, legislation and research in which multiple sectors, communicate and work together to achieve better public health outcomes*'. In the definitions of the United Nations Food and Agriculture Organization (FAO) the World Organization for Animal Health (OIE), or the Centers for Disease Control and Prevention (CDC), health outcomes depend on food safety, control of zoonoses, and combatting antibiotic resistance while recognizing the interconnection between people, animals, plants, and their shared environment. The One Health concept has now been extended beyond

public health to include the ecological and environmental dynamics of disease in systems-based frameworks such as Planetary Health and Eco-Health (2–4). However, understanding the ecology and evolution of disease agents as well as defining health for humans, wildlife, ecosystems or food systems remains challenging because these processes are highly complex especially at a systems scale. Interactions among hosts, environments and disease agents also can be difficult to measure (3). Importantly, to tackle complex ecosystem and societal dynamics, One Health programs rely on strong collaborations with varying scopes and efforts [e.g., Eco Health Alliance www.ecohealthalliance.org; One Health Initiative www.onehealthinitiative.com; Lancet One Health Commission (5)]. Some programs are more centered on regional/global efforts and inform policy like the One Health Platform (https://onehealthplatform.com/home), or One Health Regional Network for the Horn of Africa (www.onehealthhorn.net). One of the Smithsonian's flagship programs in One Health is the Global Health Program which sits within the Conservation Biology Institute (www.nationalzoo.si.edu/global-health-program). Members of this team (clinical veterinarians, public health specialists, ecologists, and epidemiologists) have been studying health and disease at the human-animal interface since 2014. The scope is to improve global health conservation of wildlife species through (1) capacity building and training (2) research and (3) wildlife health. It has been a key coalition member of the USAID's Emerging Pandemic Threats Program (EPT) PREDICT project (www.usaid.gov/news-information/fact-sheets/emerging-pandemic-threats-program).

More Actions Are Still Needed to Improve One Health

- A broader focus on all organisms and systems biology.

Beyond zoonotic diseases, the threat of non-infectious (including non-communicable) and infectious diseases of wildlife and plants must be considered. Additional threats include deforestation, urban expansion, unreasonable natural resource extractions, environmental pollution, stress, lack of fitness, invasive species, and climate change. A holistic view of interconnected life addressing these issues through different scales (from microbes to macro-organisms across local to global environments) is crucial for understanding the mechanisms by which health outcomes emerge from different environmental and anthropogenic states. However, this has not been fully achieved (6). For instance, invasive species are increasing due to globalization, environmental degradations and climate change. This leads to biodiversity loss and ecosystem impacts (e.g., pollinator decline, watershed degradation) that subsequently affect human health and livelihoods. More information is needed about co-infection dynamics within hosts and populations. More research also is required on how microbial infections impact host communities and change ecosystem functions (7). Many wildlife diseases have spread as a result of human activities (e.g., *Batrachochytrium dendrobatidis*, chytrid fungus in amphibians; *Pseudogymnoascus destructans*, white nose syndrome in bats; or *Canine morbillivirus*, canine distemper in wild carnivores). In these cases, the threat is not to humans directly, but among other species [e.g., chytrid fungus killing amphibians globally and decreasing food availability for snakes (8)].

- Incorporation of additional expertise in social/cultural and environmental science as well as museum collections.

Integration of environmental expertise, social science and behavioral aspects of health research and governance are limited or missing from many One Health programs (3). In that sense, some efforts have been made to integrate economic burdens of disease and social sciences into One Health programs (e.g., One Health Social Sciences Initiative of the One Health Commission www.onehealthcommission.org/en/programs/one_health_social_sciences_initiative/), but more could be done (9, 10). Similarly, while there is much research being done on climate and human health, more studies should be conducted on other forms of environmental change and health (e.g., land-use change) (11). In addition, efforts to improve health also must involve indigenous knowledge, local communities, governments, and educators. Importantly, socio-economic factors and societal inequities influence psychological health and risk of diseases [Chagas disease for instance (12)]. The current paradigm of limitless resource exploitation has to be replaced with more sustainable standards that better respect life on earth and strive toward well-being and social stability (13). Furthermore, more research and education are needed to address conflict resolution between stakeholders (14). Similarly, risk governance is required (irgc.org/about). Lastly, the One Health community should better leverage untapped research resources like natural history collections, libraries, and archives (15) while creating more biorepositories (16).

- Integration of formal (K-12 curriculum, undergraduate and graduate programs) and informal educational materials (exhibits, public programs, blogs).

As recommended by the National Academy of Medicine (17), it is crucial to define, develop, evaluate, improve, and continue to refine One Health education, not only in One Health degree programs but also in existing public health, environmental, agriculture, veterinary, and medical curricula. Besides improving scientific literacy of students, the general public also needs to be aware of and understand interconnections (humans are often part of the ecosystems) through museum exhibits and public programs.

Based on existing One Health definitions and current gaps, the objectives of this perspective article are to (1) propose an innovative framework to support an interconnected/integrated approach to health and (2) provide examples fostering impactful collaborations on Interconnected Health research and education (first at the Smithsonian and then in other organizations).

SMITHSONIAN ASSETS FOR A NOVEL INTERCONNECTED HEALTH FRAMEWORK

Strong conservation infrastructure and broad disciplinary expertise are increasingly critical to supporting healthy ecosystems in the era of climate change and rapid degradation

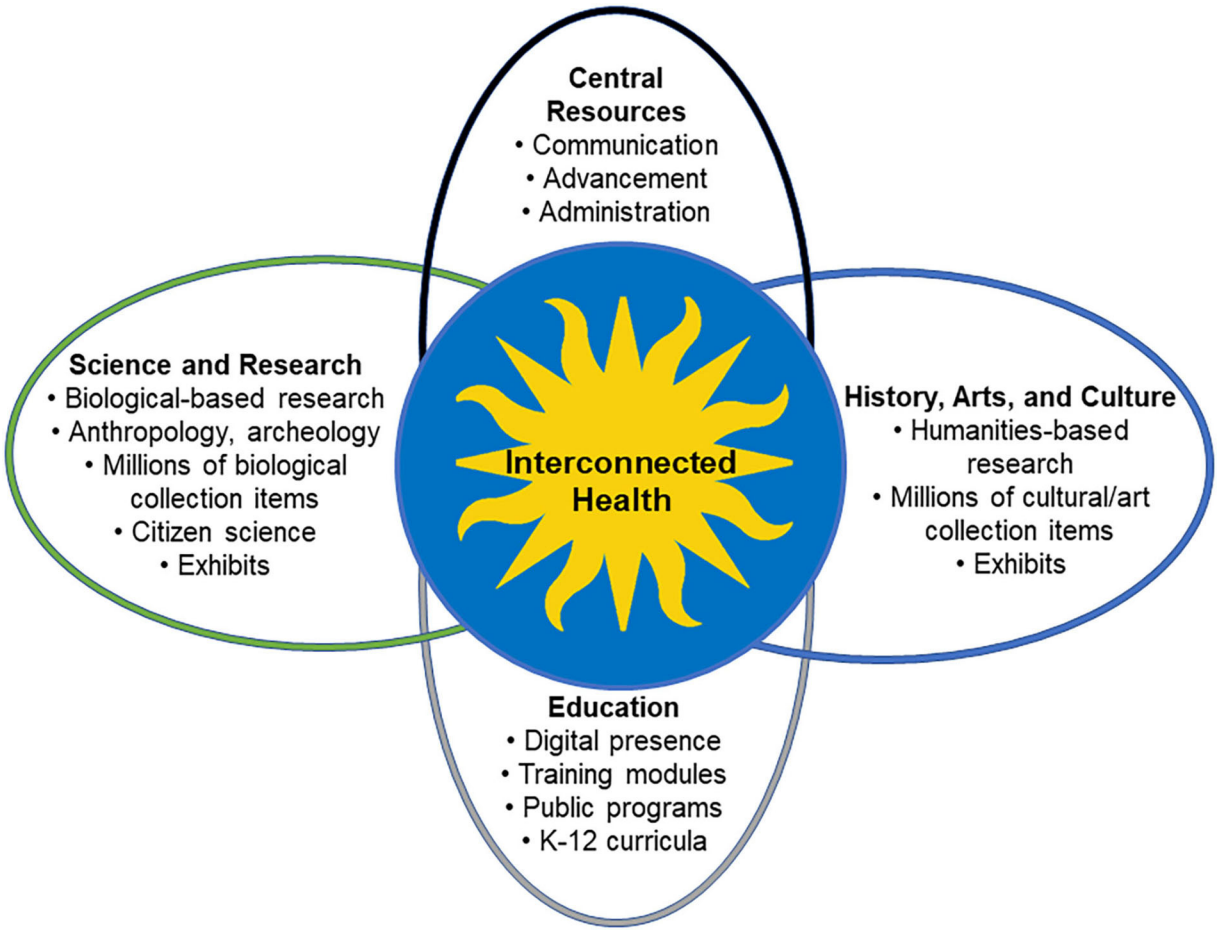

FIGURE 1 | The new framework is at the interface of key Smithsonian resources and expertise.

of biodiversity (18). An Interconnected Health framework is necessary to integrate multiple disciplines with a vision to improve health for all life through transdisciplinary research, education and rapid global action. A new framework will promote Smithsonian global leadership in One Health research and education. With 19 museums, a National Zoo, 14 education and research centers, libraries and archives, and one of the world's largest biological collections, the Smithsonian is poised to make a significant impact on health-related research and education (www.si.edu). Health is defined here in its broadest sense, as the absence of undue pathogens and unnecessary stressors for all organisms and the access to good living conditions in functional environments. This new framework that will support One Health research and education is at the interface of key resources and expertise across the institution (**Figure 1**). Here are some important details regarding the Smithsonian assets supporting the framework:

- Global presence across >140 countries and international projects (www.global.si.edu/) including research networks for forests (www.forestgeo.si.edu), marine environments (www.marinegeo.si.edu), and food systems (https://serc.si.edu/projects/oyster-reefs-and-restoration) as well as many long-term continuous research and monitoring programs across research centers and museums that started in the 20th and even 19th centuries.
- Training and education programs for students and professionals in health and disease: training in One Health and conservation medicine around the world (www.nationalzoo.si.edu/global-health-program), multidisciplinary fellowship programs in collaboration with many universities (www.smithsonianofi.com/fellowship-opportunities), practicum projects for students enrolled in Masters of Public Health, and curricula development (http://www.ssec.si.edu) (www.nmaahc.si.edu/learn/educators/stem-nmaahc).
- Large and growing citizen science programs (www.si.edu/volunteer/citizenscience) in research centers and museums (www.anacostia.si.edu/urbanwaterways/about).
- Excellence in exhibits and public engagement for tens of millions of visitors annually, in person and digitally: for example, "Outbreak: Epidemics in a Connected World" exhibit (and as a pop-up exhibit in 20 countries), "Modern Medicine and the Great War" or "Black Life in Two

Pandemics" (www.naturalhistory.si.edu/exhibits/outbreak-epidemics-connected-world; www.americanhistory.si.edu/blog/black-life-two-pandemics).
- Infrastructure facilitating internal and external communication on health and disease through existing tools and programs: SI Profiles (www.profiles.si.edu)—a system that displays data from various sources, augmented by input from individuals, to showcase the depth and breadth of expertise at the Smithsonian; Conservation Commons (www.conservationcommons.si.edu)—a network combining Smithsonian cultural and scientific strengths in conservation.
- Hundreds of diverse partnerships across government agencies, non-governmental organizations, communities, and global networks of museums and science institutions.
- The Smithsonian has been offering world class research and public programming on pandemics for many years and has been addressing impacts of Covid 19 through many lenses including; K-12 curriculum; Outbreak! Epidemics in a Connected World exhibit; socio-cultural effects on communities of color; or the Pandemic Oral History project.

PROCESS AND METHODOLOGY TO SET UP THE SMITHSONIAN FRAMEWORK

Over the past 5 years, a series of meetings and discussions around the theme of Interconnected Health have involved more than 100 experts representing almost all Smithsonian units, offices, or programs. Based on ideas and information gathered during these consultations, a 5-year strategic road map was designed for implementing the framework.

The vision is that health of all life will be improved as a result of transdisciplinary research, engagement, and rapid global action. The mission of the framework is to advance knowledge and improve the health of all life globally through illuminating the interconnectedness of all organisms. To achieve the mission, three strategic objectives will integrate new components within and across projects to enhance reach and impact. Existing expertise will be leveraged to break down silos and encourage transdisciplinary thinking and collaboration; this will be done by assembling a workforce with expertise that spans the biological and social sciences, arts and humanities, and technology, with the capacity to implement pilot projects and grow programs.

The first strategic objective is to advance transdisciplinary research to understand how the interconnectedness of life forms and their environment affect the balance of health and disease in human impacted and natural systems (particularly including the humanities in these efforts). Success will be measured against several indicators, such as workforce diversity (across biological and social sciences, arts and humanities), use of novel technologies within individual research programs, or increase in interdisciplinary research outputs.

The second strategic objective is to develop formal and informal education/engagement plans that complement the Smithsonian's research programs and effectively convey the concept of interconnectedness. New knowledge generated by those two strategic objectives will be used to enhance existing health projects, make informed decisions leading to adapted action, catalyze protection of living organisms and habitats, and thereby support health globally. Success will be evaluated in the coming years by the increase in grant proposals and funded research integrating education and engagement.

A third objective will ensure programmatic and financial sustainability to support efforts in health research and education. In addition to leveraging and linking existing expertise to break down silos and encourage transdisciplinary thinking and collaboration, this will be done by assembling a workforce with the capacity to implement pilot projects and grow programs. A steering committee and an advisory committee will coordinate efforts across Smithsonian units to provide guidance in research and education. Core organizational structures will identify and assist with funding sources (grants, contracts, philanthropy) and communicate internally and externally while promoting equality, diversity and inclusion. Success of that objective will be achieved by the creation of a central office dedicated to Interconnected Health and the ability to secure large-scale support to interdisciplinary research conducted at national and global scales.

HOW TO DEVELOP AND USE THE FRAMEWORK?

Based on the strategic objectives mentioned above, new components will be integrated within and across projects. A network of interdisciplinary practitioners will also be used to identify priorities for research and education to better prioritize and guide activities. Once existing programs, new needs, or emerging issues are identified, the use of the framework will be driven by sound questions in science, humanities and education (**Figure 2**). For instance:

- How can Smithsonian experts work more effectively across disciplines to better understand pathogen spillover into new host species and environmental pollution, and to mitigate disease impacts through health monitoring, habitat conservation, public education, and cultural knowledge?
- How do we measure the value of, and best conserve, vital ecosystem services in human impacted areas through science, community-engagement and education?
- How can we understand and mitigate the impacts of social inequity and inequality on human physiological and psychological health as well as environmental and wildlife health?

While specific questions are being formulated, key elements will be integrated into existing programs, new needs, or emerging issues (**Figure 2**). For instance:

- Providing unique expertise (cultural aspects) and professional training opportunities to ongoing One Health programs.
- Promoting community engagement, public education, and preparedness.

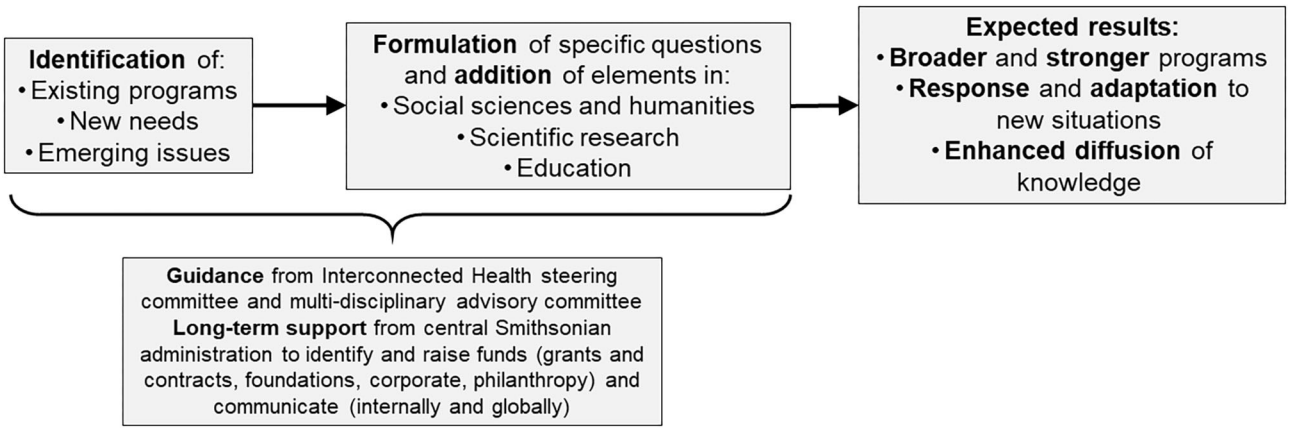

FIGURE 2 | Flowchart illustrating the use of the Smithsonian framework to extend One Health research and education.

- Creating unified messaging and education that is shared by all museums to promote interconnected/integrated approach to health among the general public and in schools.

EXPECTED RESULTS OF THE FRAMEWORK

Building Broader and Stronger Programs Upon Existing Smithsonian Projects

While the microbial disease component is crucial and already present in several Smithsonian projects, progress is being made in incorporating a focus on how to keep microbiota functional and healthy in agricultural and forest systems, conservation breeding, and reintroduction efforts. The Smithsonian also has microbe-related education projects (Outbreak exhibit) and new cultural projects (American Stories in the time of Pandemic, Art in Quarantine). However, the new framework is not solely centered on micro-organisms. Here are some examples of innovative programs within and across current activities that will help achieve a broadly integrative and holistic approach:

- Community/Species-based programs

Health and conservation of amphibians (www.nationalzoo.si.edu/center-for-species-survival/panama-amphibian-rescue-and-conservation-project) or coral reefs (www.ocean.si.edu/ecosystems/coral-reefs) are good examples that easily fit within the new framework. Both programs conduct and translate research for education and to inform policy. In the Urban Waterways Project, local communities are engaged with their environment through a holistic approach that incorporates science, art, education, religion, and the humanities (www.anacostia.si.edu/urbanwaterways/about).

- Land and seascape-based programs

Agua Salud develops approaches for tropical forest management (www.striresearch.si.edu/smartreforestation) to the mutual benefit of communities and nature. Similarly, working land and seascapes (www.wls.si.edu) aims to foster healthy and productive landscapes and seascapes. The Global Change Research Wetland houses several long-term experiments to unravel the complex ecological processes that occur in coastal wetlands and how climate change is impacting them (www.serc.si.edu/gcrew). Regarding sustainable food systems, the Marine Conservation Program combines social, ecological and economic research to protect and improve the health of marine systems that support fisheries and communities (global.si.edu/success-stories/see-how-smithsonian-working-local-fishermen-protect-marine-areas-and-create-safe). Smithsonian collections of over 35 million insect specimens have been an invaluable resource to agriculture science and management since the 19th century (agresearchmag.ars.usda.gov/2002/jun/bugs). With partners in East Africa and Asia, the Global Health Program (https://nationalzoo.si.edu/global-health-program) is studying how environmental and demographic pressures that push people and their livestock into closer contact with wildlife, create opportunities for the emergence and spread of novel diseases in people and animals. Landscape-scale experiments are being conducted in natural ecosystems, inhabited by people and their livestock, and cities, where rapid urban development—characteristic of cities in the tropics—can bring wildlife and livestock into close contact.

- Region-based programs (multiple landscapes and ecosystems)

The Mpala Research Center, in Laikipia, Kenya (www.mpala.org) —of which the Smithsonian is a founding member—provides a 'living rangeland laboratory' in which Smithsonian and international researchers conduct landscape-level experiments and training on ecology and health (e.g., wildlife endocrinology, emerging zoonotic disease, landscape connectivity, nutrient cycling), translating their findings into solutions for wildlife conservation, sustainable livelihoods and ecosystem resilience. Across land and seascapes, Movement of Life (www.movementoflife.si.edu) advances the understanding of how all

living things, big and small, move across changing land and seascapes.

Note that all of programs mentioned above integrate the well-being of humans and wildlife, economics, cultural heritage, national and global biosecurity, exhibits and public programs, and curriculum for schools.

Respond and Adapt to New Situations

A recent call for a COVID-19 One Health Research Coalition is a good illustration about the need for multidisciplinary and multilateral coalitions (19). The framework currently is helping the Smithsonian response to the COVID pandemic in research, humanities, museum exhibit and education (facilitating internal communication, enhancing exploration of museum collections, improving the search for research funds, initiating new programs). Lessons learned will also inform new pan-institutional efforts tackling global issues. Through the implementation of this framework, the Smithsonian would be well-placed to respond to future planetary challenges that threaten human, environmental and animal health.

Enhance the Diffusion of Knowledge

Smithsonian exhibits and education programs can easily incorporate new material and generate new content for public education (e.g., COVID-19 module from the Science Education Center). The Smithsonian's position in the US government provides important opportunities to participate in congressional hearings and interagency committees (including NIH, USAID, USDA, CDC) that vitally inform policy making. Many government leaders attend Smithsonian programs or visit exhibits and meet with scholars to learn about important issues. In addition to interfacing science/culture with American and foreign policy, the global reach of its staff (many of whom work in partnership with foreign governments, local institutions, and communities around the world) will help identify new and important questions for research and help design innovative educational approaches for higher impact.

CONCLUSIONS

The Smithsonian interconnected approach to health integrates science, culture and education. It allows to involve more experts, find innovative solutions, engage a wider audience, and increase impact. Although the proposed framework is specific to the Smithsonian, lessons learned (in programmatic and financial sustainability) and corrective solutions will be shared with the community to foster similar approaches in other organizations. Importantly, the approach has the possibility to change attitudes across societies and facilitate intergenerational dialogues on a global scale about vital issues. It also inspires a next generation of scientists who will diversify STEM fields and stimulate creative expertise in health and disease related research. This integrative approach should be added to the professional development of practitioners and educators. Lastly, the framework will address and help increase scientific literacy, diversity, equity, accessibility, and inclusion.

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.

AUTHOR CONTRIBUTIONS

PC, KP, CM-W, JH, and BC contributed equally to the collection of information, outline of the manuscript, and writing. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Epitope Peptide-Based Predication and Other Functional Regions of Antigenic F and HN Proteins of Waterfowl and Poultry *Avian Avulavirus Serotype-1* Isolates From Uganda

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Uganda is a Newcastle disease (ND) endemic country where the disease is controlled by vaccination using live LaSota (genotype II) and I_2 (genotype I) vaccine strains. Resurgent outbreak episodes call for an urgent need to understand the antigenic diversity of circulating wild *Avian Avulavirus serotype-1* (AAvV-1) strains. High mutation rates and the continuous emergence of genetic and antigenic variants that evade immunity make non-segmented RNA viruses difficult to control. Antigenic and functional analysis of the key viral surface proteins is a crucial step in understanding the antigen diversity between vaccine lineages and the endemic wild ND viruses in Uganda and designing ND peptide vaccines. In this study, we used computational analysis, phylogenetic characterization, and structural modeling to detect evolutionary forces affecting the predicted immune-dominant fusion (F) and hemagglutinin-neuraminidase (HN) proteins of *AAvV-1* isolates from waterfowl and poultry in Uganda compared with that in LaSota vaccine strain. Our findings indicate that mutational amino acid variations at the F protein in LaSota strain, 25 poultry wild-type and 30 waterfowl wild-type isolates were distributed at regions including the functional domains of B-cell epitopes or *N*-glycosylation sites, cleavage site, fusion site that account for strain variations. Similarly, conserved regions of HN protein in 25 Ugandan domestic fowl isolates and the representative vaccine strain varied at the flanking regions and potential linear B-cell epitope. The fusion sites, signal peptides, cleavage sites, transmembrane domains, potential B-cell epitopes, and other specific regions of the two protein types in vaccine and wild viruses varied considerably at structure by effective online epitope prediction programs. Cleavage site of the waterfowl isolates had a typical avirulent motif of ^{111}GGRQGR'L^{117} with the exception of one isolate which showed a virulent motif of ^{111}GGRQKR'F^{117}. All the poultry isolates showed the ^{111}GRRQKR'F^{117} motif corresponding to virulent strains. Amino acid sequence variations in both HN and F proteins of *AAvV-1* isolates from poultry, waterfowl, and vaccine strain

were distributed over the length of the proteins with no detectable pattern, but using the experimentally derived 3D structure data revealed key-mapped mutations on the surfaces of the predicted conformational epitopes encompassing the experimental major neutralizing epitopes. The phylogenic tree constructed using the full F gene and partial F gene sequences of the isolates from poultry and waterfowl respectively, showed that Ugandan ND aquatic bird and poultry isolates share some functional amino acids in F sequences yet do remain unique at structure and the B-cell epitopes. Recombination analyses showed that the C-terminus and the rest of the F gene in poultry isolates originated from prevalent velogenic strains. Altogether, these could provide rationale for antigenic diversity in wild ND isolates of Uganda compared with the current ND vaccine strains.

Keywords: *Avian Avulavirus serotype*-1, genotype-matched vaccine, B-cell epitopes, Uganda, Newcastle disease

INTRODUCTION

Avian Avulavirus serotype-1 (*AAvV-1*) is a member of the genus *Avulavirus* in the family of *Paramyxoviridae* that causes ND in poultry. The antigenic serotypes evolving in this group under environmental or vaccination pressure, evade the immune system of birds and account for vaccine failures (1, 2). The *AAvV-1* serotype displays a great genetic and antigenic diversity in the fusion (F) and hemagglutinin neuraminidase (HN) genes that encode for the structural envelope spike proteins playing host recognition, infection, and pathogenesis roles, but importantly influencing the antigenicity and immunogenicity of the ND viruses (3–6).

