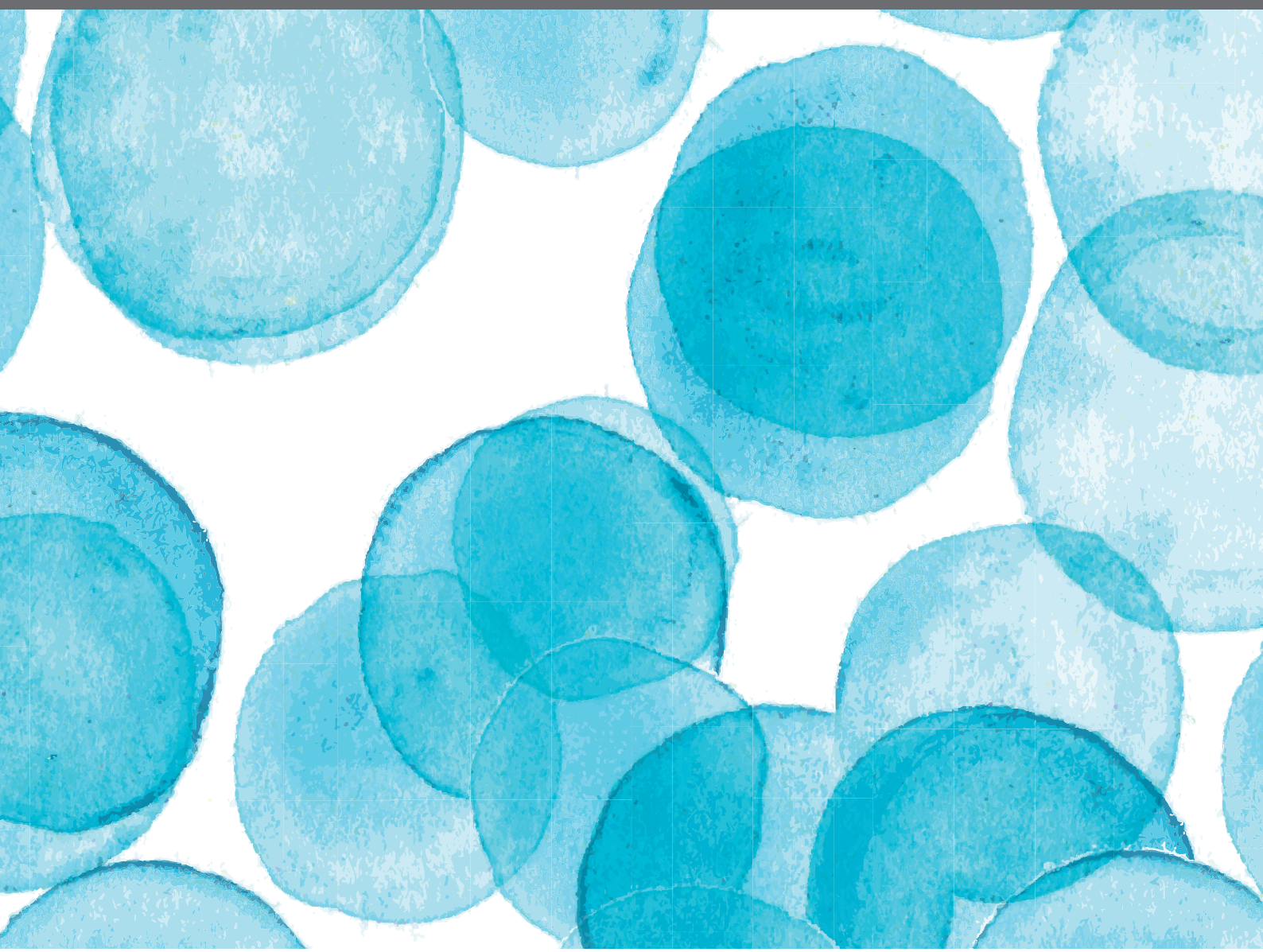
RESPIRATORY VIRUS INFECTION: RECENT ADVANCES

EDITED BY: Kelvin To, Ville Peltola and Shin-Ru Shih
PUBLISHED IN: Frontiers in Microbiology, Frontiers in Medicine and Frontiers in Public Health



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-862-8
DOI 10.3389/978-2-88963-862-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

RESPIRATORY VIRUS INFECTION: RECENT ADVANCES

Topic Editors:
Kelvin To, The University of Hong Kong, Hong Kong
Ville Peltola, Turku University Hospital, Finland
Shin-Ru Shih, Chang Gung University, Taiwan

Citation: To, K., Peltola, V., Shih, S.-R., eds. (2020). Respiratory Virus Infection: Recent Advances. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-862-8

Table of Contents

frontiers in Medicine

EDITORIAL
published: 09 June 2020
doi: 10.3389/fmed.2020.00257

Editorial: Respiratory Virus Infection: Recent Advances

Ville Peltola[1]*, *Shin-Ru Shih*[2] *and Kelvin Kai-Wang To*[3]

[1] Department of Paediatrics and Adolescent Medicine, Turku University Hospital and University of Turku, Turku, Finland, [2] Research Center for Emerging Viral Infections, Chang Gung University, Taoyuan, Taiwan, [3] Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong

Keywords: respiratory virus, influenza, epidemiology, pathogenesis, therapeutics, vaccines

Editorial on the Research Topic

Respiratory Virus Infection: Recent Advances

Respiratory virus infections cause an enormous disease burden both in children and in adults in all parts of the world. Influenza A and B viruses and respiratory syncytial virus (RSV) are responsible for the highest numbers of deaths and hospitalizations but other respiratory viruses, including rhinovirus, parainfluenza viruses, adenovirus, human bocavirus and coronaviruses, are also currently known to cause severe diseases in addition to mild upper respiratory tract infections. Furthermore, respiratory viruses of animal origin keep on emerging as novel human pathogens with epidemic or pandemic potential. Such viruses include avian influenza viruses and the Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS) coronaviruses.

Despite the high clinical importance of respiratory viruses, the mechanisms of pathogenicity, transmission and evolution are only partially known, and most respiratory virus infections lack effective treatment or prevention modalities. With this Research Topic we aimed to focus on these knowledge gaps. We sought to collect original research articles and focused reviews from leading experts all over the world on respiratory virus epidemiology, pathogenesis, diagnostics, therapeutics and vaccines. We acknowledge all authors who submitted high-quality manuscripts. We accepted nine papers including six original research articles and three reviews. Influenza viruses are well-presented in the Topic by five papers. Two articles focus on RSV and one each on rhinovirus and MERS coronavirus.

Seasonal influenza A and B virus epidemics globally cause up to 600,000 deaths per year. Influenza A viruses have an animal reservoir in avian species, which forms the basis for a permanent threat of pandemics. Vaccines and specific antiviral drugs against influenza are available but they have limitations including variable vaccine efficacies. McAuley et al. review the current knowledge on the structure of the influenza virus neuraminidase and link the structural features of this surface glycoprotein with the multiple functions of neuraminidase in virus infection and fitness. Guo et al. studied the genetic changes that occur in avian influenza A H5 viruses when they infect humans. Identification of the important steps in adaptation of these viruses to humans is needed for evaluating the pandemic risk related to avian influenza viruses.

National reporting of clinical microbiology results is an important part of surveillance for respiratory virus epidemics. Clinical microbiology services have recently been consolidated in many countries to a limited number of central laboratories. Van den Wijngaert et al. studied the effects of such laboratory merging on influenza surveillance in Belgium. They report benefits of the integration of whole genome sequencing of influenza viruses performed in centralized clinical microbiology laboratories into the public health surveillance system.

OPEN ACCESS

Edited and reviewed by:
Marc Jean Struelens, European Centre for Disease Prevention and Control (ECDC), Sweden

***Correspondence:**
Ville Peltola
ville.peltola@utu.fi

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *13 January 2020*
Accepted: *12 May 2020*
Published: *09 June 2020*

Citation:
Peltola V, Shih S-R and To KK-W (2020) Editorial: Respiratory Virus Infection: Recent Advances. Front. Med. 7:257. doi: 10.3389/fmed.2020.00257

Neuraminidase inhibitors are widely used for treatment (and sometimes prevention) of influenza infection, and they have demonstrated efficacy. However, new drugs are needed to reach better treatment results in vulnerable patient groups particularly in the scenario of developing antiviral resistance. Several new antiviral drugs against influenza have been developed or are under development, including polymerase inhibitors and monoclonal antibodies. These new drugs are reviewed in the paper by Principi et al..

Vaccines are the cornerstone in protection against influenza. However, currently used influenza vaccines have limitations and the process of production in eggs is vulnerable. Yamada et al. report preclinical results on the efficacy of a recombinant hemagglutinin protein vaccine candidate produced in human Expi293F cell culture. Interestingly, recombinant hemagglutinin protein of H1N1pdm09 virus protected mice not only against H1N1pdm09 virus but also against avian H5N1 influenza virus.

RSV occurs in yearly epidemics that cause high number of severe lower respiratory tract infections in young infants and also in the elderly. There is variation in the timing of RSV epidemics between geographic areas. Recognition of the onset of the epidemic is important in order to accurately start adequate control measures, particularly passive immunization of high-risk infants by monoclonal antibodies such as palivizumab. Yamagami et al. developed a mathematical model that was able to detect the onset of RSV season in different parts of Japan with various climate conditions. Another interesting study on RSV by Hong et al. focused on enhancing antiviral activity of alveolar macrophages by administration of *Bacillus subtilis* spores in a mouse model. Their findings may help in development of novel RSV therapeutics in the future.

Rhinovirus is frequently detected in children with pneumonia, but its clinical significance remains unclear. Hartiala et al. compared rhinovirus-positive and rhinovirus-negative children hospitalized with community-acquired pneumonia. They found that the clinical picture was rather similar in both groups. Premature birth associated with rhinovirus-positive pneumonia. More research is needed on the pathogenic role of rhinovirus in pneumonia.

Skariyachan et al. reviewed the pathogenesis, animal models and therapeutics for MERS coronavirus infections. This review is timely not only for the continuing identifications of human infections by MERS coronavirus in the Middle East but also because of the potential of other novel coronaviruses to jump from animals to humans. Most recently, a novel beta coronavirus has led to an outbreak of respiratory tract infection in Wuhan, China.

We believe that the articles published in this Research Topic provide important new knowledge on respiratory virus infections. They identify areas of lacking knowledge that need to be addressed by future research. We hope that this collection of articles is beneficial for the readers and advances the field.

AUTHOR CONTRIBUTIONS

VP drafted the editorial and all authors revised it.

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

frontiers in Microbiology

REVIEW
published: 29 January 2019
doi: 10.3389/fmicb.2019.00039

Influenza Virus Neuraminidase Structure and Functions

Julie L. McAuley[1], Brad P. Gilbertson[1], Sanja Trifkovic[1,2], Lorena E. Brown[1] and Jennifer L. McKimm-Breschkin[1]*

[1]*Department of Microbiology and Immunology, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia,* [2]*Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis, TN, United States*

With the constant threat of emergence of a novel influenza virus pandemic, there must be continued evaluation of the molecular mechanisms that contribute to virulence. Although the influenza A virus surface glycoprotein neuraminidase (NA) has been studied mainly in the context of its role in viral release from cells, accumulating evidence suggests it plays an important, multifunctional role in virus infection and fitness. This review investigates the various structural features of NA, linking these with functional outcomes in viral replication. The contribution of evolving NA activity to viral attachment, entry and release of virions from infected cells, and maintenance of functional balance with the viral hemagglutinin are also discussed. Greater insight into the role of this important antiviral drug target is warranted.

Keywords: influenza, neuraminidase, hemagglutinin, sialic acid, infection, HA:NA balance, NA

OPEN ACCESS

Edited by:
Ville Peltola,
Turku University Hospital, Finland

Reviewed by:
Kirsty Renfree Short,
The University of Queensland, Australia
Takashi Irie,
Hiroshima University, Japan

****Correspondence:***
Julie L. McAuley
jmcauley@unimelb.edu.au

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *25 October 2018*
Accepted: *10 January 2019*
Published: *29 January 2019*

Citation:
McAuley JL, Gilbertson BP, Trifkovic S, Brown LE and McKimm-Breschkin JL (2019) Influenza Virus Neuraminidase Structure and Functions. Front. Microbiol. 10:39. doi: 10.3389/fmicb.2019.00039

INTRODUCTION

Influenza viruses are members of the family *Orthomyxoviridae*, a group of enveloped viruses containing a segmented negative-sense single-stranded RNA genome. Three main types of influenza viruses (A, B, and C) infect humans, with influenza A and B viruses causing significant morbidity and mortality annually. While yearly outbreaks of influenza in the human population induce the development of neutralizing antibody against common circulating strains, new strains arise constantly in a process referred to as antigenic drift. This occurs due to errors in copying of the genome by the viral polymerase and selection of those mutants with changes in the antigenic sites that allow escape from neutralization [reviewed in (Wilson and Cox, 1990)].

The influenza virus major surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA) dominate the virion surface and form the main targets for these neutralizing antibodies. In addition to the mutations that arise due to antigenic drift, the HA and NA of influenza A viruses (IAVs) can exist in different forms. Based on HA and NA antigenicity using serologic tests with hyperimmune sera, there have been a total of 16 HA (H1-16) and 9 NA (N1-9) subtypes identified in birds. These are expressed in numerous combinations of viruses isolated from aquatic avian species, and an additional two combinations, H17N10 and H18N11, have been identified in bats (Tong et al., 2012, 2013). IAVs of subtypes H1N1 and H3N2 are endemic in humans, circulating constantly within the population and giving rise to seasonal outbreaks. Zoonotic transmission from birds and swine of viruses with certain other HA and NA combinations (e.g. H5N1, H7N9, and H9N2) sporadically occurs; however, these viruses need to accrue additional mutations in order to gain the ability to transmit readily between humans (Cox and Subbarao, 2000; Harris et al., 2017). If viruses bearing a novel HA subtype,

which is often accompanied by a novel NA, gain the ability to transmit between humans, the potential exists to cause another pandemic, as no one will have relevant pre-existing neutralizing antibody immunity to this novel virus.

The functions of both HA and NA involve interaction with sialic acid, a terminal structure bound to underlying sugar residues expressed by glycoproteins or glycolipids at the cell surface (Gottschalk, 1958; Palese et al., 1974). The binding of HA to sialic acids presented by cellular receptors triggers cell entry by clathrin-mediated endocytosis, although other endocytic routes, including micropinocytosis, may also be used [reviewed in (Lakadamyali et al., 2004)]. A major function of NA occurs in the final stage of infection. Viral NA removes sialic acids from both cellular receptors and from newly synthesized HA and NA on nascent virions, which have been sialylated as part of the glycosylation processes within the host cell (Palese et al., 1974; Basak et al., 1985). NA cleavage of sialic acids prevents virion aggregation and stops virus binding back to the dying host cell *via* the HA, enabling efficient release of virion progeny and spread to new cell targets (Palese et al., 1974).

The role of the viral HA in attachment and infection has been well explored, yet examination of the role of NA in the IAV infection cycle has been largely limited to its role in aiding exit of virion progeny from infected cells. The majority of reviews on NA have focused on viral inhibitors that target NA and block this function. While some studies have suggested NA function does not influence the early stage of IAV infection (Liu et al., 1995), arguably, the sialidase activity of NA aids the virus to gain access to cells by catalyzing the cleavage of sialic acids presented by decoy receptors, such as mucins (Kesimer et al., 2009; McAuley et al., 2017), potentially providing NA with an important role in viral entry. In addition, experiments showing a decrease in infection of cells in the presence of NA-blocking drugs provide evidence for a role of NA in a virus entry step (Matrosovich et al., 2004; Ohuchi et al., 2006; Su et al., 2009; Gulati et al., 2013). As such there is a need for better understanding of the complex role of NA in the influenza infection and replication cycle, particularly with consideration to how the disparate roles of HA and NA glycoproteins need to achieve a functional balance in order to maintain viral fitness. Therefore, we sought to review the existing literature to evaluate the NA structure and function in relation to its role in the IAV infection cycle.

NA STRUCTURE

The NA assembles as a tetramer of four identical polypeptides and, when embedded in the envelope of the virus, accounts for approximately 10–20% of the total glycoproteins on the virion surface, with about 40–50 NA spikes and 300–400 HA spikes on an average sized virion of 120 nm (Varghese et al., 1983; Ward et al., 1983; Moules et al., 2010). The four monomers, each of approximately 470 amino acids, fold into four distinct structural domains: the cytoplasmic tail, the transmembrane region, the stalk, and the catalytic head (**Figure 1**). Cryoelectron tomography studies have indicated that the NA tetramer exists in local clusters on the virion surface or as isolated spikes surrounded by HA (Harris et al., 2006). Depending on the length of the stalk region, the NA may protrude slightly more (Harris et al., 2006) or less (Matsuoka et al., 2009) above the viral envelope than the HA, which may influence the overall enzymatic activity of the virus.

Cytoplasmic Tail

Suggesting that the NA cytoplasmic tail is involved in critical viral functions, the N-terminal domain sequence is nearly 100% conserved across all IAV subtypes and consists of the sequence MNPNQK (Blok and Air, 1982). Reverse engineered viruses containing site-specific mutations in this domain exhibit altered virion morphology and reduced replicative yields (Mitnaul et al., 1996; Jin et al., 1997; Barman et al., 2004). IAV engineered to encode an NA lacking a cytoplasmic tail could still be rescued albeit with a markedly attenuated phenotype (Garcia-Sastre and Palese, 1995). The altered morphology and attenuated infectivity of viruses expressing NA lacking the cytoplasmic tail domain are thought to be due to a lack of interaction with the membrane-associated matrix M1 viral protein (Enami and Enami, 1996), which ultimately alters the efficiency of budding from the infected host cell (Jin et al., 1997; Ali et al., 2000; Barman et al., 2001; Mintaev et al., 2014). Determinants in both the cytoplasmic tail domain and the transmembrane domain contribute to the transport of the glycoprotein to the apical plasma membrane (Kundu and Nayak, 1994; Kundu et al., 1996). However, the role of the tail domain in packaging the surface NA into virions remains unclear. A complete loss of the tail domain (Garcia-Sastre and Palese, 1995) resulted in a 50% reduction in the amount of NA in infected cells. This corresponded to a reduction in the amount of NA incorporated into virions, suggesting efficient packaging of existing NA. However, the absence of all tail amino acids except for the initiating methionine gave rise to virus that also showed markedly less incorporation of NA into virions, but in this case, NA was present at the plasma membrane at similar levels to wild-type virus (Mitnaul et al., 1996).

Transmembrane Domain

The N-terminal hydrophobic transmembrane domain, which attaches the NA to the viral envelope (Bos et al., 1984), contains a variable sequence of amino acids spanning residue numbers 7–29 and is predicted to form an alpha helix (Blok and Air, 1982; Air, 2012) with interspersed polar residues driving subunit-subunit interactions (Nordholm et al., 2013). The transmembrane domain provides signals for translocation from the endoplasmic reticulum to the apical surface, as well as association with lipid rafts (Barman and Nayak, 2000). The N-terminal amino acids (positions 1–74), which include both the cytoplasmic tail, the transmembrane domain, and some of the stalk region have been reported to be sufficient to target the cell membrane and for the formation of the NA tetramer complex (Kundu et al., 1991, 1996; da Silva et al., 2013; Nordholm et al., 2013). Implicating the function of the transmembrane domain directly in the translocation of NA to the apical membrane, membrane trafficking can also occur in the absence of the stalk and head domain (Ernst et al., 2013). Mutation of specific amino acids

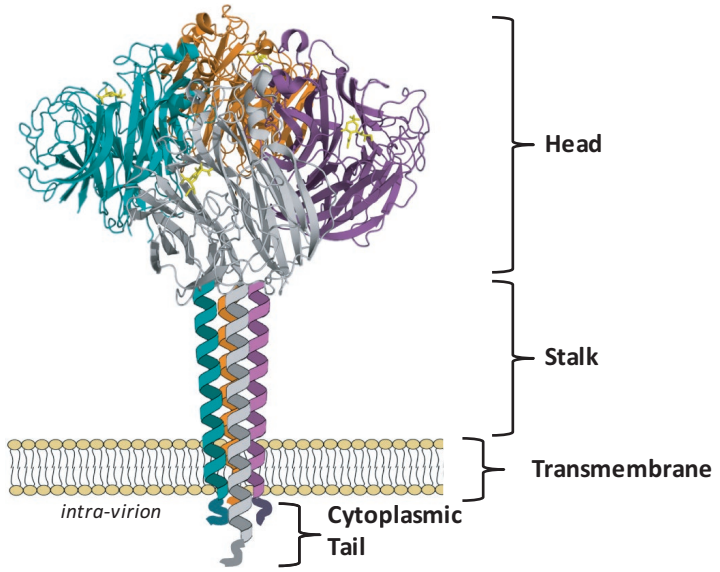


FIGURE 1 | NA exists as a tetramer of four identical monomers. Each monomer consists of four distinct structural domains known as the catalytic head, the stalk, the transmembrane region and the cytoplasmic tail. The head domain structure was generated in Pymol using structural information from Protein Data Bank code 4GZX (A/Tanzania/205/2010 N2 NA). Individual head domain monomers are shown in green, grey, purple, and orange. The NA stalk, transmembrane region, and cytoplasmic tail are yet to be resolved and are depicted here as four alpha helices.

within the transmembrane domain can induce stable architectural differences in the anchoring signal region that result in diminished transport to the plasma membrane (Ernst et al., 2013).

Stalk

The stalk domains of NAs of different IAV subtypes share some structural features, but the number and sequence of amino acid residues can vary considerably (Blok and Air, 1982). Regardless of this variability, all NA stalk domains share some structural features, including at least one cysteine residue and a potential glycosylation site (Blok and Air, 1982; Air, 2012). The cysteine residue(s) may assist with tetramer stabilization by enabling disulfide bonds to form between each monomeric NA unit (Blok and Air, 1982; Ward et al., 1983). While the cysteine residues may occur at variable positions in the NA stalks of different subtypes, the tetrameric structure of NA allows them to align, so that the disulfide bonds can form between the pairs of cysteine residues on neighboring monomers (Blok and Air, 1982). The presence of carbohydrate side chains within the stalk is thought to contribute further to the stability of the tetramer (Blok and Air, 1982).