Infectivity assays of ND viruses employ the use of animal models (1-day-old *Gallus gallus* chick) which requires a lot of time and monetary inputs. Low-virulent strains are defined by the monobasic amino acid sequences at positions 112–113 and 115–116 of the C-terminus of fusion protein cleavage site (FPCS) with leucine (L) at position 117 and/or intracerebral pathogenicity indices (ICPI) of $\leq$0.7 (7, 8). Such strains induce antibody titers sufficient to prevent ND with the infected birds frequently remaining asymptomatic or presenting mild respiratory form of ND. The FPCS together with the hypervariable regions of the F protein are the basis of phylogenetic analysis of pathotypes (9). Genotypes and lineages emerge among other typing methods that classify NDV strains (10, 11). To date, two classes I and II, are recognized and each further subdivided into three different subgenotypes (1.1.1, 1.1.2, and 1.2) and 21 clades or subgenotypes (I-XXI), respectively (9, 10, 12, 13). In order to avoid discrepancies of nomenclature systems and incorrect assigning of genotypes, the most recent classification emphasizes the use of full F gene to classify these viruses further (6, 12, 14). Notably, the class II genotype VII viruses are the most commonly reported in ND outbreaks in poultry, pet, and wild birds throughout the world, while class I which is commonly isolated from waterfowl, shore birds, and some poultry is less virulent and is exploited for potential vaccine candidates (10).

The potential targets for immune system response to ND virus during ND infection are the two membrane-anchored glycoproteins F and HN, also known for cell-binding and infection (6, 15). Genomic and antigenic differences between wild isolates and vaccine strains are among the reported causes of ND vaccine failure. These differences accrue from several cumulative mutations at the F and HN genes of the wild strains in response to vaccination pressure (1, 13, 16). Enzymatic restriction of the viral precursor F_0 molecule by the host enzymes in the respiratory and intestinal tracts facilitates replication of virulent viruses in these tissues (17). The conformational change induced in the precursor F_0 protein after proteolytic cleavage and the suspension of two disulfide-linked subunits of the hydrophobic N-terminus of F1 and the C-terminus of F_2 fragments off the membrane initiates the cellular fusion interaction. Thus, structural protein changes produced by point mutations involving one or two amino acids or the changes resulting from recombination at the F protein antigenic site of these viruses circulating in an endemic wild birds or poultry are implicated in ND outbreaks (13, 16, 18).

In ND pathogenesis, HN initiates infection while F-glycoprotein mediates viral attachment and penetration into host cells (19). Both proteins induce host-immune response and are required for producing neutralizing antibodies induced by vaccines. Antibodies against F proteins have been demonstrated in *in vivo* study as importantly necessary in neutralizing the ND infectivity (20, 21). In fact, the passive immunization of chicks using antibodies directed to the two antigenic sites I and II of F protein, which reacted with antibodies 83(F) and 313(F), respectively, have demonstrated complete suppression of viral growth and death of chicks (22). Seven major F protein neutralizing epitopes involved in fusion inhibition and neutralization are shown at specific residues 72, 74, 75, 78, 79, 157–171, and 343 for epitopes A1, A2, A3, A4, and A5, respectively. The amino acid residues show that both F_1 and F_2 are involved in the formation of a single antigenic site vital in the structure and function of the active F epitopes (23, 24). In the HN protein, five conformational and linear antigenic sites are crucial in inducing antibodies at amino acid residues of 193–201, 345–353, 494, 513–521, and 569 (25, 26). Amino acid changes in the linear epitopes of wild isolates from glutamate (E) to lysine (K) at position 347 have been known to alter the character of HN protein making some strains of ND viruses escape from epitope recognition by mABs (27).

Genomic changes in the non-segmented, negative-sense RNA viruses are known to arise from the inherent error rate of polymerase, generating a large number of variants in the non-conserved proteins upon which nature selects suitable changes in the viral genome (28). Lower recombination rates are also reported in HN and F genes of non-segmented, negative-sense *AAvV-1s*, that account for strain variation (29, 30). However, strain-specific variations in *AAvV-1s*, arising from mutations are available in the public domain repositories. Nonsynonymous mutation rates have been associated with the codons at the cleavage site motif of the F protein resulting into diversity and classification of the strains. The mono- or multibasic amino acid tendencies at this site suggest that increase in virulence increases the rate of virus evolution (28, 31). Wild aquatic birds are considered to be natural reservoirs for ND viruses and constituting a threat in spreading these recombinant viruses to the domestic birds as they come into contact with them along their migration pathways across the world (32). Studies have revealed similarities between strains recovered from aquatic birds and shorebirds with those isolated from live bird markets (LBMs) in some parts of the world (10).

ND is known to be endemic in Uganda; phylogenic analysis involving F and HN sequences of wild isolates from Uganda have indicated unique ND viruses in relation to those found elsewhere in the world (33, 34). Understanding the forces of adaptive evolution or increased genetic variation in both virulent and non-virulent *AAvV-1s* can help prevent future outbreaks. Currently, two live monovalent ND vaccines (LaSota and I_2) are available in Uganda for the control of ND infection. Due to the frequent and recent ND outbreaks, there is a renewed interest to understand the forces that drive positive or negative (purifying) pressures on specific amino acid sites within surface proteins. Importantly, the possible continued cocirculation of the virulent *AAvV-1s* in the apparently healthy-looking birds marketed at LBMs (33) needs to be studied. Recently, we isolated thermostable, low-virulent ND viruses in waterfowl and compared their immunogenicity with those of the vaccine (LaSota and I_2) strains. We challenged the immunized birds with a circulating wild virulent strain and noted some protection failure rates (35).

Solutions to ND vaccine failure rates and the observed presence of virulent viruses harbored by healthy domestic birds can only be envisaged by establishing the role of selection pressures and virulence on evolutionary change of the NDV F protein and comparing the immune-dominant epitopes of the currently used vaccine strains with that of wild viruses using *in silico* approaches. The amino acid patterns, which are apparently hidden at sequence level become evident when mapped onto experimentally derived 3D structure. Carrying out 3D mapping of the amino acid patterns provides timely and inexpensive insights into antigen homology studies for ND control. In this study, we focused on the HN and F proteins of the Ugandan *AAvV-1s*, which are the main surface proteins important in viral entry, carrying functionally important structures like signal peptide, cleavage activation sites, and B-cell-mediated antibody response. Additionally, the sequential and conformational variations at the hydrophobic and hydrophilic sites and transmembrane sites establish the diversity of wild ND viruses and the current vaccine strains.

MATERIALS AND METHODS

Viral Sequence Compilation

For analysis, 70 and 55 selected sequences of the F and HN protein were used, respectively. Of the 70 F protein, 40 were retrieved from the GenBank database, comprising 25 described earlier in our pathogenicity and evolution study of *Avulavirus serotype-1* isolated from poultry sampled from LBM in Uganda (33). Three ND viruses isolated from waterfowl in 2011 (36), one sequence described 10 years earlier in Uganda (34), and 11 sequences representing various class II NDV genotypes were used. The rest of the 30 sequences were partial F gene sequences of viruses isolated from freshly voided fecal matter of several species of waterfowl including the long-tailed cormorant, gray-headed gull, white-winged tern, little egret, greater cormorant, Egyptian goose, slender-billed gull, African open-billed stork, black-headed heron, gull-billed tern, African jacana, yellow-billed duck, black crake, yellow-billed stork, sacred ibis, pink-backed pelican, long-toed plover, pied kingfisher, African darter, hadada ibis, cattle egret, fish eagle, hamerkop, white-faced whistling duck, malachite, common squacco, Terek sandpiper, and wood sandpiper. Accordingly, all the isolates from LBM were highly pathogenic and of low evolution placed in genotype V in the new classification system described by Byarugaba et al. (33). Similarly, of the 55 HN protein sequences, 32 were retrieved from the GenBank database as part of our poultry LBM isolates during our pathogenicity study and evolutionary study (33) and 23 representing the 21 genotypes in class II.

The NDV isolates from poultry in Uganda were selected according to the districts, namely, Mukono (MUK), Masaka (MAS), Wakiso (WAK), Bugiri (BUG), Iganga (IGA), Kotido (KOT), Namutamba (NAM), Arua (ARU), Kiryandongo (KIR), Koboko (KOB), Nebbi (NEB), Gulu (GUL) Kasese (KAS), and Abim (ABI). The NDV isolates from waterfowl described in this study were collected from aquatic birds found at selected landing sites, namely, Musambwa Island (MUS), Makanaga Bay (MAK), Lutembe Bay (LUT), Mabamba Bay, Nakiwogo landing site (NAK), Samuka Island (SAM), and Queen Elizabeth National Park (QE) shown in **Table 1**.

Sampling, Isolation, Confirmation, and Sequencing of Waterfowl *AAvV-1s* Isolates

Sampling, virus isolation, confirmation, and sequencing of the waterfowl *AAvV-1* isolates were done as described by Byarugaba et al. (33) and Wanyana et al. (36). Samples of freshly voided fecal matter were swabbed using sterile Dacron swabs from land/rock surfaces into cryovials containing viral transport medium supplemented with antibiotics (isotonic PBS, 2,000 U/ml penicillin, 2 mg/ml streptomycin, 50 μg/ml gentamycin, 50 U/ml nystatin, and 0.5% BSA). The sample vials were transported in dry shippers to the laboratory and stored at −80°C until analysis. Each sample was inoculated (in triplicate) by the allantoic route into 9–10-day embryonated chicken eggs (ECEs) for virus isolation according to the OIE Manual of Standards

TABLE 1 | A list of Ugandan *AAvV*-1 strains and reference vaccines used in this study.

No	Virus (reference)	Abbreviations	Source	Genotype	F-accession number	HN-accession number
1	LaSota, vaccine strain	LaSota, vaccine strain	Vaccine^{+}	II	AY845400	AY845400
2	I-2, vaccine strain	I-2, vaccine strain	Brentek^{R}	II	AY935499	AY935499
3	NDV/Chicken/Pallisa/0405/01$^{\beta}$	NDV/C/Pallisa/0405/01	Pallisa	II	AY367559	–
4	NDV/Waterfowl/Uganda/MU116/2011	NDV/WF/UG/MU116/2011QE	Queen Elizabeth	II	ND	ND
5	NDV/Waterfowl/Uganda/MU122/2011	NDV/WF/UG/MU122/2011QE	Queen Elizabeth	II	ND	ND
6	NDV/Waterfowl/Uganda/MU125/2011	NDV/WF/UG/MU125/2011LUT	Lutembe	II	ND	ND
7	NDV/Waterfowl/Uganda/MU126/2011	NDV/WF/UG/MU126/2011LUT	Lutembe	II	ND	ND
8	NDV/Waterfowl/Uganda/MU129/2011	NDV/WF/UG/MU129/2011LUT	Lutembe	II	ND	ND
9	NDV/Waterfowl/Uganda/MU130/2011	NDV/WF/UG/MU130/2011LUT	Lutembe	II	ND	ND
10	NDV/Waterfowl/Uganda/MU131/2011	NDV/WF/UG/MU131/2011LUT	Lutembe	II	ND	ND
11	NDV/Waterfowl/Uganda/MU132/2011	NDV/WF/UG/MU132/2011LUT	Lutembe	II	ND	ND
12	NDV/Waterfowl/Uganda/MU137/2011	NDV/WF/UG/MU137/2011LUT	Lutembe	II	ND	ND
13	NDV/Waterfowl/Uganda/MU149/2011	NDV/WF/UG/MU149/2011MAK	Makanaga	II	ND	ND
14	NDV/Waterfowl/Uganda/MU151/2011	NDV/WF/UG/MU151/2011MAK	Makanaga	II	ND	ND
15	NDV/Waterfowl/Uganda/MU152/2011	NDV/WF/UG/MU152/2011MAK	Makanaga	II	ND	ND
16	NDV/Waterfowl/Uganda/MU154/2011	NDV/WF/UG/MU154/2011MUS	Musambwa	II	ND	ND
17	NDV/Waterfowl/Uganda/MU159/2011	NDV/WF/UG/MU159/2011MUS	Musambwa	II	ND	ND
18	NDV/Waterfowl/Uganda/MU162/2011	NDV/WF/UG/MU162/2011MUS	Musambwa	II	ND	ND
19	NDV/Waterfowl/Uganda/MU165/2011	NDV/WF/UG/MU165/2011MUS	Musambwa	II	ND	ND
20	NDV/Waterfowl/Uganda/MU167/2011	NDV/WF/UG/MU167/2011MUS	Musambwa	II	ND	ND
21	NDV/Waterfowl/Uganda/MU170/2011	NDV/WF/UG/MU170/2011MUS	Musambwa	II	ND	ND
22	NDV/Waterfowl/Uganda/MU171/2011	NDV/WF/UG/MU171/2011MUS	Musambwa	II	ND	ND
23	NDV/Waterfowl/Uganda/MU172/2011	NDV/WF/UG/MU172/2011MUS	Musambwa	II	ND	ND
24	NDV/Waterfowl/Uganda/MU173/2011	NDV/WF/UG/MU173/2011MUS	Musambwa	II	ND	ND
25	NDV/Waterfowl/Uganda/MU174/2011	NDV/WF/UG/MU174/2011NAK	Nakiwogo	II	ND	ND
26	NDV/Waterfowl/Uganda/MU176/2011	NDV/WF/UG/MU176/2011NAK	Nakiwogo	II	ND	ND
27	NDV/Waterfowl/Uganda/MU178/2011	NDV/WF/UG/MU178/2011NAK	Nakiwogo	II	ND	ND
28	NDV/Waterfowl/Uganda/MU181/2011	NDV/WF/UG/MU181/2011NAK	Nakiwogo	II	ND	ND
29	NDV/Waterfowl/Uganda/MU177/2011	NDV/WF/UG/MU177/2011NAK	Nakiwogo	II	ND	ND
30	NDV/Waterfowl/Uganda/MU138/2011^{e}	NDV/WF/UG/MU138/2011MAK	Makanaga	II	LT549451	ND
31	NDV/Waterfowl/Uganda/MU150/2011^{e}	NDV/WF/UG/MU150/2011MAK	Makanaga	II	LT549452	ND
32	NDV/Waterfowl/Uganda/MU186/2011^{e}	NDV/WF/UG/MU186/2011SAM	Samuka	II	LT549453	ND
33	NDV/Waterfowl/Uganda/MU118/2011	NDV/WF/UG/MU118/2011QE	Queen Elizabeth	II	ND	ND
34	NDV/Chicken/Uganda/MU001/2011	NDV/C/UG/MU001/2011MAS	Masaka	Vd	HG937567	HG937535
35	NDV/Chicken/Uganda/MU007/2011	NDV/C/UG/MU007/2011MAS	Masaka	Vd	HG937568	HG937536
36	NDV/Chicken/Uganda/MU009/2011	NDV/C/UG/MU009/2011MAS	Masaka	Vd	HG937569	HG937537
37	NDV/Chicken/Uganda/MU010/2011	NDV/C/UG/MU010/2011MAS	Masaka	Vd	HG937570	Hg937538
38	NDV/Chicken/Uganda/MU0138/2011	NDV/C/UG/MU013/2011MUK	Mukono	Vd	HG937571	HG937539
39	NDV/Chicken/Uganda/MU019/2011	NDV/C/UG/MU019/2011WAK	Wakiso	Vd	HG937572	HG937541
40	NDV/Chicken/Uganda/MU024/2011	NDV/C/UG/MU024/2011ABI	Abim	Vd	HG937573	–
41	NDV/Chicken/Uganda/MU026/2011	NDV/C/UG/MU026/2011BUG	Bugiri	Vd	HG937574	HG937543
42	NDV/Chicken/Uganda/MU032/2011	NDV/C/UG/MU032/2011IGA	Iganga	Vd	HG937575	–
43	NDV/Chicken/Uganda/MU033/2011	NDV/C/UG/MU033/2011IGA	Iganga	Vd	HG937576	–
44	NDV/Chicken/Uganda/MU035/2011	NDV/C/UG/MU035/2011IGA	Iganga	Vd	HG937577	HG937547
45	NDV/Chicken/Uganda/MU037/2011	NDV/C/UG/MU037/2011KOT	Kotido	Vd	HG937578	–
46	NDV/Chicken/Uganda/MU039/2011	NDV/C/UG/MU039/2011KUM	Kumi	Vd	HG937579	HG937548
47	NDV/Chicken/Uganda/MU040/2011	NDV/C/UG/MU040/2011KUM	Kumi	Vd	HG937580	–

(Continued)

TABLE 1 | Continued

No	Virus (reference)	Abbreviations	Source	Genotype	F-accession number	HN-accession number
48	NDV/Chicken/Uganda/MU044/2011	NDV/C/UG/MU044/2011NAM	Namutamba	Vd	HG937581	–
49	NDV/Chicken/Uganda/MU050/2011	NDV/C/UG/MU050/2011ARU	Arua	Vd	HG937582	–
50	NDV/Chicken/Uganda/MU056/2011	NDV/C/UG/MU056/2011ARU	Arua	Vd	HG937583	HG937552
51	NDV/Chicken/Uganda/MU062/2011	NDV/D/UG/MU062/2011ARU	Arua	Vd	HG937584	–
52	NDV/Chicken/UgandaMU069/2011	NDV/C/UGMU069/2011KIR	Kiryandongo	Vd	HG937585	HG937554
53	NDV/Chicken/Uganda/MU071/2011	NDV/C/UG/MU071/2011KIR	Kiryandongo	Vd	HG937586	HG937555
43	NDV/Chicken/Uganda/MU074/2011	NDV/C/UG/MU074/2011KOB	Koboko	Vd	HG937587	HG937557
44	NDV/Chicken/Uganda/MU084/2011	NDV/C/UG/MU084/2011NEB	Nebbi	Vd	HG937588	HG937560
45	NDV/Chicken/Uganda/MU090/2011	NDV/C/UG/MU090/2011GUL	Gulu	Vd	HG937589	HG937561
46	NDV/Chicken/Uganda/MU111/2011	NDV/C/UG/MU111/2011KAS	Kasese	Vd	HG937590	HG937566
47	NDV/Chicken/Uganda/MU113/2011	NDV/C/UG/MU113/2011KAS	Kasese	Vd	HG937591	–

The table presents 25 poultry isolates for which full F or HN gene or both were previously sequenced and retrieved from the GenBank database using accession numbers indicated (33), 30 isolates from waterfowl for which partial F gene was sequenced targeting the F cleavage site, including [e] Three of waterfowl F genes retrieved from the GenBank database (36), [β] One of the retrieved F gene of the chicken isolate was described 10 years earlier in Uganda (34). NDV, Newcastle disease virus; WF, waterfowl; C, chicken; ND, not done; M, Makerere University; UG, Uganda.

for Diagnostic Tests and Vaccines (37). Hemagglutinin inhibition tests, specific RT-PCR test, RNA extraction, cDNA synthesis, and sequencing were made on the harvested allantoic fluid (AF) accordingly. The AF, harvested 3 days post-inoculation was tested for hemagglutination (HA) using 1% chicken erythrocytes and ND virus confirmed by hemagglutination inhibition (HI) with an in-house rabbit-generated polyclonal anti-*AAvV-1* sera as described (37). The HI-positive samples were confirmed further by RNA extraction and cDNA synthesis by Qiagen one-step RT-PCR that extracts and amplifies the F and HN genes according to the conditions described by Byarugaba et al. (33). The amplified cDNAs of the NDV-positive extracts were separated, purified on 1% Agarose gel then visualized and documented in a Bio-Rad Gel Doc XR imager. Sequencing was done by Sanger sequencing method on a 3130XL Applied Biosystems capillary sequencer at the Plateau de Genomique GeT-Purpan, UDEAR UMR 5165 CNRS/UPS, CHU PURPAN, Toulouse France. The positive samples were also biologically characterized using standard pathogenicity tests (mean death time, intracerebral pathogenicity test, and intravenous pathogenicity test described by 33). *In vivo* evaluation of immunogenicity and protection efficacy on four of these *AAvV-1* isolates was performed previously as described in Omony et al. (35). These waterfowl *AAvV-1* isolates were sequenced for partial F gene targeting the cleavage site and the hypervariable region of the F gene. No sequenced data for the HN gene of these isolates were availed for this study.

Sequence Data and Phylogenetic Analysis

Both nucleotide and amino acid sequences of F protein were individually retrieved from the NCBI GenBank in fasta format, matching those present in viral zone UniProtKB/Swiss-Prot entries using accession numbers. These sequences were edited to equal length and aligned using ClustalW provided in Bioedit software version 7.0.9 (38). Nucleotide similarity calculated using MegAlign Pro (DNASTAR program for life sciences, version 9, Inc., Madison, WI, USA). The waterfowl partial F sequences were aligned separately and converted into protein sequences using Emboss software (EMBL-EBI). Phylogenetic analysis was performed by Bayesian methods in MEGA version X program (39) and or Geneious Prime 2020 with the NJ Kimura 2-parameter method and 1,000 bootstrap replicates. The phylogenetic analysis was done on nucleotides based on the pilot tree proposed by Dimitrov et al. (12) to maintain the tree topology and to ascertain the genotypes of the waterfowl isolates. The same nucleotide sequences were later translated into amino acid sequences in MEGA X program v10.1.8 to compare our waterfowl, poultry isolates, and vaccine (LaSota: AY845400 and I_2: AY935499) strains at amino acid levels.

Generation of Structural Homology Models

To determine the conserved regions, homology models were generated for the translated F and HN proteins by aligning sequences for each gene separately using multiple sequence alignment (MSA) with the aid of ClustalW as implemented in the Bioedit program version 7.0 (38) and or Jalview. The consensus regions for each protein in each group (poultry, waterfowl, and vaccine) were used in a BLAST search against the protein Data Bank (PDB), to identify the available homologs or orthologous of known proteins. Structural homology modeling was also done using tools of SWISS-MODEL modeling server and viewed by deep viewer v4.01 (40). Overall, PDB entries 3MAW and 3T1E were used as templates for the F protein and HN protein, respectively. The experimental 3D structure (3MAW) of F protein from ND virus had identity and similarity scores of LaSota (71.1 and 74.3%), waterfowl (71.6 and 74.3%), and poultry (72.3 and 74.5%), respectively. Selected templates were subjected to chimeras to replace amino acids with *Avulavirus* F and HN protein consensus sequence. Energy minimization was carried out with YASARA force field in YASARA version 18.4.24, to obtain the most stable local minimum protein conformation.

Later on, correctness of models was established using PROSTAT (module in homology) and PROCHECK (41), and the candidate epitopes were analyzed by different predication tools for structurally conserved regions (SCRs), loops, and accuracy.

B-Cell Epitopes, MHC Class I and MHC Class II Binding Predications

A portion of the immunogenic F or HN that interacts with B-lymphocyte was assess by hydrophilic and accessible propensity scale methods and hidden Markov model programmed software from Immune Epitope Database IEDB analysis resource (http:www.iedb.org/). For linear B-cell epitopes, BepiPred was used with default threshold value of 0.4. Surface accessibility was predicted by Emini surface accessibility tool of IEDB while the antigenicity sites by the Kolaskar and Tongaonker antigenicity method with a default threshold of 1.042 (42). Peptide binding to the MHC class I and MHC class II molecules were assessed by the IEDB tools at http://tools.iedb.org/mhci/ and http://tools.iedb/mhcii/ respectively with comparison with HLA alleles by artificial neural network (ANN), stabilized matrix network (SMN), or NetMHCII pan in addition to consensus method from combination libraries. Epitope length set as 9 mers of conserved epitopes that bind to HLA at a score $\leq$1.0 percentile rank were selected.

Mapping of Mutations, Variable Positions, and Specific Functional Regions

Following MSA, sequence variation was mapped onto the protein structures and entropy calculations with the aid of Scop3D, a tool, which visualizes variations across multiple sequences on the protein structures (43). F protein and HN protein numbering was based on LaSota using GenBank accession numbers AY845400 and P35743, respectively. The functional regions were defined based on literature. All functional regions were mapped on to the structures and Jalview or Chimera-analyzed models for diversity as visualized to the predicted structure models. Also using aligned sequences, single nucleotide polymorphisms (SNPs) were detected by trimming all gene reads and assembled to LaSota (AY845400) using Geneious assembler in Geneious Prime 2020 program (version11.0.4). During the analysis, phylogenetic tree of isolates based on selected functional regions were considered using Jalview.

Determination of F or HN Protein Recombination Among Isolates

F and HN nucleotide sequences were separately aligned using MegaAlign software (DNAStar, 5.01) and analyzed for recombination using the split decomposition method in seven local statistical recombination tools (RDP4, GeneConv, BootScan/RecScan, MaxChi, Chimera, LARD) integrated in the RDP4 program version 4 (44). These tools were used to estimate any recombination event, recombination hotspot, recombination rate plots, etc., and to detect any putative recombination breakpoint. These methods were applied using the following parameters: window size = 20, highest acceptable p-value < 0.001, and Bonferroni correction. For reliable results, any four putative recombination events were detected and translated into corresponding amino acid sequences.

Availability of Data and Materials

F and HN sequences of the isolates analyzed in this study were all retrieved from the GenBank database with indicated accession numbers including three waterfowl isolates reported by Wanyana et al. (36) with accession numbers LT549451–53. These included NDV/WF/UG/MU138/2011 representing the 22 isolates with identical sequences, NDV/WF/UG/MU150/2011 and NDV/WF/UG/MU186/2011 provided in **Table 1**. Others were the 27 F gene waterfowl isolates characterized for fusion cleavage sites and were not deposited in the GenBank database.

Animal Ethics

The College of Veterinary Medicine Animal Resources and Biosecurity Higher Degrees Research Committee and the Uganda National Council of Science and Technology (approval #HS 776) approved this study.

RESULTS

Phylogenetic Analyses Based on the Fusion Gene of *AAvV-1s*

The phylogenetic analyzes was performed on the full F gene (1,659 nucleotides) encoding for full F protein (553-amino acids), including the hypervariable and cleavage site regions of partial F gene (603 nucleotides) of isolates from waterfowl, encoding for a 201-amino acid protein. In order to compare the genetic relatedness of NDV isolates from waterfowl and poultry used in this study to the vaccine strains and other NDV genotypes, the phylogenetic analysis was done by comparing our isolates with the already known F sequences of class II genotype. The analyses inferred two subclusters of *AAvV-1* isolates from poultry and waterfowl. These virus isolates clustered differently from the commonly used vaccine strains and historic strains available in the GenBank database. Notably, the phylogenetic tree clustered *AAvV-1* isolates from waterfowl (n = 29) differently from genotype II strains, which had been historically described as genotype II. This was in exception of the virulent strain NDV/WF/MU0118/2011 isolated from Queen Elizabeth (QE) park, which clustered together with isolates from poultry (n = 25). Overall, the *AAvV-1* isolates from waterfowl clustered differently from the isolates derived from poultry, which were recently subtyped in genotype Vd as shown in **Figure 1**.

Predicted F Protein 3D Structure, Functional and Antigenic Domains

The complete translated fusion protein amino acid sequences from poultry isolates spanning cDNA coding sequence (CDS) of 553 amino acids and the partial fusion protein from waterfowl virus isolates of 182 amino acids were used to predict 3D fusion structure and to assess the functional and antigenic domains relative to LaSota vaccine strain. Comparing these sequences at hypervariable region, proteolytic cleavage site (F_0), N-terminus of the fusion protein (F2), cleavage motif, transmembrane regions, and amino acid involved in neutralization sites yielded

FIGURE 1 | Molecular phylogenetic analysis based on the nucleotide sequences of the F gene of ND viruses of class II by neighbor-joining method. The inferred ancestral F gene sequences of the Ugandan *AAvV-1* isolates from waterfowl, poultry, and known vaccine strains based on the Tamura-Nei model using the bootstrap consensus of 1,000 replicates. *AAvV-1*

(Continued)

FIGURE 1 | isolates from waterfowl clustered differently from those of the poultry already assigned to genotype V except for one virulent waterfowl isolate (NDV/WF/MU0118/2011). The isolates from waterfowl and poultry (highlighted) did not cluster together with any of the vaccine genotypes.

differences between wild isolates and the vaccine strain. The hypervariable regions of the fusion peptide had the expected three-heptad repeat regions HRa, HRb, and HRc spanning at positions 143–185, 268–299, and 471–500, respectively. Eight variable transmembrane domains were observed between isolates from poultry and LaSota strain at amino acid positions; 14–27, 15–25, 118–131, 120–128, 266–269, 429–432, 499–525, and 501–523. Notable were substitutions in the cleavage site ($n = 3$), fusion peptide ($n = 7$), and the heptad repeat regions ($n = 22$) compared with LaSota vaccine. The change of amino acid from glycine (G) to a basic amino arginine (R) or lysine (K) at specific positions of 112 and 115, respectively, together with presence of phenylalanine (F) at position 117 affects the fusogenic activity of the F protein. This change in all the poultry isolates stabilizes the F protein allowing the host-cell protease to recognize and cleave F_0 necessary for virulence in cells. These details are provided in **Table 2**. Deduced amino acid sequences of the F gene cleavage site (F_0) in all domestic poultry isolates contained multiple basic amino acid residues. The conserved amino acid residues ^{112}R-R-Q-K-R^{116}, with phenylalanine at position 117 between C-terminus of F_2 and N-terminus of F_1 fragment of this gene confirmed the poultry isolates to belong to a virulent group of viruses. All the waterfowl *AAvV-1* isolates had the conserved amino acid residues ^{112}G-R-Q-G-R'L^{117} at the cleavage site with the exception of one NDV118/WF/UG/2011QE. This was a characteristic of the avirulent strains like the vaccines strain currently in use in the country.

In both poultry and waterfowl F proteins, the six potential glycosylation sites were conserved. These sites had the motif asparagine (N)-X-serine (S)/threonine (T) where X is any amino acid except of proline (P) and aspartate (A). These six asparagine-linked glycosylation sites were at 85NRT, 191NNT, 366NTS, 447NIS, 471NNS, and 541NNT. Although the 12-cysteine residues reported at 25, 27, 76, 199, 338, 347, 362, 370, 394, 399, 401, and 424 were conserved (**Figure 2A**), some of them were not available for the partial F protein analyses in waterfowl virus isolates. Compared with the representative vaccines used in Uganda, the CDS of poultry and waterfowl NDV isolates, had a number of mutations at the fusion peptide (117–142), hydrophilic region A (143–185), hydrophilic site B (268–299), hydrophilic region C (471–500), and transmembrane domains of F protein (**Table 3**). The substitution especially at the I508A, I509V, V513L, I516V, L517T, and I520V in the transmembrane site suggests limited conservation at this site in the F gene.

At structural level, by mapping sequence variation in poultry isolates onto the homology model PDB-entry 3MAW built on, F protein which is the F_0 protein for *Avulavirus serotype−1*, > 80% sequence identity was observed. The predicted 3D structure of poultry *AAvV-1* isolates was similar to that of reference LaSota (RSMD, Cα = 3.203). To map these variations onto the PDB

TABLE 2 | Analysis of mutations in the F protein structure between LaSota vaccine and poultry/waterfowl isolates mapped on the 3MAW-Chain A model at hypervariable regions, fusion protein, cleavage site, neutralizing epitopes, and glycosylation domains.

Fusion protein structure domain	**Variable positions in fusion protein structure**																			
	Hypervariable regions/signal peptide (1–31) and predicted antigenic region (7–30)																		**ANT**	
AA positions	4	8	9	11	13	14	16	17	18	19	20	21	22	26	28	29	30	32	44	69
3MAW_A chain	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**V**	M
Chicken structure	K	R	I	A	**L**	**T**	**I**	**T**	**Q**	**I**	**T**	**L**	**T**	**V**	**M**	**T**	S	L	**I**	M
LaSota (AY845400)	R	K	N	A	**M**	**M**	**T**	**I**	**R**	**V**	**A**	**L**	**V**	**I**	**P**	**A**	N	I	**V**	L
Waterfowl structure	K	R	I	V	**L**	**T**	**I**	**T**	**Q**	**I**	**T**	**V**	**T**	**V**	**M**	**T**	S	L	**I**	L
Domain	**ANT**		**Cleavage site (112–116)**		**Fusion protein (117–142)**					**HRa (143–185)**						**Antigenic region (196–241)**		**HRb (268–299)**		
AA positions	82	106	112	115	117	118	121	124	135	145	146	159	190	192	195	203	231	232	269	286
3MAW_A chain	E	–	–	–	–	–	–	–	–	N	Q	T	F	N	Q	T	T	**Q**	**I**	**Q**
Chicken structure	E	A	R	K	**F**	**V**	**V**	**S**	V	N	K	A	F	N	R	T	T	**Q**	**V**	**Q**
LaSota (AY845400)	D	V	G	G	**L**	**I**	**I**	**G**	I	K	Q	A	L	K	Q	A	N	**K**	**I**	**R**
Waterfowl structure.	E	V	G	G	**L**	**I**	**I**	**G**	I	K	Q	A	–	–	–	–	–	**–**	**–**	–
Domain	**HRb (268–299)**			**Antigenic region (315–332)**		**Antigenic region (380–394)**				**Antigenic region (413–437)**					**Antigenic region (447–460)**		**HRc (471–500)**			
AA positions	288	292	312	330	341	364	385	386	402	403	421	422	430	442	451	453	457	476	479	482
3MAW_A chain	**T**	V	K	**I**	T	N	**T**	L	A	D	R	H	D	A	**L**	**S**	**V**	N	D	E
Chicken structure	**N**	I	K	**L**	S	S	**A**	L	A	D	K	H	D	V	**L**	**T**	**V**	S	D	A
LaSota (AY845400)	**T**	V	R	**I**	T	S	**T**	I	V	N	K	Q	G	V	**Q**	**S**	**I**	N	N	E
Waterfowl structure	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Domains	**HRc (471–500)**			**Major transmembrane domain (501–521)**																
AA positions	486	489	494	508	509	513	516	520	548	550	552	553								
3MAW_A chain	S	D	–	–	–	–	–	–	–	–	–	–								
Chicken structure	N	N	**N**	**A**	**V**	**L**	T	**V**	K	A	R	T								
LaSota (AY845400)	R	D	**K**	**T**	**I**	**V**	L	**I**	R	T	K	M								
Waterfowl structure	–	–	–	–	–	–	–	–	–	–	–	–								
Domains	**Potential B-cell epitopes[T] (157–171)**																			
AA positions	157	158	160	161	162	164	165	167	168	196	170	171								
3MAW_A chain	S	I	A	T	N	A	V	E	V	T	D	G								
Chicken structure	S	I	A	T	N	A	V	E	V	T	D	G								
LaSota (AY845400)	S	I	A	T	N	A	V	E	V	T	D	G								
Waterfowl structure	N	M	P	P	K	P	G	K	G	I/P	N	E								

Variable positions found in the fusion protein mutated in poultry and waterfowl compared with LaSota vaccine virus strain—AY845400 used as a reference. Bold positions are surface-exposed residues in the variable situation predicted by discrimination of protein secondary structure class. Underlined positions contribute to the hydrophobic stability of the protein. ANT, antigenic region. [T]All these mutations were in only two waterfowl isolates but conserved in other waterfowl and in all poultry isolates.

TABLE 3 | Comparison of amino acid substitutions within the functional domains of fusion protein sequences of Ugandan *AAvV-1* and the historical vaccine strains.

Strains	Fusion peptide 117–141					HRa 143–185		HRb 268–299			HRc 471–500					Transmembrane domain (TM) 500–521								
Amino acid positions	117	118	121	124	135	139	145	146	286	288	476	479	482	486	489	494	508	509	513	514	516	517	520
All genotypes (Cons.)	F/L	I	I	G	I	A	N	Q	Q	N	N	D	A	S	D	K	T	V	V	F	I	L	V
NDV strain LaSota	L	–	–	–	–	–	K	–	R	T	–	N	E	R	–	–	–	I	–	–	–	–	I
B1 vaccine strain	L	–	–	–	–	–	K	–	–	T	–	N	E	R	–	–	–	I	–	–	–	–	I
F-strain	L	–	–	–	–	–	K	–	–	T	–	N	E	–	–	–	–	I	–	–	–	–	–
NDV strain I2	L	–	–	–	–	S	–	–	–	T	–	–	E	–	–	–	–	–	I	C	–	–	–
V4	L	–	–	–	–	S	–	–	–	T	–	–	E	–	–	–	–	–	–	C	–	–	–
Mukteswar	L	–	–	S	–	S	–	–	–	T	–	–	E	–	–	R	–	–	–	L	M	–	–
All waterfowl isolates (Cons.)	L	–	–	–	–	–	K	–															
All chicken isolates (Cons.)	F	V/I	V	S	V	–	–	K	–	–	S	–	–	N	N	N	A	–	L	–	V	T	–

No change in amino acid compared with the CONSENSUS (–); comparison not done ().

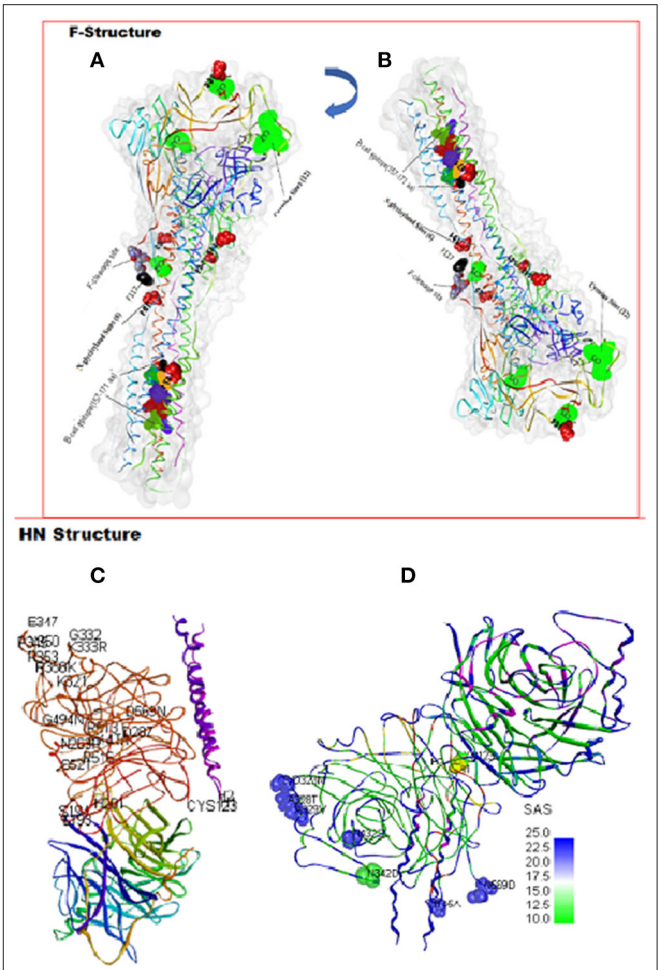

FIGURE 2 | Amino acid substitutions in the predicted F and HN proteins. A PDB-3maw model for predicting variations on LaSota vaccine strain on chain A represented by a ribbon. The *N*-glycosylation, cysteine, cleavage site, and variable B-cell epitopes are labeled. The structures **(A)** and **(B)** are different orientations of the same F protein molecule rotated as shown. A PDB-3T1E chain A model HN protein ectodomain. **(C)** One dimer of the HN neuraminidase (NA) domain flanks the 2HB in the stalk side-on view packing, conserved amino acids, and substitutions positions at the antigenic sites on the A-chain are indicated. **(D)** Side-on view packing of HN dimer rotated showing the Cys-123 (yellow), Helices 1 and 2 (H_1 and H_2), and eight substituted amino acids (blue/green) in the antigenic sites of the A-chain displayed by solvent accessibility surface (SAS).

model and perform entropy calculations, we used Scop3D and Chimeras for sequence manipulations and final visualization was done in Discovery Studio Visualize v20.1.0. We confirmed that the seven residues involved in virus neutralization from a stretch of 157–171 and individual residues D72, E74, A75, K78, A79, and L343 were conserved across all poultry virus isolates compared to LaSota. Two *AAvV-1* isolates from waterfowl; NDV/WF/MU131/LUT and NDV/WF/MU159/MUS showed 13 amino acids variability at a stretch of 157–171 site with a shift from more hydrophobic to hydrophilic amino acid residues seen

in LaSota/poultry and to the two waterfowl isolates respectively (**Table 3**; **Figures 2A,B**).

We predicted linear B-cell epitopes and their surface accessibility and antigenicity scores using the BepiPred linear Epitope prediction method, Emini surface accessibility tool, and Kolaskar and Tongaonkar antigenicity, which use epitope scores and immunogenicity predictions through IEDB online (www.iedb.org) facilities. Both BepiPred-2.0 prediction tool and Vaxijen 2.0 tool gave 13 effective B-cell epitopes for LaSota vaccine and 11 for chicken isolates. Two effective epitopes at position 271–293 and 451–458 were undetected in chicken. Overall, these gave seven nonoverlapping sites above antigenicity score (0.8) and surface accessibility score (0.6); $_{33}$DGR$_{35}$, $_{164}$AVHE$_{167}$, $_{222}$GPQITSP$_{228}$, $_{223}$PQITSP$_{228}$, $_{407}$IISQNYG$_{413}$, $_{407}$IISQN$_{411}$, and $_{483}$ESN$_{485}$. All these were conserved in all the vaccine strain, poultry and waterfowl ND virus isolates except for the two waterfowl isolates NDV/WF/MU159/MUS and NDV/WF/MU131/LUT having substitution at amino acid 164–167. However, structure-based antibody prediction based on DiscoTope of the F protein consensus in poultry isolates showed four B-cell epitope locations (**Table 4**). Linear epitopes of F consensus for poultry and waterfowl isolates at (1–182 amino acid) indicated 16 substituted positions; L32I, M68L, E82D, R112G, K115G, S/D124G, V135I, N145K, K146Q, L451Q, T453S, V457I, D479N, A482E, N486R, and N489D, suggesting that these surface-exposed amino acid residues do modify the effectiveness of the predicted epitopes thus are speculated for the antigenic differences between these wild viruses and vaccine group.

HN Protein Structural and Functional Domains and Predicted 3D Structure

Analysis of the complete nucleotide and the corresponding protein sequences of HN from our domestic poultry *AAvV-1s* isolates, retrieved from the GenBank database was done to identify any structural differences between them to that of the available vaccine. The ClustalW MSA of the HN protein showed 93.5% identity and 97.0% similarity among the 32 domestic poultry ND viral isolate sequences. Comparing these 32 entries, all were identical except for amino acid changes at 36 positions from majority to minority shown as R3C, V9A, I27V, I34V, M35V, L37F, T48R/A, A57S, S58G, A62V, T66A, V70I, D79E, D82N, I102V, S121G, V136I, S146N, S181C, S269P, D309E, N323N, R333K, N341K, V352A, G362R, V392I, A424V, H432Y, N440S, T443I, V477I, H482R, N494D, I514T, and I548L. On sequence analysis, 77 amino acid positions showed variations between LaSota vaccine (AY845400) and the HN chicken consensus at 50% protein level. Importantly, we first focused our analysis on the structural positions of the extracellular domain consisting of surface-exposed amino acid residues since more exposed residues on the surface of the molecule influences the overall charge and antigenicity than the ambivalent or internal amino acid. Twenty-six amino acid positions were varying with LaSota vaccine observed in this category, namely, Q7R, D13E, R62A, N63I, R65K, E68D, K69R, D79E, K98S, N120T, Y203H, N263R, R269S, G293K, S310D, D342N, R356K, V387E, S432H, S440N, H474Y, Y479H, G494D, V495E, D569N, and G570K. Importantly, five of these E68D, K69R, K356R, D569N, and G570K were noted to lie within antipeptides previously used to produce monoclonal antibodies for differentiating birds infected by vaccine isolates and virulent (Exotic Newcastle disease) strains.

Expectedly, the HN protein for the domestic poultry ND virus isolates had the predicted stalk and globular head regions of the HN protein, comprising 1–143 and 125–571 amino acids, respectively. Three transmembrane domains were obtained at positions 24–47, 25–45, and 557–563. Predicted amino acid residues had the 12 cysteine (C) positions conserved at 172, 186, 196, 238, 247, 251, 455, 461, 465, 531, and 542. In addition, the reported cysteine at position 123 in certain known isolates was present and not replaced by tryptophan (W) like in some vaccine strains. The key biologically active residues for receptor (sialic acid) binding site were conserved at positions R174, E401, R416, and Y526; the hydrophobic core of the stalk 4HB was conserved at positions Y85, V88, S92, L96, T99, I103, I107, L110, and I114 together with the stalk residues at R83, A89, L90, L94, and L97 involved in direct interaction with F protein.

Organizing these variations and others into functional domains like cytosolic, transmembrane, and flanking regions are shown in **Table 5**. However, assembling the HN nucleotide sequences on the CDS of LaSota vaccine (AY845400), all the 32 reads predicted only four polymorphic amino acid changes that had effect on the HN protein shown as V35M (75.8%), M48V (36.4%), A66T (81.8%), and S440N (48.5%) with codon changes G103A, A142G, G196A, and G1319A, respectively. Importantly, 28 charge position variations observed were due to substitutions between the poultry isolates and LaSota strain sequences from the model (PDB-3T1E). Positions 42A→S and 43S→I are located in the transmembrane domain affecting the charge and physiochemical properties of HN protein in poultry isolates. 84I→V and 98K→S lie proximately at the hydrophobic and hydrophilic sites predicted at MHC I alleles and linked to known T-cell epitope affecting the charge of the peptide. Comparison of the HN antigen-exposed surfaces and the predicted potential B-cell epitopes of consensus sequence of isolates from poultry and LaSota strain using the BepiPred-2.0 prediction tool on IEDB server, 25 epitopes were discovered. Analysis of their antigenicity with Vaxijen 2.0 tool and accessibility with Emini Surface Accessibility Prediction tool showed 15 out of 25 effective epitopes. Six (positions: 555–566, 108–115, 526–537, 236–254, 370–381, and 147–166) out of 15 effective epitopes were similar to those in LaSota vaccine strain. The other

TABLE 4 | DiscoTope structure-based B-cell antibody prediction of chicken F protein consensus.

Rank	Location	B-cell epitopes	Score[a]	Immunogenicity
1	1–12	MGSKPSTRIPVP	0.700	
2	105–115	SVTTSGGGRQG	1.000	
3	476–485	SALDKLAESN	1.000	
4	511–520	SLLFGVTSLV	0.808	

[a] *Score given at a default threshold (–3.7) of DiscoTope v 2.0.*

TABLE 5 | Variation of amino acid in the HN antigenic, transmembrane, and its flanking regions.

Site	Cytoplasmic region AA (1-20)				Transmembrane domain/signal cleavage region (28-40) AA (21-48)									Antigenic site				Flanking region			
Residues	4	7	9	13	29	31	33	34	35	36	38	42	43	45	48	57	58	60	62	63	65
LaSota strain	A	Q	A	D	I	F	T	V	V	T	A	A	S	L	M	V	G	P	R	N	R
B1 vaccine strain	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	I	–
F-strain	–	–	–	–	–	L	–	I	–	–	–	I	–	V	–	–	–	–	–	I	K
I2 strain	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	A	I	–
V4 strain	–	–	–	–	–	L	–	–	–	–	–	–	A	A	–	–	–	–	A	I	–
Komarov strain	–	–	–	–	–	L	–	–	–	–	–	–	–	V	–	–	–	–	R	I	–
Mukteswar strain	–	–	–	–	A	L	M	–	I	–	–	V	A	A	–	–	S	–	A	I	–
Chicken (50% Con.)	V	R	V	E	V	S	I	I	M	I	V	S	I	V	T	A	S	S	A	I	K
Site										Antigenic				Antigenic						Antigenic	
Residues	66	68	69	70	73	120	123	126	127	136	145	156	182	265	266	269	290	293	307	310	315
LaSota strain	A	E	K	I	T	N	W	L	I	I	A	F	A	A	I	R	T	G	F	S	S
B1 vaccine strain	–	–	–	–	–	–	–	P	–	–	–	–	–	–	–	–	–	G	–	–	–
F-strain	–	–	–	–	V	–	–	P	–	–	–	–	–	–	–	P	–	E	–	–	–
I2 strain	–	–	–	–	A	S	C	P	–	–	D	–	–	–	A	S	–	E	–	N	P
V4 strain	–	–	–	–	A	S	C	P	–	–	–	–	–	–	I	S	–	E	–	N	P
Komarov strain	–	–	–	–	A	–	–	P	–	–	–	–	–	–	–	L	–	E	–	–	S
Mukteswar strain	–	–	R	–	A	–	C	P	V	–	T	–	–	V	I	S	–	R	–	N	P
Chicken (50% Con.)	T	D	R	V	L	T	C	P	V	V	I	Y	T	V	A	S	V	K	L	D	P
Site							Ant	Ant				Antigenic site						Antigenic			
	328	329	342	369	387	404	432	440	453	464	466	474	476	477	479	484	495	502	508	540	570
LaSota strain	T	V	D	I	V	I	S	S	I	P	V	H	L	I	Y	L	V	A	S	T	G
B1 vaccine strain	–	–	–	–	–	–	–	–	–	S	–	Y	–	–	–	–	E	A	–	–	–
F-strain	–	L	–	–	–	–	–	–	–	S	–	Y	–	–	–	–	–	A	–	–	–
I2 strain	–	A	–	V	–	V	–	–	–	S	–	Y	–	V	–	–	–	V	G	–	–
V4 strain	–	A	–	V	–	V	–	–	V	S	–	Y	–	V	–	–	K	V	–	–	–
Komarov strain	–	V	–	I	–	I	–	–	I	S	–	Y	–	–	–	–	E	A	–	–	–
Mukteswar strain	–	A	–	V	–	V	N	N	V	S	–	Y	–	V	H	–	K	V	N	–	–
Chicken (50% Con.)	A	A	N	V	E	V	H	N/S	V	S	I	Y	I	V	H	V	E	V	Y	V	K

The underline amino acid positions alter markedly the charge of the HN protein of the 32 poultry isolates analyzed. Variations at Cysteine-123 and N-glycosylation-508 positions are high-lightened.

nine epitopes (positions: 539–547, 452–481, 283–291, 493–508, 263–269, 183–213, 404–430, 437–445, and one transmembrane motif 23–48) were dissimilar in structure and epitope threshold effectiveness due to mutations at positions 203Y→H, 263N→R, 265A→V, 266V→A, 269R→S, 290T→V, 356R→K, 352I→V/A, 404I→V, 440S→N, 443T→I, 453I→V, 464P→S, 466V→I, 476L→I, 477I→V, 494G→N/D, 495V→E, 502A→V, 508S→Y, and 569D→N which all lie at the predicted conformational B-cell epitope in the 3D structure (**Figures 2C,D**). In addition, the linear ^{343}TCPDEQDYQIRMA^{355} and ^{493}DGV^{495} epitopes determining the monoclonal antibody reactivity ability of HN protein of NDVs showed mutations. Majorly the above substitutions altering the nine epitopes could be implicated in the vaccination failures while using LaSota vaccine.

At structural level, the homology model of the *AAvV-1* HN protein was based on the Australia-Victoria/32 strain (PDB-3T1E chain A). Our homology model using chicken isolates consensus HN protein was similar to 3D experimental PDB model (root mean square deviations (RMSD) of Cα = 1.2A). Several positions of amino acids in the HN protein were variable between the mapped 3D LaSota and the consensus chicken isolate HN protein structures (**Figures 2C,D**). Structurally, NDV HN protein is a multifunctional glycoprotein playing a key role in viral infectivity. The amino acid residues R174, I175, D198, K236, E258, Y299, Y317, E401, R416, R498, R516, Y526, and E547 essential for receptor recognition, were conserved across all the 32 ND virus isolates from poultry in this study, suggesting they infect the similar hosts (ducks and chicken) from where they were isolated.