The length of the stalk region of different subtypes can have significant impact on particular virus characteristics. Using reverse engineering techniques, mutant viruses unable to produce the NA stalk were able to be rescued in tissue culture cells and replicate to the equivalent titer as the unmodified parent virus, but could not replicate in eggs or mice (Castrucci and Kawaoka, 1993). Using a series of NA mutants differing only in stalk length, studies showed that while there was no correlation between the stalk length and the ability to cleave fetuin or a small substrate *in vitro*, enhanced virus replication in eggs correlated closely with increasing stalk length (Els et al., 1985; Castrucci and Kawaoka, 1993). Viruses presenting NAs with shortened stalk domains have also been reported to elute less efficiently from chicken erythrocytes (Els et al., 1985; Castrucci and Kawaoka, 1993). Reduced stalk length has been commonly thought to impact NA activity of virions because the diminished height may hinder access to cellular sialic acid expressing receptors and that the towering HA blocks the shorter NA catalytic domain from gaining access to the sialic acids (Baigent and McCauley, 2001) (**Figure 2**).

More recently, it has been postulated that the limited access to substrate theory may not fully explain the reduced sialidase activity of stalk-deletion mutants, with the argument that the clustering of NAs on the virion surface would diminish any blocking effects of nearby HA. This view was backed up by molecular dynamics simulations providing evidence that a reduced stalk size also alters the geometry and dynamics of the enzymatic pocket itself, modifying its affinity for sialic acids (Durrant et al., 2016). Further evidence for the impact of the stalk structure on the activity of the NA catalytic domain comes from experiments where the same NA head domain was expressed with different tetramerizing domains as artificial stalks (Schmidt et al., 2011). A tetrabrachion stalk conferred similar properties as the native NA, whereas the yeast stalk (GCN4-pLI) reduced substrate affinity and inhibitor binding. Others have also demonstrated that a single mutation in the stalk can significantly affect enzyme activity, presumably affecting the stability of the tetramer (Zanin et al., 2017).

There is also a mounting evidence for a relationship between NA stalk length and virulence in mammalian models of infection. NA stalk truncation mutants of the 2009 pandemic virus

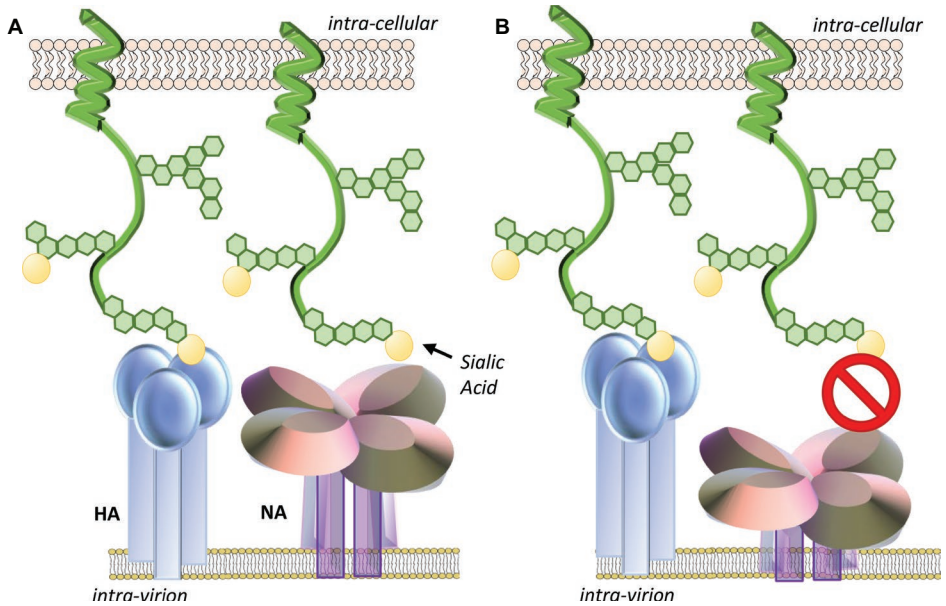


FIGURE 2 | (A) Optimal NA stalk length enables the NA catalytic head access to sialic acid-expressing receptors. **(B)** Reduced stalk length may impact the ability of NA to contact sialic acids on mucins or cellular receptors as neighboring HA may sterically hinder its approach. The degree of impact may be dependent on the ratio and spacing of HA and NA glycoproteins on the virion surface. Reduced stalk size may also shift the geometry and dynamics of the enzymatic pocket altering its affinity for sialic acid.

A(H1N1)pdm09 showed greater lethality in mice and virulence in ferrets than the untruncated counterpart (Park et al., 2017). Loss of the glycosylation site in the stalk likewise enhanced virulence in mice (Park et al., 2017). A deletion of 20 amino acids is frequently observed upon transmission of IAV from waterfowl to domestic poultry (Matsuoka et al., 2009; Hoffmann et al., 2012; Blumenkrantz et al., 2013; Sun et al., 2013; Bi et al., 2015). As such, the length of the stalk domain may be a species-specific determinant of viral adaptation and virulence that we are yet to fully understand (Li et al., 2014; Stech et al., 2015; Park et al., 2017).

Head Domain

Peptide maps from crystallized NA catalytic heads were first detailed in 1978 (Laver, 1978). However, the actual structure of this domain was first described in 1983 for IAVs and in 1992 for IBVs (Varghese et al., 1983; Burmeister et al., 1992). The catalytic head of all NAs consists of a box-shaped structure comprising four monomers (**Figure 3**). Each monomer is in the form of a six-bladed propeller structure, with each blade having four anti-parallel β-sheets that are stabilized by disulfide bonds and connected by loops of variable length (Varghese et al., 1983). A functional catalytic site is present on the surface of each monomer and is directed sideward rather than upward, a property consistent with the ability to cleave sialic acids from nearby membrane glycoproteins to prevent virus trapping (Colman et al., 1983; Burmeister et al., 1992). These catalytic sites are characterized by a large cavity with an unusually large number of charged residues in the pocket and around its rim (Colman et al., 1983; Varghese et al., 1992). The tetrameric form of NA is considered optimal for enzyme activity, and mutations that lead to instability of the tetramer lead to decreased enzyme activity (McKimm-Breschkin et al., 1996b; Fujisaki et al., 2012; McKimm-Breschkin et al., 2013). While it has been reported that monomers alone have no enzyme activity (Air, 2012) and usually expression of recombinant soluble NA heads requires a synthetic tetramerization domain for active NA (Schmidt et al., 2011), there are reports of expression of soluble recombinant monomeric influenza NA heads in both yeast and mammalian cells that have comparable properties to the native enzyme (Yongkiettrakul et al., 2009; Nivitchanyong et al., 2011). When the head domain of NA is proteolytically cleaved from the remaining NA tetrameric stalk embedded in the virion, the enzymatic properties remain active and the heads retain the tetrameric state of purification (Laver, 1978; McKimm-Breschkin et al., 1991).

The NA active site consists of an inner shell of eight highly conserved residues that interact directly with sialic acids (Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371, and Tyr406) (**Figure 4**). In addition, there is an outer shell of 10 residues, which do not contact sialic acid, but which have an important structural role and are defined as framework residues. These comprise Glu119, Arg156, Trp178, Ser179, Asp198, Ile222, Glu227, Glu277, Asn294, and Glu425 (Colman et al., 1983, 1993; Burmeister et al., 1992). Three arginine residues (Arg118, 292, 371) interact with the carboxylate of the sialic acid substrate. Arg152 binds to the acetamido group on the sugar ring, while Glu276 interacts with the 8- and 9-hydroxyl groups on the glycerol side chain. The enzyme active site is said to be highly conserved in both spatial orientation and sequence properties, making it an ideal target for drug inhibition.

NA active site variants that either occur naturally or are created *via* genetic modification have shown that both framework

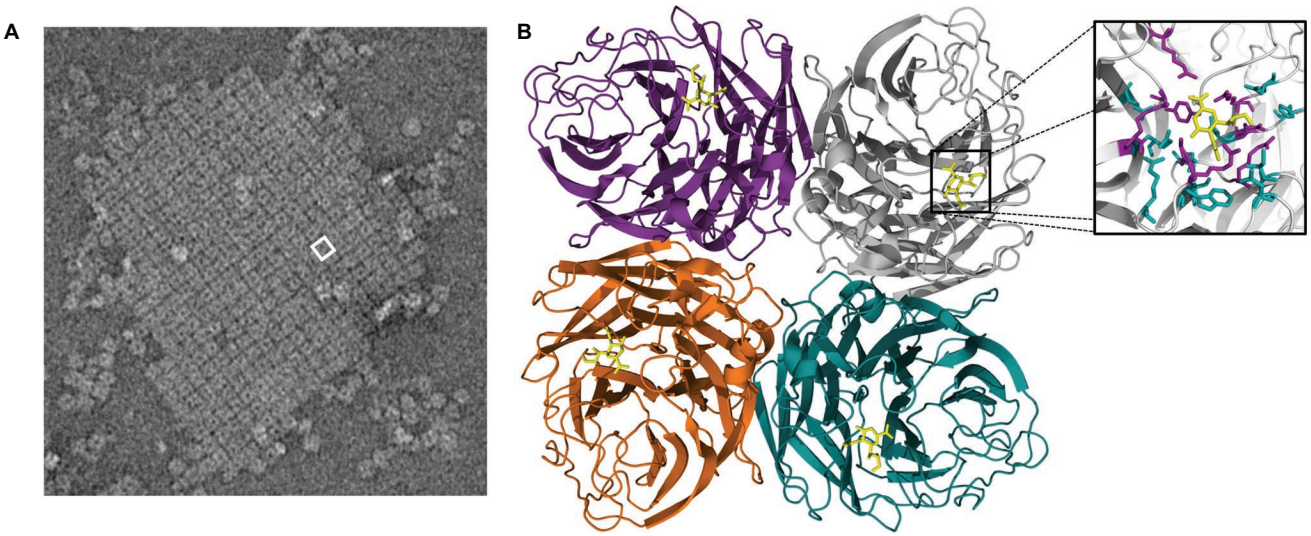


FIGURE 3 | (A) An electron micrograph of a two-dimensional crystal array formed by NA heads, generated as described by Oakley et al. (2010). One square-shaped structure = one tetramer head, with the hole in the center of each tetramer. White box represents one tetramer head, which is depicted in the structural cartoon in B. **(B)** The catalytic head of NA consists of a six-bladed propeller structure, with each blade of the propeller having four anti-parallel β-sheets that are stabilized by disulfide bonds and connected by loops of variable length. Sialic acid (yellow structure) is observable on the head of each monomer. The boxed region is magnified in the inset and shows the receptor-binding pocket to which sialic acid (yellow compound) binds. The structure was generated in Pymol using structural information from Protein Data Bank code 4GZX.

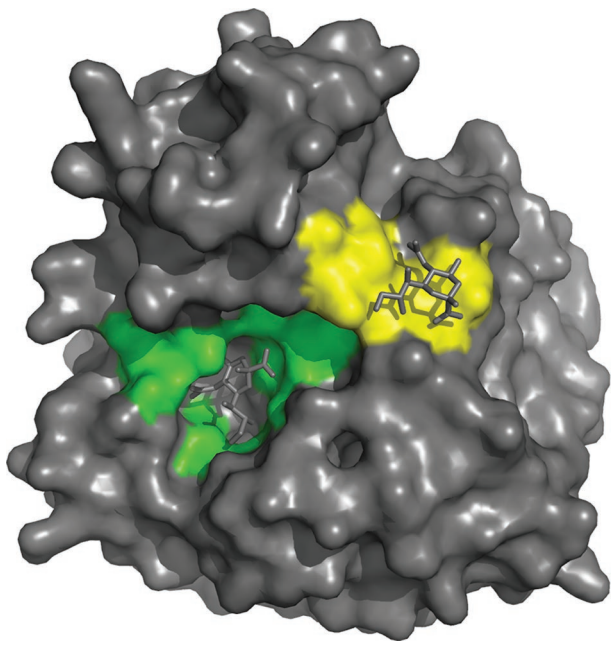


FIGURE 4 | On the basis of interaction with sialic acid, residues Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371 and Tyr406 are considered as the catalytic sites that mediate cleavage from the underlying sugar residues presented by glycoproteins and are highlighted in green. The second site binds sialic acid by making contacts with Ser367, Ser370, Ser372, Asn400, Trp403 and Lys432 (highlighted in yellow), but bound sialic acid at this site is not released by the NA activity. The structure was generated in Pymol using structural information from Protein Data Bank code 1MWE for the A/tern/Australia/G70C/75 NA/Neu5Ac complexed at 4°C.

and catalytic residues can alter the viral replicative ability, transmissibility, and susceptibility to antiviral inhibitors to varying degrees (Lentz et al., 1987; McKimm-Breschkin, 2000, 2013; Abed et al., 2006; Meijer et al., 2009; Richard et al., 2011; Chao et al., 2012; Govorkova, 2013). An H1N1 virus that emerged in 2007–2008 had a single H275Y amino acid change conferring oseltamivir resistance (Hurt et al., 2011; Takashita et al., 2015). This virus showed no decrease in fitness, spreading globally over the next year. Therefore, it remains important to monitor antiviral susceptibility of viruses circulating in the community.

STRUCTURAL RELATIONSHIPS BETWEEN NA SUBTYPES

Phylogenic mapping, which included comparisons of genetic and structural relationships between NAs from different viruses (not including the recently discovered bat viruses) revealed that IAV NAs fall into two distinct groups, regardless of their serotype identification (i.e. N1–N9) (Russell et al., 2006). Group 1 contains N1, N4, N5, and N8 serotypes, while group 2 contains N2, N3, N6, N7, and N9 serotypes. Crystal structures of the group 1 and group 2 NAs reveal a particularly striking feature in the catalytic domain referred to as the '150 loop.' This loop comprises residues 147–152, which forms one corner of the enzyme active site, and is able to exist in at least two conformations (Russell et al., 2006). Group 1 NA structures have an additional cavity next to the active site, which is created by the movement of the 150 loop during conformational changes brought about by substrate binding within the active site. Structurally, the group 2 NAs do not appear to have the cavity. By X-ray crystallography,

the A(H1N1)pdm09 NA also appears to lack the 150-cavity and has more structural similarities to the group 2 NAs (Li et al., 2010; Amaro et al., 2011). However, molecular dynamics studies suggest that the A(H1N1)pdm09 NA and the group 2 NAs do have the 150-loop flexibility, but there may be fewer of the population of the open 150-loop conformation, hence not seen in the static structures in crystals (Amaro et al., 2011). As NA inhibitors have been developed based on crystal structures of group 2 NAs, there is now a great interest in the development of novel inhibitors that target the cavity of group 1 NAs (Russell et al., 2006; Li et al., 2010; Amaro et al., 2011). Sialic acid derivatives that selectively lock the 150 loop in the open cavity conformation inhibit the group 1 sialidases, including the A(H1N1)pdm09 NA, further supporting the fact that this pandemic NA does have an accessible 150 cavity that is exposed to some extent upon the binding of an appropriate inhibitor (Rudrawar et al., 2010).

NA Hemadsorption Site

X-ray crystal structures of N6 and N9 that have revealed sialic acids can also bind at a discrete second site on the NA head (Varghese et al., 1997; Air, 2012; Streltsov et al., 2015), resulting in the binding of red blood cells (hemadsorption) to the NA. The second site, a shallow pocket located next to the catalytic site, is made up of three surface loops and binds sialic acid by making contacts with Ser367, Ser370, Ser372, Asn400, Trp403, and Lys432 (**Figure 4**), but bound sialic acids at this site are not released by the NA activity (Varghese et al., 1997). The second site appears to be a common feature of avian NAs of different subtypes, while swine influenza strains have lost several of these conserved residues, so theoretically they do not have the second site (Kobasa et al., 1997; Varghese et al., 1997). However, the Brownian dynamics simulation of human N2 from the 1957 pdmIAV and the A(H1N1)pdm09 NA suggests that some of the key structural features are retained (Sung et al., 2010), and NMR studies subsequently added support to the presence of the second site in these viruses (Lai et al., 2012). Substitutions at different positions in the three loops of the second sialic acid-binding site on the NA of human H1N1 IAV have been shown to have a profound effect on binding and cleavage of multivalent, but not monovalent, receptors and significantly affect virus replication (Du et al., 2018). Linking the second sialic acid-binding site with host tropism, it has been reported that for avian viruses that have succeeded in zoonotic transmission to man, such as the pandemic 1918 and 1968 IAVs and the emerging H7N9 IAV, this site contains point mutations (Uhlendorff et al., 2009; Dai et al., 2017). It has been hypothesized that substitutions in the second sialic acid-binding site enable the enzymatic activity of the NA of newly emerging viruses to be compatible with an HA that is evolving toward human receptor-binding specificity (Du et al., 2018).

Understanding the biological function of the NA second sialic acid-binding site has been challenging. One study has shown that the ability of NA to bind red blood cells correlated with the cleavage efficiencies of multivalent substrates like fetuin (Uhlendorff et al., 2009). The observation that substrate binding *via* the second sialic acid-binding site in H7N9 viruses enhances NA catalytic efficiency against the same substrate (Dai et al., 2017) is possibly achieved by recruiting and keeping multivalent sialosides close to the active site (Uhlendorff et al., 2009; Dai et al., 2017). The second site has also been shown to be a highly conserved target for a novel influenza inhibitor found in the saliva of mice that impact the ability of the infection to progress to the lungs of infected mice when the inoculum is initially confined to the upper respiratory tract (Ivinson et al., 2017). The amino acids at positions 368–370 (N2 numbering) on the rim of the second site dictated the *in vitro* susceptibility of different viral strains to the salivary inhibitor and their ability to progress to the lungs of mice (Gilbertson et al., 2017).

NA FUNCTIONAL ROLES IN REPLICATION

Virus Entry

NA activity and cleavage of sialic acids have long been thought to enable movement of the virion through mucus (Burnet, 1948). Airway mucus is comprised of a large network of sialylated glycoproteins known as gel-forming mucins (MUC5AC and MUC5B in particular), which interconnect and serve as a scaffold to present secreted antiproteases, antioxidants, antimicrobial proteins, secretory immunoglobulins (IgA), cytokines, and other innate defensive molecules (Lillehoj and Kim, 2002). Hypersecretion of mucus during the course of respiratory tract infection can occlude the airways and increase the lung pathology (Rose and Voynow, 2006). In murine models of influenza infection, overexpression of the major respiratory tract gel-forming mucin, Muc5ac (non-human form of MUC5AC), revealed that this glycoprotein presents SAα2-3Gal, which can bind virus and limit infection of the underlying epithelia (Ehre et al., 2012). This supports the proposal that the physical properties of the gel-forming mucins serve as a trap by presenting decoy receptors to which the inhaled pathogen binds and is then cleared by way of the mucociliary escalator (Button et al., 2012). IAV has been shown to interact with secreted mucus on frozen human trachea and bronchus tissue sections, and bead-bound mucins inhibited the NA cleavage of substrate (Cohen et al., 2013). To add credence to the hypothesis that NA functions to aid viral movement through the respiratory mucus layer, NA inhibitors have been shown to block IAV entry into differentiated human tracheobronchial and nasal epithelial cells, as well as porcine cells that secrete mucus (Matrosovich et al., 2004; Yang et al., 2014). Exogenous NA also enhanced passage through the mucus layer (Yang et al., 2014). This suggests that NA is needed to remove decoy sialic acids presented on mucins, cilia, and cellular glycocalyx in order for virus to efficiently access functional receptors on the surface of target cells.

Receptor Binding

In addition to the non-catalytic sialic-binding site that is structurally distinct from the NA active site in N6 and N9 NAs (Varghese et al., 1997; Air, 2012; Streltsov et al., 2015), more recent studies have shown that the NAs of human H3N2 viruses isolated since 1994 can also demonstrate agglutination

of red blood cells after passage in MDCK cells, but not in eggs (Lin et al., 2010; Hooper and Bloom, 2013; Mohr et al., 2015). This property of the more recent N2 NAs was first noted because many H3N2 isolates showed weak HA-mediated binding to chicken red blood cells allowing NA-dependent hemagglutination to be detected. NA agglutination is inhibited by NA inhibitor drugs (NAIs) but only poorly inhibited by post-infection ferret antisera, thus distinguishing it from HA binding. In contrast to the N6 and N9, both sialidase and receptor-binding functions reside in the N2 active site, yet the catalytic and receptor-binding sites do not appear to be identical since relative sensitivity to inhibition of the two functions varies with oseltamivir, zanamivir, and peramivir (Mohr et al., 2015). Substitution of aspartate at position 151 near the active site to glycine, alanine or asparagine, or threonine 148 to isoleucine in H3N2 NAs (Lin et al., 2010; Mohr et al., 2015) or glycine 147 to arginine in N1 NAs (Hooper and Bloom, 2013) correlates with the acquisition of receptor binding. An H150R substitution has also recently been shown to correlate with NA receptor binding and has been found in both clinical samples and passaged viruses (Mogling et al., 2017). Interestingly, the affinity of the NA receptor-binding site to sialyl lactose is much stronger than the corresponding affinities of HA with its sialylated receptors (Zhu et al., 2012). The fact that entry and infection of MDCK cells with viruses having NA D151G can be blocked by NAIs (Gulati et al., 2013) suggests this NA active site-associated receptor binding function may play an important biological role for these H3N2 isolates.