The original five *N*-glycosylation sites in the HN protein of the isolates from poultry were all conserved between themselves at positions 119NTS, 341NNT, 433NKT, 481NHT, and 538NKV but with differences in the patterns of amino acids at three positions 119NSS, 341NDT, and 538NKT when compared with LaSota and I_2 vaccine strains commonly used in Uganda. *N*-Glycosylation reported at amino acid position 508 was absent in all our isolates from poultry but replaced by 508N→Y. Comparisons of the HN protein total length of vaccine strain with wild isolates revealed several point mutations at transmembrane domains including the hydrophobic (HRa) and hydrophilic (HRb) sites and in the six continuum of antigenic sites 2, 3, 4, 12, 14, and 23 that form 3D conformation of the HN molecule (**Table 6**).

TABLE 6 | Variations in structurally and functionally important residues of the HN protein in poultry and vaccine strains mapped on the 3T1E-chain A model structure.

Virus	Hydrophobic/hydrophilic sites							Antigenic neutralizing epitopes residues																															
	HRa					HRb		1			2						3					4					12					14					23				
	(74–88)					(96–110)					(513–569)						(363–321)					(332–356)					(494–516)					(347–353)					(193–302)				
	75	81	84	98	101		102	345			513	514	521	569			263	287	321			332	333	356			494	516	347			350	352	353			193	194	201			203
Lasota	G	V	I	K	T		T	P			R	I	S	D			N	D	K			G	K	R			G	R	E			Y	I	R			L	S	H			Y
B1	–	–	–	N	–		–	–			–	–	–	–			–	–	–			–	–	–			–	–	–			–		–			–	–	–			–
F-strain	–	–	–	S	–		–	–			–	–	–	–			–	–	–			–	–	–			–	–	–			–		–			–	–	–			H
I2	–	–	–	N	S		–	–			–	–	–	–			–	–	R			–	–	–			D	–	–			–		–			–	–	–			H
V4	–	–	–	N	S		–	–			–	–	–	–			–	–	–			–	–	–			D	–	–			–		Q			–	–	–			H
Komarov	–	–	–	N	–		–	–			–	–	–	–			–	–	–			E	–	–			G	–	–			–		–			S	–	–			
Mukteswar	–	–	–	N	S		I	–			–	–	–	G			K	–	–			–	R	–			D	–	–			–		–			–	–	–			–
Chicken	S	I	V	S	S		I	–			–	T/I	–	N			R	–	–			–	R	K			N/D	–	–			–		V			–	–	–			H

The F and HN Protein Recombination Among Isolates

Using all recombination detection methods in RPD4, there was no recombination detected while analyzing the HN gene of both isolates from waterfowl and poultry compared within, among themselves, or with vaccine strain used in Uganda. However, using F gene of individual groups or combined in the presence of LaSota, I_2 and I_2 progenitor as reference vaccines, there were two breakpoints that signaled the recombination events which were attributable to an evolutionary process in isolates of waterfowl using the stringent criteria of any four recombination detection methods. The seven statistical methods used in the RPD4 were automated RPD(R), GeneConv (G), Bootscan (B),

MaxChi(M), Chimaera (M), SiScan (S), and 3SEQ (T). Several potential recombination points were detected; two of these in the isolates from waterfowl and one isolate from poultry are shown in **Table 7**.

Using only GeneConv to search for the possibility of significant recombination, which uses the homologous segments, we detected few recombination breakpoints in the isolates of poultry. By using less-stringent criteria of two recombination detection methods, we found 25 statistically significant recombinant fragments in isolates from poultry, observed at lengths 11–437 nt and 439–1,659 derived from major parent (LaSota: 93.9% similarity) and 12–438 nt regions derived from minor parent (NDV/C/UG/MU032/2011IGA: 100% similarity) in the fusion gene producing a recombinant strain NDV/C/UG/MU074/2011KOB. At the same time, 30 recombinant fragments were observed in isolates from waterfowl at lengths 10–438 to 438–546 nt and 439–1,654 nt derived from minor parent (LaSota: 99.2% similarity) and major parent (NDV/WF/MU159/MUS: 100% similarity) respectively producing a recombinant strain NDV/WF/MU131/LUT. However, only two most important breakpoint events compatible with recombination with a beginning breakpoint at nucleotide positions 316 to 436 nt and 317 to 435 nt using the five recombination detection methods (**Figure 3A**: A–E) were identified in the F gene of waterfowl NDV isolate NDV/WF/MU118/QE isolated from a conservation area (Queen Elizabeth National park). Both FPCS and intracerebral pathogenicity indices showed the daughter virus to be virulent. They also indicated that it appeared to have arisen from a recombination between a non-virulent NDV/WF/MU159/MUS and LaSota vaccine with statistically significant probabilities ($p < 0.01$). The lines in open rectangles seen in the GeneConv plots (**Figure 3A**: B) connect the probable beginnings and ends of the fragments that underwent recombination.

Recombination signals were also detected between I_2 and I_2 progenitor vaccines and the NDV isolates from waterfowl at varying lengths along the fusion gene. GeneConv in the RDP sorted and scored all the significant fragments in the alignment and listed them in decreasing Bonferroni-corrected *p*-values. Testing this assortment of sequences provided a maximum parsimony pedigree suggestive of recombination. However, the exact point of fragmentation together with the relationships of the ND viruses waterfowl isolates involved can be identified by the tree-like network. The *p*-values produced guided in the identification of the fragments and locations of recombination as shown in **Figure 3B**.

DISCUSSION

The economic impact of ND on both the backyard and commercial poultry industry in Uganda and elsewhere is significant. The annual disease resurgence, vaccine failures, and potentially virulent strains circulating in apparently healthy birds become an issue. The concerns of viral evolution, type of vaccine used to protect flocks, and post-vaccine evaluation are always raised. This study evaluated the predicted B-cell epitopes and functional domains of F and HN proteins in *AAvV-1* isolated from Ugandan aquatic birds and poultry and compared them with those of the vaccines to determine variations between the two groups that might explain ND vaccine failures. To date, *AAvV-1* vaccine evaluation is based on empirical crossprotection of birds. Many of such studies indicate an increasing evidence that antigenically matched ND vaccines provide better immunity (45, 46). However, such studies are costly and laborious and require extended field studies. Notwithstanding other factors that might lead to vaccine failures, insufficient cold-chain maintenance, insufficient immunity titer, hygiene status, etc. have been of great concern. Here, we focused on *in silico* study of the neutralizing epitopes of HN and F-glycoproteins of endemic wild ND viruses and vaccine (genotype II) to ascertain the presence of virus variants and any recombinants at these two antigenic and surface glycoproteins in our *AAvV-1* isolates that could result into inefficient vaccination.

In Uganda, vaccination using live avirulent LaSota and recently introduced I_2 strain, belonging to genotypes II and I, respectively, of class II offers the best ND control. Phylogenetic analysis of *AAvV-1s* reported in the two previous independent studies showed that the partial and full F gene sequences of *AAvV*-1 isolates from waterfowl (36) and poultry (33) clustered Ugandan viruses with those of genotypes II and Vd viruses, respectively. This is consistent with previous reports of a predominance of genotypes II and V viruses in wild and domestic birds. However, based on the current unified system of classification described by Dimitrov et al. (12), the subgenotype Vd becomes V due to lack of the branch support. However, phylogenetic analysis of the same viruses combined in the current study showed two subclusters; one cluster of genotype V where poultry belonged and other (unassigned) where waterfowl isolates belonged. No virus isolates from waterfowl clustered with genotype II as previously reported except that one virulent (NDV/WF/MU118/2011QE) strain clustered with poultry isolates (genotype V). Although knowledge of circulating genotypes established through phylogenetic analyses is important in epidemiology or evolutionary changes of NDV, little about the antigenic diversity at the B-cell epitopes is gained from these tree analyses, suggesting that selection of neutralizing variants arising from mutations or fragments of gene recombination implicated in vaccine failures have to be identified. Besides, the proper genotype to where the *AAvV-1* isolates from waterfowl are placed in class II viruses warrants investigation.

The importance of antibodies directed toward both F and HN epitopes that strongly neutralizes ND infectivity is important for understanding the diversity of these genes in wild virus isolates and vaccine strains. In this study, we demonstrated amino acid substitutions in the entire length of F and HN proteins in *AAvV-1* isolates from waterfowl and poultry as compared with vaccine strains used in Uganda. Analysis of the hypervariable site of F protein revealed two groups, one with multiple basic acid residues at the proteolytic cleavage site $_{112}$RRQKR$_{116}$ F$_{117}$ at the N-terminus of F2 and F1, respectively, where viruses from poultry belonged. The other group had $_{112}$GRQGR$_{116}$L$_{117}$ where those from waterfowl and vaccine strains belonged. This confirms the incidences of *AAvV-1s* in susceptible domestic and

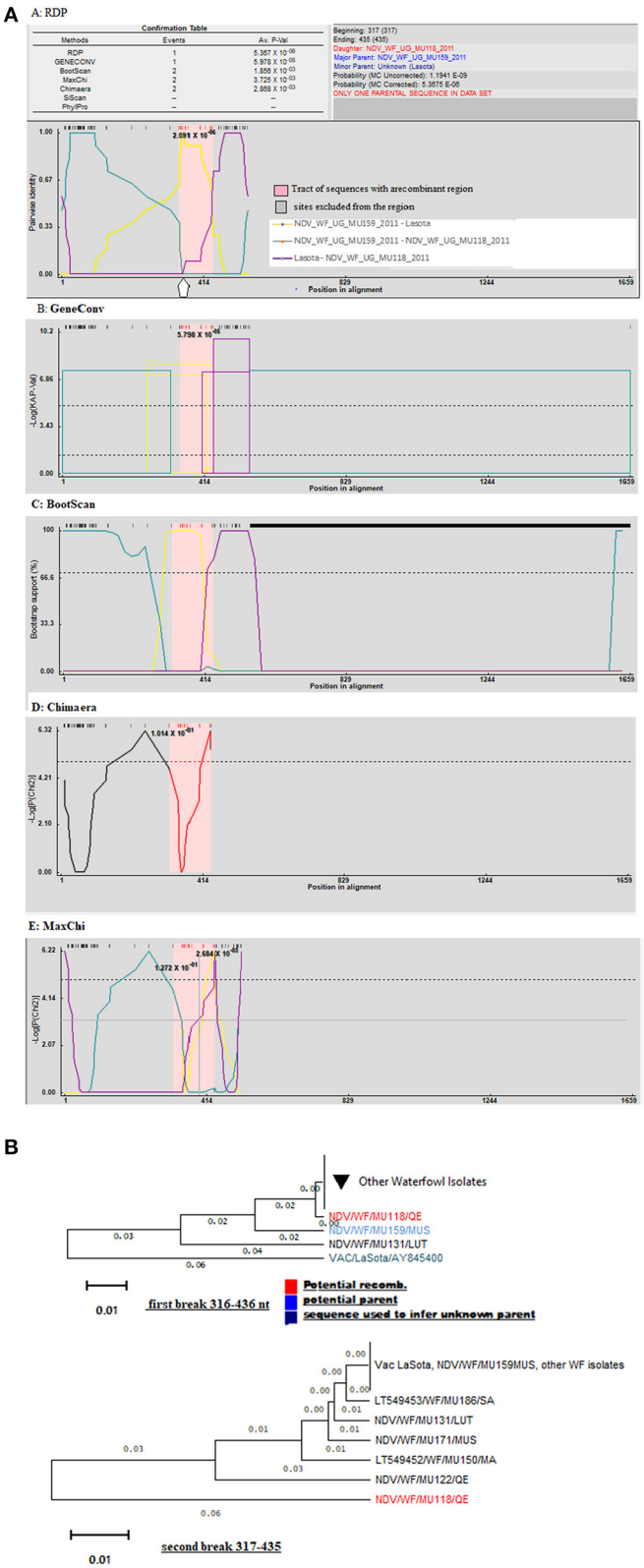

FIGURE 3 | Graphic representation of recombination in fusion gene of *Avian Avulaviruses*. **(A)** Lines forming an open rectangle connects the beginning and the end of a fragment that underwent recombination. The height of the open rectangle is proportional to –log10 of the probability. **(B)** Phylogenetic trees for different regions of F gene showing the movement of recombinant fragments (red colored) in the NDV isolates from waterfowl. Trees were generated with the RDP program (version 4) by using UPGMA analysis of region derived from both major and minor parents. Bootstrap values (percentage from 1,000 replicates) were shown for the relevant nodes.

TABLE 7 | Fusion gene recombination confirmation by split decomposition method using local statistical methods.

Detection method	**Poultry viral isolate pairs**		
	Recombinant NDV/C/UG/MU074/2011KOB	**Major parent LaSota**	**Minor parent NDV/WF/UG/MU032/2011IGA**
	Sequence detected in		**Av. *p*-value**
RDP	1		3.537×10^{-39}
GENECONV	25		9.107×10^{-1}
BOOTSCAN	1		0.100
MAXCHI	5		6.309×10^{-1}
CHIMAERA	5		–
SISCAN	–		–
3SCAQ	1		7.771×10^{-15}
Detection method	**Waterfowl viral isolate pairs**		
	Recombinant NDV/WF/UG/MU131/2011LUT	**Major parent LaSota**	**Minor parent NDV/WF/UG/MU159/2011MUS**
	Sequences detected in		**Av. *p*-values**
RDP	1		3.282×10^{-2}
GENECONV	4		1.850×10^{-6}
BOOTSCAN	1		6.872×10^{-7}
MAXCHI	3		3.588×10^{-10}
CHIMAERA	2		2.387×10^{-5}
SISCAN	–		–
3SCAQ	28		3.54×10^{-15}
Detection method	**Recombinant NDV/WF/UG/MU118/2011MUS**	**Minor parent LaSota**	**Minor parent NDV/WF/UG/MU159/2011MUS**
RDP	1		5.367×10^{-6}
GENECONV	1		5.978×10^{-5}
BOOTSCAN	2		1.856×10^{-3}
MAXCHI	2		3.72×10^{-3}
CHIMAERA	2		2.868×10^{-3}
SISCAN	–		–
3SCAQ	–		–

feral bird species and suggestive of the potential role of feral birds in transmitting these viruses to poultry. The reported (RK/RQRR↓F) motif cleavage site among *AAvV-1* isolates within the same genotype from domestic poultry and other wild hosts have been attributed to native selection, host pressure selection, viral evolution, or wide vaccination (14). There was no evidence of variation of pathogenicity among strains in paired samples isolated from cloaca and oropharynx of a single bird (33), and the presence of *AAvV-1s* with different pathogenicity based on the cleavage site motif within one geographical area suggests a long time geographical isolation of these different pathotypes. Waterfowl harboring viruses with lentogenic F-cleavage motif site generally, except for one isolate, and healthy-looking poultry harboring virulent *AAvV-1* strains confirmed by the RRQKR↓F F-cleavage motif site, suggests that the two groups differ in their fusion activity and virulence in their hosts (31). This is of great importance and should be noted by both epidemiologists and vaccinators since variability in amino acid residues at the FPCS of viruses existing in the same geographical location suggests presence of different viral genotypes and pathotypes and potential for vaccine failure (47). The presence of avirulent ND viruses in waterfowl indicates that wild birds may be a potential source of virulent *AAvV-1* for poultry due to possible point mutations at FPCS (7, 48). At the same time, these wild birds might therefore serve as a source of potential vaccine isolates (35).

Conservation of F and HN proteins at *N*-glycosylation, cysteine sites, and other specific structural and functional sites like receptor (sialic acid) binding sites between *AAvV-1* isolates from waterfowl and poultry together with vaccine strains indicate these are required for biological active state especially among amino acids residues involved at the direct interactions that form a high-level structural integrity (49, 50). The substitutions particularly in the surface-exposed amino acid residues in signal peptide (position: 13–29 aa) is not surprising considering that the amino acids in this region undergo constant positive selection making them highly variable. However, the mutations in the fusion peptide (position: 117–142 aa) is least expected except for the two nucleotides necessary for a change from a trypsin-like to a furin-like site. The exceptional changes at the nearby position of this site especially at V118I, V121I, S124G, V134I,

and N145K in isolates from poultry and D124G and A129V in *AAvV*-1 isolates from waterfowl warrant further investigation in view of recombination detected in this region. The hypervariable hydrophobic regions (HRa, HRb, and HRc) especially I269V, T288N, I330L, T385A, Q451L, S453T, I457V, and K494N whose side chains are surface-exposed (51), together with T508A, I509V, V513L, and I520V of the transmembrane region of F protein are implicated in structural variations between the wild and vaccine strains. The 13 observed mutations in the F protein of the two *AAvV-1* isolates from waterfowl at the known antigenic aa position 157–171 account for variation. These favored more of hydrophilic aa residues ($n = 5$) at positions N157S, K162N, K167E, N170D, and E171G than hydrophobic aa residues ($n = 2$) at positions M158I and I169T while other aa were neutral in nature ($n = 6$). Only K167E and E171G of the hydrophilic aa decreased the protein stability by the predictor of effects of single-point protein mutation in I-Mutant Suite. Taken together, increased antigenicity and the neutralizing epitopes of F protein of these two viruses could result into altered fusion activity and neutralizing escape variants (52, 53). The implications of these changes in the critical regions of F and HN genes (neutralizing epitopes) could be serious as they may mean escape of the virus from immunity.

Structurally, the seven and five major epitopes on the F protein and HN proteins, respectively, have been mapped on to experimental 3D structure in the PDB. Our findings located these functional residues and found those of F protein to be more conserved at positions 72, 78, 79, 343, and 157–171 in all virus isolates from poultry and vaccine strains emphasizing the role of mABs elicited by this glycoprotein in the neutralization of NDV (54). However, the 13 substitutions occurring in two virus isolates from waterfowl at a stretch of 157–171 aa between LaSota strain, displaying mutations N157S, K162N, K167E, N170D, and E171G, which are hydrophilic and exposed on the outer periphery of the protein surface had improved antigenicity of these epitope which are necessary for antibody binding and are crucial for the *AAvV-1* isolate antigen diversity. This suggests cocirculation of these novel viruses similar to those identified earlier as vaccine candidates (35). On the other hand, the eight substitutions in the HN protein-neutralizing epitopes together with other functional sites between LaSota strain and *AAvV-1* isolates in this study could explain why these are antigenic variants which fail to be neutralized by antibodies produced in healthy poultry birds vaccinated with LaSota vaccine and sold in the market.

However, our previously reported vaccination results in Omony et al. (35) did not fully prevent ND infection under our experimental conditions where we used four avirulent NDV isolates from waterfowl as vaccine strains and challenged the immunized birds with a virulent NDV isolates from poultry. In any case, excretion of the challenge virus might have occurred. Not ignoring other factors of immune status of the vaccinated flocks, level of hygiene, and cold-chain maintenance to safeguard vaccine doses, protection against ND is dependent on the genotype and immunogenicity of the vaccine virus (35). Therefore, it would be important to develop an effective vaccine that is able to produce and maintain a high antibody level in vaccinated flocks. In our case, the variations in amino acid between the wild isolates and vaccine strains could be implicated in antigenic variants and vaccine failures leading to annual ND incidence.

Evolution of *AAvV-1* isolates has continuously posed threats of emergence of new virulent strains and challenges of diagnosis of ND (55). Many studies have suggested that different strains of *AAvV-1* evolve through various degrees of accumulation of point mutations and gene exchange by recombination (23). However, our analysis did not detect any recombination in the HN gene of wild *AAvV-1* isolates and from the LaSota vaccine strain, but we obtained signals of recombination in the F gene confirming previous findings in the genotype V viruses (29, 30). The virulent *AAvV-1* isolates from poultry possessing the virulent motif ^{112}RRQKRF^{117} which is different from that of LaSota strain ^{112}GRQGRL^{117} can be explained by the acquisition of the F2-terminus of F gene from virulent viruses retaining their avirulent F1-terminus giving a novel clade of genotype (V) of Ugandan ND-viruses. On the other hand, *AAvV-1* isolates from waterfowl maintain in nature their avirulent consensus of F1-terminus. This is again corroborated by studies that have showed that *AAvV-1* isolates from poultry highly select pathogenic derivatives of the virus from nonpathogenic precursors (56). The N-terminal of F gene and the rest of the genes in the *AAvV-1* isolated from waterfowl had high degree of similarity with those of the genotype II (LaSota strain). Therefore, it was more likely that the F2 terminal of F gene in *AAvV-1* isolates from poultry originated from one of the prevalent LaSota (genotype II) strain or isolates from waterfowl. Although it has been noted that the F cleavage site of ancestor viruses, such as the long used live LaSota vaccine strain tends to be conserved, the potential of these viruses mutating to highly virulent strains with passage in chicken is a notable concern. Our current study noted recombination at 316–436 nt region that codes for cleavage site and its surrounding F1-terminus region (106–145 aa) which produced the only virulent daughter strain among the low virulent strains. It is not surprising considering that this region was predicted as a potential B-cell epitope by structural-based DiscoTope tool in *AAvV-1* isolates from waterfowl. However, such event suggests that further work is warranted to investigate this role in the waterfowl NDV virus isolates. Recombination in F gene *AAvV-1* viruses is suggested here as an important parameter to be considered in vaccination with live, attenuated viruses. The F gene recombination in NDV might be a potential reason for immunization failure.

CONCLUSION

We have evaluated *AAvV-1s* isolates originally assigned to genotypes V and II isolated from chicken and waterfowl, respectively, in Uganda. Based on the deduced amino acid analysis, structures mapping various mutations were found throughout F and HN genes of the isolates that affect the predicted B-cell epitopes; some were exclusive to either *AAvV-1* isolates from waterfowl or poultry. Recombination was also confirmed in the F gene of this non-segmented negative-strand RNA virus. These changes could have real impact on the

immunity and disease dynamics of the virus in vaccinated as well as immunologically naïve flock. Mixed or closed feeding of poultry with waterfowl in NDV-prevalent areas and frequent and uncontrolled use of vaccines could have serious implications in the control of ND where vaccination was done. Whether all these changes individually or combined influence the antigenicity of the virus leading to serious consequences of vaccine efficacy need to be established.

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 below: https://www.ncbi.nlm.nih.gov/genbank/, LT549451-53, HG937535-91.

ETHICS STATEMENT

The animal study was reviewed and approved by College of Veterinary Medicine Animal Resources and Biosecurity Higher Degrees Research Committee and Uganda National Council of Science and Technology (Approval #HS 776) approved this study. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JBO, DKB, HK, JLN, and MO-O designed the study. JBO and DKB analyzed the sequenced data. AW, JBO, and KKM collected the samples, isolated *AAvV-1* strains, and identified the viruses. All authors read and approved the final manuscript.

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High-Throughput Sequencing for Examining *Salmonella* Prevalence and Pathogen—Microbiota Relationships in Barn Swallows

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Studies in both humans and model organisms suggest that the microbiome may play a significant role in host health, including digestion and immune function. Microbiota can offer protection from exogenous pathogens through colonization resistance, but microbial dysbiosis in the gastrointestinal tract can decrease resistance and is associated with pathogenesis. Little is known about the effects of potential pathogens, such as *Salmonella*, on the microbiome in wildlife, which are known to play an important role in disease transmission to humans. Culturing techniques have traditionally been used to detect pathogens, but recent studies have utilized high throughput sequencing of the 16S rRNA gene to characterize host-associated microbial communities (i.e., the microbiome) and to detect specific bacteria. Building upon this work, we evaluated the utility of high throughput 16S rRNA gene sequencing for potential bacterial pathogen detection in barn swallows (*Hirundo rustica*) and used these data to explore relationships between potential pathogens and microbiota. To accomplish this, we first compared the detection of *Salmonella* spp. in swallows using 16S rRNA data with standard culture techniques. Second, we examined the prevalence of *Salmonella* using 16S rRNA data and examined the relationship between *Salmonella*-presence or -absence and individual host factors. Lastly, we evaluated host-associated bacterial diversity and community composition in *Salmonella*-present vs. -absent birds. Out of 108 samples, we detected *Salmonella* in six (5.6%) samples based on culture, 25 (23.1%) samples with unrarefied 16S rRNA gene sequencing data, and three (2.8%) samples with both techniques. We found that sex, migratory status, and weight were correlated with *Salmonella* presence in swallows. In addition, bacterial community composition and diversity differed between birds based on *Salmonella* status. This study highlights the value of 16S rRNA gene

sequencing data for monitoring pathogens in wild birds and investigating the ecology of host microbe-pathogen relationships, data which are important for prediction and mitigation of disease spillover into domestic animals and humans.

Keywords: barn swallows, microbiome, *Salmonella*, culture, 16S rRNA sequencing, disease surveillance

INTRODUCTION

The gut microbiome, defined as the community of living microorganisms (e.g., bacteria) and non-living genetic elements (e.g., relic DNA) inhabiting the gastrointestinal tract (Berg et al., 2020), plays an important role in an individual's development, digestion, and immune function (van der Waaij, 1989; Kohl, 2012). Intestinal microbial communities provide different functions for the host and are influenced by host diet, physiology, environment, and taxonomy (Kohl, 2012; Hird et al., 2015). Further, the microbiome is a key within-host trait that is associated with host-pathogen interactions. Infection with pathogens is linked to changes in the microbiome, as seen in mallards infected with low-pathogenic avian influenza virus (Ganz et al., 2017), as well as Marek's disease virus (Perumbakkam et al., 2014) and *Salmonella* infection in chickens (Videnska et al., 2013). It is unclear whether microbial dysbiosis is a result of or a precursor to pathogen infection: commensal microbiota can promote colonization resistance, but changes in the normal microbial community can decrease colonization resistance allowing for pathogen infection (Sorbara and Pamer, 2019). Alternatively, pathogen infection can disrupt the microbial balance in the gut, or a state of dysbiosis (Lupp et al., 2007). In either case, the relationship between pathogen infection and microbiome composition highlights the need to improve our understanding of the how and when the microbiome is influenced by pathogen infections.