Virus Internalization

NAIs were found to reduce infection efficiency of cell lines without inhibiting virus binding or fusion activity, supporting a role for the NA during the viral entry process (Ohuchi et al., 2006). It was proposed that the NA facilitated movement of the virus across the cell surface by repeated binding and release steps from an endocytosis inactive site on the cell, to an active site, thereby increasing the efficiency of viral uptake. With the recent development of a biolayer interferometry assay, there is now clear evidence that viral NA plays a major role in driving virus particles over sialylated receptor surfaces. Using this method, Guo et al. (2018) showed that NA contributed to the initial rate of virus binding to sialoglycoproteins after which multiple low-affinity HA-sialic acid interactions take place. The rapid association and dissociation of these allow the NA to remove sialic acids and create a receptor density gradient that enables the rolling of virus particles across the surface.

NA has also been shown to enhance HA-dependent influenza virus fusion and infectivity using a cell-cell fusion assay and an HIV-based pseudotype infectivity assay (Su et al., 2009). When the NA gene from H9N2, H5N1, or A(H1N1)pdm09 virus was expressed on a PR8 background, the replication kinetics were similar *in vitro* (MDCK cells) and *in vivo* (mice) (Chen et al., 2013), yet the initial infection kinetics and virus-induced fusion and elution from erythrocytes were affected, implicating a role for NA during the early stage of infection (Chen et al., 2013).

Catalytic Activity

By far, the most characterized function of NA is its action as a sialidase enzyme, enabling release of new virion progeny by enzymatically cleaving sialic acids from cell surface receptors and from carbohydrate side chains on nascent virions (Gottschalk, 1958; Palese et al., 1974). When NA activity is inhibited by the use of antivirals that target the enzymatic site, or through alteration of key amino acid residues, such as those identified to be integral in the catalytic process, the budding virions aggregate on the cell surface instead of being released (Lentz et al., 1987; Tarbet et al., 2014; Yang et al., 2016). This clumping of virions is due to HA on newly released virus binding to the sialic acids expressed on receptors in the vicinity of the budding site and to carbohydrate side chains on the HA and NA of progeny viruses that still contain terminal sialic acids in the absence of NA activity.

The catalytic mechanism of NA has not yet been completely resolved but is expected to begin with the binding of substrate to the active site *via* interactions with the catalytic residues and involves salt-bridge formation between the carboxylate of the sialic acid and the three arginine cluster at one end of the active site. Functional and structural evidence for the formation of a covalent intermediate between the C-2 on the sugar ring and the Tyr406 was obtained using a 2,3-difluoro sialic acid derivative (DFSA), which exhibits slow turnover, permitting accumulation of the covalent intermediate (Kim et al., 2013; Vavricka et al., 2013). This confirmed that the Tyr406 functions as the catalytic nucleophile. This leads to a change in the chair conformation of sialic acid with the carboxylate in the axial position, to a boat conformation with the carboxylate rotated into the pseudo equatorial position, then eventual cleavage of the sialic acid molecule from the preceding galactose residue. This cleavage first results in the release of sialic acid in an α-anomer conformation, then conversion into a β-anomer state shortly thereafter (Air, 2012).

The optimal activity of NA occurs at a pH range of 5.5–6.5 (Mountford et al., 1982; Lentz et al., 1987; McKimm-Breschkin et al., 2013); however, some viruses have been reported to have a stable NA activity at a lower pH range of 4–5, which has been shown to enhance replication kinetics (Takahashi and Suzuki, 2015). The presence of Ca^{2+} is thought to be essential during the reaction for both thermostability and enzyme activity of the NA. Using common fluorometric activity assays, increasing calcium ion concentration was shown to increase NA activity (Dimmock, 1971; Potier et al., 1979; Chong et al., 1991; Johansson and Brett, 2003). In crystal structures of NA bound to sialic acid, up to 5 Ca^{2+} ions per subunit that forms each tetramer are observed (Varghese et al., 1983; Russell et al., 2006; Xu et al., 2008; Lawrenz et al., 2010). An X-ray crystal structure of a whale N9 NA revealed that there were structural alterations near the substrate-binding site in the absence of calcium (Smith et al., 2006).

NA Substrate Specificity

Avian IAVs express HAs that typically bind to galactose in an α2-3 linkage (SAα2-3Gal). For avian influenza viruses to undergo human to human transmission, the HA must acquire the capacity to bind SAα2-6Gal through mutations within the receptor-binding pocket (Gambaryan and Matrosovich, 2015). In some

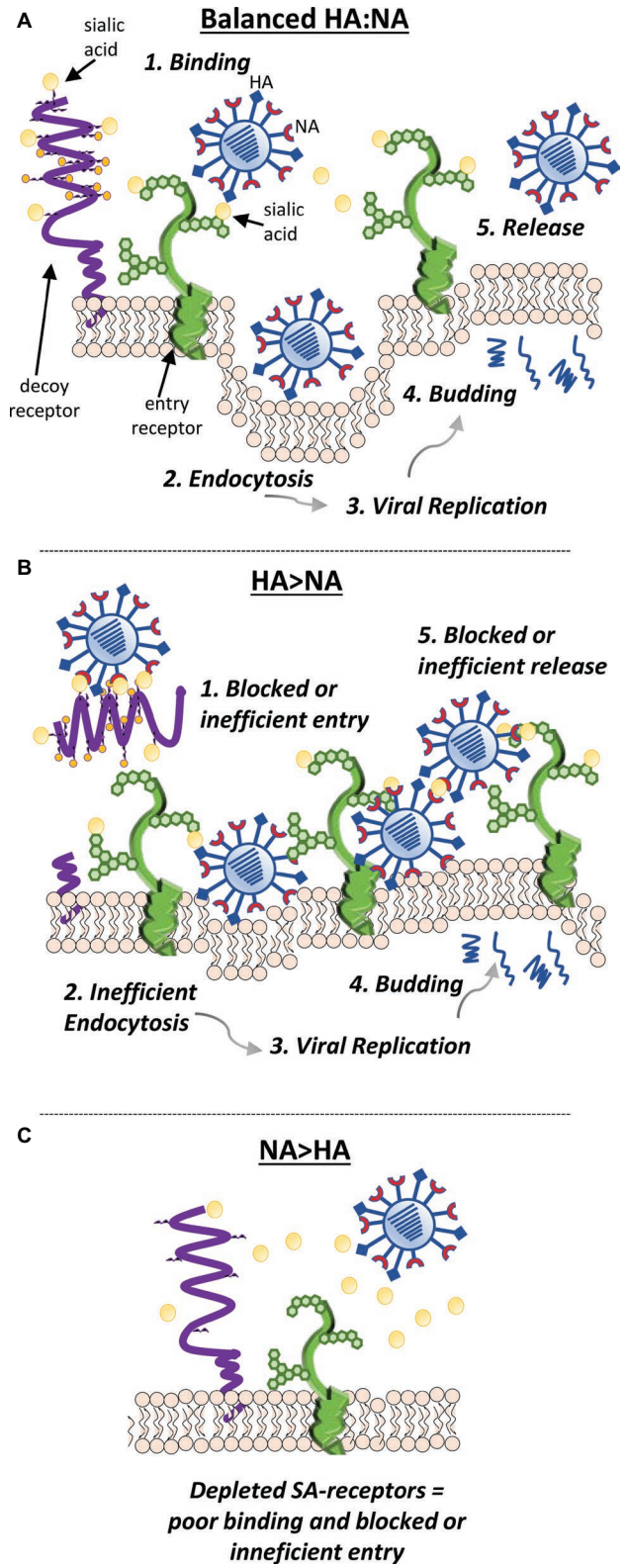


FIGURE 5 | The relative activity of the HA and NA needs to be balanced to maintain the ability of the virion to efficiently infect and be released from cells. **(A)** Efficient cleavage of sialic acids from decoy receptors (such as

(Continued)

FIGURE 5 | cell-surface mucins) by NA enables HA access to sialic acids expressed by entry receptors and efficient endocytosis. After endosome escape of the viral genome and its migration to the nucleus, replication of the genome, synthesis of viral mRNAs, and translation of viral proteins take place. New virions assemble at the cell surface and are released from the cell by budding. As the viral components bud from the cell, NA cleaves sialic acids from receptors near the budding site to prevent virions binding back to the dying cell. NA cleavage of sialic acids from the carbohydrate side chains of nascent HA and NA also prevents newly budded virus from clumping together. Both these functions enable efficient release of the nascent virions from the cell. **(B)** If the HA and NA are mismatched and have mutations in important binding or catalytic sites that alter function, the relative activity of the two proteins may be imbalanced. If the sialidase function of NA is suboptimal, virus may remain bound by decoy receptors, which may shed and block virus entry into the cell. As the virus buds from the cell, an imbalance of HA and NA function may result in the lack of release of the virions due to the binding of HA to the sialic acids expressed at the cell surface that have not been removed by the NA. **(C)** Alternatively, if the sialidase activity of NA is too strong when compared to the HA-binding activity, sialic acids may be removed from receptors at the expense of the HA being able to bind and trigger endocytosis.

instances, the mutations are such that the HA will retain SAα2-3Gal binding and have dual specificity. Through further evolution in humans, the HA can become solely specific for SAα2-6Gal (Couceiro et al., 1993; Matrosovich et al., 1997, 2007). Similar to HA, the specificity of the active site of the viral NA evolves with time in the human host toward SAα2-6Gal (Kobasa et al., 1999; Gambaryan and Matrosovich, 2015). However, unlike the HA, it always maintains the ability to cleave SAα2-3Gal, even in viruses whose sole HA specificity is for SAα2-6Gal (Baum and Paulson, 1991; Kobasa et al., 1999). This evolution of specificity for sialic acid bound to galactose in different conformations is most likely due to the presentation of these glycoproteins on the surface of the target epithelium. In the upper airways, human tracheal epithelium expresses sialylated glycoproteins that are bound in an SAα2-6Gal linkage (Couceiro et al., 1993). In contrast, human bronchial mucus secretions contain large glycoproteins that express SAα2-3Gal, and as such, maintenance of NA activity for this linkage may be necessary for virion movement through the mucus barrier.

Functional evolution of NA has been shown to occur by amino acid substitutions that subtly alter the conformation of the NA catalytic domain to enable a different form of sialic acid to bind to the active site (Kobasa et al., 1999). A single change of isoleucine 275 to valine in N2 NA enables the shift in NA specificity toward increased activity for SAα2-6Gal, while other mutations are thought to subtly alter the conformation of the active site to accommodate this linkage of sialic acid (Kobasa et al., 1999).

HA:NA BALANCE

With respect to the ability for IAV to circumnavigate the mucosal environment and successfully infect underlying epithelial cells, the HA and NA need to have complementary receptor and ligand-binding specificity. It is also imperative that the relative activity of the two proteins is balanced to maintain the ability to infect and to release from cells efficiently (**Figure 5**). The

importance of this functional balance was initially demonstrated when the first NA inhibitor resistant mutants were analyzed. Unexpectedly, rather than mutations in the NA, these drug resistant viruses had mutations in the HA (McKimm-Breschkin et al., 1996a,b, 1998). The HA mutations were found to reduce the affinity for receptors, so that less NA activity was required for virion release. However, while they had a fitness advantage in the presence of NA inhibitors, in the absence of NA inhibitors, the receptor binding was so poor for some of these mutants that the NA was able to cleave off the receptors before HA binding could take place. Such viruses are thus drug dependent (McKimm-Breschkin et al., 1996a; Blick et al., 1998). Others subsequently confirmed the need for balanced HA and NA activities (Wagner et al., 2002). The relevance to *in vivo* adaptation of influenza virus was shown by the isolation of several H3N2 viruses from patients that were reported to have little or no NA activity (Ferraris et al., 2006). A weak-binding HA was found to compensate for the absence of NA activity (Richard et al., 2012). Obviously, evolution of an HA or NA that negatively impacts viral attachment, replication, and transmission results in a less fit virus. In order to survive, compensatory mutations are needed to restore fitness (Lin et al., 2010; Mohr et al., 2015). Thus, for a human virus to gain efficient access to the cell surface *in vivo*, it needs to have a combination of HA and NA activities that enable escape from inhibition in the mucus layer by having either an HA with low avidity for mucin-bound SAα2-3Gal or an NA with greater activity for SAα2-3Gal, or a combination of the two. To attach and enter a cell, HA avidity for SAα2-6Gal must be strong enough to enable binding before the NA can cleave receptors. However, HA binding cannot be too strong, since release of progeny virions and prevention of aggregation at the cell surface need access of the NA to cleave the SAα2-6Gal.

Traditionally, incubation of IAV with red blood cells at different temperatures enables the functional balance of the HA and NA to be explored. As NA is inactive at 4°C but active at 37 °C, the ability of HA to agglutinate red cells at 4°C and of NA to elute virus at 37°C can be measured. However, this is no longer so clear cut. The isolation of the NA inhibitor-resistant HA mutants revealed that elution can occur rapidly with little NA activity, due to a weak HA (McKimm-Breschkin et al., 2012, 2013). More insight into the relative roles can be obtained by further modifications of the HA elution assay. When the eluted virus/red blood cells are mixed and re-incubated at 4 °C, if elution is due to a weak HA, virus will rebind. If the NA has cleaved the receptors, virus cannot rebind as in the traditional reaction. Alternatively, the addition of NA inhibitors will prevent elution if it is by the NA, but not by the HA (McKimm-Breschkin et al., 2012, 2013). Furthermore, NA inhibitors will also prevent agglutination if it is through the NA active site, as seen for the H3N2 viruses (Lin et al., 2010; Mohr et al., 2015). However, even with these variations on the assay, binding and elution vary depending on the species from which the red blood cells are derived from and are due to different sialic acid linkages presented at their surface (Ferraris et al., 2006; Richard et al., 2012).

Thus, the optimal functional balance of the HA and NA glycoproteins is difficult to measure as a number of physical characteristics of the virus can influence the properties of the HA- and NA-mediated receptor interactions. HA and NA affinity for and kinetics of interaction with sialic acids are the foremost determinants for the ability of the virion to infect a cell. On an average virion, there are 300–400 HA spikes and 40–50 NA spikes (Harris et al., 2006). The excess of HA over NA is perhaps reflective of the weak interaction of HA for sialic acids (Sauter et al., 1989) and the need to form several connections to form a stable interaction. The position and amount of NA present on the virion then plays an important role in gaining access to the cell surface sialic acids; its catalytic activity can directly influence efficiency of viral release, movement through mucus, and potential infection of new cells. Virion morphology can also contribute to altering the protein distribution and amount of NA and HA on the virion surface, potentially altering viral fitness and replication kinetics (Wasilewski et al., 2012). Finally, the ability for NA to access substrate either by the distribution of the NA on the virion or by variation in the length of protrusion of the NA spike can significantly influence both HA binding to a receptor and cleavage by NA from the receptor (Els et al., 1985; Castrucci and Kawaoka, 1993; Baigent and McCauley, 2001; Durrant et al., 2016). The recent study of Guo et al. (2018) further implicates the critical importance of HA:NA functional balance on virion movement through the mucus layer and over epithelial surfaces, a dynamic rolling process that may also be involved in cell-to-cell spread across the respiratory epithelium.

CONCLUSION

Rather than just a sialidase that facilitates virus release from infected cells, the NA is a complicated multifunctional protein with an important role at many stages of the infectious process. While the NA is the main target for current antiviral therapies (Ison, 2015), recent approaches to new influenza therapy include targeting the HA with monoclonal antibodies (Nachbagauer and Krammer, 2017). However, given the NA also has the capacity to bind receptors, there needs to be caution in this approach, as it is possible that compensating mutations in the NA may allow escape from inhibition of the HA. As antibody levels against NA in children, adults, and the elderly correlate well with functional neuraminidase inhibition titers (Rajendran et al., 2017), altering vaccine strategies to enable efficient boosting of broadly cross-reactive antibodies against neuraminidase (Sandbulte et al., 2007; Marcelin et al., 2011; Liu et al., 2015; Chen et al., 2018) may be an important consideration in the campaign against the incredibly adaptable influenza virus.

AUTHOR CONTRIBUTIONS

JM and JM-B contributed to the conception and design of the review. JM wrote the first draft of the manuscript and designed figures. BG, ST, LB, and JM-B contributed to various sections of

the manuscript, including figures. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the Australian Government National Health and Medical Research Council grant 1079924 and program grant 1071916.

ACKNOWLEDGMENTS

The authors would like to acknowledge Dr. Victor Streltsov (Florey Institute of Neuroscience and Mental Health, Victoria, Australia) for his technical assistance in developing the structural images and Dr. Lynne Waddington, CSIRO Melbourne, Australia for provision of the 2D-electron micrograph image.

REFERENCES

Abed, Y., Baz, M., and Boivin, G. (2006). Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. *Antivir. Ther.* 11, 971–976.

Air, G. M. (2012). Influenza neuraminidase. *Influenza Other Respir. Viruses* 6, 245–256. doi: 10.1111/j.1750-2659.2011.00304.x

Ali, A., Avalos, R. T., Ponimaskin, E., and Nayak, D. P. (2000). Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. *J. Virol.* 74, 8709–8719. doi: 10.1128/JVI.74.18.8709-8719.2000

Amaro, R. E., Swift, R. V., Votapka, L., Li, W. W., Walker, R. C., and Bush, R. M. (2011). Mechanism of 150-cavity formation in influenza neuraminidase. *Nat. Commun.* 2:388. doi: 10.1038/ncomms1390

Baigent, S. J., and McCauley, J. W. (2001). Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. *Virus Res.* 79, 177–185. doi: 10.1016/S0168-1702(01)00272-6

Barman, S., Adhikary, L., Chakrabarti, A. K., Bernas, C., Kawaoka, Y., and Nayak, D. P. (2004). Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza a virus neuraminidase in raft association and virus budding. *J. Virol.* 78, 5258–5269. doi: 10.1128/JVI.78.10.5258-5269.2004

Barman, S., Ali, A., Hui, E. K., Adhikary, L., and Nayak, D. P. (2001). Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses. *Virus Res.* 77, 61–69. doi: 10.1016/S0168-1702(01)00266-0

Barman, S., and Nayak, D. P. (2000). Analysis of the transmembrane domain of influenza virus neuraminidase, a type II transmembrane glycoprotein, for apical sorting and raft association. *J. Virol.* 74, 6538–6545. doi: 10.1128/JVI.74.14.6538-6545.2000

Basak, S., Tomana, M., and Compans, R. W. (1985). Sialic acid is incorporated into influenza hemagglutinin glycoproteins in the absence of viral neuraminidase. *Virus Res.* 2, 61–68. doi: 10.1016/0168-1702(85)90060-7

Baum, L. G., and Paulson, J. C. (1991). The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. *Virology* 180, 10–15. doi: 10.1016/0042-6822(91)90003-T

Bi, Y., Xiao, H., Chen, Q., Wu, Y., Fu, L., Quan, C., et al. (2015). Changes in the length of the neuraminidase stalk region impact H7N9 virulence in mice. *J. Virol.* 90, 2142–2149. doi: 10.1128/JVI.02553-15

Blick, T. J., Sahasrabudhe, A., McDonald, M., Owens, I. J., Morley, P. J., Fenton, R. J., et al. (1998). The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. *Virology* 246, 95–103. doi: 10.1006/viro.1998.9194

Blok, J., and Air, G. M. (1982). Variation in the membrane-insertion and "stalk" sequences in eight subtypes of influenza type A virus neuraminidase. *Biochemistry* 21, 4001–4007.

Blumenkrantz, D., Roberts, K. L., Shelton, H., Lycett, S., and Barclay, W. S. (2013). The short stalk length of highly pathogenic avian influenza H5N1 virus neuraminidase limits transmission of pandemic H1N1 virus in ferrets. *J. Virol.* 87, 10539–10551. doi: 10.1128/JVI.00967-13

Bos, T. J., Davis, A. R., and Nayak, D. P. (1984). NH2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. *Proc. Natl. Acad. Sci. U. S. A.* 81, 2327–2331.

Burmeister, W. P., Ruigrok, R. W., and Cusack, S. (1992). The 2.2 A resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.* 11, 49–56. doi: 10.1002/j.1460-2075.1992.tb05026.x

Burnet, F. M. (1948). Mucins and mucoids in relation to influenza virus action; inhibition of virus haemagglutination by glandular mucins. *Aust. J. Exp. Biol. Med. Sci.* 26, 371–379. doi: 10.1038/icb.1948.38

Button, B., Cai, L. H., Ehre, C., Kesimer, M., Hill, D. B., Sheehan, J. K., et al. (2012). A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. *Science* 337, 937–941. doi: 10.1126/science.1223012

Castrucci, M. R., and Kawaoka, Y. (1993). Biologic importance of neuraminidase stalk length in influenza A virus. *J. Virol.* 67, 759–764.

Chao, D. L., Bloom, J. D., Kochin, B. F., Antia, R., and Longini, I. M. Jr. (2012). The global spread of drug-resistant influenza. *J. R. Soc. Interface* 9, 648–656. doi: 10.1098/rsif.2011.0427

Chen, Q., Huang, S., Chen, J., Zhang, S., and Chen, Z. (2013). NA proteins of influenza A viruses H1N1/2009, H5N1, and H9N2 show differential effects on infection initiation, virus release, and cell-cell fusion. *PLoS One* 8:e54334. doi: 10.1371/journal.pone.0054334

Chen, Y. Q., Wohlbold, T. J., Zheng, N. Y., Huang, M., Huang, Y., Neu, K. E., et al. (2018). Influenza infection in humans induces broadly cross-reactive and protective neuraminidase-reactive antibodies. *Cell* 173, 417–429:e410. doi: 10.1016/j.cell.2018.03.030

Chong, A. K., Pegg, M. S., and Von Itzstein, M. (1991). Influenza virus sialidase: effect of calcium on steady-state kinetic parameters. *Biochim. Biophys. Acta* 1077, 65–71.

Cohen, M., Zhang, X. Q., Senaati, H. P., Chen, H. W., Varki, N. M., Schooley, R. T., et al. (2013). Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virol. J.* 10:321. doi: 10.1186/1743-422X-10-321

Colman, P. M., Hoyne, P. A., and Lawrence, M. C. (1993). Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. *J. Virol.* 67, 2972–2980.

Colman, P. M., Varghese, J. N., and Laver, W. G. (1983). Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature* 303, 41–44. doi: 10.1038/303041a0

Couceiro, J. N., Paulson, J. C., and Baum, L. G. (1993). Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res.* 29, 155–165. doi: 10.1016/0168-1702(93)90056-S

Cox, N. J., and Subbarao, K. (2000). Global epidemiology of influenza: past and present. *Annu. Rev. Med.* 51, 407–421. doi: 10.1146/annurev.med.51.1.407

da Silva, D. V., Nordholm, J., Madjo, U., Pfeiffer, A., and Daniels, R. (2013). Assembly of subtype 1 influenza neuraminidase is driven by both the transmembrane and head domains. *J. Biol. Chem.* 288, 644–653. doi: 10.1074/jbc.M112.424150

Dai, M., McBride, R., Dortmans, J. C., Peng, W., Bakkers, M. J., De Groot, R. J., et al. (2017). Mutation of the second sialic acid-binding site, resulting in reduced neuraminidase activity, preceded the emergence of H7N9 influenza A virus. *J. Virol.* 91. doi: 10.1128/JVI.00049-17

Dimmock, N. J. (1971). Dependence of the activity of an influenza virus neuraminidase upon Ca^{2+}. *J. Gen. Virol.* 13, 481–483. doi: 10.1099/0022-1317-13-3-481

Du, W., Dai, M., Li, Z., Boons, G. J., Peeters, B., Van Kuppeveld, F. J. M., et al. (2018). Substrate binding by the 2(nd) sialic acid-binding site of influenza

A virus N1 neuraminidase contributes to enzymatic activity. *J. Virol.* 92, e01243–e01218. doi: 10.1128/JVI.01243-18

Durrant, J. D., Bush, R. M., and Amaro, R. E. (2016). Microsecond molecular dynamics simulations of influenza neuraminidase suggest a mechanism for the increased virulence of stalk-deletion mutants. *J. Phys. Chem. B* 120, 8590–8599. doi: 10.1021/acs.jpcb.6b02655

Ehre, C., Worthington, E. N., Liesman, R. M., Grubb, B. R., Barbier, D., O'Neal, W. K., et al. (2012). Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs. *Proc. Natl. Acad. Sci. U. S. A.* 109, 16528–16533. doi: 10.1073/pnas.1206552109

Els, M. C., Air, G. M., Murti, K. G., Webster, R. G., and Laver, W. G. (1985). An 18-amino acid deletion in an influenza neuraminidase. *Virology* 142, 241–247. doi: 10.1016/0042-6822(85)90332-0

Enami, M., and Enami, K. (1996). Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein. *J. Virol.* 70, 6653–6657.

Ernst, A. M., Zacherl, S., Herrmann, A., Hacke, M., Nickel, W., Wieland, F. T., et al. (2013). Differential transport of influenza A neuraminidase signal anchor peptides to the plasma membrane. *FEBS Lett.* 587, 1411–1417. doi: 10.1016/j.febslet.2013.03.019

Ferraris, O., Kessler, N., Valette, M., and Lina, B. (2006). Evolution of the susceptibility to antiviral drugs of A/H3N2 influenza viruses isolated in France from 2002 to 2005. *Vaccine* 24, 6656–6659. doi: 10.1016/j.vaccine.2006.05.050

Fujisaki, S., Takashita, E., Yokoyama, M., Taniwaki, T., Xu, H., Kishida, N., et al. (2012). A single E105K mutation far from the active site of influenza B virus neuraminidase contributes to reduced susceptibility to multiple neuraminidase-inhibitor drugs. *Biochem. Biophys. Res. Commun.* 429, 51–56. doi: 10.1016/j.bbrc.2012.10.095

Gambaryan, A. S., and Matrosovich, M. N. (2015). What adaptive changes in hemagglutinin and neuraminidase are necessary for emergence of pandemic influenza virus from its avian precursor? *Biochemistry* 80, 872–880. doi: 10.1134/S000629791507007X

Garcia-Sastre, A., and Palese, P. (1995). The cytoplasmic tail of the neuraminidase protein of influenza A virus does not play an important role in the packaging of this protein into viral envelopes. *Virus Res.* 37, 37–47. doi: 10.1016/0168-1702(95)00017-K

Gilbertson, B., Ng, W. C., Crawford, S., McKimm-Breschkin, J. L., and Brown, L. E. (2017). Mouse saliva inhibits transit of influenza virus to the lower respiratory tract by efficiently blocking influenza virus neuraminidase activity. *J. Virol.* 91, e00145–e00117. doi: 10.1128/JVI.00145-17

Gottschalk, A. (1958). The influenza virus neuraminidase. *Nature* 181, 377–378. doi: 10.1038/181377a0

Govorkova, E. A. (2013). Consequences of resistance: in vitro fitness, in vivo infectivity, and transmissibility of oseltamivir-resistant influenza A viruses. *Influenza Other Respir. Viruses* 7 (Suppl. 1), 50–57. doi: 10.1111/irv.12044

Gulati, S., Smith, D. F., Cummings, R. D., Couch, R. B., Griesemer, S. B., St George, K., et al. (2013). Human H3N2 influenza viruses isolated from 1968 to 2012 show varying preference for receptor substructures with no apparent consequences for disease or spread. *PLoS One* 8:e66325. doi: 10.1371/journal.pone.0066325

Guo, H., Rabouw, H., Slomp, A., Dai, M., Van Der Vegt, F., Van Lent, J. W. M., et al. (2018). Kinetic analysis of the influenza A virus HA/NA balance reveals contribution of NA to virus-receptor binding and NA-dependent rolling on receptor-containing surfaces. *PLoS Pathog.* 14:e1007233. doi: 10.1371/journal.ppat.1007233

Harris, A., Cardone, G., Winkler, D. C., Heymann, J. B., Brecher, M., White, J. M., et al. (2006). Influenza virus pleiomorphy characterized by cryoelectron tomography. *Proc. Natl. Acad. Sci. U. S. A.* 103, 19123–19127. doi: 10.1073/pnas.0607614103

Harris, K. A., Freidl, G. S., Munoz, O. S., Von Dobschuetz, S., De Nardi, M., Wieland, B., et al. (2017). Epidemiological risk factors for animal influenza A viruses overcoming species barriers. *EcoHealth* 14, 342–360. doi: 10.1007/s10393-017-1244-y

Hoffmann, T. W., Munier, S., Larcher, T., Soubieux, D., Ledevin, M., Esnault, E., et al. (2012). Length variations in the NA stalk of an H7N1 influenza virus have opposite effects on viral excretion in chickens and ducks. *J. Virol.* 86, 584–588. doi: 10.1128/JVI.05474-11

Hooper, K. A., and Bloom, J. D. (2013). A mutant influenza virus that uses an N1 neuraminidase as the receptor-binding protein. *J. Virol.* 87, 12531–12540. doi: 10.1128/JVI.01889-13

Hurt, A. C., Hardie, K., Wilson, N. J., Deng, Y. M., Osbourn, M., Gehrig, N., et al. (2011). Community transmission of oseltamivir-resistant A(H1N1) pdm09 influenza. *N. Engl. J. Med.* 365, 2541–2542. doi: 10.1056/NEJMc1111078

Ison, M. G. (2015). Optimizing antiviral therapy for influenza: understanding the evidence. *Expert Rev. Anti-Infect. Ther.* 13, 417–425. doi: 10.1586/14787210.2015.1018183

Ivinson, K., Deliyannis, G., McNabb, L., Grollo, L., Gilbertson, B., Jackson, D., et al. (2017). Salivary blockade protects the lower respiratory tract of mice from Lethal influenza virus infection. *J. Virol.* 91, e00624–e00717. doi: 10.1128/JVI.00624-17

Jin, H., Leser, G. P., Zhang, J., and Lamb, R. A. (1997). Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. *EMBO J.* 16, 1236–1247. doi: 10.1093/emboj/16.6.1236

Johansson, B. E., and Brett, I. C. (2003). Variation in the divalent cation requirements of influenza a virus N2 neuraminidases. *J. Biochem.* 134, 345–352. doi: 10.1093/jb/mvg151

Kesimer, M., Scull, M., Brighton, B., Demaria, G., Burns, K., O'Neal, W., et al. (2009). Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. *FASEB J.* 23, 1858–1868. doi: 10.1096/fj.08-119131

Kim, J. H., Resende, R., Wennekes, T., Chen, H. M., Bance, N., Buchini, S., et al. (2013). Mechanism-based covalent neuraminidase inhibitors with broad-spectrum influenza antiviral activity. *Science* 340, 71–75. doi: 10.1126/science.1232552

Kobasa, D., Kodihalli, S., Luo, M., Castrucci, M. R., Donatelli, I., Suzuki, Y., et al. (1999). Amino acid residues contributing to the substrate specificity of the influenza A virus neuraminidase. *J. Virol.* 73, 6743–6751.

Kobasa, D., Rodgers, M. E., Wells, K., and Kawaoka, Y. (1997). Neuraminidase hemadsorption activity, conserved in avian influenza A viruses, does not influence viral replication in ducks. *J. Virol.* 71, 6706–6713.

Kundu, A., Avalos, R. T., Sanderson, C. M., and Nayak, D. P. (1996). Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells. *J. Virol.* 70, 6508–6515.

Kundu, A., Jabbar, M. A., and Nayak, D. P. (1991). Cell surface transport, oligomerization, and endocytosis of chimeric type II glycoproteins: role of cytoplasmic and anchor domains. *Mol. Cell. Biol.* 11, 2675–2685. doi: 10.1128/MCB.11.5.2675

Kundu, A., and Nayak, D. P. (1994). Analysis of the signals for polarized transport of influenza virus (A/WSN/33) neuraminidase and human transferrin receptor, type II transmembrane proteins. *J. Virol.* 68, 1812–1818.

Lai, J. C., Garcia, J. M., Dyason, J. C., Bohm, R., Madge, P. D., Rose, F. J., et al. (2012). A secondary sialic acid binding site on influenza virus neuraminidase: fact or fiction? *Angew. Chem. Int. Ed. Eng.* 51, 2221–2224. doi: 10.1002/anie.201108245

Lakadamyali, M., Rust, M. J., and Zhuang, X. (2004). Endocytosis of influenza viruses. *Microbes Infect.* 6, 929–936. doi: 10.1016/j.micinf.2004.05.002

Laver, W. G. (1978). Crystallization and peptide maps of neuraminidase "heads" from H2N2 and H3N2 influenza virus strains. *Virology* 86, 78–87. doi: 10.1016/0042-6822(78)90009-0

Lawrenz, M., Wereszczynski, J., Amaro, R., Walker, R., Roitberg, A., and McCammon, J. A. (2010). Impact of calcium on N1 influenza neuraminidase dynamics and binding free energy. *Proteins* 78, 2523–2532. doi: 10.1002/prot.22761

Lentz, M. R., Webster, R. G., and Air, G. M. (1987). Site-directed mutation of the active site of influenza neuraminidase and implications for the catalytic mechanism. *Biochemistry* 26, 5351–5358.

Li, Q., Qi, J., Zhang, W., Vavricka, C. J., Shi, Y., Wei, J., et al. (2010). The 2009 pandemic H1N1 neuraminidase N1 lacks the 150-cavity in its active site. *Nat. Struct. Mol. Biol.* 17, 1266–1268. doi: 10.1038/nsmb.1909

Li, Y., Chen, S., Zhang, X., Fu, Q., Zhang, Z., Shi, S., et al. (2014). A 20-amino-acid deletion in the neuraminidase stalk and a five-amino-acid deletion in the NS1 protein both contribute to the pathogenicity of H5N1 avian influenza viruses in mallard ducks. *PLoS One* 9:e95539. doi: 10.1371/journal.pone.0095539

Lillehoj, E. R., and Kim, K. C. (2002). Airway mucus: its components and function. *Arch. Pharm. Res.* 25, 770–780. doi: 10.1007/BF02976990

Lin, Y. P., Gregory, V., Collins, P., Kloess, J., Wharton, S., Cattle, N., et al. (2010). Neuraminidase receptor binding variants of human influenza A(H3N2) viruses resulting from substitution of aspartic acid 151 in the catalytic site: a role in virus attachment? *J. Virol.* 84, 6769–6781. doi: 10.1128/JVI.00458-10

Liu, C., Eichelberger, M. C., Compans, R. W., and Air, G. M. (1995). Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. *J. Virol.* 69, 1099–1106.

Liu, W. C., Lin, C. Y., Tsou, Y. T., Jan, J. T., and Wu, S. C. (2015). Cross-reactive neuraminidase-inhibiting antibodies elicited by immunization with recombinant neuraminidase proteins of H5N1 and pandemic H1N1 influenza A viruses. *J. Virol.* 89, 7224–7234. doi: 10.1128/JVI.00585-15

Marcelin, G., Dubois, R., Rubrum, A., Russell, C. J., McElhaney, J. E., and Webby, R. J. (2011). A contributing role for anti-neuraminidase antibodies on immunity to pandemic H1N1 2009 influenza A virus. *PLoS One* 6:e26335. doi: 10.1371/journal.pone.0026335

Matrosovich, M., Matrosovich, T., Uhlendorff, J., Garten, W., and Klenk, H. D. (2007). Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. *Virology* 361, 384–390. doi: 10.1016/j.virol.2006.11.030

Matrosovich, M. N., Gambaryan, A. S., Teneberg, S., Piskarev, V. E., Yamnikova, S. S., Lvov, D. K., et al. (1997). Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology* 233, 224–234. doi: 10.1006/viro.1997.8580

Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A., and Klenk, H. D. (2004). Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J. Virol.* 78, 12665–12667. doi: 10.1128/JVI.78.22.12665-12667.2004

Matsuoka, Y., Swayne, D. E., Thomas, C., Rameix-Welti, M. A., Naffakh, N., Warnes, C., et al. (2009). Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. *J. Virol.* 83, 4704–4708. doi: 10.1128/JVI.01987-08

McAuley, J. L., Corcilius, L., Tan, H. X., Payne, R. J., McGuckin, M. A., and Brown, L. E. (2017). The cell surface mucin MUC1 limits the severity of influenza A virus infection. *Mucosal Immunol.* 10, 1581–1593. doi: 10.1038/mi.2017.16

McKimm-Breschkin, J. L. (2000). Resistance of influenza viruses to neuraminidase inhibitors – a review. *Antivir. Res.* 47, 1–17.

McKimm-Breschkin, J. L. (2013). Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza Other Respir. Viruses* 7 (Suppl. 1), 25–36. doi: 10.1111/irv.12047

McKimm-Breschkin, J. L., Blick, T. J., Sahasrabudhe, A., Tiong, T., Marshall, D., Hart, G. J., et al. (1996a). Generation and characterization of variants of NWS/G70C influenza virus after in vitro passage in 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en. *Antimicrob. Agents Chemother.* 40, 40–46.

McKimm-Breschkin, J. L., Caldwell, J. B., Guthrie, R. E., and Kortt, A. A. (1991). A new method for the purification of the influenza A virus neuraminidase. *J. Virol. Methods* 32, 121–124. doi: 10.1016/0166-0934(91)90192-3

McKimm-Breschkin, J. L., McDonald, M., Blick, T. J., and Colman, P. M. (1996b). Mutation in the influenza virus neuraminidase gene resulting in decreased sensitivity to the neuraminidase inhibitor 4-guanidino-Neu5Ac2en leads to instability of the enzyme. *Virology* 225, 240–242.

McKimm-Breschkin, J. L., Rootes, C., Mohr, P. G., Barrett, S., and Streltsov, V. A. (2012). In vitro passaging of a pandemic H1N1/09 virus selects for viruses with neuraminidase mutations conferring high-level resistance to oseltamivir and peramivir, but not to zanamivir. *J. Antimicrob. Chemother.* 67, 1874–1883. doi: 10.1093/jac/dks150

McKimm-Breschkin, J. L., Sahasrabudhe, A., Blick, T. J., McDonald, M., Colman, P. M., Hart, G. J., et al. (1998). Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. *J. Virol.* 72, 2456–2462.

McKimm-Breschkin, J. L., Williams, J., Barrett, S., Jachno, K., McDonald, M., Mohr, P. G., et al. (2013). Reduced susceptibility to all neuraminidase inhibitors of influenza H1N1 viruses with haemagglutinin mutations and mutations in non-conserved residues of the neuraminidase. *J. Antimicrob. Chemother.* 68, 2210–2221. doi: 10.1093/jac/dkt205

Meijer, A., Lackenby, A., Hungnes, O., Lina, B., Van-Der-Werf, S., Schweiger, B., et al. (2009). Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. *Emerg. Infect. Dis.* 15, 552–560. doi: 10.3201/eid1504.181280

Mintaev, R. R., Alexeevski, A. V., and Kordyukova, L. V. (2014). Co-evolution analysis to predict protein-protein interactions within influenza virus envelope. *J. Bioinforma. Comput. Biol.* 12:1441008. doi: 10.1142/S021972001441008X

Mitnaul, L. J., Castrucci, M. R., Murti, K. G., and Kawaoka, Y. (1996). The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *J. Virol.* 70, 873–879.

Mogling, R., Richard, M. J., Vliet, S. V., Beek, R. V., Schrauwen, E. J. A., Spronken, M. I., et al. (2017). Neuraminidase-mediated haemagglutination of recent human influenza A(H3N2) viruses is determined by arginine 150 flanking the neuraminidase catalytic site. *J. Gen. Virol.* 98, 1274–1281. doi: 10.1099/jgv.0.000809

Mohr, P. G., Deng, Y. M., and McKimm-Breschkin, J. L. (2015). The neuraminidases of MDCK grown human influenza A(H3N2) viruses isolated since 1994 can demonstrate receptor binding. *Virol. J.* 12:67. doi: 10.1186/s12985-015-0295-3

Moules, V., Ferraris, O., Terrier, O., Giudice, E., Yver, M., Rolland, J. P., et al. (2010). In vitro characterization of naturally occurring influenza H3NA- viruses lacking the NA gene segment: toward a new mechanism of viral resistance? *Virology* 404, 215–224. doi: 10.1016/j.virol.2010.04.030

Mountford, C. E., Grossman, G., Holmes, K. T., O'Sullivan, W. J., Hampson, A. W., Raison, R. L., et al. (1982). Effect of monoclonal anti-neuraminidase antibodies on the kinetic behavior of influenza virus neuraminidase. *Mol. Immunol.* 19, 811–816. doi: 10.1016/0161-5890(82)90007-4

Nachbagauer, R., and Krammer, F. (2017). Universal influenza virus vaccines and therapeutic antibodies. *Clin. Microbiol. Infect.* 23, 222–228. doi: 10.1016/j.cmi.2017.02.009

Nivitchanyong, T., Yongkiettrakul, S., Kramyu, J., Pannengpetch, S., and Wanasen, N. (2011). Enhanced expression of secretable influenza virus neuraminidase in suspension mammalian cells by influenza virus nonstructural protein 1. *J. Virol. Methods* 178, 44–51. doi: 10.1016/j.jviromet.2011.08.010

Nordholm, J., Da Silva, D. V., Damjanovic, J., Dou, D., and Daniels, R. (2013). Polar residues and their positional context dictate the transmembrane domain interactions of influenza A neuraminidases. *J. Biol. Chem.* 288, 10652–10660. doi: 10.1074/jbc.M112.440230

Oakley, A. J., Barrett, S., Peat, T. S., Newman, J., Streltsov, V. A., Waddington, L., et al. (2010). Structural and functional basis of resistance to neuraminidase inhibitors of influenza B viruses. *J. Med. Chem.* 53, 6421–6431. doi: 10.1021/jm100621s

Ohuchi, M., Asaoka, N., Sakai, T., and Ohuchi, R. (2006). Roles of neuraminidase in the initial stage of influenza virus infection. *Microbes Infect.* 8, 1287–1293. doi: 10.1016/j.micinf.2005.12.008

Palese, P., Tobita, K., Ueda, M., and Compans, R. W. (1974). Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* 61, 397–410. doi: 10.1016/0042-6822(74)90276-1

Park, S., Il Kim, J., Lee, I., Bae, J. Y., Yoo, K., Nam, M., et al. (2017). Adaptive mutations of neuraminidase stalk truncation and deglycosylation confer enhanced pathogenicity of influenza A viruses. *Sci. Rep.* 7:10928. doi: 10.1038/s41598-017-11348-0

Potier, M., Mameli, L., Belisle, M., Dallaire, L., and Melancon, S. B. (1979). Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. *Anal. Biochem.* 94, 287–296. doi: 10.1016/0003-2697(79)90362-2

Rajendran, M., Nachbagauer, R., Ermler, M. E., Bunduc, P., Amanat, F., Izikson, R., et al. (2017). Analysis of anti-influenza virus neuraminidase antibodies in children, adults, and the elderly by ELISA and enzyme inhibition: evidence for original antigenic sin. *MBio* 8, e02281–e02316. doi: 10.1128/mBio.02281-16

Richard, M., Erny, A., Care, B., Traversier, A., Barthelemy, M., Hay, A., et al. (2012). Rescue of a H3N2 influenza virus containing a deficient neuraminidase protein by a hemagglutinin with a low receptor-binding affinity. *PLoS One* 7:e33880. doi: 10.1371/journal.pone.0033880

Richard, M., Ferraris, O., Erny, A., Barthelemy, M., Traversier, A., Sabatier, M., et al. (2011). Combinatorial effect of two framework mutations (E119V and I222L) in the neuraminidase active site of H3N2 influenza virus on resistance to oseltamivir. *Antimicrob. Agents Chemother.* 55, 2942–2952. doi: 10.1128/AAC.01699-10

Rose, M. C., and Voynow, J. A. (2006). Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol. Rev.* 86, 245–278. doi: 10.1152/physrev.00010.2005

Rudrawar, S., Dyason, J. C., Rameix-Welti, M. A., Rose, F. J., Kerry, P. S., Russell, R. J., et al. (2010). Novel sialic acid derivatives lock open the 150-loop of an influenza A virus group-1 sialidase. *Nat. Commun.* 1:113. doi: 10.1038/ncomms1114

Russell, R. J., Haire, L. F., Stevens, D. J., Collins, P. J., Lin, Y. P., Blackburn, G. M., et al. (2006). The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature* 443, 45–49. doi: 10.1038/nature05114

Sandbulte, M. R., Jimenez, G. S., Boon, A. C., Smith, L. R., Treanor, J. J., and Webby, R. J. (2007). Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. *PLoS Med.* 4:e59. doi: 10.1371/journal.pmed.0040059

Sauter, N. K., Bednarski, M. D., Wurzburg, B. A., Hanson, J. E., Whitesides, G. M., Skehel, J. J., et al. (1989). Hemagglutinins from two influenza virus variants bind to sialic acid derivatives with millimolar dissociation constants: a 500-MHz proton nuclear magnetic resonance study. *Biochemistry* 28, 8388–8396.