The introduction of massive-parallel genetic sequencing methods has dramatically advanced the field of microbial ecology, allowing for a deeper examination of the microbial communities in both humans and other animals. Traditional culture techniques are time-intensive and tend to favor microbial species that thrive in laboratory settings (Davies et al., 2000), thereby excluding the vast majority of microbial diversity present within a community (Rhoads et al., 2012). Molecular techniques, such as low- and high-throughput 16S rRNA gene metabarcoding, have helped address the underestimation of community microbial diversity using culture methods, because they increase sensitivity through sequencing a small DNA region from all bacteria, and are more time efficient (Felske et al., 1998; Schwieger and Tebbe, 1998). The advent of next-generation sequencing has greatly accelerated the number of studies characterizing the microbiome of specific organisms and environments, with some projects achieving this at a global scale (e.g., The Earth Microbiome Project, Thompson et al., 2017). This has led to many new insights into host-microbe interactions at the molecular, individual, and community levels (Rosario and Breitbart, 2011), as well as the role of the microbiome in fighting disease and stimulating the host immune response (Kohl, 2012).

Recent studies have expanded the use of 16S rRNA gene amplicon data beyond the characterization of host-associated microbiomes to the detection of bacterial pathogens with greater taxonomic specificity (Srinivasan et al., 2014; Banskar et al., 2016). Many studies comparing traditional culture techniques to sequencing methods have found that 16S rRNA gene sequencing is more sensitive and can capture a greater proportion of the microbial diversity than culture techniques (Westergren et al., 2009; Rhoads et al., 2012; Park et al., 2014; Gupta et al., 2019), although comparative results were more equivocal (Wilson et al., 2018) with differing results based on the study. In part, 16S rRNA gene amplicon sequencing might increase sensitivity because DNA can be detected from living and dead cells, as well as from residual DNA present in the environment, whereas culturing is restricted to living cells. Most studies comparing culture to 16S sequencing have been limited to humans and have focused on commensal bacteria (Rhoads et al., 2012; Gupta et al., 2019), rather than bacterial pathogens (Westergren et al., 2009). Those studies outside of humans have focused on livestock (Park et al., 2014; Wilson et al., 2018) and not wildlife, despite wild populations being important reservoirs of zoonotic disease that can spill over into humans or livestock (e.g., COVID-19 likely originated from a bat host; Lu et al., 2020; Zhou et al., 2020).

Salmonella is a genus of bacteria that contains several pathogenic strains capable of being transmitted among wildlife, domestic animals, and humans as well as through the environment where it naturally resides in the soil (Wiedemann et al., 2014; Aung et al., 2020). *Salmonella* may be naturally occurring in the gastrointestinal tract and harmless in small quantities, but can become pathogenic when significantly increased in abundance, as is the case with some strains of *Escherichia coli* (Tizard, 2004). When *Salmonella* becomes pathogenic, it causes the disease known as salmonellosis and can result in symptoms such as diarrhea, fever, and lethargy, although some infected individuals may be asymptomatic. Infections can occur through fecal-oral contact via contaminated food or water, direct animal contact, and occasionally from person to person (Tizard, 2004). Wild avian hosts often serve as reservoirs for *Salmonella* (Gargiulo et al., 2018), and sometimes are the source of outbreaks in human and livestock populations (e.g., Foti et al., 2009). Furthermore, *Salmonella* outbreaks have been known to rapidly emerge in songbird (Passeriformes) populations largely due to the use of bird feeders, where high-density aggregations of birds increase the likelihood of transmission to other wild and domestic species (Tizard, 2004; Giovannini et al., 2012). A recent *Salmonella* outbreak resulted in die-offs of songbirds across multiple states within the United States (Machemer, 2021;

Mansfield and Lehman, 2021). Therefore, pathogen monitoring in birds is critical for reducing the likelihood of spillover events to susceptible wildlife, domestic animals, and humans. Monitoring can entail not just estimating the prevalence of a bacterial pathogen, but also examining its relationship with the host's microbial community.

A healthy microbiome may affect host health and immune response by preventing successful colonization of invading bacteria (colonization resistance), acting as a first line of defense against pathogens, and by modulating immune signaling (Sorbara and Pamer, 2019; Kogut et al., 2020; Rogers et al., 2020). Infection by pathogens, such as *Salmonella*, triggers an immune response leading to inflammation within the gut that alters the microbiome composition to favorable conditions (e.g., changes in pH) for pathogen colonization and reproduction, reducing competitors and decreasing the overall community diversity (i.e., α-diversity; Lupp et al., 2007). This dysbiosis allows invading pathogens to persist and may lead to differences in the microbiome between infected and uninfected individuals, as seen in waterfowl infected with avian influenza viruses (Ganz et al., 2017; Hird et al., 2018). While studies on the relationship between *Salmonella* infections and the microbiome are extensive in vertebrates (Bratburd et al., 2018, reviewed by Rogers et al., 2020), little is known on the effects of the presence of *Salmonella* itself on the microbiome of wild species regardless of pathogen status (i.e., carriers of *Salmonella* that may or may not be diseased). With this in mind, we sought to explore the microbiome of barn swallows (*Hirundo rustica*) with or without the presence of *Salmonella*. We hypothesized that the microbiome of *Salmonella*-present (pathogenic or non-pathogenic) barn swallows will have decreased alpha diversity compared to the microbiome of *Salmonella*-absent birds.

The barn swallow is a widespread ubiquitous passerine species well studied in terms of its life history (Balbontin et al., 2012; Møller, 2014), behavior (Saino et al., 2002; Lifjeld et al., 2011), physiology (de Ayala et al., 2006; Schmidt-Wellenburg et al., 2007; Safran et al., 2008), migration ecology (Altwegg et al., 2011; Liechti et al., 2014; Pancerasa et al., 2018), and host-associated microbiomes (Kreisinger et al., 2015, 2017; Ambrosini et al., 2019; Turjeman et al., 2020), which make the species an ideal exemplar for this study. The breadth of knowledge available on barn swallows allows for the integration of information across fields and more in-depth conclusions from our findings. In particular, the migratory distance of barn swallows could serve as an indicator of the potential for transmission of *Salmonella* spp. across broad geographic scales, as seen in bar-headed geese (*Anser indicus*) infected with highly pathogenic avian influenza virus H5N1 (Prosser et al., 2011) and passerine birds with the parasite *Babesia venatorum* (Hasle et al., 2011). Although *Salmonella* spillover into humans and domestic animals has not been linked with barn swallows, one study found barn swallows carried strains of the bacterial pathogen, *Clostridium difficile*, that were also found in humans and farm animals, implicating swallows as a potential source of spillover (Bandelj et al., 2014). Further, barn swallows migrate along the Palearctic-African flyway, the world's largest bird migration network. Israel, our sampling area, serves as a migratory bottleneck along the flyway linking Eurasia and Africa, where birds are able to avoid crossing large ecological barriers along their migration route (Collins-Kreiner et al., 2013). More specifically, an estimated 500 million birds travel through the Hula Valley region of Israel each season (Gophen, 2015) where dense congregations of birds create opportunities for pathogen transmission and spillover to agricultural operations in the area and beyond.

In this study, we aimed to: (1) explore the prevalence of *Salmonella* from fecal microbiome data within barn swallows, (2) assess the utility of microbiome data for accurately detecting potentially pathogenic bacteria (*Salmonella* spp.), by comparing results from traditional culture techniques to those from 16S rRNA gene sequencing, (3) evaluate different host ecological factors predicting *Salmonella* presence or absence, and (4) analyze the diversity of total (living and relic) microbial communities with and without the presence of *Salmonella* in barn swallows. Our results highlight the value of 16S rRNA gene sequencing not just for monitoring potential bacterial pathogens, but also for better understanding the ecology and role of microbial communities in pathogen infections.

MATERIALS AND METHODS

Study Area and Sample Collection

We captured 159 adult birds with mist-nets from four sites (Beit She'an, Haifa, Hula, Shefayim, **Figure 1**) in the northern half of Israel from November of 2016 to the end of November 2017. All fieldwork was conducted using permit number 2017/41764 issued by the Israel National Protection Authority and approved by Hebrew University ethics committee according to institutional and national guidelines. We placed captured birds in clean plastic cups inside individual bags to collect fecal samples for microbiome characterization. All fecal samples were collected using a sterile swab and stored in 95% EtOH and immediately frozen in a −20°C portable freezer for up to 7 days in the field before transfer to a −80°C freezer for long-term storage until extraction. For all individuals, an additional swab (fecal) was taken and stored in a glycerol-LB mixture for culture detection of *Salmonella* spp. Where possible, we collected associated data on age, sex, season of capture, and site for all the birds. Sexing was completed according to standard protocols described in Turjeman et al. (2020). We estimated, as described below, *Salmonella* prevalence for each site using a filtered dataset (n = 108) and visualized the prevalence distribution over our study area by generating a map using QGIS version 2.16.3 with the Apple iPhoto basemap (QGIS Development Team, 2009). Sample sizes, summary statistics on the number of *Salmonella* reads, and GPS location details for each site are available in **Supplementary Table 1**.

Culture Techniques for *Salmonella* Detection

Fecal swabs were stored in glycerol-LB and cultured to test for *Salmonella* spp. through one of two culturing workflows. The first workflow (workflow 1), done at the Kimron Veterinary Institute, Israel, used a culture-based approach and was performed

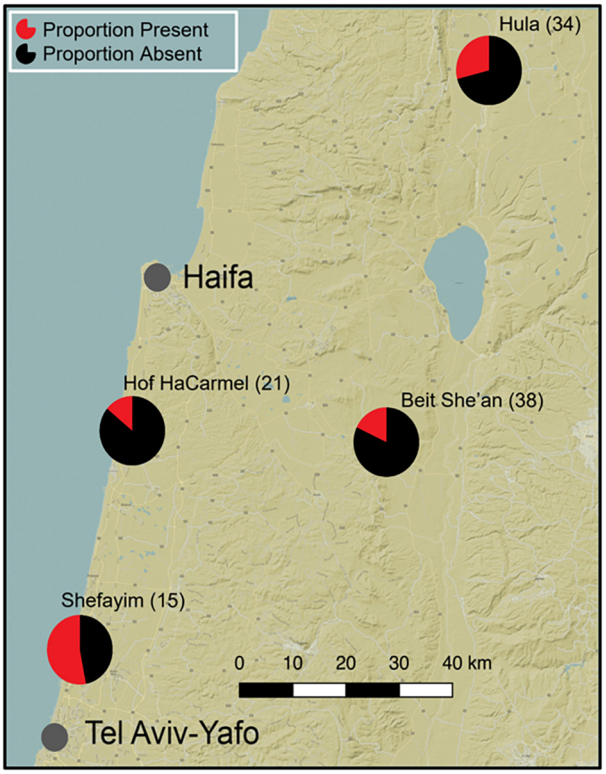

FIGURE 1 | The distribution of *Salmonella* in barn swallows (*Hirundo rustica*) across four sampling locations in Israel. The proportion of birds with *Salmonella* (red) and without *Salmonella* (black), as determined by 16S rRNA gene sequencing (pre-rarefying), is shown for each site, with samples sizes in parentheses.

on a subset of samples (n = 74) prior to 16S rRNA gene sequencing and did not detect *Salmonella* in any of the samples. However, sequencing revealed the presence of *Salmonella* in some samples and therefore we utilized a second culturing workflow (workflow 2) on the remaining samples (n = 34) to determine if *Salmonella* could be detected using a different culturing protocol. For the workflow 1, swabs were incubated in 10 mL buffered peptone water, followed by 1 mL tetrathionate-brilliant green broth, then XLT4 agar and Enteroplus slant agar. Each step included a 24 h incubation at 37°C. Cultures were considered *Salmonella*-present if they exhibited a characteristic tri-color appearance of *Salmonella* (red, black, yellow; ISO 6579-1 2017). Workflow 2 was completed at the Hebrew University of Jerusalem, Israel. Swabs were incubated in 10 mL buffered peptone water, followed by 1 mL Rappaport-Vassiliadis broth, then brilliant green agar, and triple sugar iron agar slants. Each step included an 18–24 h incubation step at 37°C. Colonies suspected of being *Salmonella* were verified through PCR. DNA was extracted using DNeasy Blood and Tissue kits (Qiagen Inc., Germantown, MD) following the gram-negative bacteria protocol. PCR was conducted following Halatsi et al. (2006) using primer pair SdiA1 (AATATCGCTTCGTACCAC) and SdiA2 (GTAGGTAAACGAGGAGCAG). Reaction volume was 20 μL: 3 μL DNA template, 1 μL of each primer, 10 μL of OneTaq Master Mix, and 5 μL of molecular grade water. Cycling conditions were as follows: 5 min of denaturation at 94°C, then 30 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 30 s ending with a final extension at 72°C for 7 min. PCR products were visualized by gel electrophoresis for the determination of the presence/absence of *Salmonella*.

Microbiome Extraction and Sequencing

A detailed protocol for 16S rRNA gene sequencing and processing of resulting reads is available in Corl et al. (2020) and Turjeman et al. (2020). Briefly, frozen swabs stored in 95% EtOH were extracted using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Germantown, MD). Each DNA extraction contained a set of individuals randomized across sites and times of collection to control for any batch effects during extraction. Along with the barn swallow samples, three negative control samples were processed through the DNA extraction workflow in the same manner as for the fecal samples. These negative controls were included to offset the ubiquitous nature of bacteria and to account for possible contamination from laboratory plastics and consumables used throughout the collection and extraction processes. DNA extractions were shipped to Argonne National Laboratory (Lemont, IL) for PCR-amplification and DNA sequencing. Primers 515F and 806R (Caporaso et al., 2012), targeting the variable V4 region of the bacterial 16S rRNA gene were used for Illumina sequencing. Paired-end reads of 151 base pairs (bp) sequences were generated on two runs of an Illumina MiSeq.

Microbiome Quality Control, and Data Filtering

Sequences were demultiplexed using QIIME2 (Bolyen et al., 2019). Analyses of the data were conducted in R 3.6.3 (R Core Team, 2020) following the R workflow for processing 16S data published by Callahan et al. (2016b). The first 10 bases were removed from all reads and DADA2 (Callahan et al., 2016a) was used to identify amplicon sequence variants (ASVs), which were unique sequences that were statistically unlikely to be due to sequencing error. The SILVA 132 taxonomy database was used to assign taxonomy, DECIPHER (Wright, 2015) was used to align sequences and the package phangorn (Schliep, 2010) was used to build a maximum likelihood phylogenetic tree. All parts of the data (sequence variant table, taxonomy table, phylogentic tree) were combined along with the metadata using the phyloseq package (McMurdie and Holmes, 2013) for statistical analyses. Contaminants were removed from the data set using the decontam package (Davis et al., 2018). The prevalence filter was used with a threshold set at 0.5 to remove all sequences that were more prevalent in the three negative controls than in samples from birds. The negative control samples did not have any *Salmonella* ASVs within them. In total, 518 ASVs were removed as contaminants. In addition, any ASVs not of the kingdom Bacteria were also removed, as well as any sequences matching mitochondria or chloroplasts. Lastly, we removed samples with poor quality PCR (n = 45) and samples

without culture results (n = 2). The remaining 108 samples were analyzed to compare the sensitivity of culturing to 16S rRNA gene sequencing in detecting *Salmonella*. Throughout this manuscript, we use *Salmonella* presence/absence to indicate *Salmonella* spp. ASVs present/absent in the microbiome and note that it is unknown whether the *Salmonella* strains detected are pathogenic.

We examined the bacterial community diversity within and across samples after rarefying the data to a sampling depth of 12,000 reads. A threshold of 12,000 reads was chosen after examining rarefaction curves (**Supplementary Figure 1**, rngseed = 711) that showed even the most diverse samples leveled off at this threshold and we would only lose seven samples, five of which had fewer than 5,000 reads. Therefore, our analyses of microbial diversity were conducted on 101 samples, after removing these seven samples from the dataset. To test for sampling effects during rarefaction, we ran the rarefaction analyses 100 times using different random seeds (1:100) and then determined the consistency across runs in detecting *Salmonella*.

Salmonella Prevalence and Comparison of Detection Methods

We estimated the prevalence of *Salmonella* spp. by detection method for all swallows with paired culture and 16S rRNA gene data (n = 108). Given that we used two different culture workflows, we ran separate sets of analyses for each workflow ($n_{\text{workflow 1}}$ = 74, $n_{\text{workflow 2}}$ = 34). A McNemar's exact test, assuming non-independence of samples that is appropriate for smaller sample sizes (Fagerland et al., 2013), was used to compare detection probabilities of culture and 16S rRNA gene sequencing for both workflow one and two. We used a generalized linear model (GLM) with a quasipoisson distribution from the car package (Fox and Weisberg, 2011) followed by a Type II ANOVA with the smaller dataset of workflow 2 and the combined dataset to test two hypotheses. First, we tested whether culture success or failure could predict the absolute abundance of *Salmonella* as measured by the number of 16S rRNA sequencing reads, including the total number of reads per sample as a covariate to account for sequencing depth. We used absolute abundance for this model to maximize detection power while acknowledging that samples did not have equal sequencing effort (unrarefied). Second, to address how sequencing depth affects our detection probability, we tested whether *Salmonella*-present vs. -absent status in the host, as measured by the 16S data, was correlated with the total number of reads sequenced for the sample. We do not report the results for rarefied data as the results did not differ for either the culture success and absolute abundance or *Salmonella* presence and total reads tests. To determine the sensitivity of *Salmonella* detection to 16S sequencing depth, we used all samples with at least a single read for *Salmonella* and greater than 45,000 reads (n = 18). We chose 45,000 as a cutoff because at higher depths many samples were lost during rarefaction as they did not meet the required number of reads. We rarefied the data for these 18 *Salmonella*-positive samples between depths of 5,000–45,000, in increments of 5,000 reads, using the default seed (711). For each depth, we calculated the proportion of samples with at least a single read of *Salmonella*. *Salmonella* detection, prevalence estimation, and statistical analyses were conducted in R 3.6.3 (R Core Team, 2020). Figures displaying prevalence between detection methods were generated using the ggplot2 (Wickham, 2016) and phyloseq (McMurdie and Holmes, 2013) packages in R.

Relationships Between Host Ecology and *Salmonella* Status

We explored whether *Salmonella* presence in the gut microbiome was correlated with host characteristics to highlight how 16S data can enhance our understanding of host ecology in relation to the presence of potential pathogens. We tested whether the presence or absence of *Salmonella* was dependent on three host factors: migratory status, weight, and sex. A generalized linear model with a binomial distribution was run for each factor, with the total number of reads for every sample included as a covariate to control for sequencing depth, which likely influences the detection of *Salmonella* in the hosts. For this analysis and all subsequent analyses, we initially assigned *Salmonella*-present samples as samples that had one or more *Salmonella* reads. We also repeated each analysis with a higher threshold of two or more *Salmonella* reads to assign a *Salmonella*-present sample. We did this to account for possible false positives due to PCR or sequencing error. When using a two read threshold, we removed seven samples that had only a single *Salmonella* read from the analyses. We were not able to explore thresholds greater than two reads due to the need to maintain a sufficient sample size of *Salmonella*-present samples. Data was available for 92 samples to test for the effects of weight and sex at read threshold one, while 88 samples were used for read threshold two. Data for migratory status was available for 37 (one read) and 33 (two reads) samples that were classified as migrant or resident using both feather molt pattern evaluated during field capture and stable isotope analyses (see Turjeman et al., 2020). For each model, we used an ANOVA to identify significant relationships.

Relationships Between the Microbiome and *Salmonella* Status

We estimated the diversity and bacterial species composition in all samples post rarefaction (rngseed = default of 711, n = 101) to examine host microbiome relationships with the presence of *Salmonella*. We used the Chao1 estimator of the number of species (Chao, 1984) to measure alpha diversity and used a Mann-Whitney-Wilcoxon test (MWW) to test for differences in alpha diversity of the microbiota between birds with and without *Salmonella* sequence reads. We chose to use Chao1 as an estimator for alpha diversity, because it accounts for rare and missing species (Chao and Shen, 2003). To test for confounding factors that may also influence alpha diversity, we used a linear model and ANOVA, including sex, site, and season as additional factors. Migration status was not included because our previous work showed no differences in alpha diversity between migrants and residents in our study population (Turjeman et al., 2020).

Differences in bacterial communities between *Salmonella*-present and -absent birds were visualized using bar plots and principal coordinates analysis (PCoA) with the phyloseq package.

PCoA chooses axes that explain most of the variation in the entire dataset without reference to the particular factors that may distinguish two groups of samples. Permutational multivariate analysis of variance (PERMANOVA) tests were calculated with adonis from the vegan package (Oksanen et al., 2019) with 9,999 permutations. This was done on both weighted and unweighted UniFrac metrics. UniFrac is a distance metric comparing bacterial communities that uses phylogenetic information to measure distance between samples (Lozupone and Knight, 2005). The unweighted UniFrac uses only presence/absence data, whereas weighted UniFrac also incorporates relative abundance of ASVs to measure distance between samples (Lozupone et al., 2007). To account for potentially significant differences based on location or dispersion, we tested for homogeneity of group dispersions using the betadisper function in the vegan package. When dispersions are significantly different, then significant differences in communities as found by PERMANOVA can be the result of differences in either dispersion or both dispersion and location.

RESULTS

16S rRNA Gene Sequencing Detected Higher *Salmonella* Prevalence Than Culture-Based Methods

Culture results revealed a relatively low prevalence of *Salmonella,* with zero out of 74 birds positive for *Salmonella* presence in workflow 1 (0%, **Figure 2A**) and six out of 34 birds positive in workflow 2 (17.6%; **Figure 2B**). In comparison, results based on unrarefied 16S rRNA gene data identified *Salmonella* in ten swallows (13.5%, **Figure 2A**) for workflow 1 and 15 swallows (44.1%, **Figure 2B**) in workflow 2. The McNemar's exact test revealed a significant difference in *Salmonella* detection probability between culturing vs. 16S rRNA gene sequencing for both culture workflow 1 (**Figure 2A**, $p = 0.002$) and workflow 2 (**Figure 2B**, $p = 0.035$).

A GLM showed no significant relationship between *Salmonella* culture results and the absolute abundance of *Salmonella* as measured by unrarefied reads of 16S rRNA data for workflow 2 ($p = 0.58$) and both workflows combined ($p = 0.96$). We did not use workflow 1 for this analysis as there were no culture positive samples for comparison. Samples that were identified as having at least one *Salmonella* sequence read in the 16S rRNA data had significantly higher sequencing depths than samples where no *Salmonella* sequences were detected (**Supplementary Figure 2**, $p = 0.002$). The mean absolute abundance of *Salmonella*, as measured by unrarefied 16S rRNA in culture-positive samples for workflow 2 (mean = 3.67, std dev = 7.12) and both workflows combined (same as workflow 2 as culture-positive samples did not change), did not significantly differ from mean abundance in culture-negative samples for workflow 2 (mean = 10.71, std dev = 38.87) (ANOVA, $F = 0.19$, $p = 0.66$) and the combined workflows (mean = 3.44, std dev = 20.83) (ANOVA, $F = 0.00069$, $p = 0.98$), respectively. *Salmonella* abundance was low, ranging from 1–199 reads (<0.007%, mean = 14.92) for samples positive for its presence based on 16S rRNA sequencing (**Supplementary Figure 3A**). Seven out of the 25 samples with *Salmonella* present in the microbiome had only a single *Salmonella* read.

We explored how *Salmonella* was affected by rarefaction, given that *Salmonella* presence status was related to sequencing depth. After rarefying to 12,000 reads, we detected *Salmonella* in an average of 12.95 samples (down from 25 samples pre-rarefying), with variability across runs (9–17 samples positive per run) due to random sampling (**Supplementary Figure 3**). The median value was 13 positive samples with a 95% confidence interval range of 12.6–13.3 samples after 100 iterations, so rarefaction reduced the number of *Salmonella*-present samples by about half. Samples with higher numbers of *Salmonella* reads before rarefying consistently had higher numbers of reads after rarefying. Post-rarefaction, one to three cultured samples were confirmed for *Salmonella* presence with 16S rRNA data, whereas three of the six culture positive samples were confirmed prior to rarefaction. In terms of sequencing depth and *Salmonella* detection, a high depth (>32,000) was required to obtain 80% detection within this system (**Supplementary Figure 4**) suggesting that a very high sequencing depth is required to consistently detect *Salmonella* in our data.

Salmonella Presence Differed Between Birds by Migratory Status and Weight

Using a *Salmonella* detection threshold of one read, we found a marginally significant difference in *Salmonella* presence ($p = 0.056$) between male and female birds. For host weight, *Salmonella*-present birds were slightly heavier (mean = 19.1 grams) in weight than those without *Salmonella* (mean = 18.1 grams) ($p = 0.012$). For migratory status, migrant swallows were significantly more likely to have *Salmonella* in their microbiome than resident swallows ($p = 0.041$), with a *Salmonella* prevalence of 52% in migrants and 16.7% in resident swallows.

When applying a *Salmonella* detection threshold of two reads, there was similarly a significant difference in *Salmonella* presence ($p = 0.015$) between male and female birds. *Salmonella* was present in seventeen of 37 (31.5%) male birds, in comparison to four of 39 (9.3%) females. For both weight and migratory status, there was no significant difference between *Salmonella*-present and -absent birds ($p = 0.081$ and 0.099, respectively).

Alpha Diversity Differed Among Birds Based on *Salmonella* Presence and Absence

When examining relationships between *Salmonella* and microbial communities, our set of positive samples were all samples with *Salmonella* sequence reads identified by 16S rRNA sequencing (pre-rarefaction; $n = 25$), plus the samples that were only determined to be positive by culture ($n = 3$). One sample with *Salmonella* identified pre-rarefaction did not meet the required threshold inclusion and was removed during rarefaction; this left 27 *Salmonella*-present samples for the remainder of the analyses, when applying a detection threshold of one read. For a detection threshold of two reads, six

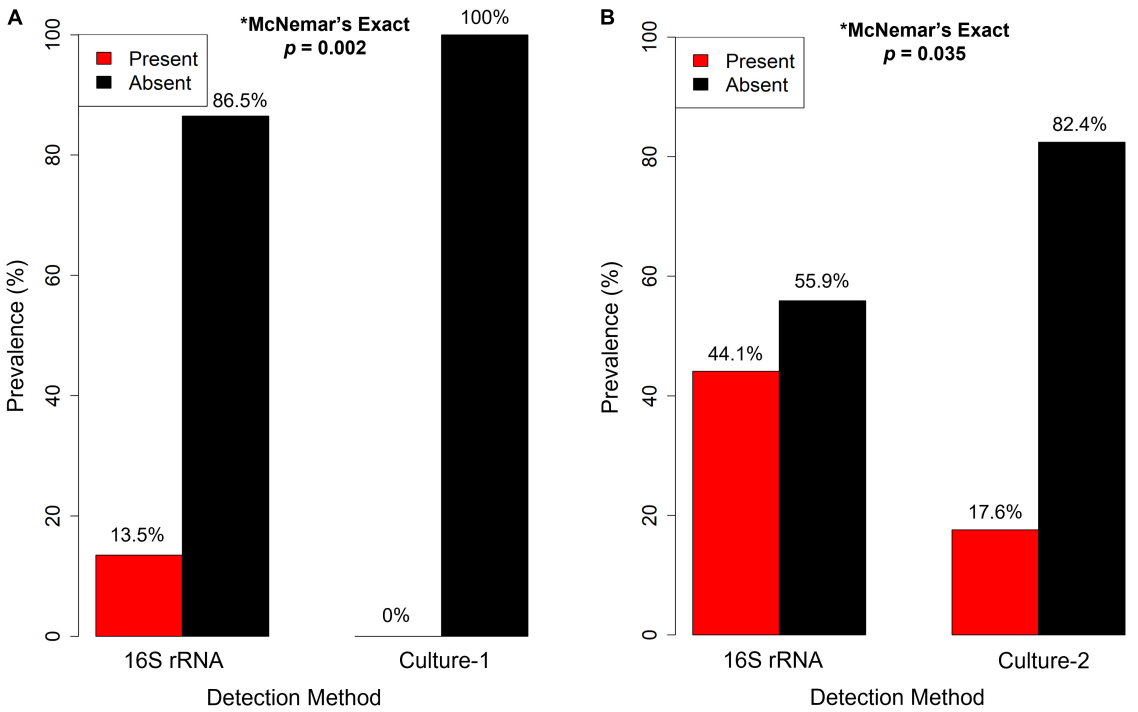

FIGURE 2 | *Salmonella* spp. prevalence in barn swallows (*Hirundo rustica*) from Israel, estimated using unrarefied 16S rRNA gene sequencing (16S rRNA) and two culture methods: **(A)** culture workflow 1 (Culture-1; $n = 74$) and **(B)** culture workflow 2 (Culture-2; $n = 34$). The proportion of samples positive for the presence of *Salmonella* (red bars) detected by 16S rRNA gene sequencing was significantly higher than those obtained by culturing. This analysis was conducted on paired samples from the same individuals that had both culture and 16S rRNA gene data collected.

additional samples were removed, leaving 21 *Salmonella*-present samples in the remaining analyses. At *Salmonella* read threshold one, individuals with *Salmonella* ($n = 27$) had higher levels of alpha diversity than individuals without *Salmonella* (**Figure 3**, MWW, $p = 0.001$). Both *Salmonella* presence/absence (ANOVA, $p = 0.047$, $F = 4.044$, df = 1) and site (ANOVA, $p = 0.048$, $F = 2.739$, df = 3) were significant predictors of microbial community alpha diversity (Model $R^2 = 0.18$). Sex (ANOVA, $p = 0.509$, $F = 0.44$, df = 1), age (ANOVA, $p = 0.55$, $F = 0.553$, df = 1) and season (ANOVA, $p = 0.92$, $F = 0.084$, df = 2) were not significantly correlated with alpha diversity. *Salmonella* read threshold two yielded similar results: individuals with *Salmonella* had higher levels of alpha diversity than individuals without *Salmonella* (**Supplementary Figure 5**, MWW, $p = 0.003$), and both *Salmonella* presence/absence (ANOVA, $p = 0.028$, $F = 4.970$, df = 1) and site (ANOVA, $p = 0.042$, $F = 2.852$, df = 3) were significant predictors of microbial community alpha diversity (Model $R^2 = 0.112$). In addition, sex (ANOVA, $p = 0.225$, $F = 1.49$, df = 1), age (ANOVA, $p = 0.60$, $F = 0.280$, df = 1), and season (ANOVA, $p = 0.93$, $F = 0.074$, df = 2) were not significantly correlated with alpha diversity.

Across all samples, 16S rRNA data revealed that bacterial communities were comprised mainly of the phyla Proteobacteria, Tenericutes, Firmicutes, and Actinobacteria (**Supplementary Figure 6**). The microbial communities of *Salmonella*-present and -absent birds were significantly different in PERMANOVA tests using both unweighted (**Figure 4A**, $p = 0.001$, $F = 2.637$, df = 1, $R^2 = 0.260$) and weighted (**Figure 4B**, $p = 0.013$, $F = 2.821$, df = 1, $R^2 = 0.028$) UniFrac distances at read threshold one. Homogeneity of dispersion between the two groups was rejected for both unweighted and weighted UniFrac distances (unweighted: $F = 24.786$, df = 1, $p = 0.001$; weighted: $F = 12.506$, df = 1, $p = 0.001$). Thus, significant differences in the bacterial communities of *Salmonella*-present and -absent birds are driven at least in part by different dispersion of the two groups. However, the principal coordinates analysis plot for the unweighted UniFrac (**Figure 4A**) suggests that there may also be differences in the location of points within the plot for the two groups, because many birds with *Salmonella* reads have bacterial communities that occupy a space that is distinct from birds without *Salmonella* reads. Read threshold two yielded similar results and thus are not reported here (**Supplementary Figure 7**).

DISCUSSION

Salmonella is a bacterial genus that includes several pathogenic serotypes of concern to both public health and agriculture. As wild bird species can act as reservoirs or carriers for these pathogenic bacteria, continued *Salmonella* surveillance is critical for preventing spillovers into humans and domestic animals (Giovannini et al., 2012). In barn swallows, *Salmonella* was not previously detected when using a culture diagnostic approach for screening (Haemig et al., 2008). However, our data suggest that

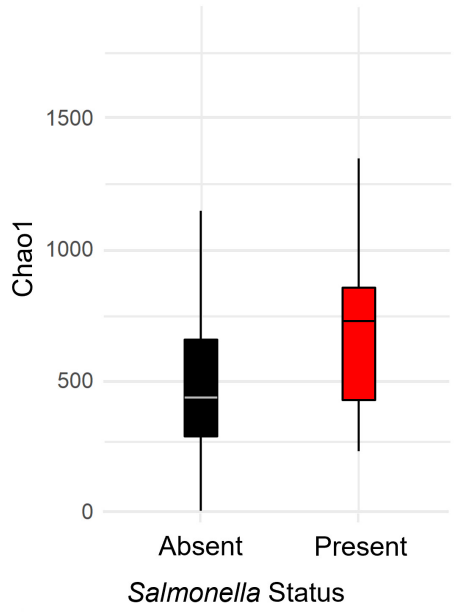

FIGURE 3 | Bacterial alpha diversity, as estimated by the Chao1 statistic, by *Salmonella* spp. status (absent, present) in barn swallow (*Hirundo rustica*) fecal samples collected in Israel. *Salmonella*-absent samples had significantly lower diversity than -present samples (Mann-Whitney-Wilcoxon test, $p = 0.008$).

Salmonella can be missed by culturing (e.g., culture workflow 1), and therefore microbiome data could enhance efforts to monitor *Salmonella* and other bacterial pathogens in wild birds for the purposes of both human and avian health. Further, our results demonstrate an approach that can be used not only for monitoring potential pathogens, but also for addressing key questions in disease ecology, such as the relationship between individual host factors (e.g., age, sex, and microbial community diversity) and pathogen infection.

We found that detection of *Salmonella* was significantly better with 16S rRNA gene sequencing data than using culture techniques (**Figure 2**). Our finding that 16S rRNA sequence data detected *Salmonella* in more birds than either respective culture workflow suggests that sequencing approaches could be an important tool for the detection and surveillance of bacterial pathogens like *Salmonella* in wildlife populations. Higher sensitivity of microbe detection using 16S rRNA gene sequencing data could be due to the detection of both residual or dead cells. For the purposes of this study, dead cell DNA is still of interest in terms of the potential for carrier status, because these cells could represent bacteria that are alive in the gastrointestinal tract, but unable to survive the semi-aerobic conditions of the cloaca (Grond et al., 2018), where fecal material passes prior to sample collection. However, if detection of only live potential pathogens is desired, a pre-enrichment step using ethidium monoazide (Nogva et al., 2003) or propidium monoazide (Nocker et al., 2006) can be included to remove non-viable DNA.

The detection of *Salmonella* or other bacteria in 16S rRNA sequencing data will depend on the sequencing depth and the abundance of the bacteria in the sample. We found that *Salmonella* presence in barn swallows was related to sequencing depth (**Supplementary Figure 2**), that rarefaction to 12,000 reads resulted in *Salmonella* detection in only half the total

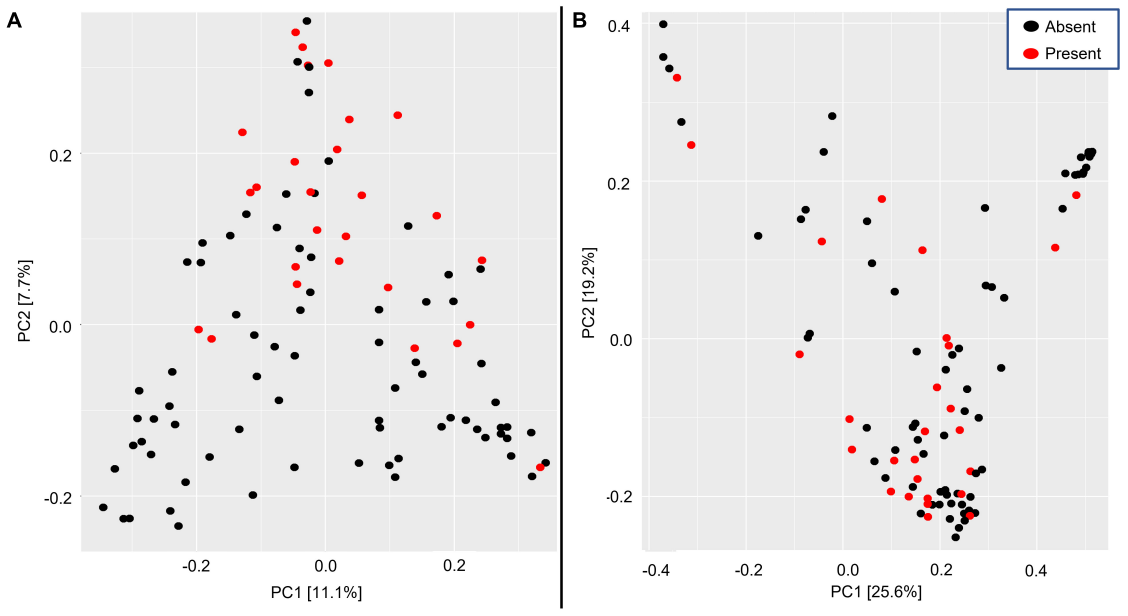

FIGURE 4 | Principal coordinate analysis plots showing the bacterial communities in barn swallow (*Hirundo rustica*) fecal samples with *Salmonella* (present: red dots) and without (absent: black dots) by **(A)** unweighted UniFrac and **(B)** weighted UniFrac metrics. The amount of the variation explained by each axis is in brackets. Bacterial beta diversity significantly differed for both metrics ($p = 0.001$ for both) between *Salmonella*-present and -absent birds.

samples observed when using the full data set, and that a high sequencing depth was required for reliable detection of *Salmonella* spp. (**Supplementary Figures 4, 5**). This high sequencing depth requirement may explain the discrepancy in *Salmonella* detection for the three false negative samples that were culture positive, but 16S negative. Further, these data highlight two vital factors to keep in mind when using 16S rRNA gene data for disease detection. First, sequencing depth will determine the power to detect taxa, especially those in low abundance, as we observed here when increasing the *Salmonella* detection threshold. The minimum sequencing depth would change for other host species, which may not have a similarly low level of *Salmonella* abundance, especially if they are in a diseased state. Our results are consistent with these swallows potentially being infected or carriers of *Salmonella*, rather than experiencing disease, due to extremely low levels of abundance. Thus, we recommend high sequencing depths unless it is known *a priori* that the pathogen is abundant. A cost-effective approach would be to first sequence a subset of samples at high sequencing depth to determine the optimal level of sequencing for the remaining samples. Second, we recommend that all reads be used for detection of rare pathogens, because this approach maximizes the power for detection within a dataset. If this approach is taken, it could be useful to withhold some DNA before 16S library preparation that could be used for further PCR or sequencing of the target pathogen to confirm positive samples that have very few reads from the bacteria of interest. A previous study showed that with a proper enrichment step, PCR detected *Salmonella* from as little as one colony forming unit (CFU) in food (Ferretti et al., 2001), suggesting that even in low abundance, *Salmonella* can be detected. PCR and Sanger sequencing of 16S positive samples could also be useful for determining whether the bacterial strain is pathogenic, which often requires longer reads than are typical with 16S rRNA amplicon sequencing.

Our data showed that the presence of *Salmonella* was correlated with multiple differences in host ecology and microbiota. When using a detection threshold of one read, birds with *Salmonella* weighed more than birds without *Salmonella*, and *Salmonella* was present in more migrants than resident barn swallows. These two factors were related to one another, because migrant birds were heavier than resident barn swallows (mean 20.2 vs. 17.5 grams). Barn swallows have been known to accumulate fat in preparation for migration (Pilastro and Spina, 1999), which suggests that migrants might have differing foraging behaviors that could lead to a higher prevalence of *Salmonella.* Another potential reason why migrants have a higher prevalence of *Salmonella* is that they travel through multiple environments, which could increase their encounters with *Salmonella* species. This sort of pattern has been observed in barn owls (*Tyto alba*), where owls that traveled greater distances from their nests had more diverse microbiota (Corl et al., 2020). However, migration does not necessarily lead to changes in the microbiota as microbial diversity did not vary between fecal samples collected from common cranes (*Grus grus*) before and after migration (Pekarsky et al., 2021). Dietary, physiological, or subspecies differences might also explain differences in *Salmonella* prevalence between migrants and resident barn swallows. A caveat to the models correlating *Salmonella* presence with host ecological variables is they were sensitive to the read threshold and/or sample size. With read threshold two, the relationships with both migratory status and weight were not significant (migratory status, $p = 0.099$, weight $p = 0.081$), whereas a read threshold of one revealed a significant relationship ($p < 0.05$) between these factors and *Salmonella* status. In contrast, the relationship with sex changed from marginally significant ($p = 0.056$) to significant ($p = 0.012$) when increasing the *Salmonella* detection threshold to two reads. Thus, similar trends were found for all three traits, but the statistical significance of the correlations is tentative. However, these results do highlight a set of traits to target for future study to elucidate the relationship between host ecology, microbiota, and *Salmonella* status. In addition, these results underscore the need for high sequencing depths and sufficient numbers of positive samples when investigating relationships between *Salmonella* status and host ecology.

We found that birds with *Salmonella* had more diverse bacterial communities, suggesting that *Salmonella* may alter community level interactions among bacterial taxa in the gut microbiome or that *Salmonella*-presence is correlated with other factors that alter bacterial communities. Increased diversity in *Salmonella*-present birds may be related to the vital role the gut microbiome plays in immune response and health. Hosts and their microbiota can work together to promote colonization resistance (Sorbara and Pamer, 2019; Rogers et al., 2020), while infection by *Salmonella* spp. can cause dysbiosis in favor of *Salmonella* growth (Lupp et al., 2007) and a decrease in diversity that is not seen here. For example, microbial diversity was reduced in American white ibis (*Eudocimus albus*) shedding *Salmonella enterica,* relative to the diversity observed in healthy ibis (Murray et al., 2020). Similarly, microbial diversity was reduced in mallard ducks infected with avian influenza viruses (Ganz et al., 2017). However, pathogen relationships with microbial diversity are species-specific, as seen in waterfowl where influenza A virus infection was negatively correlated with alpha diversity in only two of five species (Hird et al., 2018). However, our results contrast with these previous studies and the apparent increased microbial diversity in individuals with *Salmonella* may be due to avirulent or low abundance strains failing to trigger colitis, and thus being outcompeted by other bacterial species (Stecher et al., 2007). Another explanation is that hosts with *Salmonella* could have more varied diets, resulting in an increased chance of being colonized by diverse bacteria, including *Salmonella*. In addition, hosts may differ in their movement patterns (e.g., migrant vs. resident), which could affect their exposure and colonization by a particular bacterium: the use of more diverse habitats (long-distance migrants) may lead to more exposures and colonization by diverse bacteria as supported by our finding that migrants were more likely to have *Salmonella*.

We found that bacterial communities differed in individuals with and without *Salmonella*, but we were unable to disentangle whether the significance of these tests is driven in part by

community-level differences or by significant differences in dispersion between *Salmonella*-present and -absent groups. With that caveat in mind, the PcoA of unweighted UniFrac suggests that there are both differences in location (i.e., position of the points) and dispersion (i.e., the spread of the points) of the two bacterial communities. Therefore, dispersion might not be the only factor separating bacterial communities with and without *Salmonella,* and *Salmonella*-present hosts may vary in their bacterial communities.

Our work demonstrates the potential for 16S rRNA gene sequencing data collected for microbiome studies to also be used for monitoring *Salmonella* and other pathogens in wild bird populations. The observed variation in microbial communities and host traits by *Salmonella* status also suggests that studies on pathogen transmission and host microbial ecology can mutually inform one another. Pathogens should be considered as one of the many plausible causal explanations for the differences in the host microbiota. The few studies focused on pathogen-microbiome interactions in wildlife highlight a need for further research to elucidate the relationships between microbiota, pathogen infections, and disease. We propose that future microbiome studies of wild animals have great potential to be used to better understand disease epidemiology and ecology in wild populations, as well as aid in the identification of potential reservoir species for pathogenic bacteria. Microbiome studies could thus be a rich, but untapped source of data for better understanding the distribution and ecological dynamics of pathogens in the environment.

DATA AVAILABILITY STATEMENT

Part of the sequence data generated for this study were previously deposited in the Sequence Read Archive (BioProject ID: PRJNA578383, accession nos. SRX9094853 and SRX9094908) along with associated metadata (Turjeman et al., 2020) and the remainder of the sequence in the same Sequence Read Archive BioProject (PRJNA578383) along with associated metadata (BioSamples SAMN21551599 and SAMN21551658, accession numbers SRX12293439 and SRX12293498). Both datasets can be found at https://www.ncbi.nlm.nih.gov/sra/PRJNA578383.

ETHICS STATEMENT

The animal study was reviewed and approved by the Israel National Protection Authority (#2017/41764) and approved by an ethics committee according to institutional and national guidelines.

AUTHOR CONTRIBUTIONS

PK, RB, RN, and WG obtained funding and provided resources and facilities that were used in this study. AC, OC, RB, and PK designed the study. AW collected samples. ST developed sampling protocols, helped coordinate field and laboratory work, and prepared sampling material and media. AC, AL, OC, and ST completed laboratory work (AC: microbiome extractions, AL and OC: culture workflows, ST: determining the sex of the samples). AC completed bioinformatics processing. AC and SI contributed code for analyses. OC completed statistical analyses and wrote the first draft of the manuscript with help from AC, SI, and PK. All authors read and revised the 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/fevo.2021.683183/full#supplementary-material

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Identifying Edaphic Factors and Normalized Difference Vegetation Index Metrics Driving Wildlife Mortality From Anthrax in Kenya's Wildlife Areas

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Anthrax, an acute disease of homeotherms caused by soil-borne *Bacillus anthracis* is implicated in dramatic declines in wildlife mainly in sub-Saharan Africa. Anthrax outbreaks are often localized in space and time. Therefore, understanding predictors of the spatial and temporal occurrence of anthrax in wildlife areas is useful in supporting early warning and improved response and targeting measures to reduce the impact of epizootic risk on populations. Spatial localization of anthrax is hypothesized to be driven by edaphic factors, while the temporal outbreaks are thought to be driven by extreme weather events including temperature, humidity, rainfall, and drought. Here, we test the role of select edaphic factors and normalized difference vegetation index (NDVI) metrics driven by vegetation structure and climate variability on the spatial and temporal patterns of wildlife mortality from anthrax in key wildlife areas in Kenya over a 20-year period, from 2000 to 2019. There was a positive association between the number of anthrax outbreaks and the total number of months anthrax was reported during the study period and the nitrogen and organic carbon content of the soil in each wildlife area. The monthly occurrence (timing) of anthrax in Lake Nakuru (with the most intense outbreaks) was positively related to the previous month's spatial heterogeneity in NDVI and monthly NDVI deviation from 20-year monthly means. Generalized linear models revealed that the number of months anthrax was reported in a year (intensity) was positively related to spatial heterogeneity in NDVI, total organic carbon and cation exchange capacity of the soil. These results, examined in the light of experimental studies on anthrax persistence and amplification in the soil enlighten on mechanisms by which these factors are driving anthrax outbreaks and spatial localization.

Keywords: anthrax, wildlife, NDVI, Kenya, spatio-temporal

INTRODUCTION

Anthrax is an acute, febrile disease of homeotherms, caused by the gram-positive, non-motile, spore-forming, soil-borne bacteria; *Bacillus anthracis* and *Bacillus cereus* biovar *anthracis* (Koch, 1937; Hoffmaster et al., 2004; Leendertz et al., 2006b). The disease has a global distribution (Schild et al., 2006; Turnbull, 2008; Revich et al., 2012; Barro et al., 2016; Chen et al., 2016; Hoffmann et al., 2017; New et al., 2017), and infects a wide range of vertebrate species (Mollel, 1977; Lindeque, 1998; Leendertz et al., 2006a; Clegg et al., 2007; Beyer and Turnbull, 2009). Anthrax epizootics have caused significant declines in wildlife species by 10–30% in most outbreaks and by 80–90% in severe outbreaks (Creel et al., 1995; Hugh-Jones and De Vos, 2002; Leendertz et al., 2006a; Clegg et al., 2007; Muoria et al., 2007; Kaitho et al., 2013; Salb et al., 2014; Hoffmann et al., 2017).

Bacillus anthracis is endemic in many parts of the world with an epizootic transmission cycle (van Ness, 1971; Hugh-Jones and Blackburn, 2009). Areas that *B. anthracis* is deemed endemic to are characterized by high content of soil calcium, pH (pH $\geq$ 6.1), moisture, and organic carbon and nitrogen (Joyner et al., 2010; Chikerema et al., 2013; Kracalik et al., 2017; Steenkamp et al., 2018; Romero-Alvarez et al., 2020) and occur at low-lying topography (Dragon and Rennie, 1995; Munang'andu et al., 2012). *B. anthracis* consists of clade A and clade B among others, but clade A thrives in soil with wider ranges in pH and calcium whereas anthrax clade B is more common in soil rich in calcium and high pH (Smith et al., 2000). Regarding topology, seasonally flooded plains and low lying areas are known to be prone to anthrax outbreaks (Dragon and Rennie, 1995; Munang'andu et al., 2012).

Anthrax outbreaks are driven by a synergy of intrinsic and extrinsic factors linked to both the host and the pathogen. The occurrence and severity of anthrax are highly variable across seasons and years and appear to be driven by mean temperature, and precipitation in the temperate regions while in the tropics, precipitation is a dominant factor (Joyner et al., 2010; Chikerema et al., 2013; Kracalik et al., 2017; Steenkamp et al., 2018; Romero-Alvarez et al., 2020). Most anthrax epizootics in endemic tropical regions occur in the dry season and it is speculated that exposure to hot weather and subsequent heat stress and malnutrition from forage scarcity play a role in decreasing host resistance to infections (Hugh-Jones and Blackburn, 2009). However, this hypothesis does not address cases in which anthrax mortalities occur in the wet season or where the disease begins at the end of the rains or beginning of dry seasons. Nevertheless, recent evidence suggest that anthrax outbreaks are driven by extreme weather events (Hampson et al., 2011; Anttila et al., 2015) that may be caused by El Nino and La Nina and associated ENSO phenomenon. Similar timing of anthrax outbreaks has been observed in temperate and arctic climates, where outbreaks are associated with hot-dry summers preceded by heavy spring rain (Blackburn and Goodin, 2013; Maksimović et al., 2017). Extreme weather events may induce anthrax epizootics because precipitation and temperature are known to modulate ecological dynamics of soil-dwelling microbes known experimentally to amplify or inhibit *B. anthracis* multiplication in the soil (Clarholm, 1981; Rutherford and Juma, 1992; Anderson, 2000; Ritz and Young, 2011; Dey et al., 2012).