Schmidt, P. M., Attwood, R. M., Mohr, P. G., Barrett, S. A., and McKimm-Breschkin, J. L. (2011). A generic system for the expression and purification of soluble and stable influenza neuraminidase. *PLoS One* 6:e16284. doi: 10.1371/journal.pone.0016284

Smith, B. J., Huyton, T., Joosten, R. P., McKimm-Breschkin, J. L., Zhang, J. G., Luo, C. S., et al. (2006). Structure of a calcium-deficient form of influenza virus neuraminidase: implications for substrate binding. *Acta Crystallogr. D Biol. Crystallogr.* 62, 947–952. doi: 10.1107/S0907444906020063

Stech, O., Veits, J., Abdelwhab El, S. M., Wessels, U., Mettenleiter, T. C., and Stech, J. (2015). The neuraminidase stalk deletion serves as major virulence determinant of H5N1 highly pathogenic avian influenza viruses in chicken. *Sci. Rep.* 5:13493. doi: 10.1038/srep13493

Streltsov, V. A., Pilling, P., Barrett, S., and McKimm-Breschkin, J. L. (2015). Catalytic mechanism and novel receptor binding sites of human parainfluenza virus type 3 hemagglutinin-neuraminidase (hPIV3 HN). *Antivir. Res.* 123, 216–223. doi: 10.1016/j.antiviral.2015.08.014

Su, B., Wurtzer, S., Rameix-Welti, M. A., Dwyer, D., Van Der Werf, S., Naffakh, N., et al. (2009). Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). *PLoS One* 4:e8495. doi: 10.1371/journal.pone.0008495

Sun, Y., Tan, Y., Wei, K., Sun, H., Shi, Y., Pu, J., et al. (2013). Amino acid 316 of hemagglutinin and the neuraminidase stalk length influence virulence of H9N2 influenza virus in chickens and mice. *J. Virol.* 87, 2963–2968. doi: 10.1128/JVI.02688-12

Sung, J. C., Van Wynsberghe, A. W., Amaro, R. E., Li, W. W., and McCammon, J. A. (2010). Role of secondary sialic acid binding sites in influenza N1 neuraminidase. *J. Am. Chem. Soc.* 132, 2883–2885. doi: 10.1021/ja9073672

Takahashi, T., and Suzuki, T. (2015). Low-pH stability of influenza A virus sialidase contributing to virus replication and pandemic. *Biol. Pharm. Bull.* 38, 817–826. doi: 10.1248/bpb.b15-00120

Takashita, E., Kiso, M., Fujisaki, S., Yokoyama, M., Nakamura, K., Shirakura, M., et al. (2015). Characterization of a large cluster of influenza A(H1N1) pdm09 viruses cross-resistant to oseltamivir and peramivir during the 2013-2014 influenza season in Japan. *Antimicrob. Agents Chemother.* 59, 2607–2617. doi: 10.1128/AAC.04836-14

Tarbet, E. B., Hamilton, S., Vollmer, A. H., Luttick, A., Ng, W. C., Pryor, M., et al. (2014). A zanamivir dimer with prophylactic and enhanced therapeutic activity against influenza viruses. *J. Antimicrob. Chemother.* 69, 2164–2174. doi: 10.1093/jac/dku127

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D. A., Chen, L. M., et al. (2012). A distinct lineage of influenza A virus from bats. *Proc. Natl. Acad. Sci. U. S. A.* 109, 4269–4274. doi: 10.1073/pnas.1116200109

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., et al. (2013). New world bats harbor diverse influenza A viruses. *PLoS Pathog.* 9:e1003657. doi: 10.1371/journal.ppat.1003657

Uhlendorff, J., Matrosovich, T., Klenk, H. D., and Matrosovich, M. (2009). Functional significance of the hemadsorption activity of influenza virus neuraminidase and its alteration in pandemic viruses. *Arch. Virol.* 154, 945–957. doi: 10.1007/s00705-009-0393-x

Varghese, J. N., Colman, P. M., Van Donkelaar, A., Blick, T. J., Sahasrabudhe, A., and McKimm-Breschkin, J. L. (1997). Structural evidence for a second sialic acid binding site in avian influenza virus neuraminidases. *Proc. Natl. Acad. Sci. U. S. A.* 94, 11808–11812.

Varghese, J. N., Laver, W. G., and Colman, P. M. (1983). Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. *Nature* 303, 35–40. doi: 10.1038/303035a0

Varghese, J. N., McKimm-Breschkin, J. L., Caldwell, J. B., Kortt, A. A., and Colman, P. M. (1992). The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. *Proteins* 14, 327–332. doi: 10.1002/prot.340140302

Vavricka, C. J., Liu, Y., Kiyota, H., Sriwilaijaroen, N., Qi, J., Tanaka, K., et al. (2013). Influenza neuraminidase operates via a nucleophilic mechanism and can be targeted by covalent inhibitors. *Nat. Commun.* 4:1491. doi: 10.1038/ncomms2487

Wagner, R., Matrosovich, M., and Klenk, H. D. (2002). Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev. Med. Virol.* 12, 159–166. doi: 10.1002/rmv.352

Ward, C. W., Colman, P. M., and Laver, W. G. (1983). The disulphide bonds of an Asian influenza virus neuraminidase. *FEBS Lett.* 153, 29–33. doi: 10.1016/0014-5793(83)80113-6

Wasilewski, S., Calder, L. J., Grant, T., and Rosenthal, P. B. (2012). Distribution of surface glycoproteins on influenza A virus determined by electron cryotomography. *Vaccine* 30, 7368–7373. doi: 10.1016/j.vaccine.2012.09.082

Wilson, I. A., and Cox, N. J. (1990). Structural basis of immune recognition of influenza virus hemagglutinin. *Annu. Rev. Immunol.* 8, 737–771. doi: 10.1146/annurev.iy.08.040190.003513

Xu, X., Zhu, X., Dwek, R. A., Stevens, J., and Wilson, I. A. (2008). Structural characterization of the 1918 influenza virus H1N1 neuraminidase. *J. Virol.* 82, 10493–10501. doi: 10.1128/JVI.00959-08

Yang, J., Liu, S., Du, L., and Jiang, S. (2016). A new role of neuraminidase (NA) in the influenza virus life cycle: implication for developing NA inhibitors with novel mechanism of action. *Rev. Med. Virol.* 26, 242–250. doi: 10.1002/rmv.1879

Yang, X., Steukers, L., Forier, K., Xiong, R., Braeckmans, K., Van Reeth, K., et al. (2014). A beneficiary role for neuraminidase in influenza virus penetration through the respiratory mucus. *PLoS One* 9:e110026. doi: 10.1371/journal.pone.0110026

Yongkiettrakul, S., Boonyapakron, K., Jongkaewwattana, A., Wanitchang, A., Leartsakulpanich, U., Chitnumsub, P., et al. (2009). Avian influenza A/H5N1 neuraminidase expressed in yeast with a functional head domain. *J. Virol. Methods* 156, 44–51. doi: 10.1016/j.jviromet.2008.10.025

Zanin, M., Duan, S., Wong, S. S., Kumar, G., Baviskar, P., Collin, E., et al. (2017). An amino acid in the stalk domain of N1 neuraminidase is critical for enzymatic activity. *J. Virol.* 91, e00868–e00916. doi: 10.1128/JVI.00868-16

Zhu, X., McBride, R., Nycholat, C. M., Yu, W., Paulson, J. C., and Wilson, I. A. (2012). Influenza virus neuraminidases with reduced enzymatic activity that avidly bind sialic acid receptors. *J. Virol.* 86, 13371–13383. doi: 10.1128/JVI.01426-12

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

frontiers in Public Health

ORIGINAL RESEARCH
published: 07 March 2019
doi: 10.3389/fpubh.2019.00039

Detection of the Onset of the Epidemic Period of Respiratory Syncytial Virus Infection in Japan

Hidetomi Yamagami[1], Hirokazu Kimura[2], Takafumi Hashimoto[3], Isao Kusakawa[4] and Satoshi Kusuda[5]*

[1] Medical Affairs, AbbVie GK, Tokyo, Japan, [2] Department of Health Science, Gunma Paz University Graduate School of Health Science, Gunma, Japan, [3] Data Science and Stat, AbbVie GK, Tokyo, Japan, [4] Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan, [5] Department of Pediatrics, Faculty of Medicine, Kyorin University, Tokyo, Japan

OPEN ACCESS

Edited by:
Sunil Dhiman, Defence Research and Development Establishment (DRDE), India

Reviewed by:
Karen Bohmwald, Pontificia Universidad Católica de Chile, Chile
Bernhard Resch, Medical University of Graz, Austria

***Correspondence:**
Hidetomi Yamagami
hidetomi.yamagami@abbvie.com

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *20 December 2018*
Accepted: *14 February 2019*
Published: *07 March 2019*

Citation:
Yamagami H, Kimura H, Hashimoto T, Kusakawa I and Kusuda S (2019) Detection of the Onset of the Epidemic Period of Respiratory Syncytial Virus Infection in Japan. Front. Public Health 7:39. doi: 10.3389/fpubh.2019.00039

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infection in young children worldwide. An annual epidemic of RSV infection generally begins around autumn, reaching a peak at the end of year in Japan, but in 2017 it started in early July and peaked in September. As the onset timing of RSV season varies, it is important to detect the beginning of an epidemic, to enable the implementation of preventive measures. However, there are currently no specified criteria or methods to determine the onset of RSV season in a timely manner. Therefore, we developed a model to detect the epidemic onset, based on data from the Infectious Diseases Weekly Report from 2012 to 2017. The 47 prefectures of Japan span 11 climate zones, which affect the timing of epidemic onset. Therefore, the onset of RSV season was assessed separately in each prefecture. Non-linear regression analysis was performed to generate a mathematical model of the annual epidemic cycle for each prefecture. A search index was used to determine the onset of RSV season, which was estimated using the number of RSV reports per week within an epidemic period (RSV-reports/w) and the number of reported cases included within an epidemic period relative to the total number of RSV reports (capture rate). A number of RSV-reports/w, which was used as a threshold (a number at onset line) to determine the condition of the onset of RSV season, was then estimated based on the search index. The mean number at the onset of RSV season for 47 prefectures was 29.7 reports/week (median 21.0, range 6.0–121.0 reports/ week). The model also showed that the onset of RSV season in 2017 was more than 1 month earlier than the previous year. In conclusion, the model detected epidemic cycles and their onset conditions in all prefectures, despite the 11 climate zones of Japan. The results are expected to contribute to infant medical care by allowing medical personnel to take preventive measures promptly at the beginning of the epidemic RSV season.

Keywords: respiratory syncytial virus infection, palivizumab, IDWR, epidemic period, the onset of RSV season

INTRODUCTION

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection in infants, and more than 50% of infants are infected for the first time by the age of 1 year, and almost 100% of infants by the age of 2 years (1, 2). Lifelong immunity is not acquired after the primary infection; therefore, reinfection with RSV is universally observed in nearly 50% of infants by the age of 2 years (1). In infants, about 50% of cases of pneumonia, and around 50% to 90% of cases of bronchiolitis are attributed to RSV infection (3). In particular, in premature infants and infants with chronic respiratory diseases such as bronchopulmonary dysplasia, RSV infection tends to be severe (4–7). Also, RSV infection tends to be severe even in infants with congenital heart diseases (8, 9)[1] and infants with immunodeficiency or Down's syndrome (10). It is important to take preventive measures for these high-risk infants, as RSV infection may take a fatal course in some cases (11).

Because the medicine for RSV infection has not been developed, patients receive supportive treatment mainly by transfusion and respiratory management. In Japan, public health insurance coverage was approved in 2002 for palivizumab (product name: Synagis®), an anti-RSV humanized monoclonal antibody, which is used as a prophylactic medicine to prevent severe lower respiratory tract disease associated with RSV infection in high-risk infants. As palivizumab needs to be administered once a month throughout the epidemic period of RSV infection, for its appropriate use, it is important to accurately detect the onset of the epidemic period.

RSV infection is monitored via pediatric sentinel sites under the Act on Prevention of Infectious Diseases and Medical Care for Patients Suffering from Infectious Diseases (11). In this program, physicians at designated notification facilities (∼3,000 pediatric medical institutions at fixed points all around Japan to cover 10% of pediatric institutions in Japan) are required to report patients diagnosed with RSV infection based on their symptoms and findings of RSV antigen or by PCR, and the information is published in the Infectious Diseases Weekly Report (IDWR) as national surveillance data by the National Institute of Infectious Diseases (NIID)[1]. In Japan, the epidemic of RSV infection had generally begun around autumn, and the number of reported cases had increased rapidly in autumn, reached a peak at the end of the year, and continued until spring. No significant fluctuations were observed in the epidemic tendency nationwide until 2015; however, the number of reported cases increased from early August in 2016. In 2017, the number of reported cases increased rapidly from early July and reached a peak in September. Tendencies of fluctuations in the onset of the epidemic period are evident, as the number of RSV reports started to increase earlier than in the past several seasons (**Figure 1**). To resolve the issue, the guideline for the use of palivizumab in Japan was immediately revised in accordance with the observed epidemical change of RSV. As the onset of RSV season changes every year and there are no specified decision criteria for the onset of the epidemic RSV season, the onset of the RSV season should be determined on the basis of the IDWR and other included reports[2].

In response to this, we investigated the onset of the epidemic period of RSV infection based on the IDWR surveillance data using a newly developed algorithm. The main objective of this study was to detect the onset weeks of the annual RSV epidemics in Japan to enable timely access to preventive measures for severe RSV disease.

METHODS

Data Source

Six epidemic periods with a 1-year cycle of RSV infection for 46 prefectures and five epidemic periods with a 1-year cycle of RSV infection for Okinawa were analyzed using the IDWR surveillance data from the first week of 2012 to the 24th week of 2018[3]. The numbers of sentinel medical institutions (SMI) in each prefecture were used on the basis of the average numbers of sentinel medical institutions in each prefecture in 2016. Weeks ending logs were referenced from the NIID report[4].

Modeling the RSV Season as a Periodic Oscillation

RSV incidence is reported weekly in each prefecture in Japan. Since epidemic cycles vary among regions in Japan, we generated a mathematical model of the annual epidemic cycle for each prefecture by applying non-linear regression analysis to data reported from the first week of 2012 to the 24th week of 2018. The following sine function was used to model the cyclical nature of the annual epidemics, where "Y" represents the number of RSV reports each week, "w" represents the week of the year (1–52) in which the cases were reported, and a_0, a_1, a_2, and a_3 represent fixed parameters.

$$Y = a_0 + (a_1 \times \sin(a_2 + a_3 \times (w \times \pi/180))). \quad (1)$$

Because we focused on the rhythm of the epidemics, year-to-year variations in RSV reporting numbers (the amplitude of the function) were not incorporated into the model, and an upper limit was applied to variable Y. The model parameters were calculated using the "NLIN" non-linear regression procedure in SAS software (version 9.4; SAS Institute, Cary, NC, USA), with Gauss-Newton iteration. We confirmed that the convergence criterion was met for each prefecture. Also, visual confirmation of graphs of each data set confirmed that the incidence of RSV cases in each prefecture of Japan had sinusoidal periodicity.

When there was more than one peak between the 1st week and the 52nd week in a plot, the peak corresponding to the highest reported number of cases was selected as the main peak for calculation purposes. When there was more than one trough between the 1st week and the 52nd week in a plot, the trough falling just before the main peak was selected as the central trough for calculation purposes. The range of a 1-year cycle based on the sinusoidal curve was identified between one trough and next

[1]https://www.niid.go.jp/niid/ja/rs-virus-m/rs-virus-idwrc/7509-idwrc-1734.html

[2]https://www.jpeds.or.jp/modules/news/index.php?content_id=345

[3]https://www.niid.go.jp/niid/en/survaillance-data-table-english.html

[4]https://www.niid.go.jp/niid/en/calendar-e.html

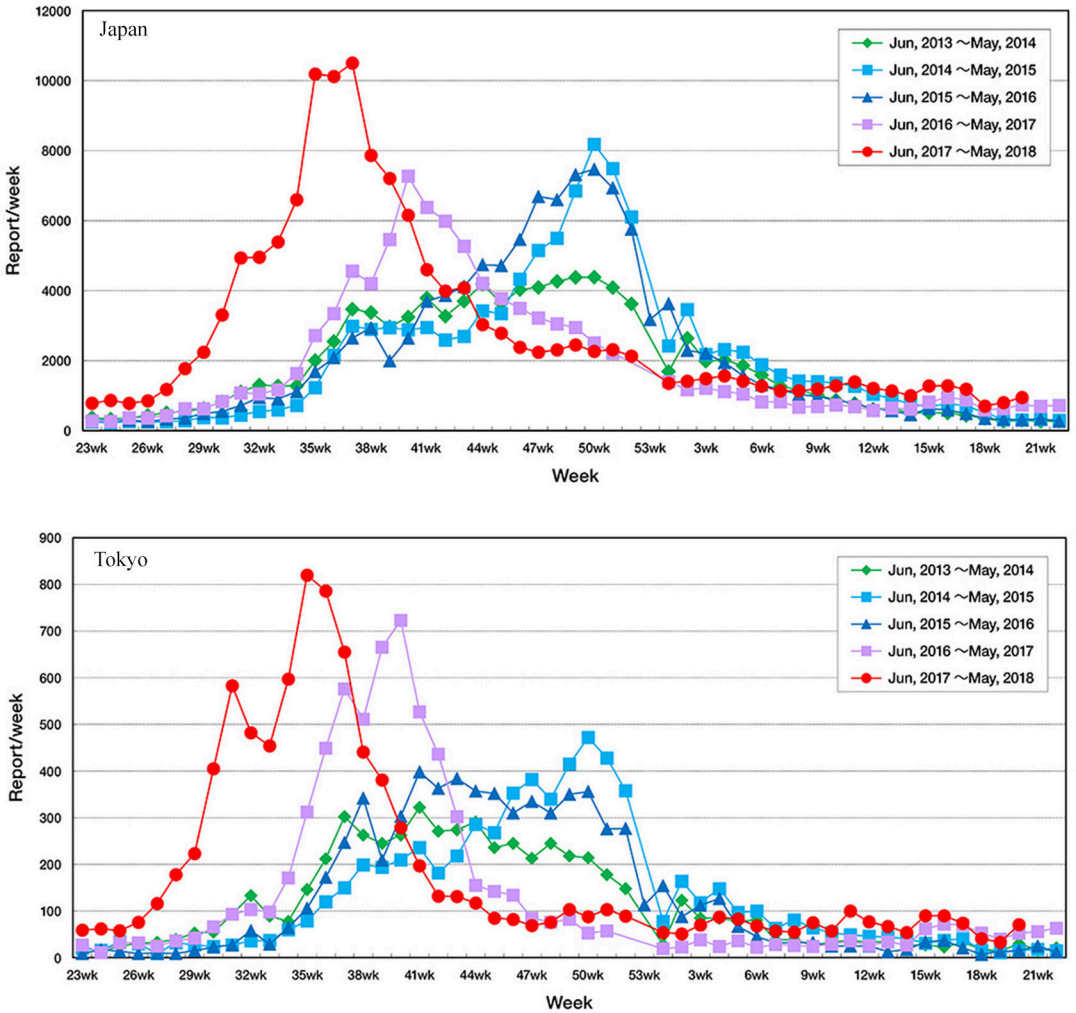


FIGURE 1 | Fluctuation in the number of reported cases of respiratory syncytial virus infection throughout Japan according to the Infectious Diseases Weekly Report nationwide surveillance data in Tokyo and in Japan.

trough. The range of data used to identify epidemic starting points was a 6-year cycle for 46 prefectures, and a 5-year cycle for 1 prefecture (Okinawa). An epidemic period was defined as the period between the onset and the next trough.

The validity of the model was confirmed by determining the proportion of the number of RSV reports falling below the onset line (horizontal line of RSV reporting number at onset) of the RSV season and within the bounds of the sine function.

Determining the Onset of RSV Season

The main objective of this study was to determine the number of RSV reports at the onset of RSV season in Japan to enable timely access to preventive treatment. Ideally, the determination of period for the epidemic should include as many patients as possible. However, the higher number of RSV reports included, the longer the specified epidemic period becomes. From a health economics perspective, more information about a short epidemic period with more RSV reports is desirable. Accordingly, both the number of RSV reports included within epidemic periods relative to the total number of RSV reports (the capture rate) and the number of RSV reports per week within an epidemic period (RSV-reports/w) must be taken into account when determining epidemic starting points. The search index for both the capture rate and RSV-reports/w was then calculated to determine the onset of the epidemic cycle (see the formula below).

The search index was expressed by the following equation:

$$A(x) = \frac{\Sigma_{i=1}^{s} \Sigma_{j=onset(x)_i}^{Through_i} k_j}{\Sigma_{i=1}^{s} (Trough_i - onset(x)_i + 1)}$$

$$B(x) = \frac{\Sigma_{i=1}^{s} \Sigma_{j=onset(x)_i}^{Trough_i} k_j}{\Sigma_{j=Trough(0)}^{Trough(s)} k_j}$$

$$INDEX(x) = \frac{A(x)}{max\left\{x \middle| A(x)\right\}} + \frac{B(x)}{max\{x \middle| B(x)\}}$$

A(x) denotes RSV-reports/w (the number of RSV reported per week)

B(x) denotes capture rate (the number of RSV reports included within onset—trough periods relative to the total number of RSV reports in the dataset).