Another driver of temporal patterns of anthrax epizootics is vegetation as measured through NDVI. A few studies have confirmed a positive relationship between NDVI and anthrax outbreaks (Kracalik et al., 2017; Steenkamp et al., 2018) or variance in NDVI and occurrence of anthrax (Chikerema et al., 2013). Plant growth is experimentally known to enhance the proliferation of *B. anthracis* (Saile and Koehler, 2006; Ganz et al., 2014) and influence the dynamics of organisms such as *Acanthamoeba* (Rodriguez-Zaragoza et al., 2005; Fernández, 2015), which are speculated to amplify anthrax presence in the soil based on experimental results (Dey et al., 2012).

In this study, we examined the influence of edaphic factors particularly total organic carbon, total nitrogen, pH, total carbonate equivalent, cation exchange capacity, gypsum content and available water capacity on the frequency and duration of anthrax outbreaks. We also examined the influence of normalized difference vegetation index (NDVI) metrics - surrogates for climate variability, vegetation structure, plant diversity, plant growth or phenology and plant cover – on the monthly occurrence (timing) and the number of months of anthrax when anthrax mortality was reported each year (intensity) in selected wildlife areas in Kenya. Lastly, we combined both edaphic variables and NDVI metrics as predictors of anthrax mortality in wildlife in multilevel model analyses.

MATERIALS AND METHODS

Wildlife Areas: Sizes, Climate, and Vegetation

The focal wildlife areas selected are those with endangered species, such as Rothschilds giraffe (*Giraffa camelopardalis rothschildi* Lydekker), black rhinoceros (*Diceros bicornis* Linn.) and white rhinoceros (*Ceratotherium simum*, Burchell) where veterinary investigations on causes of mortality are prioritized and likely to be reported. These areas included Hell's Gate National Park (HGNP), Lake Nakuru National Park (LNNP), Nairobi (NNP), Tsavo East National Park (TENP), Tsavo West National Park (TWNP), Sibiloi National Park (SNP) and Mwea National Reserve (MNR) (**Figure 1**). Areal coverage, climate, and elevation for each of these protected areas are summarized in **Table 1**.

The vegetation of LNNP consists of wooded and bushed grassland. The dominant grasses include *Cynodon niemfluencis*, *Chloris gayana*, *Sporobolus spicatus,* and *Themeda triandra* while bushlands and woodlands are dominated by closed stands of *Euphorbia candelabrum* and *Acacia xanthophloea*. NNP has a vegetation composed of lightly wooded grasslands on the plains to the east and dry forest in the upland areas to the west. The wooded grasslands are dominated by *Acacia drepanolobium* trees, and *Themeda triandra*, *Pennisetum mezianum*, *Bothriochloa insculpta*, and *Digitaria macroblepharu* grasses whereas the highland dry forest is dominated with *Olea africana*, *Croton dichogamus*, *Brachylaena hutchinsii*, and *Calodendrum* (Boutton et al., 1988). The vegetation of HGNP is predominantly open

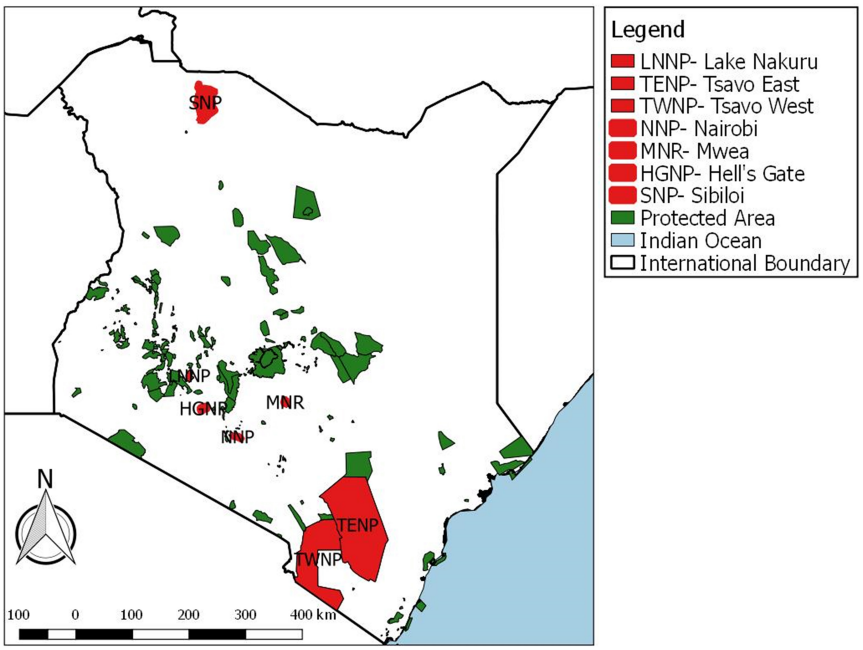

FIGURE 1 | The spatial distribution of Kenya's protected wildlife areas (NP, National Park; NR, National Reserve) showing focal wildlife areas examined for anthrax outbreaks. Focal wildlife areas are indicated in red and other wildlife areas are indicated by green.

TABLE 1 | Areal coverage, climatic and topographic variables of focal wildlife areas in Kenya examined.

Wildlife area	Area (km^2)	Mean annual rainfall (mm)	Minimum and maximum rainfall	Rain season(s)	Mean annual temperature	Minimum and maximum temperature	Elevation	Sources
Mwea National Reserve (MNR)	42	783	650–1000	March–May and October–December	22.1°C	14–30°C	950–1150	Chira and Kinyamario, 2009; Kaitho et al., 2013
Hell's Gate National Park (HGNP)	68	626	443–939	March–May and October–December	19°C	8–30°C	2000	Getonto, 2018
Nairobi National Park (NNP)	117	808	366–1697	March–May and November–December	19.6°C	12–28°C	1600–1800	Deshmukh, 1986; Ogutu et al., 2013
Lake Nakuru National Park (LNNP)	188	869	363–1146	March–June and October–December	18°C	8.2–25.6°C	1760–2080	Ng'weno et al., 2010; Ogutu et al., 2012
Sibiloi National Park (SNP)	1570	192	18.5–472.3	March-May & October-December	32°C	26–37°C	360–560	Mbaluka and Brown, 2016; Avery and Tebbs, 2018
Tsavo West National Park (TWNP)	7065	600	400–900	March–May and November–January	28°C	20–30°C	600–1800	Waweru and Githaiga, 2014
Tsavo East National Park (TENP)	13747	538	184–1201	March–May and November–January	27.9°C	22.1–33.6°C	150–1200	Coe, 1978; Kyale et al., 2011

For MNR, additional information accessed from: https://en.climate-data.org/africa/kenya/embu/mwea-sub-location-1024021/ and for TENP, https://en.climate-data.org/africa/kenya/makueni/tsavo-103115/.

to dense scrubland, and open grassland. Scrubland is dominated by *Acacia drepanolobium* and *Tarchonanthus camphoratus*, while grasslands are dominated by *Themeda triandra* (Lovart and Lucherin, 1992). MNR has three vegetation types including bushlands dominated by *Acacia mellifera*, *Commiphora africana*, *Grewia bicolor* and *Acacia ataxacantha*, woodlands dominated by *A. mellifera* and *C. africana* and wooded grasslands covered by mainly under *Terminalia brownie* trees (Chira and Kinyamario, 2009). SNP has an arid vegetation dominated by grassland and dwarf shrubland, with riparian forest and riparian woodland at the shores (Mbaluka and Brown, 2016). The Vegetation of TWNP is dominated by *Acacia–Commiphora* bushland and *Hyphaene compressa* woodlands in the north and open grassy plains in the south (Wato et al., 2006). TENP has a semi-arid vegetation

consisting mainly of bushland–grassland savannah and *Acacia–Commiphora* woodlands interspersed with *Premna* sp., *Bauhinia* sp. and *Sericocomorpsis* sp., *Delonix elata* and *Melia volkensii* trees in open plains (Kyale et al., 2011).

The Occurrence of Anthrax in Focal Wildlife Areas in Kenya

We extracted data on months when anthrax outbreaks occurred from disease surveillance reports at the Kenya Wildlife Service as well as published literature for seven wildlife areas in Kenya for the period 2000 to 2019 (Kaitho et al., 2013; Muturi et al., 2018). An Anthrax outbreak was defined as an occurrence of wildlife deaths more than what would normally be expected in a defined geographical area within a span of less than 2 weeks that warranted a disease investigation. We used only data from reports in which anthrax was determined by clinical examination of fresh carcasses and through microscopic examination of bacilli in blood-stained smears (Examples: Muoria et al., 2007; Aminu et al., 2020). Typically, thin blood smears were prepared in the field, stained with polychrome methylene blue and examined under a light microscope. The presence of blue square-ended rods, usually in short chains, surrounded by a pinkish-red capsule was used to diagnose the presence of *B. anthracis* (M'Fadyean, 1903). For each outbreak, we extracted the months, and records of wildlife species affected. The number of animals that died from each outbreak were opportunistically counted following confirmation of positive cases. There were no systematic surveys to determine detection probability and to objectively estimate the number of carcasses. The number of animals reported are therefore unreliable as estimates of the magnitude of outbreaks and are lower than the true number of animals that died. More generally, wildlife cases are usually underestimates compared to human or livestock anthrax cases.

Normalized Difference Vegetation Index Variables

Normalized difference vegetation index is a measure of differential reflectance and absorbance of the light spectra by vegetation and depends on the phenology and density of vegetation. NDVI is emerging as an important predictor of anthrax outbreaks as it integrates soil moisture, plant phenology, spatial variability in vegetation structure, species composition, topography, and variability in rainfall (e.g., Norris and Blackburn, 2019). The NDVI data used for this study covered January 2000 to December 2019 and was sourced from satellite images at the MODIS NDVI, a 16-day, 250-m resolution database[1]. Shape files of the study areas were obtained from the http://datasets.wri.org. The zonal statistic tool in ArcGIS 10.1 (ESRI, 2012) was used to calculate the mean, minimum, maximum, and standard deviation of NDVI values based on the number of 250-m pixels within each polygon and two time periods within each month. From these statistics, the spatial variation in NDVI, a metric which is positively correlated with plant diversity, vegetation structure and vegetation coverage was obtained (Gould, 2000; Fairbanks and McGwire, 2004; Oldeland et al., 2010; Rocchini et al., 2010; Pau et al., 2012), whereas temporal variability is correlated with climatic variability particularly precipitation and temperature that drive plant phenology (Hawinkel et al., 2016; Kalisa et al., 2019). Estimates of mean monthly NDVI and monthly standard deviation of NDVI across pixels for each wildlife area (spatial NDVI heterogeneity) were used as independent covariates. In addition, we calculated monthly NDVI deviation from long-term monthly average over a 20-year period and temporal heterogeneity in NDVI or the standard deviation from the annual average.

Edaphic Variables

The soil data were obtained from the ISRIC- an International World Soil Reference and Information Centre[2]. We extracted the following soil variables using data from soil classification maps for our focal areas; soil type, soil type specific mass fraction (g/kg^{-1}) of total organic carbon, total nitrogen and alkalinity or pH, a measure of hydrogen ions [H+] concentration expressed on a negative logarithmic scale of the hydrogen ion concentration [H+] in water from SOTER-based soil parameter estimates (SOTWIS) for Kenya[3]. Further, we extracted soil-type specific average; total carbonate equivalent (g/kg^{-1}), cation exchange capacity ($cmolc/kg^{-1}$), gypsum content (g/kg^{-1}), and available water capacity (vol.% –33 to –1500 kPa, USDA standards). We clipped soil data to each wildlife area shape file polygon. The area of coverage of each soil type was calculated using ArcGIS 10.1 software (ESRI, 2012). To obtain a single measure for each soil variable above for each wildlife area, the value for each soil type was multiplied by the areal coverage of that soil type and summed across soil types and divided by total wildlife area.

Statistical Analyses

First, we examined the influence of edaphic factors [total organic carbon (g/kg^{-1}), total nitrogen (g/kg^{-1}), pH, lime, or total carbonate equivalent (g/kg^{-1}), cation exchange capacity ($cmolc/kg^{-1}$), gypsum content (g/kg^{-1}), and available water capacity] on anthrax outbreaks across protected areas. The total number of months anthrax was reported and the number of outbreaks for each wildlife area during the study period were used as dependent variables in a Poisson regression using a generalized linear model (GLM) framework. We performed univariate and multivariate relationships among dependent and independent variables. In a multivariate GLM, we selected the most important variables using AIC implemented in the MuMIn package (Barton and Barton, 2015).

Second, we examined the influence of NDVI metrics on anthrax outbreaks at the monthly level and at the annual level. To identify the possible drivers of the monthly timing of anthrax, we used data for Lake Nakuru National Park for which there were many months when anthrax was reported. We compared this with monthly timing of anthrax outbreaks for all wildlife

[1]http://ivfl-info.boku.ac.at

[2]https://www.isric.org/projects/world-inventory-soil-emission-potentials-wise

[3]https://data.isric.org/geonetwork/srv/eng/catalog.search;jsessionid=A3C1451B0F8C364D00CCF9A61ECDC69A#/metadata/4648929a-8031-49cc-9d56-9f3aeff2f8d9

TABLE 2 | Anthrax outbreaks from 2000 to 2019 in selected wildlife areas (NP, National Park and NR, National Reserve) in Kenya.

Focal wildlife area	Number of months	Number of outbreaks	Year, month (Season) for each outbreak	Wildlife species affected (minimum number)
Hell's gate NP	2	1	2006, January-February (Early dry)	Eland, *Taurotragus oryx* (3); African buffalo, *Syncerus caffer* (2); Plain's zebra, *Equus quagga* (3); Hartebeest, *Alcelaphus buselaphus* (3)
Lake Nakuru NP	12	5	2019, March–May (Early wet)	African buffalo, *S. caffer* (145)
			2017, July (Early dry)	African buffalo, *S. caffer* (2)
			2015, June-September (Early dry)	African buffalo, *S. caffer* (669), black rhinoceros, *Diceros bicornis* (5), white rhinoceros, *Ceratotherium simum* (3), Rothschild's giraffe, *Giraffa camelopardalis rothschildi* (1), eland, *T. oryx* (4), impala, *Aepyceros melampus* (4), Thomson's gazelle, *Eudorcas thomsonii* (2), warthog, *Phacochoerus africanus* (1), waterbuck, *Kobus ellipsiprymnus* (1)
			2006, January–March (Early dry)	Thomson's gazelle, *E. thomsonii* (13), plain's zebra, *E. quagga* (2), African buffalo, *S. caffer* (1), eland, *T. oryx* (1)
			2001, April (Early wet)	Hippo, *Hippopotamus amphibius* (1), white rhinoceros, *C. simum* (1)
Mwea NR	3	1	2011, May-July (Late wet)	Rothschild's giraffe, *G. c. rothschildi* (11), Lesser kudu, *Tragelaphus imberbis* (1)
Nairobi NP	7	5	2001, June (Early dry)	African buffalo, *S. caffer* (1)
			2005, May (Late wet)	Black rhinoceros, *D. bicornis* (1)
			2005, December (Late wet)	Eland, *T. oryx* (1)
			2006, June–July (Early dry)	African buffalo, *S. caffer* (2), black rhinoceros, *D. bicornis* (2)
			2017, April (Early wet)	African buffalo, *S. caffer* (3)
Sibiloi NP	1	1	2006, January (Early dry)	Plain's zebra, *E. quagga* (3)
Tsavo East NP	0	0	NA	NA
Tsavo West NP	2	1	2019, August–September (Late dry)	African buffalo, *S. caffer* (5)

TABLE 3 | Average values of selected edaphic covariates of the focal wildlife areas (NP, National Park; NR, National Reserve) in Kenya.

Focal wildlife area	pH in aqueous media	Total organic carbon (g/kg^{-1})	Total nitrogen (g/kg^{-1})	Total carbonate equivalent (g/kg^{-1})	Total available water capacity (vol.%)	Gypsum (g/kg^{-1})	Cation exchange capacity ($cmolc/kg^{-1}$)
Hells' Gate NP	7.41	6.44	0.87	79.14	19.79	0.013	33.86
Lake Nakuru NP	6.61	18.68	1.54	8.08	11.47	0.031	21.17
Mwea NR	6.05	14.44	1.35	1.90	9.14	0.175	20.50
Nairobi NP	6.81	13.59	1.27	5.71	11.86	0.429	32.58
Sibiloi NP	7.84	9.05	0.78	55.73	14.15	0.191	24.99
Tsavo East NP	6.23	9.37	0.98	8.85	9.62	0.105	15.72
Tsavo West NP	5.90	11.47	1.13	2.63	8.91	0.142	16.96

TABLE 4 | Average values of NDVI metrics across focal wildlife areas (NP, National Park; NR, National Reserve) in Kenya from 2000–2019.

Focal wildlife areas	Mean NDVI.	Extreme NDVI deviation	Spatial heterogeneity	Temporal heterogeneity	Mean deviation from long-term average
Hell's Gate NP	0.477 ± 0.054	0.070 ± 0.026	0.061 ± 0.005	0.050 ± 0.014	−1.30 – 19 ± 0.054
Lake Nakuru NP	0.574 ± 0.056	0.087 ± 0.025	0.117 ± 0.029	0.070 ± 0.026	−5.00 – 11 ± 0.056
Mwea NR	0.525 ± 0.043	0.054 ± 0.016	0.087 ± 0.010	0.071 ± 0.017	1.50 – 19 ± 0.043
Nairobi NP	0.468 ± 0.049	0.077 ± 0.024	0.047 ± 0.004	0.062 ± 0.025	−1.00 – 10 ± 0.049
Sibiloi NP	0.162 ± 0.022	0.032 ± 0.013	0.042 ± 0.007	0.026 ± 0.013	−1.35 – 06 ± 0.022
Tsavo East NP	0.353 ± 0.031	0.057 ± 0.017	0.084 ± 0.010	0.071 ± 0.016	−5.00 – 11 ± 0.031
Tsavo West NP	0.388 ± 0.034	0.073 ± 0.019	0.087 ± 0.007	0.073 ± 0.018	−4.74 – 05 ± 0.034

areas. The presence of anthrax cases reported in a month was used as a binary dependent variable and monthly NDVI metrics, including monthly NDVI, spatial NDVI heterogeneity, monthly NDVI deviation from a 20-year mean were used as dependent variables. Additionally, we used lagged NDVI variables above (values from the previous month) as independent variables. We performed both univariate and multivariate relationships using a logistic regression in a GLM framework for Lake Nakuru NP and a generalized mixed model (GLMM) framework for all wildlife areas combined. For GLMM, wildlife area ID was entered as

a random effect and edaphic variables were also incorporated in a set of multivariate analyses. The best temporal predictors for monthly timing of anthrax in Lake Nakuru and all wildlife areas combined were selected using the AIC implemented in the MuMIn package in R software for statistical computing (Barton and Barton, 2015).

At the annual level, we used the number of months that anthrax deaths were recorded each year in a wildlife area as a dependent variable. We made this aggregation for two reasons: (1) to reduce data sparseness and enhance model stability and convergence and (2) it is a better proxy for intensity or size of outbreak in wildlife where mortality estimates are not reliable indicator of intensity, but the duration of an outbreak is a better indicator of the intensity. As independent variables, we obtained annual means for spatial NDVI heterogeneity, NDVI, and monthly deviations from 20-year means for each month and the annual standard deviation of NDVI for annual heterogeneity. We used a Poisson regression under a generalized linear mixed model framework (GLMM) with a log link function for statistical analysis with the number of months in a year when anthrax occurred as a dependent variable. Wildlife area ID was incorporated into the model as a random effect. All GLMMs were performed using the glmmTMB package in the R software for statistical computing (Brooks et al., 2017). Conditional effects of covariates were visualized using the R package ggeffects (Lüdecke, 2018). We also tested the effect of edaphic factors, total organic carbon (g/kg^{-1}), total nitrogen (g/kg^{-1}), pH, lime, or total carbonate equivalent (g/kg^{-1}), cation exchange capacity ($cmolc/kg^{-1}$), gypsum content (g/kg^{-1}), and available water capacity and annual NDVI metrics as independent covariates and number of months anthrax is reported in a year as a dependent variable.

In all multivariate analyses undertaken above, we tested for collinearity as it can lead to unstable parameter estimates and inaccurate variances which affects confidence intervals, hypothesis tests and model selection in a multivariable model (Shen and Gao, 2008). To address this, we took the following steps. First, we checked for multicollinearity among covariate using the performance package in R (Lüdecke et al., 2021) and confirmed that multicollinearity was pervasive (**Supplementary Table 1**). Second, we performed univariate analyses with a single covariate using maximum likelihood estimation and ranked the importance of our covariates using Akaike Information Criteria (AIC). Thirdly, we used the MuMIn package (Barton and Barton, 2015) to compare all possible permutation of independent variables and selected the best model based on AIC.

All AIC model selection details are provided in the **Supplementary Material 1**.

RESULTS

Anthrax Outbreaks, Edaphic Factors and Normalized Difference Vegetation Index Variables in Focal Wildlife Areas

Fourteen anthrax outbreaks were recorded during the study period from 2000 to 2019 in six of the seven focal wildlife areas. The average number of months per outbreak was 1.92 ± 0.97. The longest outbreaks lasted 3–4 months but most outbreaks did not last more than a month (**Table 2**). LNNP and NNP recorded the highest number of outbreaks at five outbreaks each, during the years examined (**Table 2**). TWNP, HGNP, SNP, and MNR experienced a single outbreak each while TENP did not experience an outbreak during the years examined. However, anthrax is known to have occurred in TENP and TWNP in 1996. Most anthrax outbreaks (71% or 10/14) occurred at the beginning of the dry season or end of the wet season. In terms of years, 2006 had a very widespread occurrence of anthrax outbreaks, occurring in four of seven wildlife areas considered in this study (**Table 2**). Several species suffered

TABLE 5 | The influence of selected edaphic variables on the number of months and the number of anthrax outbreaks in focal wildlife areas of Kenya 2000–2019.

Selected edaphic variables	Number of months					Number of outbreaks				
	Estimate	*SE*	*z*-value	*P*-value	AIC	Estimate	*SE*	*z*-value	*P*-value	AIC
Intercept	−3.094	1.221	−2.534	0.0113	32.40	−2.628	1.526	−1.722	0.0851	28.55
Total nitrogen	3.578	0.899	3.978	0.0001		2.728	1.157	2.357	0.0184	
Intercept	−1.596	0.814	−1.961	0.0499	32.61	−1.610	1.051	−1.532	0.1256	28.31
Total organic carbon	0.218	0.052	4.167	<0.0001		0.175	0.070	2.492	0.0127	
Intercept	1.625	0.228	7.125	<0.0001	47.84	0.924	0.320	2.891	0.0039	33.42
Total carbonate equivalent	−0.015	0.009	−1.694	0.0903		−0.012	0.012	−1.048	0.2944	
Intercept	0.743	0.726	1.023	0.3060	50.71	−0.594	1.053	−0.564	0.5730	32.99
Cation exchange capacity	0.025	0.028	0.888	0.3750		0.052	0.039	1.325	0.1850	
Intercept	1.645	0.702	2.342	0.0192	51.29	0.779	0.956	0.815	0.4150	34.73
Total available water capacity	−0.025	0.057	−0.431	0.6662		−0.007	0.076	−0.093	0.9260	
Intercept	1.976	1.963	1.007	0.3140	51.38	0.054	2.677	0.020	0.9840	34.68
pH	−0.094	0.294	−0.320	0.7490		0.095	0.396	0.241	0.8100	
Intercept	1.276	0.306	4.177	<0.0001	51.39	0.263	0.453	0.581	0.5610	33.10
Gypsum	0.464	1.456	0.318	0.7500		2.426	1.817	1.335	0.1820	

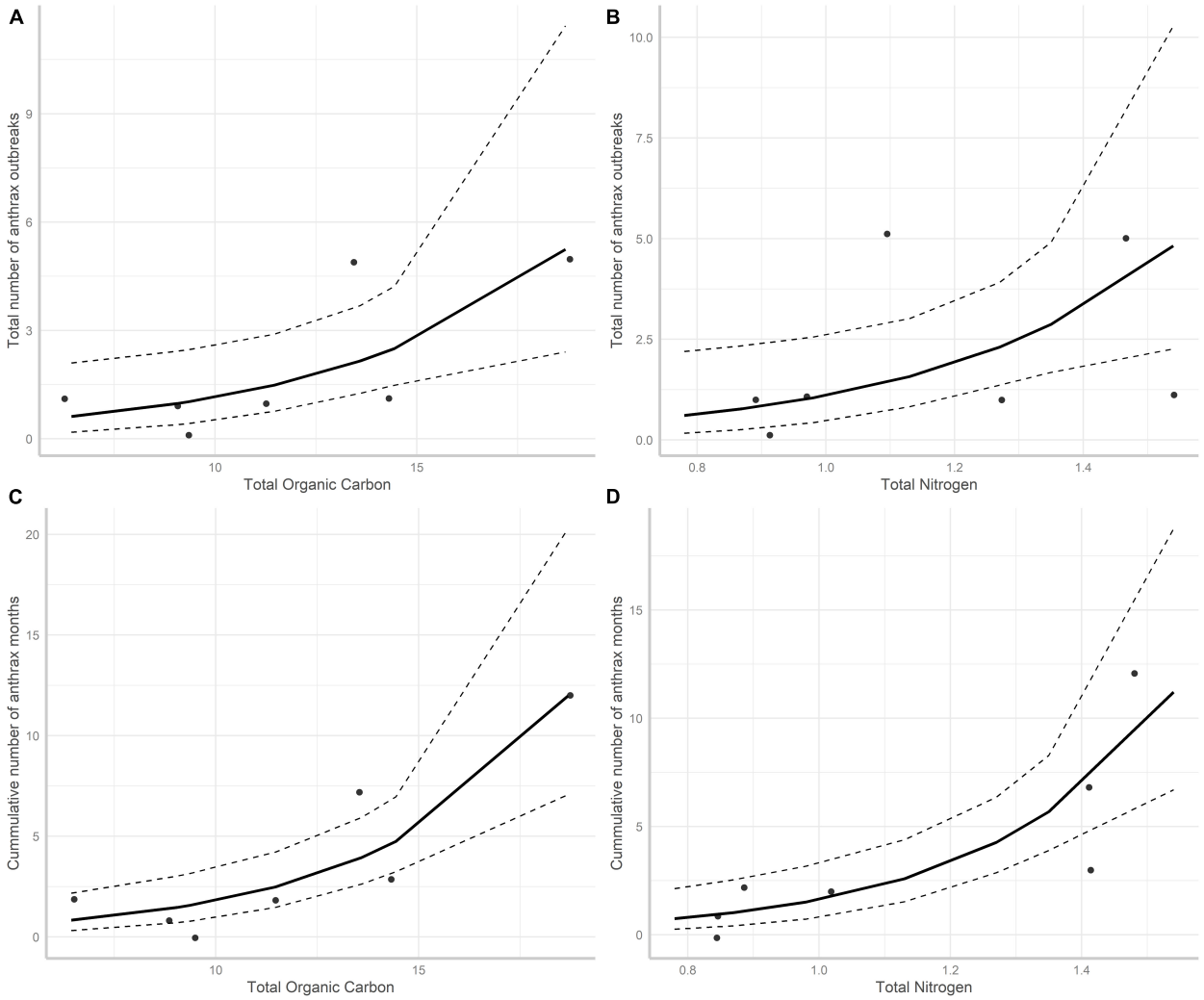

FIGURE 2 | The relationships between total organic carbon (g/kg^{-1}), total nitrogen (g/kg^{-1}), with the total number of anthrax outbreaks **(A,B)** and cumulative total months anthrax mortality was recorded **(C,D)** in Kenya's wildlife focal areas. The predicted values and their confidence intervals were obtained from a Poisson GLM analysis.

anthrax mortality. The dominant species documented in terms of frequency and numbers are the African buffalo (*Syncerus caffer* Sparrman), Thomson's gazelle (*Eudorcas thomsonii* Günther), Rothchild's giraffe (*Giraffa camelopardalis rothschildi* Lydekker), and common zebra (*Equus quagga burchellii* Gray). Others documented include white rhinoceros (*Ceratotherium simum*, Burchell), black rhinoceros (*Diceros bicornis* Linn.), and lesser kudu (*Tragelaphus imberbis* Blyth) (**Table 2**).

The average soil pH across most wildlife areas was greater than 6.0 except for TWNP (**Table 3**). The average organic carbon content across all wildlife areas ranged from 6.44 g/kg of soil in HGNP to 18.68 g/kg of soil in LNNP while Nitrogen content ranged from 0.78 g/kg of soil in SNP to 1.58 g/kg of soil in LNNP (**Table 3**). Lime content measured as total carbonate equivalent was highest in HGNP and SNP at 79.14 and 55.73 respectively and lowest in MNR and TWNP (**Table 3**). Cation exchange capacity (cmolc/kg^{-1}), and available water capacity were highest in HGNP and lowest in TENP and TWNP respectively.

The wildlife areas in Kenya were typically dry with mean monthly NDVI varying from 0.574 in LNNP to a low of 0.162 in SNP over 20 years (**Table 4**). The mean of monthly standard deviation was highest in LNNP at 0.117 and lowest in NNP at 0.047 and SNP at 0.042 (**Table 4**).

Variables Influencing Spatial Variation in Anthrax Outbreaks in Focal Wildlife Areas

Univariate GLM models revealed that the total number of recorded anthrax outbreaks in each wildlife area was positively influenced by total organic soil carbon and total organic nitrogen (**Table 5** and **Figures 2A,B**). Similarly, the number of cumulative month when anthrax outbreaks were reported for each wildlife

TABLE 6 | Univariate and multivariate logistic models showing the influence of NDVI metrics on the monthly occurrence of anthrax outbreaks in wildlife from LNNP, Kenya

NDVI covariates	Estimate	*SE*	*Z*-value	Probability	AIC
Univariate models					
Intercept	−3.152	0.352	−8.964	<0.001	
Spatial NDVI heterogeneity	0.664	0.293	2.269	0.0233	93.9
Intercept	−3.167	0.357	−8.861	<0.001	
[1]Lagged spatial NDVI heterogeneity	0.695	0.297	2.338	0.0194	93.4
Intercept	−2.985	0.307	−9.736	<0.001	98.2
NDVI deviation from a 20-year mean for each month	0.292	0.269	1.086	0.277	
Intercept	−3.018	0.316	−9.566	<0.001	
[1]Lagged NDVI deviation from a 20-year mean for each month	0.403	0.260	1.550	0.121	97.0
Intercept	−2.960	0.300	−9.856	<0.001	98.9
Monthly NDVI	−0.183	0.278	−0.658	0.511	
Intercept	−3.010	0.314	−9.597	<0.001	
[1]Lagged monthly NDVI	−0.380	0.261	−1.454	0.146	97.2
Best multi-variate models					
Intercept	−3.319	0.414	−8.028	<0.001	93.2
[1]Lagged spatial NDVI heterogeneity	0.756	0.332	2.274	0.023	
[1]Lagged NDVI deviation from a 20-year mean for each month	0.466	0.308	1.514	0.130	

[1]*Variables are lagged by a month.*

area was also positively influenced by total organic carbon and organic nitrogen in the soil (**Table 5** and **Figures 2C,D**). All other edaphic variables were not statistically significant (**Table 5**). No multivariate model was selected as the most parsimonious in multivariate model selection of the edaphic factors affecting both the total number of months and number of anthrax outbreaks in wildlife areas. Instead, total soil organic carbon was the best predictor, but nitrogen was also supported (Number of months ΔAIC = 0.28, Number of outbreaks; ΔAIC = 0.38). Total soil organic carbon and total nitrogen were highly correlated (r = 0.95, t = 6.98, df = 5, P = 0.0009, **Supplementary Table 1**) when the number of months (VIF = 22.78) and number of outbreaks (VIF = 18.36) were used as dependent variables.

Factors Influencing Monthly Timing of Anthrax Outbreaks in Lake Nakuru National Park and All Focal Wildlife Areas

Univariate GLM analyses revealed that monthly occurrence (timing) of anthrax mortality in LNNP was positively associated with spatial heterogeneity in NDVI from the current and previous months (**Table 6** and **Figure 2**). Monthly NDVI, and monthly NDVI deviations from 20-year mean for each month both from the current and previous months did not influence the occurrence of anthrax mortality in LNNP (**Table 6**). However, in a multivariable GLM analyses, both spatial NDVI heterogeneity and monthly NDVI deviations from a 20-year mean for each month from the previous months were included as important predictors of the monthly timing of anthrax mortality in LNNP (**Table 6** and **Figures 3A,B**).

Moreover, multivariate GLMM analyses and model selection on anthrax mortality in all focal wildlife areas in Kenya revealed that monthly NDVI deviation from 20-year mean for each month and spatial NDVI heterogeneity both from the previous month positively influenced monthly occurrence of mortality from anthrax (**Table 7** and **Figures 3C,D**). Combined models including edaphic variables and NDVI metrics indicated that the previous month's spatial NDVI heterogeneity, and NDVI deviation from long-term average, total organic carbon and cation exchange capacity influenced the monthly occurrence of wildlife mortality in wildlife focal areas (**Table 8**).

Influence of Normalized Difference Vegetation Index-Metrics and Edaphic Variables on the Annual Intensity of Anthrax in Focal Wildlife Areas

Univariate GLMM analyses revealed statistically significant positive relationship between the number of months when anthrax mortality was reported per year and mean spatial heterogeneity in NDVI among NDVI metrics (**Table 9**). There was no multivariate model more parsimonious than the best univariate model. Among edaphic factors, total organic carbon in the soils and cation exchange capacity were the most parsimonious predictors (**Table 10**). Alkalinity or pH, gypsum, available water capacity and total carbonate equivalent were not significantly correlated with occurrence and duration of anthrax outbreaks (**Table 10**).

Multivariable GLMM analyses and model selection of combined edaphic variables and NDVI metrics, indicated that spatial NDVI heterogeneity, total organic carbon and cation exchange capacity were the best predictors of the number of months anthrax mortality was recorded in a year (**Table 11**).

DISCUSSION

In this study, we found a statistically significant positive relationship between monthly occurrence of anthrax (timing) and monthly NDVI deviation from a 20-year mean for each calendar month. These findings suggest that rainfall fluctuation is an important driver of anthrax outbreaks in endemic areas as rainfall has a major influence on NDVI variability in East Africa (Kalisa et al., 2019; Dagnachew et al., 2020). Specifically, anthrax mortality in wildlife was associated with above average rainfall as inferred from a positive relationship between anthrax occurrence and the previous month's NDVI deviations from each month 20-year mean. These results are consistent with empirical data showing that anthrax outbreaks occur following heavy rains (Hampson et al., 2011; Maksimović et al., 2017; Rume et al., 2020) and in locations with high NDVI variability (Chikerema et al., 2013). For example, studies in the Serengeti Ecosystem observed an association between anthrax outbreaks in wildlife with cumulative extremes in weather conditions; two outbreaks followed heavy rains, while two other outbreaks occurred during

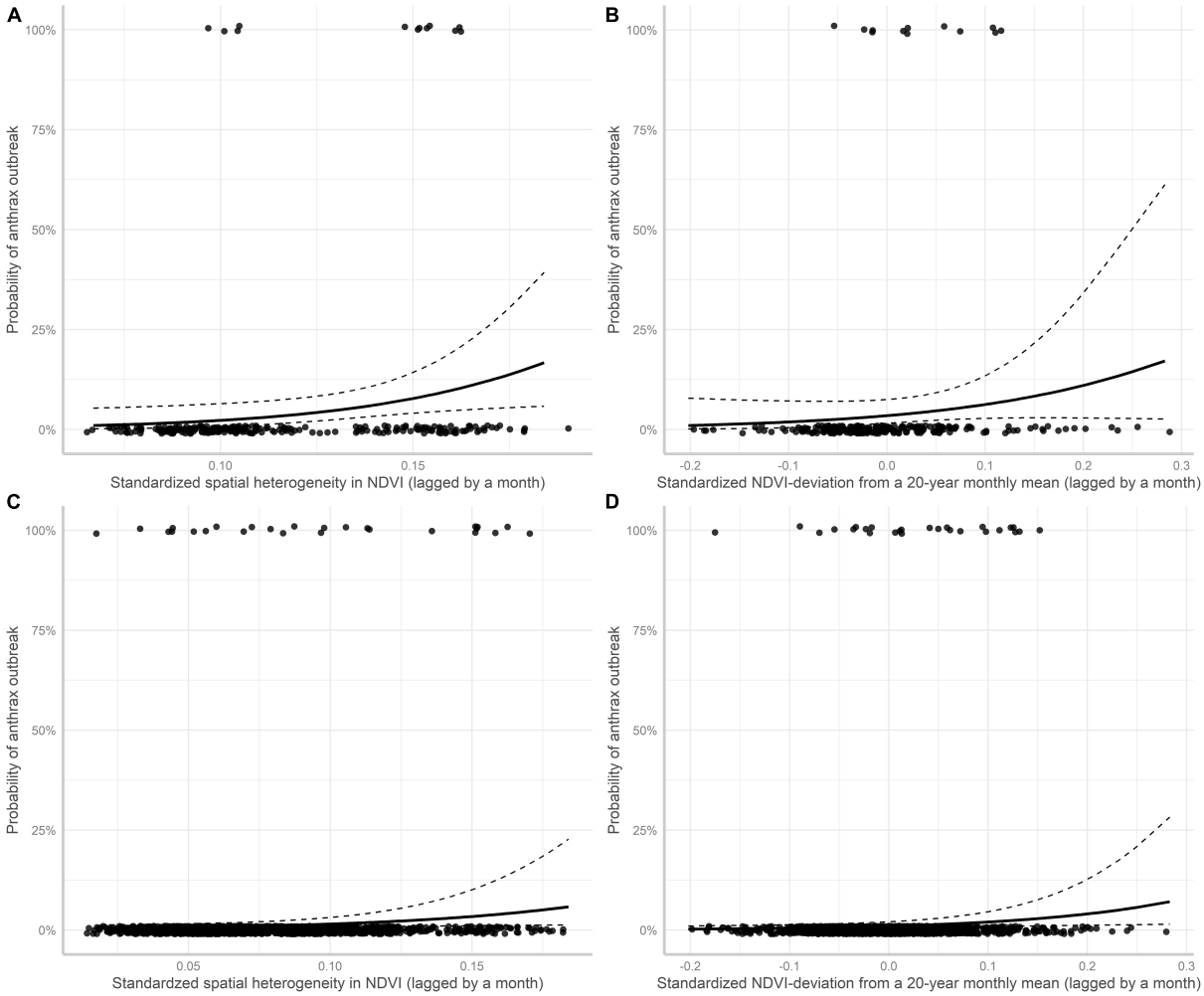

FIGURE 3 | The influence of NDVI metrics on the probability of monthly anthrax mortality in Lake Nakuru NP **(A,B)** and combined focal wildlife areas **(C,D)**. The predicted values and their confidence intervals are from a GLM **(A,B)**, and GLMM **(C,D)** binomial regressions respectively.

prolonged dry periods (Hampson et al., 2011). Anthrax niche model for Zimbabwe indicated that large variance in NDVI was positively correlated with anthrax occurrence in some locations in that country (Chikerema et al., 2013).

However, anthrax outbreaks are often associated with extreme weather events but the mechanism by which this enhances outbreaks is unclear. One possible hypothesis is that extreme weather events modulate multiple environmental factors as well as ecological dynamics of soil dwelling organisms known to amplify or inhibit anthrax multiplication in the soil. Experimental analyses have shown that protists particularly amoebas contribute to the persistence and amplification of *B. anthracis* in natural environments (Dey et al., 2012). These studies have revealed that spores germinate within phagosomes of amoebas, where vegetative bacilli multiply and sporulate in the extracellular milieu following the demise of amoeba. Interestingly, the most important factor driving protists abundance is precipitation (Anderson, 2000). Specifically, precipitation after a prolonged drought has been shown to cause a 20-fold increase in the abundance of naked amoebae few days after rainfall (Clarholm, 1981). This is because protists essentially depend on the water layer connecting soil pores to move, feed and multiply (Rutherford and Juma, 1992; Ritz and Young, 2011) but when soils are moist, there is strong chemical warfare between abundant bacteria and protist grazers (Matz and Kjelleberg, 2005; Bonkowski and Clarholm, 2012; Jousset, 2012) and drought inactivates this inhibitory effect (Foissner, 1987). In support, most of the anthrax outbreaks we observed occurred at the beginning of the dry season, when conditions may be favorable for protists.

Monthly timing of anthrax in LNNP and all focal wildlife areas or the number of months in a year when there was an anthrax mortality were positively related to the spatial heterogeneity in NDVI. This result suggests that diversity and heterogeneity in vegetation cover or species richness has an important influence on anthrax outbreaks. Spatial and temporal heterogeneity in vegetation cover can influence the distribution and migratory behavior of herbivores (Winnie et al., 2008; Peters et al., 2017;

Duparc et al., 2020), enhancing aggregation in greener habitats. Such aggregations can lead to overgrazing of rich patches and an increased risk of anthrax infection (Gainer, 1987). For example, Turner et al. (2014) found improved vegetation vigor on zebra carcass sites and persistence of *Bacillus anthracis* on grasses for up to 2 years after death, thereby demonstrating

TABLE 7 | Univariate and the best multivariate NDVI models from GLMM analyses showing their influence on monthly occurrence of anthrax in focal wildlife area, Kenya.

NDVI covariates	Estimate	*SE*	*z*-value	Probability	AIC
Univariate models					
Intercept	−4.512	0.389	−11.592	<0.0001	
Spatial NDVI heterogeneity	0.419	0.217	1.932	0.0534	261.3
Intercept	−4.513	0.389	−11.628	<0.0001	
[1]Lagged spatial NDVI heterogeneity	0.478	0.216	2.215	0.027	260.0
Intercept	−4.555	0.434	−10.501	<0.0001	
NDVI deviation from a 20-year mean	0.296	0.166	1.782	0.0748	261.6
Intercept	−4.578	0.430	10.641	<0.0001	
[1]Lagged NDVI deviation a 20-year mean for each month	0.390	0.162	2.412	0.0159	258.9
Intercept	−4.524	0.453	−9.996	<0.0001	
Monthly NDVI	−0.030	0.376	−0.080	0.9360	264.6
Intercept	4.565	−0.504	−9.063	<0.0001	
[1]Lagged monthly NDVI	−0.214	0.383	−0.559	0.576	264.1
Best multivariate models					
Intercept	−4.586	0.374	−12.257	<0.0001	
[1]Lagged spatial NDVI heterogeneity	0.507	0.219	2.319	0.0204	
[1]Lagged NDVI deviation from 20-year average for each month	0.422	0.171	2.474	0.0134	256.2

[1]Variables are lagged by a month.

TABLE 8 | Best multivariate logistic model combining edaphic and NDVI-metric predictors of the monthly occurrence of anthrax in wildlife focal areas showing lagged and unlagged NDVI metrics.

NDVI and edaphic covariates	Estimate	*SE*	*Z*-value	Probability	AIC
Edaphic covariates and lagged NDVI metrics					
Intercept	−4.737	0.304	−15.57	<0.0001	248.2
[1]Lagged spatial NDVI heterogeneity	0.467	0.235	1.986	0.0470	
[1]Lagged NDVI deviation from 20-year average for each month	0.380	0.163	2.333	0.0197	
Total organic carbon	0.700	0.265	2.642	0.0082	
Cation exchange capacity	0.771	0.311	2.477	0.0133	
Edaphic covariates and unlagged NDVI metrics					
Intercept	−4.697	0.299	−15.689	<0.0001	251.7
Spatial NDVI heterogeneity	0.398	0.233	1.707	0.0878	
NDVI deviation from 20-year average	0.286	0.167	1.715	0.0863	
Total organic carbon	0.753	0.265	2.841	0.0045	
Cation exchange capacity	0.738	0.308	2.391	0.0168	

[1]Variables are lagged by a month.

TABLE 9 | Univariate NDVI-metric Poisson models and their influence on the number of months in a year (intensity) anthrax was reported from wildlife areas in Kenya.

NDVI covariates	Estimate	*SE*	Z-value	Probability	AIC
Univariate models					
Intercept	−2.066	0.376	−5.499	<0.0001	151.8
Spatial NDVI heterogeneity	0.571	0.205	2.781	0.0054	
Intercept	−2.054	0.407	−5.045	0.0001	156.7
Temporal heterogeneity	0.299	0.198	1.506	0.1320	
Intercept	−1.984	0.316	−6.279	<0.0001	157.1
Mean NDVI (Annual)	0.551	0.366	1.505	0.1320	
Intercept	−2.054	0.429	−4.783	<0.0001	158.1
Mean NDVI deviation from a 20-year mean	0.146	0.163	0.896	0.3700	

There was no multivariate model better than the best univariate model-based on AIC.

TABLE 10 | Univariate models and the best multivariate model for the influence of edaphic factors on the number of months anthrax mortality occurred in a year in focal wildlife areas.

Independent covariates	Estimate	*SE*	*Z*-value	Probability	AIC
Univariate models					
Intercept	−2.092	0.277	−7.554	<0.0001	149.3
Total nitrogen	0.925	0.234	3.952	<0.0001	
Intercept	−2.053	0.264	−7.787	<0.0001	149.4
Total organic carbon	0.844	0.203	4.152	<0.0001	
Intercept	−2.039	0.400	−5.095	<0.0001	158.0
Total carbonate equivalent	−0.383	0.404	−0.949	0.3430	
Intercept	−2.062	0.433	−4.758	<0.0001	158.4
Cation exchange capacity	0.268	0.416	0.644	0.5190	
Intercept	−2.044	0.429	−4.763	<0.0001	158.8
pH	−0.089	0.427	−0.209	0.8350	
Intercept	−2.043	0.429	−4.765	<0.0001	158.8
Total available water capacity	−0.067	0.419	−0.159	0.8740	
Intercept	−2.050	0.433	−4.735	<0.0001	158.8
Gypsum	0.102	0.378	0.269	0.7880	
Best multivariate model					
Intercept	−2.165	0.293	−7.386	<0.0001	147.6
Cation exchange capacity	0.491	0.255	1.927	0.0540	
Total organic carbon	0.984	0.232	4.235	<0.0001	

TABLE 11 | The best multivariate GLMM model for the influence of edaphic variables and NDVI metrics on the number of months an anthrax outbreak was recorded in a year in Kenya's focal wildlife areas.

Independent covariates	Estimate	*SE*	*Z*-value	Probability
Intercept	−2.222	0.299	−7.426	<0.0001
Spatial NDVI heterogeneity	0.518	0.205	2.526	0.0115
Total organic carbon	0.644	0.265	2.429	0.0151
Cation exchange capacity	0.861	0.314	2.744	0.0061

that animal carcasses enhance local vegetation heterogeneity and attract grazing hosts to *B. anthracis* hotspots and thus increasing transmission risk and potential for anthrax outbreaks. In dry areas, presence of certain species of shrubs was found to be correlated with high densities of *Acanthamoeba* (Rodriguez-Zaragoza et al., 2005), protist species speculated to amplify anthrax presence in the soil based on experimental results (Dey et al., 2012). Similarly, areas covered with vegetation are known to be rich in protists compared to areas devoid of vegetation (Fernández, 2015). A more direct association between vegetation and *B. anthracis* proliferation has been demonstrated experimentally using simple grass plant-soil model system, that have revealed that *B. anthracis* can survive as a saprophyte outside of a mammalian host, germinating and growing characteristic long filaments on and around plant roots (Saile and Koehler, 2006). Another experiment using a local, virulent strain of *B. anthracis* in an enclosure within a grassland savanna, revealed that *B. anthracis* increased the rate of establishment of a native grass (*Enneapogon desvauxii*) by 50% and that grass seeds exposed to blood attained heights that were 45% taller than controls (Ganz et al., 2014). Interactions between *B. anthracis* and plants may result in increased host grazing and subsequently increased transmission to hosts. These studies agree with observations that anthrax outbreaks are positively correlated with NDVI, a measurement of vegetation growth vigor. For example in temperate and arctic climates, anthrax epizootics are associated with hot-dry summers preceded by heavy spring rain (Blackburn and Goodin, 2013). Monitoring of annual trajectories of vegetation indices has revealed that early green-up in the spring in contrast to later and less intense spring green-up signals the occurrence of anthrax epizootic in the approaching summer.

We observed a statistically significant positive association between the number of anthrax outbreaks, or the total number of months anthrax was during the study period and the nitrogen, organic carbon content and cation exchange capacity of the soil. These results confirm previous findings that the geographical localization of anthrax outbreaks are associated with specific soil factors, specifically organic matter content (Hugh-Jones and Blackburn, 2009). Soil cation exchange capacity, CEC, is a measure of the soil's storage capacity for nutrients and is estimated from the sum of exchangeable major cations (Ca2+, Mg2+, K+, Na+) expressed in centimoles of positive charge per kilogram of soil [cmol(+)/kg]. Soil organic matter and clay content correlate with measures of soil cation exchange capacity, CEC (Peverill et al., 1999; Liang et al., 2006), because cations are attracted to the negatively charged sites in clay minerals and organic matter. Soils with high organic carbon content or high CEC content contribute to greater soil microbial abundance and diversity (Stotzky, 1966; Drenovsky et al., 2004; Docherty et al., 2015). Although pH is known to influence *B. anthracis* persistence, we did not find any influence perhaps because nearly all focal wildlife areas had pH $>$ 6.1. Locations with alkaline pH ($\geq$6.1), high soil moisture, and organic matter are conducive for the persistence and proliferation of *B. anthracis* spores, through repeated cycles of spore germination, vegetative cell outgrowth, and re-sporulation which can cause an overall increase in spore numbers and probability of an anthrax outbreak (West et al., 1985). Further support comes from a study in Badin district of Pakistan, which found a positive correlation between anthrax prevalence in soil with percent organic matter and calcium contents of soil with prevalence of *B. anthracis* (Mari et al., 2017). Similarly, a study in Minnesota indicate that soil calcium and magnesium concentrations, soil pH, and sand content are the most important properties for predicting soil suitability for *B. anthracis* (Nath and Dere, 2016). Although we found a spatial association of anthrax outbreaks with edaphic factors, we cannot entirely rule out the influence of non-edaphic spatial effects.

Empirical studies on anthrax outbreaks and their association with population dynamics of soil microbiota, plant diversity, and vegetation growth with climate variability and edaphic factors are urgently needed. This will enhance our understanding of the environmental ecology of *Bacillus anthracis* and will provide a more accurate and refined variables for the prediction of anthrax outbreaks in wildlife areas.

Our findings support previous research indicating that incidences of anthrax outbreaks are positively correlated with indices of climate variability. These have implications for re-emergence of anthrax under projected current climate change scenarios where climatic extremes in precipitation are predicted for the East African region including Kenya (Case, 2006; CDKN, 2014). Our findings also suggest soil factors can be used for spatial disease risk assessment across wildlife areas in Kenya to guide strategic anthrax surveillance and aid with timely vaccination of endangered species that are vulnerable to anthrax and susceptible to local extinction.

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

Ethical review and approval was not required for the animal study because we did not handle animals but used data collected during anthrax surveillance conducted by Kenya Wildlife Service, a government parastatal with the mandate to manage and protect wildlife.

AUTHOR CONTRIBUTIONS

VO and PC conceived the idea. VO, DN, and EK compiled the anthrax outbreak data. VAO, OL, and PC assembled the soil and NDVI data. All authors contributed equally in drafting, editing, and analysis of the 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/fevo.2021.643334/full#supplementary-material

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