INDEX(x) denotes the search index. The capture rate (A(x)) and RSV- reports/w (B(x)) were converted into the same scale and summed.

The other variables were as follows:

- *x*, a percentile value ranging from 0 to 100 in integer units;
- *s*, the number of epidemic cycles in the dataset;
- *Onset(x)*, the ordinal number of each onset -week* within the dataset from 1 to *x* in integer units;
- *Trough*, the ordinal number of each trough-week within the dataset from 0 to *x* in integer units;
- *k*, the number of RSV reports in a given week.

*The procedure to set the onset was as follows: The number of RSV reports for each prefecture was transposed into percentile rank, and the minimum percentile rank that was greater than the percentile value was selected. For the 101 percentile values, 101 percentile ranks were selected for each prefecture. The reporting week corresponding to the intersection of these percentile ranks and the increasing slope of the sinusoidal curve was taken as the onset.

Percentile rank of the highest index was set as the onset line, which provided a number of RSV reports/w and was used as a threshold horizontal line to detect the onset of the RSV season. The onset of each epidemic was identified from the intersection of the onset line of the epidemic and the increasing slope of the sinusoidal curve.

The validity of the determined onset was confirmed by calculating a capture rate.

The analyses were first performed on the data reported in Tokyo, the model was then validated using the data for 47 prefectures.

RESULTS

Determination of the Onset of RSV Season in Tokyo

The number of RSV reports/w at the onset of RSV season in Tokyo was 81 reports/week (**Figure 2**). When 81 reports/week was set as the onset line of the epidemic period in Tokyo, the onset of epidemic periods was detected through all the years from 2012 to 2017.

The onset of the epidemic period was the 32nd week (August 6–12) in 2012, the 31st week (July 29–August 4) in 2013, the 36th week (September 1–7) in 2014, the 35th week (August 24–30) in 2015, and the 31st week (August 1–7) in 2016. The onset was the 27th week (July 3–9) in 2017, which was earlier as compared to those previously observed (**Figure 2**, **Table 1**).

When converting the value to the number of cases reported per SMI with the view to generalize the findings to other prefectures, 81 reports/week was converted to 0.31 reports/week per SMI for 259 pediatric medical institutions at fixed locations in Tokyo.

Application of the Approach to All 47 Prefectures in Japan

As in the case of Tokyo, the model identified the RSV epidemic cycles as periodic oscillations in other prefectures as well, despite the 11 climate zones of Japan (**Table 1**, **Supplemental Figures**). The minimum proportion of RSV reports falling below the onset line of RSV season and within the bounds of the sine function was 83.1% (mean 91.3%, median 91.4%, and max 97.1%). The

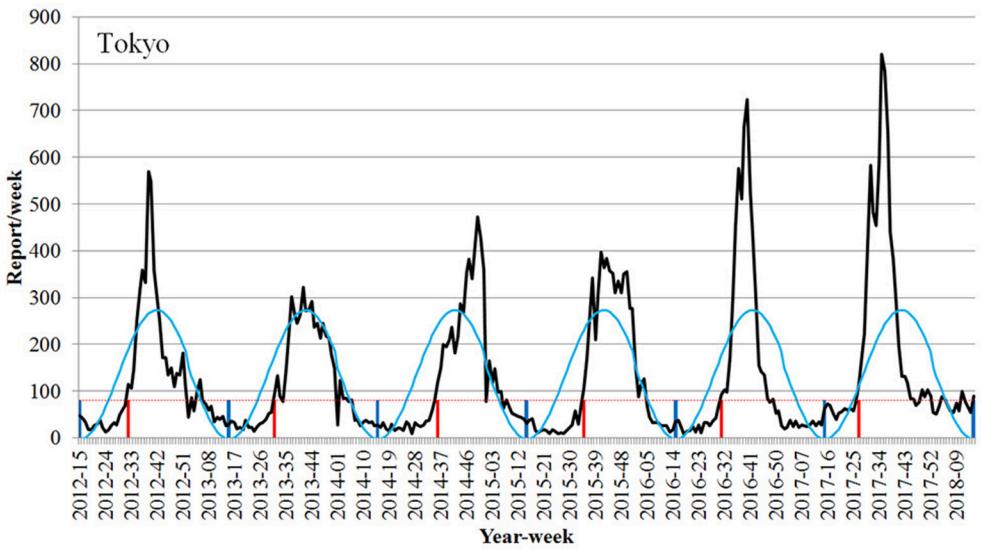


FIGURE 2 | Detection of the onset week of the epidemic season of respiratory syncytial virus infection using the Infectious Diseases Weekly Report nationwide surveillance data in Tokyo (2012–2017 seasons). Black line, number of RSV report; light blue line, RSV epidemic cycle; dotted red line, onset line of RSV season; vertical red line, onset week; vertical blue line, trough of epidemic cycle.

TABLE 1 | The onset of RSV season in 47 prefectures.

Prefecture	Number at a onset line of RSV season	Number of weekly report/SMI	Onset week of RSV season					
			2012	2013	2014	2015	2016	2017
Hokkaido	71	0.50	Week 40 (Oct.1–7)	Week 28 (Jul.8–14)	Week 43 (Oct.20–26)	Week 36 (Aug.31–Sep.6)	Week 29 (Jul.18–24)	Week 21 (May.22–28)
Aomori	20	0.49	Week 31 (Jul.30–Aug.5)	Week 37 (Sep.9–15)	Week 42 (Oct.13–19)	Week 36 (Aug.31–Sep.6)	Week 34 (Aug.22–28)	Week 27 (Jul.3–9)
Iwate	21	0.53	Week 37 (Sep.10–16)	Week 37 (Sep.9–15)	Week 39 (Sep.22–28)	Week 30 (Jul.20–26)	Week 34 (Aug.22–28)	Week 27 (Jul.3–9)
Miyagi	28	0.47	Week 28 (Jul.9–15)	Week 31 (Jul.29–Aug.4)	Week 45 (Nov.3–9)	Week 34 (Aug.17–23)	Week 34 (Aug.22–28)	Week 25 (Jun.19–25)
Akita	11	0.31	Week 30 (Jul.23–29)	Week 36 (Sep.2–8)	Week 39 (Sep.22–28)	Week 39 (Sep.21–27)	Week 37 (Sep.12–18)	Week 31 (Jul.31–Aug.6)
Yamagata	21	0.72	Week 35 (Aug.27–Sep.2)	Week 35 (Aug.26–Sep.1)	Week 39 (Sep.22–28)	Week 36 (Aug.31–Sep.6)	Week 34 (Aug.22–28)	Week 31 (Jul.31–Aug.6)
Fukushima	35	0.76	Week 35 (Aug.27–Sep.2)	Week 32 (Aug.5–11)	Week 43 (Oct.20–26)	Week 35 (Aug.24–30)	Week 34 (Aug.22–28)	Week 24 (Jun.12–18)
Ibaraki	25	0.33	Week 36 (Sep.3–9)	Week 36 (Sep.2–8)	Week 41 (Oct.6–12)	Week 42 (Oct.12–18)	Week 35 (Aug.29–Sep.4)	Week 28 (Jul.10–16)
Tochigi	22	0.46	Week 36 (Sep.3–9)	Week 36 (Sep.2–8)	Week 44 (Oct.27–Nov.2)	Week 40 (Sep.28–Oct.4)	Week 35 (Aug.29–Sep.4)	Week 32 (Aug.7–13)
Gunma	21	0.37	Week 37 (Sep.10–16)	Week 40 (Sep.30–Oct.6)	Week 44 (Oct.27–Nov.2)	Week 41 (Oct.5–11)	Week 33 (Aug.15–21)	Week 31 (Jul.31–Aug.6)
Saitama	56	0.35	Week 35 (Aug.27–Sep.2)	Week 32 (Aug.5– 11)	Week 37 (Sep.8–14)	Week 36 (Aug31–Sep.6)	Week 34 (Aug.22–28)	Week 27 (Jul.3–9)
Chiba	35	0.26	Week 35 (Aug.27–Sep.2)	Week 30 (Jul.22–28)	Week 36 (Sep.1–7)	Week 35 (Aug.24–30)	Week 34 (Aug.22–28)	Week 29 (Jul.17–23)
Tokyo	81	0.31	Week 32 (Aug.6–12)	Week 31 (Jul.29–Aug.4)	Week 36 (Sep.1–7)	Week 35 (Aug.24–30)	Week 31 (Aug.1–7)	Week 27 (Jul.3–9)
Kanagawa	47	0.22	Week 36 (Sep.3–9)	Week 32 (Aug.5–11)	Week 36 (Sep.1–7)	Week 35 (Aug.24–30)	Week 31 (Aug.1–7)	Week 24 (Jun.12–18)
Niigata	43	0.74	Week 39 (Sep.24–30)	Week 34 (Aug.19–25)	Week 36 (Sep.1–7)	Week 34 (Aug.17–23)	Week 33 (Aug.15–21)	Week 24 (Jun.12–18)
Toyama	17	0.61	Week 36 (Sep.3–9)	Week 37 (Sep.9–15)	Week 39 (Sep.22–28)	Week 41 (Oct.5–11)	Week 35 (Aug.29–Sep.4)	Week 29 (Jul.17–23)
Ishikawa	17	0.59	Week 35 (Aug.27–Sep.2)	Week 35 (Aug.26–Sep.1)	Week 40 (Sep.29–Oct.5)	Week 37 (Sep.7–13)	Week 35 (Aug.29–Sep.4)	Week 31 (Jul.31–Aug.6)
Fukui	15	0.68	Week 36 (Sep.3–9)	Week 32 (Aug.5–11)	Week 35 (Aug.25–31)	Week 40 (Sep.28–Oct.4)	Week 37 (Sep.12–18)	Week 31 (Jul.31–Aug.6)
Yamanashi	6	0.25	Week 40 (Oct.1–7)	Week 38 (Sep.16–22)	Week 37 (Sep.8–14)	Week 41 (Oct.5–11)	Week 36 (Sep.5–11)	Week 33 (Aug.14–20)
Nagano	21	0.40	Week 45 (Nov.5–11)	Week 43 (Oct.21–27)	Week 40 (Sep.29–Oct.5)	Week 41 (Oct.5–11)	Week 39 (Sep.26–Oct.2)	Week 34 (Aug.21–27)
Gifu	16	0.31	Week 38 (Sep.17–23)	Week 33 (Aug.12–18)	Week 36 (Sep.1–7)	Week 38 (Sep.14–20)	Week 36 (Sep.5–11)	Week 31 (Jul.31–Aug.6)
Shizuoka	35	0.39	Week 36 (Sep.3–9)	Week 37 (Sep.9–15)	Week 38 (Sep.15–21)	Week 35 (Aug.24–30)	Week 36 (Sep.5–11)	Week 30 (Jul.24–30)
Aichi	73	0.40	Week 38 (Sep.17–23)	Week 36 (Sep.2–8)	Week 36 (Sep.1–7)	Week 40 (Sep.28–Oct.4)	Week 37 (Sep.12–18)	Week 30 (Jul.24–30)
Mie	29	0.64	Week 38 (Sep.17–23)	Week 40 (Sep.30–Oct.6)	Week 40 (Sep.29–Oct.5)	Week 37 (Sep.7–13)	Week 34 (Aug.22–28)	Week 31 (Jul.31–Aug.6)
Shiga	14	0.45	Week 38 (Sep.17–23)	Week 41 (Oct.7–13)	Week 37 (Sep.8–14)	Week 41 (Oct.5–11)	Week 37 (Sep.12–18)	Week 32 (Aug.7–13)
Kyoto	17	0.23	Week 36 (Sep.3–9)	Week 35 (Aug.26–Sep.1)	Week 36 (Sep.1–7)	Week 37 (Sep.7–13)	Week 35 (Aug.29–Sep.4)	Week 30 (Jul.24–30)
Osaka	121	0.61	Week 36 (Sep.3–9)	Week 35 (Aug.26–Sep.1)	Week 35 (Aug.25–31)	Week 37 (Sep.7–13)	Week 33 (Aug.15–21)	Week 28 (Jul.10–16)

(Continued)

TABLE 1 | Continued

Prefecture	Number at a onset line of RSV season	Number of weekly report/SMI	Onset week of RSV season					
			2012	2013	2014	2015	2016	2017
Hyogo	58	0.45	Week 39 (Sep.24–30)	Week 37 (Sep.9–15)	Week 36 (Sep.1–7)	Week 40 (Sep.28–Oct.4)	Week 35 (Aug.29–Sep.4)	Week 31 (Jul.31–Aug.6)
Nara	19	0.56	Week 37 (Sep.10–16)	Week 37 (Sep.9–15)	Week 38 (Sep.15–21)	Week 37 (Sep.7–13)	Week 35 (Aug.29–Sep.4)	Week 30 (Jul.24–30)
Wakayama	15	0.48	Week 38 (Sep.17–23)	Week 36 (Sep.2–8)	Week 33 (Aug.11–17)	Week 42 (Oct.12–18)	Week 36 (Sep.5–11)	Week 27 (Jul.3–9)
Tottori	11	0.58	Week 39 (Sep.24–30)	Week 37 (Sep.9–15)	Week 36 (Sep.1–7)	Week 37 (Sep.7–13)	Week 36 (Sep.5–11)	Week 29 (Jul.17–23)
Shimane	15	0.65	Week 36 (Sep.3–9)	Week 33 (Aug.12–18)	Week 35 (Aug.25–31)	Week 35 (Aug.24–30)	Week 36 (Sep.5–11)	Week 34 (Aug.21–27)
Okayama	15	0.28	Week 37 (Sep.10–16)	Week 37 (Sep.9–15)	Week 37 (Sep.8–14)	Week 35 (Aug.24–30)	Week 36 (Sep.5–11)	Week 33 (Aug.14–20)
Hiroshima	33	0.46	Week 32 (Aug.6–12)	Week 30 (Jul.22–28)	Week 35 (Aug.25–31)	Week 33 (Aug.10–16)	Week 31 (Aug.1–7)	Week 30 (Jul.24–30)
Yamaguchi	29	0.60	Week 35 (Aug.27–Sep.2)	Week 30 (Jul.22–28)	Week 36 (Sep.1–7)	Week 29 (Jul.13–19)	Week 31 (Aug.1–7)	Week 28 (Jul.10–16)
Tokushima	23	1.00	Week 40 (Oct.1–7)	Week 37 (Sep.9–15)	Week 37 (Sep.8–14)	Week 36 (Aug.31–Sep.6)	Week 35 (Aug.29–Sep.4)	Week 31 (Jul.31–Aug.6)
Kagawa	17	0.61	Week 37 (Sep.10–16)	Week 36 (Sep.2–8)	Week 39 (Sep.22–28)	Week 40 (Sep.28–Oct.4)	Week 37 (Sep.12–18)	Week 32 (Aug.7–13)
Ehime	20	0.54	Week 35 (Aug.27–Sep.2)	Week 33 (Aug.12–18)	Week 36 (Sep.1–7)	Week 37 (Sep.7–13)	Week 35 (Aug.29–Sep.4)	Week 29 (Jul.17–23)
Kochi	13	0.43	Week 36 (Sep.3–9)	Week 37 (Sep.9–15)	Week 37 (Sep.8–14)	Week 34 (Aug.17–23)	Week 35 (Aug.29–Sep.4)	Week 31 (Jul.31–Aug.6)
Fukuoka	64	0.53	Week 30 (Jul.23–29)	Week 28 (Jul.8–14)	Week 32 (Aug.4–10)	Week 29 (Jul.13–19)	Week 28 (Jul.11–17)	Week 28 (Jul.10–16)
Saga	16	0.70	Week 35 (Aug.27–Sep.2)	Week 33 (Aug.12–18)	Week 32 (Aug.4–10)	Week 34 (Aug.17–23)	Week 39 (Sep.26–Oct.2)	Week 32 (Aug.7–13)
Nagasaki	17	0.39	Week 35 (Aug.27–Sep.2)	Week 33 (Aug.12–18)	Week 33 (Aug.11–17)	Week 32 (Aug.3–9)	Week 32 (Aug.8–14)	Week 29 (Jul.17–23)
Kumamoto	26	0.53	Week 35 (Aug.27–Sep.2)	Week 34 (Aug.19–25)	Week 36 (Sep.1–7)	Week 35 (Aug.24–30)	Week 30 (Jul.25–31)	Week 30 (Jul.24–30)
Oita	17	0.47	Week 33 (Aug.13–19)	Week 38 (Sep.16–22)	Week 35 (Aug.25–31)	Week 30 (Jul.20–26)	Week 38 (Sep.19–25)	Week 30 (Jul.24–30)
Miyazaki	29	0.81	Week 31 (Jul.30–Aug.5)	Week 31 (Jul.29–Aug.4)	Week 36 (Sep.1–7)	Week 31 (Jul.27–Aug.2)	Week 27 (Jul.4–10)	Week 31 (Jul.31–Aug.6)
Kagoshima	21	0.38	Week 27 (Jul.2–8)	Week 26 (Jun.24–30)	Week 33 (Aug.11–17)	Week 23 (Jun.1–7)	Week 24 (Jun.13–19)	Week 26 (Jun.26–Jul.2)
Okinawa	17	0.50	-	Week 7 (Feb.11–17)	Week 8 (Feb.17–23)	Week 8 (Feb.16–22)	Week 4 (Jan.25–31)	Week 5 (Jan.30–Feb.5)

RSV, respiratory syncytial virus.

A number at an onset line of RSV season was used as a threshold to determine an onset of RSV season.

mean number of RSV reports/w at onset of the RSV season for all prefectures was 29.7 reports/week (median 21.0, min 6.0, and max 121.0 reports/week), and the mean number of weekly reports/SMI to define the onset of the RSV season was 0.52 (median 0.53, min 0.22, and max 1.0, **Table 1**). In general, the onset of the epidemic RSV season in 2017 was more than 1 month earlier than in the previous year, as demonstrated by the data.

DISCUSSION

Japan is under 11 significantly different climate zones due to its geographical location between the Sea of Japan and the Pacific Ocean, and shape, which spans from north to south. There are 47 prefectures in Japan; the epidemic of RSV infection begins from the south/western area and then tends to spread to the eastern area[1]. The epidemic curve based on RSV reports reaches a peak during summer in Okinawa, the southernmost prefecture and a subtropical region, unlike other prefectures in Japan (12). For this reason, it may be difficult to define the integrated conditions of the onset of the RSV season on a national basis in Japan. Therefore, the conditions of the onset of the RSV season were investigated separately for each prefecture in this study.

First, periodicities of the RSV season in each year were explored for each prefecture using a sinusoidal curve. The

fluctuation of the recent epidemic period was found to be periodic every year in all prefectures (**Table 1**). We then explored the onset of the RSV season, assuming it would come after the trough of the sinusoidal curve for each prefecture.

Previous studies have shown similar modeling approaches for the epidemics of RSV (13–16), in which non-linear regression analyses were applied. In the present study, in addition to the non-linear regression analyses, the model was developed taking account of the following factors and their balance for the epidemic period: (1) the number of RSV reports per week within epidemic periods (a high rate of RSV-reports/w), and (2) the majority of the reports included within the RSV season (a high capture rate).

Generally, if the epidemic period is narrowed down to the vicinity of the peak, although the number of RSV-reports/w will be high, the capture rate will be low, because many RSV infections have been reported before it. Thus, it may be too late to define the epidemic start based on this period definition. Conversely, when the epidemic period is set too early, the capture rate will be sufficiently high, but the number of RSV-reports/w will be too low, and therefore it may be too early to define the onset of the RSV season. Consequently, we considered that a balance between RSV-reports/w and the capture rate of reports is important to detect the onset of the RSV epidemic. To accomplish this task, the RSV-reports/w and capture rate in the epidemic period were quantified to the same scale from 0.0 to 1.0, so that the sum of both could be used as an index. The timing of the maximum value was taken as the epidemic start.

It is worth mentioning that it is mandatory for about 10% of medical institutions (SMIs) to report the RSV-positive cases, and the data are disseminated by the IDWR in Japan, indicating that the weekly reports well represent the real epidemic RSV status in Japan, whereas other case data (hospitalization and RSV-positive rates) used in the previous studies for the modeling in the US were obtained in certain geographical regions (13, 15, 16). Such difference in the data coverage used for the modeling development, as well as that in the modeling methods, make a direct comparison difficult between our study and previous studies.

Criteria for the onset of RSV season for each prefecture were set in the present study, and the results showed a remarkable change in the onset of RSV season by years. The model detected that the onset was 1 or 2 months earlier in 2017 than in 2016. The 2017 RSV epidemic began in July in 34 (72%) prefectures and in June in 4 (8.5%) prefectures (**Table 1**). The National Institute of Infectious Disease and Tokyo Metropolitan Government announced that the RSV epidemic in 2017 started in July, indicating that our results support the findings of the NIID and the Tokyo Metropolitan Government.

The model also revealed that the changes were small in the southern prefectures. Furthermore, Miyazaki and Kagoshima prefectures, which are located between the temperate zone and the subtropical zone in the south, showed two peaks where RSV prevails, in winter and summer. In Okinawa prefecture, which is located in the subtropical zone at the south end, the epidemic period was in summer. Because of the prefecture-specific tendency in the patterns of RSV cycles, the application of our model for each prefecture will provide more precise and timely information regarding the pattern of RSV seasons.

A limitation of the study is that the model does not predict the onset of the RSV season in advance, and therefore it may not provide timely information regarding its start. However, the model enables us to detect the onset at an early stage so that we can prepare, e.g., to administer palivizumab to infants to prevent the severe RSV infection. Another limitation is that the model may not identify potential onset timing if the number of reported cases fluctuates substantially or if high numbers of cases are observed throughout a year.

The result would be used to prepare for the RSV season by estimating the onset of the next RSV season from the past onsets.

Converting to the reported number per SMI, the differences in the pediatric population by prefecture are corrected, so it would be able to refer to the situation in the neighboring prefecture. For example, many people in the Ibaraki prefecture are commuting to Tokyo.

The result would contribute timely detection of the onset of RSV season even if the season starts earlier than the presumption. It would be expected to help reduce the severe RSV infection in infants by signaling the need for the prompt start of treatment with palivizumab and hence contribute to pediatric and neonatal medical practice.

CONCLUSION

In this study, our model detected the onset week of RSV season in 47 prefectures based on the national surveillance data in Japan for 6 years (2012–2017). The model also verified that the onset of RSV season was in early July in 2017. A timely detection of the onset of RSV season is expected to help reduce severe RSV infection in infants by signaling the need for the prompt start of treatment with palivizumab, and thereby contribute to pediatric and neonatal medical practice.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

All authors prepared the manuscripts and approved the manuscript for publication. HY and TH designed the study. TH conducted data analysis. HK, IK, and SK provided efficient advice, from the data analysis planning to obtainment of results.

FUNDING

The design, study conduct, and financial support for the study were provided by AbbVie. AbbVie participated

in the interpretation of data, review, and approval of the publication.

ACKNOWLEDGMENTS

Mayumi Saotome, Ryo Yano, and Ayumi Kodama of DOT WORLD Co., Ltd., Tokyo, Japan, provided medical writing and editing services in the development of this manuscript. AbbVie provided funding to DOT WORLD Co., Ltd. for this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2019.00039/full#supplementary-material

Data Sheet 1 | Detection of the start of the epidemic period of respiratory syncytial virus (RSV) infection using the Infectious Diseases Weekly Report surveillance data (2012-2017 seasons) of 46 prefectures. Black line, number of RSV report; light blue line, RSV epidemic cycle; dotted red line, onset line of RSV season; vertical red line, onset week; vertical blue line, trough of epidemic cycle.

REFERENCES

1. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child*. (1986) 140:543–6. doi: 10.1001/archpedi.1986.02140200053026
2. Hall CB. Respiratory syncytial virus: what we know now. *Contemp Pediatr*. (1993) 10:92–110.
3. Hall CB, McBride J. Bronchiolitis. In: Mandell GL, editor. *Principles and Practice of infectious Disease* 4th ed. New York, NY: Churchill Livingstone. (1999). p. *1501*.
4. Takeuchi Y, Nishida H, Fujimura M. Prospective clinical epidemiological surveillance on respiratory syncytial virus infection in premature infants. *J Jpn Pediatr Soc*. (2003) 107:898–904.
5. Committee to Develop and Review Guidelines for the Use of Palivizumab. Prevention of respiratory syncytial virus infection, guidelines for the use of palivizumab in Japan. *J Jpn Pediatr Soc*. (2002) 106:1288–92.
6. Tsuda T, Sawada Y, Ikeda K, Fujibayashi S, Mito K, Chiba Y, et al. Respiratory syncytial virus infection in patients with left-to-right shunt congenital heart disease complicated with pulmonary hypertension. *J Jpn Pediatr Soc*. (1982) 86:2076–82.
7. Horn SD, Smout RJ. Effect of prematurity on respiratory syncytial virus hospital resource use and outcomes. *J Pediatric*. (2003) 143:S133–41. doi: 10.1067/S0022-3476(03)00509-2
8. Ohki H, Tamame T, Naganuma T, Chinen S, Matsuoka M, Miura M. Respiratory syncytial virus infection in patients with congenital heart disease: comparison before and after expanded indication of palivizumab. *J Pediatr Pract*. (2009) 72:1741–6.
9. Nakazawa M, Saji T, Ichida F, Oyama K, Kusuda S, Harada K. Guidelines for the use of palivizumab in infants with congenital heart disease. *J Jap Soc Premat Newborn Med*. (2005) 17:152–5.
10. Mori M, Morio T, Ito S, Morimoto A, Ota S, Mizuta K, et al. Guidebook for the use of palivizumab in patients with immunodeficiency and Down syndrome. *J Clin Pediatr Rheumatol*. (2014) 5:5–8.
11. Ministry of Health, Labour and Welfare, National Institute of Infectious Diseases. Infectious diseases requiring attention - respiratory syncytial virus infection. *IDWR*. (2017) 19:8–10.
12. Shobugawa Y, Takeuchi T, Hibino A, Hassan MR, Yagami R, Kondo H, et al. Occurrence of human respiratory syncytial virus in summer in Japan. *Epidemiol Infect*. (2017) 145:272–84. doi: 10.1017/S095026881600220X
13. Leecaster M, Gesteland P, Greene T, Walton N, Gundlapalli A, Rolfs R, et al. Modeling the variations in pediatric respiratory syncytial virus seasonal epidemics. *BMC Infect Dis*. (2011) 11:105. doi: 10.1186/1471-2334-11-105
14. Moore HC, Jacoby P, Hogan AB, Blyth CC, Mercer GN. Modelling the seasonal epidemics of respiratory syncytial virus in young children. *PLoS ONE*. (2014) 9:e100422. doi: 10.1371/journal.pone.0100422
15. Noveroske DB, Warren JL, Pitzer VE, WeinbergerDM. Local variations in the timing of RSV epidemics. *BMC Infect Dis*. (2016) 16:674. doi: 10.1186/s12879-016-2004-2
16. Zachariah P, Shah S, Gao D, Simões EA. Predictors of the duration of the respiratory syncytial virus season. *Pediatr Infect Dis J*. (2009) 28:772–6. doi: 10.1097/INF.0b013e3181a3e5b6

Conflict of Interest Statement: AbbVie GK planned the study design and conducted the study. HK, IK, and SK are advisors to AbbVie GK. HY and TH are employees of AbbVie GK.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 March 2019
doi: 10.3389/fmicb.2019.00447

Alveolar Macrophages Treated With *Bacillus subtilis* Spore Protect Mice Infected With Respiratory Syncytial Virus A2

Ji Eun Hong[1†], Yoon-Chul Kye[1†], Sung-Moo Park[1,2], In Su Cheon[1,3], Hyuk Chu[4], Byung-Chul Park[5], Yeong-Min Park[6], Jun Chang[7], Jae-Ho Cho[8], Man Ki Song[3], Seung Hyun Han[9] and Cheol-Heui Yun[1,2,5]*

[1] Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, South Korea, [2] Center for Food and Bioconvergence, Seoul National University, Seoul, South Korea, [3] Laboratory Science Division, Department of Molecular Vaccinology, International Vaccine Institute, Seoul, South Korea, [4] Division of Zoonoses, Center for Immunology and Pathology, Korea Center for Disease Control and Prevention, National Institute of Health, Cheongju, South Korea, [5] Institute of Green Bio Science and Technology, Seoul National University, Seoul, South Korea, [6] Department of Immunology, School of Medicine, Konkuk University, Chungju, South Korea, [7] Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea, [8] Academy of Immunology and Microbiology, Institute for Basic Science, Pohang, South Korea, [9] Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, Seoul, South Korea

OPEN ACCESS

Edited by:
Ville Peltola,
Turku University Hospital, Finland

Reviewed by:
Diana Boraschi,
Istituto di Biochimica delle Proteine (IBP), Italy
Vahid Salimi,
Tehran University of Medical Sciences, Iran

***Correspondence:**
Cheol-Heui Yun
cyun@snu.ac.kr;
cyun@snu.kr

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *16 September 2018*
Accepted: *20 February 2019*
Published: *12 March 2019*

Citation:
Hong JE, Kye Y-C, Park S-M, Cheon IS, Chu H, Park B-C, Park Y-M, Chang J, Cho J-H, Song MK, Han SH and Yun C-H (2019) Alveolar Macrophages Treated With Bacillus subtilis Spore Protect Mice Infected With Respiratory Syncytial Virus A2. Front. Microbiol. 10:447. doi: 10.3389/fmicb.2019.00447

Respiratory syncytial virus (RSV) is a major pathogen that infects lower respiratory tract and causes a common respiratory disease. Despite serious pathological consequences with this virus, effective treatments for controlling RSV infection remain unsolved, along with poor innate immune responses induced at the initial stage of RSV infection. Such a poor innate defense mechanism against RSV leads us to study the role of alveolar macrophage (AM) that is one of the primary innate immune cell types in the respiratory tract and may contribute to protective responses against RSV infection. As an effective strategy for enhancing anti-viral function of AM, this study suggests the intranasal administration of *Bacillus subtilis* spore which induces expansion of AM in the lung with activation and enhanced production of inflammatory cytokines along with several genes associated with M1 macrophage differentiation. Such effect by spore on AM was largely dependent on TLR-MyD88 signaling and, most importantly, resulted in a profound reduction of viral titers and pathological lung injury upon RSV infection. Taken together, our results suggest a protective role of AM in RSV infection and its functional modulation by *B. subtilis* spore, which may be a useful and potential therapeutic approach against RSV.

Keywords: respiratory syncytial virus, *Bacillus subtilis*, spore, alveolar macrophage activation, MyD88

INTRODUCTION

Respiratory syncytial virus (RSV) causes serious disease symptoms of bronchiolitis and acute lower respiratory tract infection resulting in up to 200,000 annual deaths worldwide, especially for the infants and elderly (Nair et al., 2010). Recent study has also suggested that children younger than 6 months old suffering from RSV amounted to 1.4 million hospital admissions and

27,300 for in-hospital death (Shi et al., 2017). Despite the numerous trials, effective preventive strategies against RSV infection have been limited and turned out to be unsuccessful. This is because of the unique ability of RSV to disarm host of both innate and adaptive immunity (Meng et al., 2014) causing overall failure of host immune defense mechanisms, which is mainly due to non-structural 1 and 2 proteins. Indeed, a poor innate response has previously been well documented particularly at the initial stage of RSV infection (Marr et al., 2013) and several attempts have been made to improve this stage in RSV infection. In this regard, it has been suggested that successful protection against RSV can be mediated by activation of innate immune cells via toll-like receptor (TLR) 2 and 4 stimulation (Kurt-Jones et al., 2000; Murawski et al., 2009), but not by caspase-1 pathway (Shim and Lee, 2015).

One of the primary innate immune cell types in the lung is alveolar macrophages (AM), which play a pivotal role to maintain homeostasis and to induce effective defense mechanism. AMs have unique properties compared to other macrophages in different tissues because they have direct contact with external environment, which could allow rapid and direct recognition of antigens derived from invading pathogens or allergens followed by the initiation of immune responses (Hashimoto et al., 2011; Herold et al., 2011). Recent studies have shown that failure of early AM-mediated defense response caused insufficient protection against various viral pathogens infecting respiratory tracts and led to diminished recruitment of immune cells followed by disrupted lung homeostasis (Maus et al., 2002; Snelgrove et al., 2008). Despite the importance of AMs in various respiratory viral diseases, the precise role and protective mechanisms of AM in RSV infection are yet to be investigated.

Previous attempts using probiotics have gained an insight for the priming innate immune system where the administration of such probiotics induced a rapid activation of innate immunity and consequently enhanced protective efficacy against respiratory viral infection (Garcia-Crespo et al., 2013; Park et al., 2013; Lee et al., 2016). Furthermore, spore derived from probiotics, as we used in this study, is known to be one of the alternatives not only for inducing enhanced innate immune responses but also for safety and stability (Hong et al., 2008). *Bacillus subtilis* has been known to change its status into spore form under the harsh circumstances such as starvation. After sporulation, it can survive with resistance to heat, cold, or other enzymatic assaults (Tan and Ramamurthi, 2014). Because of their stability, several researchers have tried to use spores as probiotics or adjuvants to enhance the health and to protect against infections caused by microbes such as influenza virus (Song et al., 2012) or *Clostridium difficile* (Colenutt and Cutting, 2014) in mice and human trials (Lefevre et al., 2015, 2017).

In this study, we tried to use *B. subtilis*-derived spore to overcome RSV infection and investigated whether and how *B. subtilis*-derived spore would influence on the AMs to induce effective innate immunity and subsequently to protect host from RSV infection.

RESULTS

Intranasal Delivery of *B. subtilis* Spore Enhanced Anti-viral Immunity to RSV Infection

It has been reported that *in vivo* administration of prebiotics or probiotics such as *Lactobacillus* can facilitate protective immunity against influenza virus infection (Kiso et al., 2013; Lee et al., 2013). These previous reports prompted us to test the potential of bacterial particular substance, *B. subtilis*-derived spore to protect against RSV infection. To address this, a group of mice was administered with or without spore intranasally (i.n.) prior to RSV infection and then infected them with RSV. The effect of spore was clear in body weight loss; untreated control mice lost 10–20% total body weight with a peak reduction of day 2 post-infection (p.i.), which is well-known symptom of RSV infection in mouse model, whereas mice treated with spore did not lose their body weight (**Figure 1A**). It should be noted, however, that the weight loss after RSV infection appeared to be transient, which is a limitation of RSV mouse infection model, so the control mice began to gradually gain weight back to near normal level by day 4 post-infection. To support the body weight data, there was about 4–5-fold decrease in viral loads in the lung of the spore-treated mice compared to those of control mice (**Figure 1B**). These results suggest that delivery of *B. subtilis* spore not only prevent the pathology, but also help clearance of virus against RSV infection.

I.n. Administration of *B. subtilis* Spore Increases the Number of Alveolar Macrophages

To identify the cellular mechanism by which *B. subtilis* spore influences on anti-viral immunity, we analyzed various innate immune cells in the lung with or without spore treatment. We found that there was the most notable increase in AM both in the lung and bronchoalveolar lavage (BAL) along with inflammatory monocytes or neutrophils (**Figures 1C,D**), implicating AMs might play an important role in mediating innate immunity to RSV. In line with increased numbers of AM, spore-treated mice showed increased gene expression of GM-CSF and well-known M1 macrophage-related cytokines (Murray et al., 2014) including TNF-α, IFN-γ, IL-12p40, and IL-6 (**Supplementary Figure S1**). The cytokine producing ability of spore was also apparent in protein level of effector cytokines such as IFN-β and IL-12p40 (**Figure 1E**), which are also categorized in M1 macrophage-related cytokines. These results suggest that administration of *B. subtilis* spore induced significantly enhanced number of AMs, and augmented expression of effector cytokines which are related to M1 macrophage differentiation.

Depletion of Alveolar Macrophages Aggravates Disease Severity in Mice Infected With RSV

In viral infection condition, it is known that AM have a critical role during the early phase of infection (Schneider et al., 2014a)

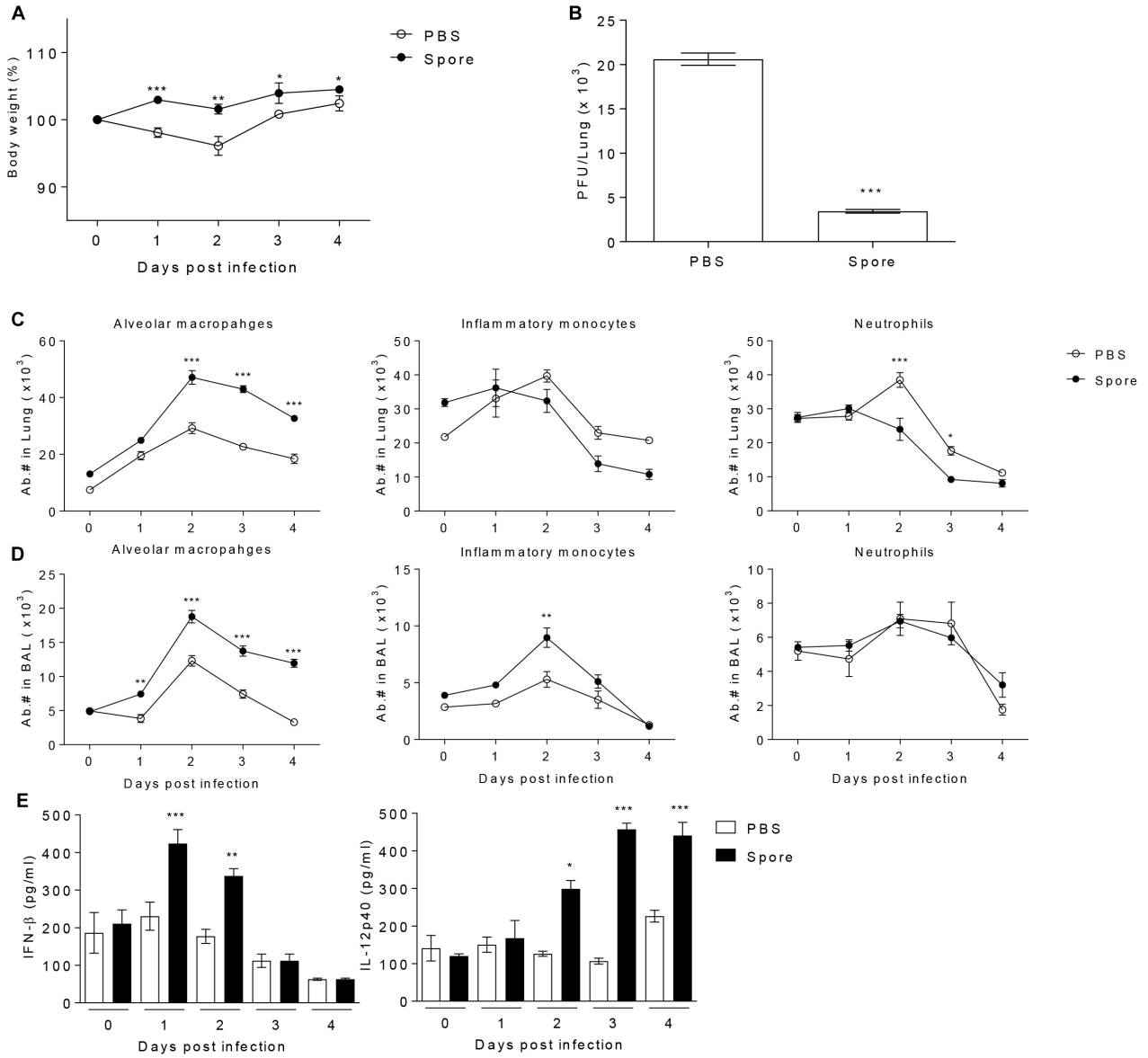


FIGURE 1 | Pre-treatment with spore through intranasal route reduces the disease severity following RSV infection and induces the population change of alveolar macrophages (AM) and enhances antiviral effector molecules. Mice were administered with 1×10^9 CFU of spore via i.n. route at 5 days prior to RSV infection with 2×10^6 PFU per mouse ($n = 3$). **(A)** Body weight was monitored daily after the infection and **(B)** viral load in the lungs was analyzed by plaque assay at 4 days post-infection (DPI). Change of various innate immune cells in the **(C)** post-lavaged lung and **(D)** BAL fluid was analyzed by flow cytometry at 0 to 4 DPI. Empty and filled circles indicate PBS and spore pre-treated mice, respectively. **(E)** IFN-β and IL-12p40 in BAL fluid were measured by ELISA. Data are expressed as mean ± S.E.M. for the group. Significant differences from results with the PBS control are $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$, respectively.

because of the alarming failure and lack of scavenger activity against infected cells (Kumagai et al., 2007; Pribul et al., 2008). Moreover, AM-depleted mice are unable to deliver the antigen to dendritic cells for the antigen presentation (Ugonna et al., 2014) in order to strengthen the following adaptive immune response. To examine the particular role of AM in RSV infection, we conducted the depletion of AM by injection of clodronate-encapsulated liposome (Clod) through the intratracheal (i.t.) route. As a result, absolute number of AM in the lungs and BAL from Clod-treated mice declined by approximately 85% compared to that in control (**Supplementary Figure S2**) without significant histological changes (**Figure 2C**). At day 1 post-infection of RSV, AM-depleted mice showed a sharp decline in body weight (**Figure 2A**) and at day 4, they have significantly higher viral loads in lung than control (**Figure 2B**). Pathological results also showed the severity of infection with displayed thickened alveolar epithelium, destruction of epithelial walls, overall alveolar swelling, and the accumulation of immune cells in the lung (**Figure 2C**). Furthermore, the accumulation of inflammatory cells in the interstitial space, bronchi, and vessel

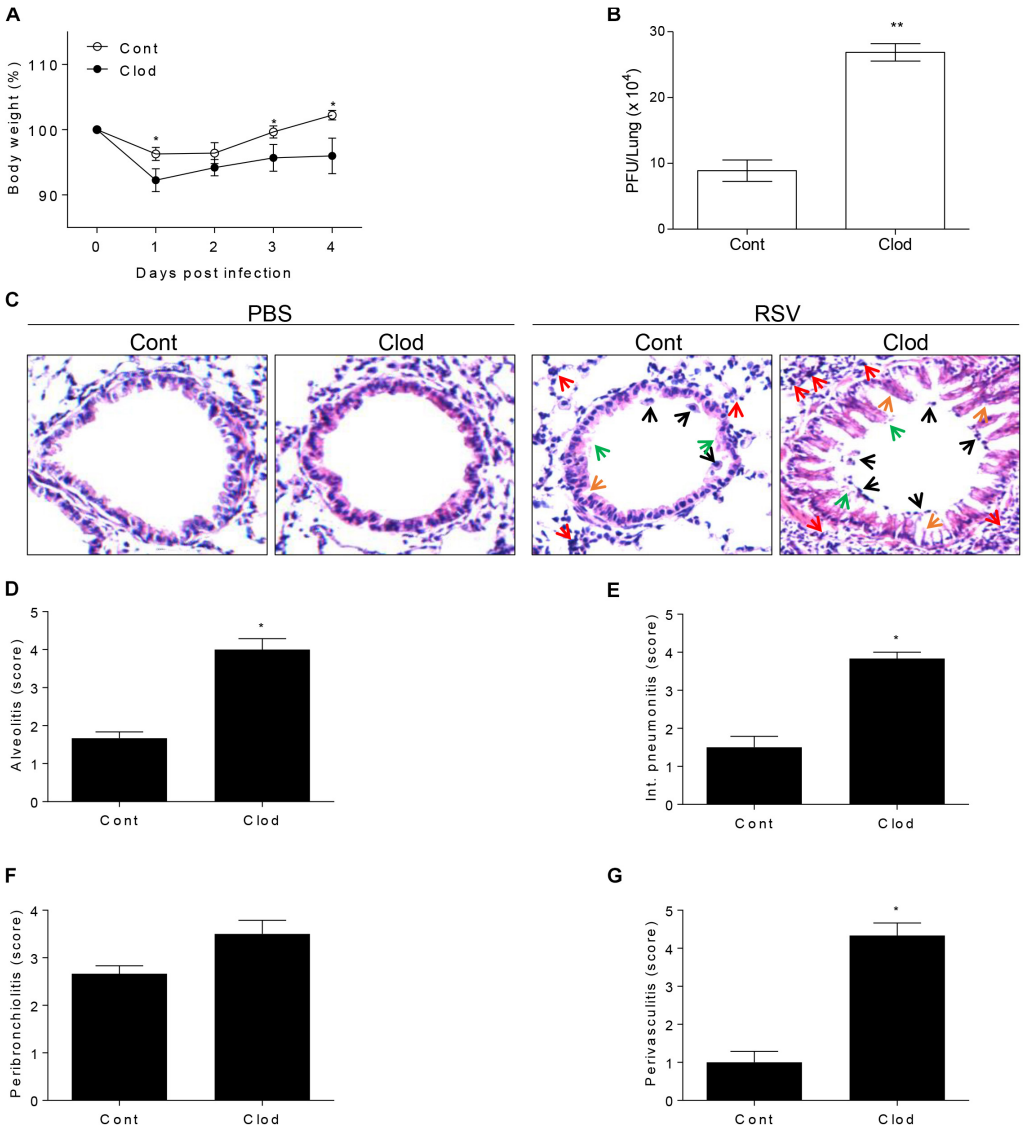


FIGURE 2 | Mice selectively depleted with AMs fail to protect RSV infection. Mice were injected i.t. with clodronate-encapsulated liposome twice on days 1 and 3 prior to RSV infection. **(A)** Body weight was monitored daily after the infection and **(B)** viral load in the lungs was analyzed by plaque assay at 4 DPI (n = 3). At 4 DPI, perfused lungs were stained with H&E **(C)** for histological examination by microscopy at 200 × magnifications and **(D–G)** scored for histopathology. "Cont" indicates the mice injected with control liposome and "Clod" indicates the mice injected with clodronate-encapsulated liposome. Arrows indicated as follows; orange, epithelium thickness and destruction; green, pulmonary edema; red, inflammatory cells; and black, cell death. Data are expressed as mean ± S.E.M. for the group. Significant differences from results with the PBS control are $^*P < 0.05$ and $^{**}P < 0.01$, respectively.

was apparent in lungs from AM-depleted mice (**Figures 2D–G**). Taken together, depletion of AM at the initial phase of RSV infection leads to exacerbated disease severity coincident with increased pulmonary inflammation.

Spore Directly Enhances the Antiviral Function of Alveolar Macrophages

To explore direct role of AM in cellular level and the effect of *B. subtilis* spore, AM cell line, MH-S, was treated with spore for 24 h and then infected with RSV. The number of plaques of RSV was significantly reduced when the cells were incubated with spore in a dose-dependent manner (**Figure 3A**). Inflammatory cytokines, IL-12p40 and IL-6, in the supernatant from the cells treated with spore followed by RSV infection were measured as an initial assessment for their antiviral activity (Ugonna et al., 2014). The results showed that the spore treatment led to substantial increase of IL-6 and, to a lesser extent, IL-12p40 (**Figure 3B**). These results demonstrated that *B. subtilis* spore directly promotes the anti-viral activity of AM, especially at the early time point after the infection, in a dose-dependent manner.

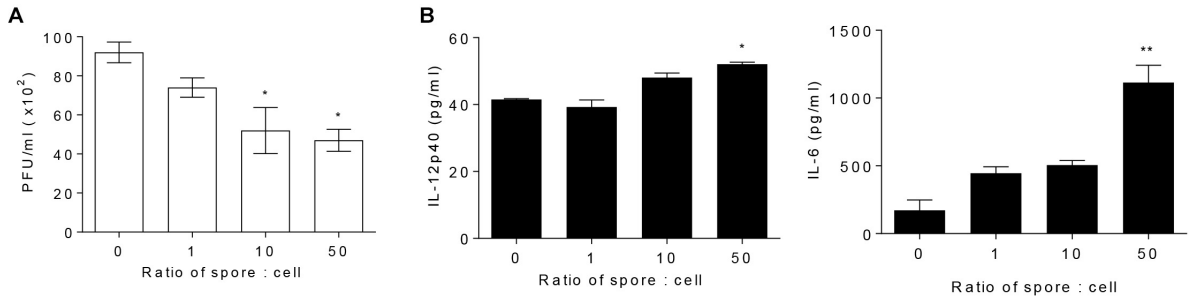


FIGURE 3 | Alveolar macrophages pre-treated with spore extend their antiviral effects. MH-S cells were stimulated with spore for 24 h, with a ratio of MH-S:Spore at = 1:0, 1:1, 1:10, and 1:50. Then, the cells were washed and infected with 1 MOI RSV for 12 h. **(A)** The viral load was analyzed by using plaque assay and **(B)** the production of IL-12p40 and IL-6 was measured by ELISA. Data are expressed as mean ± S.E.M. of three replicates. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Alveolar Macrophages Activated by Spore Have a Pivotal Protective Role in Mice Infected With RSV

To validate direct involvement of spore-activated AM for the protection from RSV infection *in vivo*, we depleted the AMs in spore pre-administered mice before RSV infection. As mentioned above, mice treated with spore did not have any significant weight loss (**Figure 4A**), in line with the ability of spore to clear the virus (**Figure 4B**). However, when we depleted AM by clodronate in spore-treated mice, they showed a moderate decrease of body weight (**Figure 4A**) and led to viral counts be increased in the lung (**Figure 4B**), suggesting that AM play an important role in the protection against RSV infection by *B. subtilis* spore treatment. The spore treatment can efficiently protect the epithelium and prevent infiltration of immune cells in alveolus (**Figure 4C**) and bronchi (**Supplementary Figure S3**), which is seen in the pathological score (**Figures 4D–G**) in support of the effect of AM. These data suggested that spore treatment prevents RSV infection efficiently, the effect is partially dependent on the AM. Taken together, these results support that the *B. subtilis* spore enhances host protection against RSV infection with support of AM.

The Effects of Spore on Protection Against RSV Infection Are Dependent on MyD88 Signaling

The next question was which signaling pathways are involved in the capacity of spore to modulate anti-viral functions of AM. Since previous reports suggest TLR2 or 4 is in charge of viral infection as mentioned above, we presumed that spore might trigger MyD88 signaling upon direct engagement with TLRs on AM. In support of this idea, we used MyD88$^{-/-}$ mice for RSV infection model with *B. subtilis* spore administration. MyD88$^{-/-}$ mice were unable to induce protective response to RSV infection with reduced body weight (**Figure 5A**) and significantly high viral load (**Figure 5B**). More importantly, the administration of spore did not work on the recovery of MyD88$^{-/-}$ mice at all. Consistent with these data, infiltration of AM in the lung (**Figure 5C**) and BAL (**Figure 5D**) was far less than wild-type mice and the inflammation with severe destruction of epithelium was also shown in alveolus (**Figures 5E–I**) and bronchi (**Supplementary Figure S3**) of MyD88$^{-/-}$ mice even with *B. subtilis* spore treatment.

To confirm the direct engagement of MyD88 signaling upon *B. subtilis* treatment in cellular level, we used bone marrow-derived macrophages (BMMs) from wild type or MyD88$^{-/-}$ mouse and treated spore *in vitro* with RSV infection and conduct plaque assay with culture supernatant. In the same context with *in vivo* data, significantly higher viral loads were observed in MyD88-deficient BMMs compared to wild-type BMMs (**Figure 6A**). As expected, the capability of spore to reduce viral load was prominent in wild-type BMMs in a dose-dependent manner; unexpectedly, however, the effect of spore was, albeit less, also apparent even in MyD88-deficient BMMs. Consistent with data from viral loads, IL-12p40 production was markedly augmented by spore treatment and substantially higher in wild-type BMMs than MyD88-deficient BMMs (**Figure 6B**).

Taken together, these results suggest that *B. subtilis* spore can enhance the protective capacity against RSV infection affecting directly on the AMs, and these activation and M1-related effector cytokine production is dependent on MyD88 signal.

DISCUSSION

Importance of AM for host defense mechanism during early viral infection in respiratory tract is well established, however, the precise role of AM, especially in mice infected with RSV is less clear. In the present study, we focused on the specific role of AM treated with *B. subtilis* spore in RSV infected mice for their protection and brought the following four notable findings: (1) AM have a pivotal role for protection during the initial stage of RSV infection; (2) Administration of *B. subtilis* spore induces the activation of AM with up-regulation of GM-CSF and M1 macrophage-related cytokines; (3) The i.n. delivery of *B. subtilis* spore induces the increase of antiviral effector molecules against RSV infection; and (4) MyD88 signals in the AM are critical for the protection against RSV.

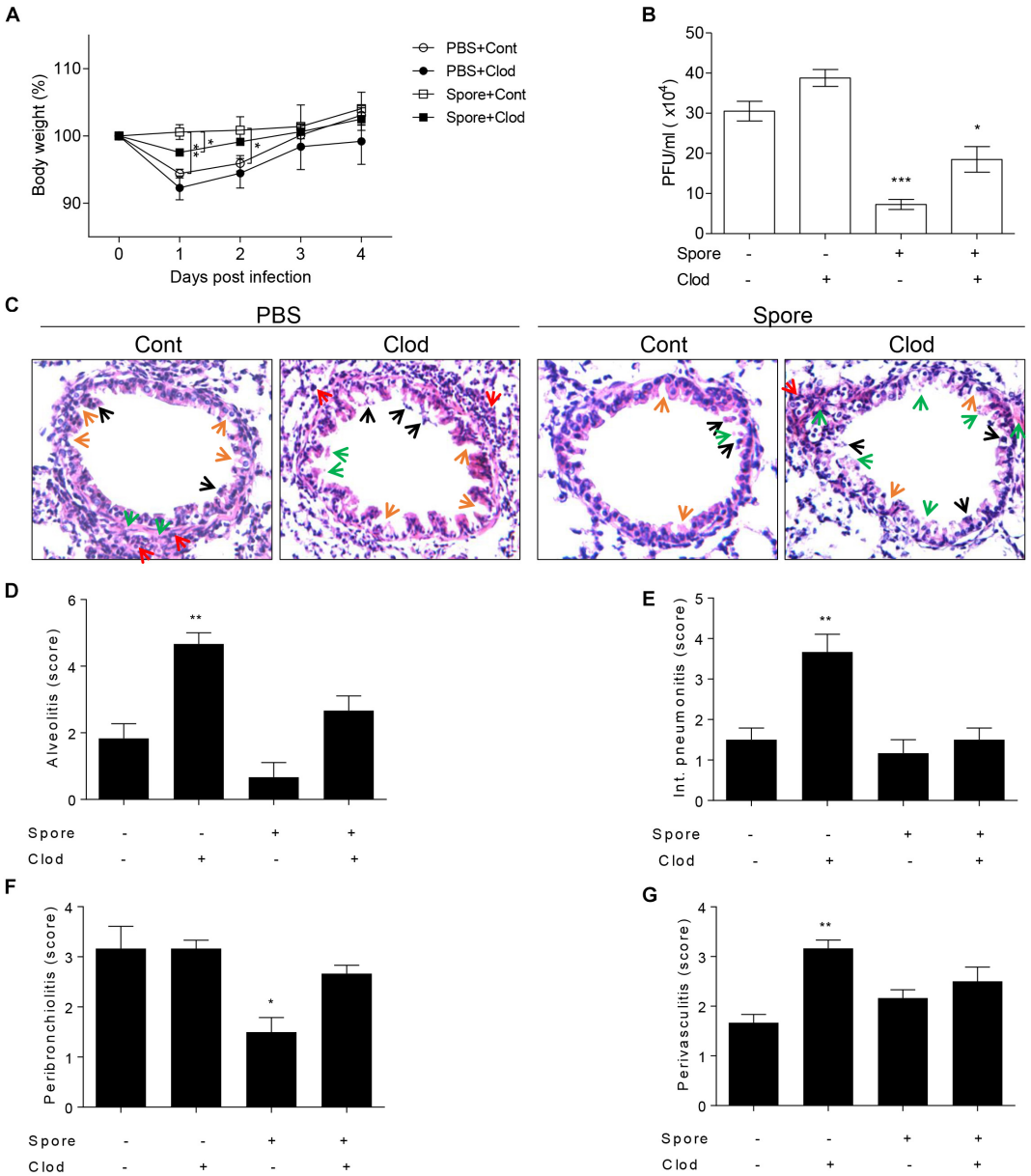


FIGURE 4 | Alveolar macrophages in mice intranasally administered with spore play a key role in RSV infection. Mice were intranasally administered with spore at 5 days prior to RSV infection and injected with control or clodronate-encapsulated liposome through intratracheal on days 3 and 1 before the infection and sacrifice on 4 DPI. **(A)** Body weight was monitored daily after the infection and **(B)** viral load in the lung was examined at 4 DPI, respectively ($n = 3$). **(C)** At 4 DPI, perfused lungs were stained with H&E for histological examination by microscopy at 200 × magnifications and **(D–G)** scored for histopathology. Arrows indicated are as follows; orange, epithelium thickness and destruction; green, pulmonary edema; red, inflammatory cells; and black, cell death. Significant differences from results with the PBS control are $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$, respectively.

Induction of innate immune response in the early phase of viral infection is necessary for host immunity (McGill et al., 2009; Copenhaver et al., 2014), and thus over the years various attempts have been tried to improve the innate immunity in order to achieve the better protection against viral infection. Delivery of probiotics and their related materials is one of the strategies (Kiso et al., 2013; Yaqoob, 2014) and in the present study we sought a potential ability of spore to enhance innate immunity against RSV in line with other studies (Hong et al., 2008; McKenney et al., 2013).

It been reported that AM play an important function in diverse respiratory infections such as a primary recognition of antigens and scavenger to infected cells (Tate et al., 2010) and that the absence of AM-mediated defense mechanism led to the failure of protective immune responses, the exacerbation of pathology, and the accumulation of debris following pulmonary infection

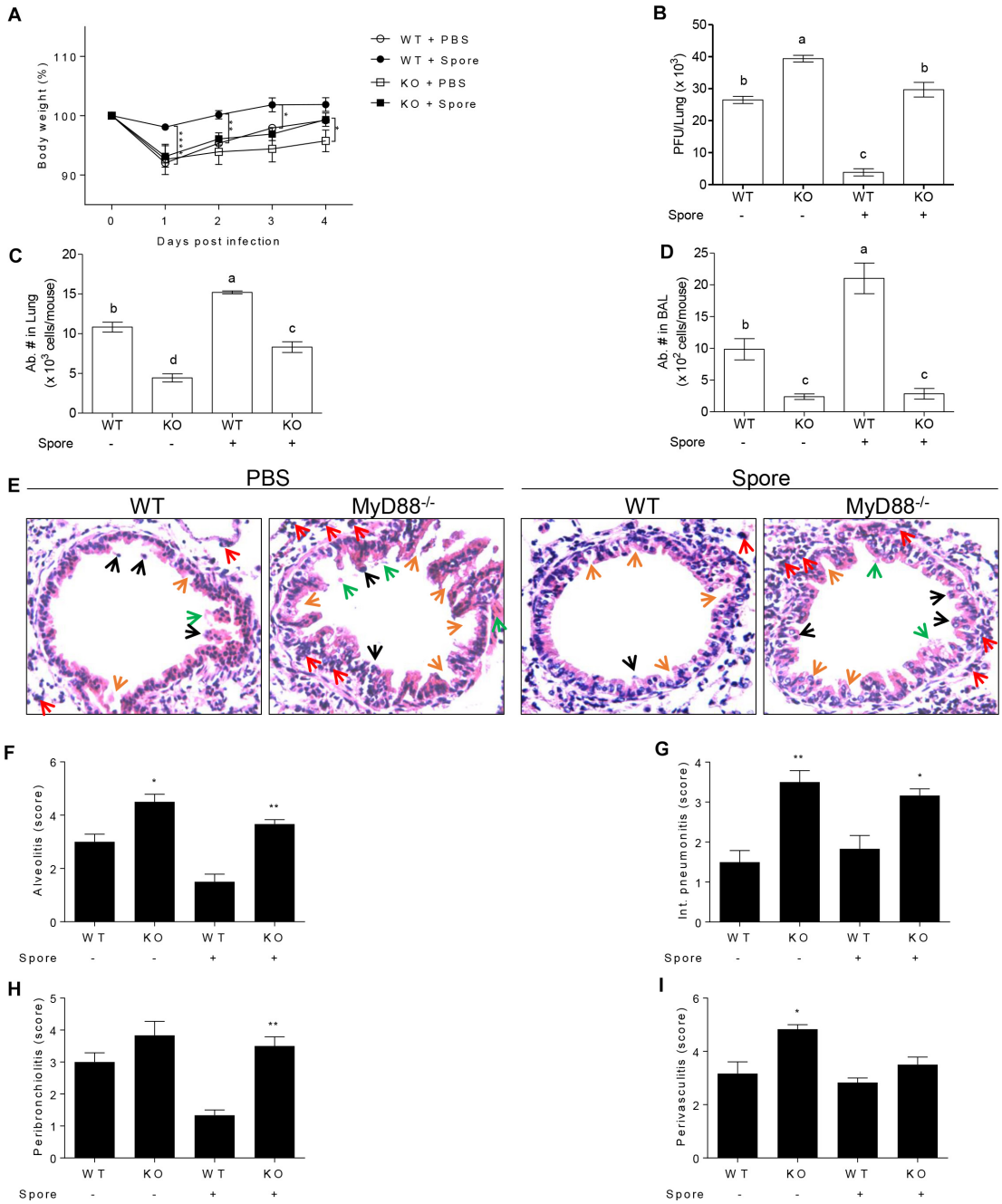


FIGURE 5 | Protective mechanism of spore pre-treated mice infected with RSV is dependent on MyD88 signaling in AMs. Wild type or MyD88 knockout mice were administered i.n. with spore at 5 days prior to RSV infection. **(A)** Body weight was monitored daily after the infection (n = 3). At 4 DPI, **(B)** viral load in the lungs was analyzed by plaque assay and absolute number of AMs in **(C)** post lavaged lungs, and **(D)** BAL cells were acquired by flow cytometry. Values with different letters (a, b, c, d) are significantly different one from another ($P < 0.05$). **(E)** Blood-perfused lungs were stained with H&E for histological examination by microscopy at 200× magnification and **(F–I)** scored for histopathology at 4 DPI. Arrows indicated are as follow; orange, epithelium thickness and destruction; green, pulmonary edema; red, inflammatory cells; and black, cell death. Significant differences from results with the PBS control are $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$, respectively.

(Kolli et al., 2014) but the precise role and protective mechanism of AM in RSV infected mice are yet to be illuminated. Direct evidence showing important role of AM in the present study came from *in vivo* experiment with depletion of AM. The results showed that the AM-depleted mice suffered from high viral load and intense pathological episode as shown by the accumulation of dead cells and debris in bronchi and blood vessels, indicative of early pulmonary inflammation. All these findings clearly support the critical role of AM in controlling disease severity of mice infected with RSV. AM treated with *B. subtilis* spore rendered

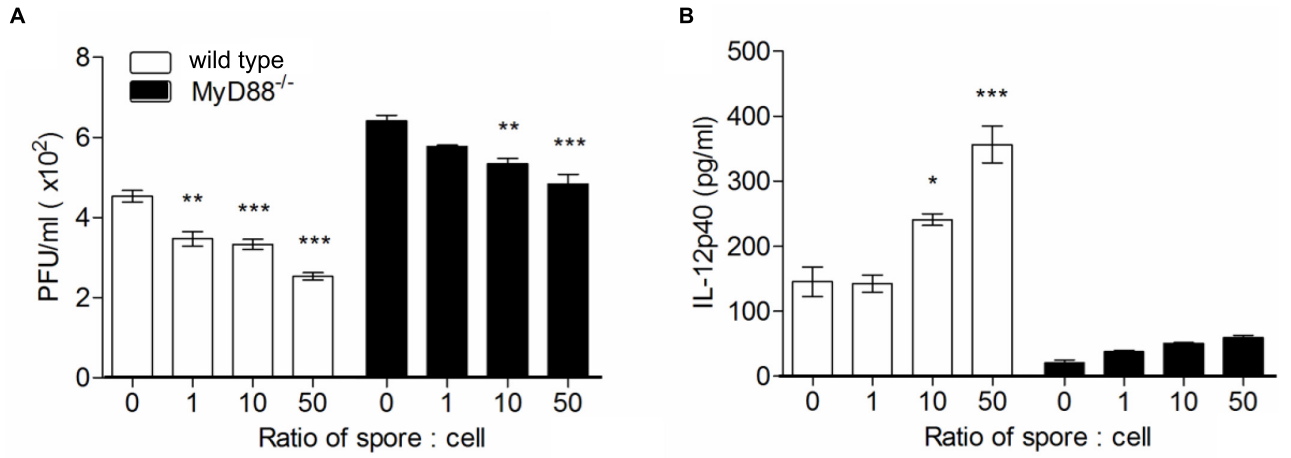


FIGURE 6 | Bone marrow-derived macrophages pre-treated with spore develop antiviral effects through MyD88-dependent pathway. Wild type or MyD88 knockout BMMs were treated with spore for 24 h at ratio of spore per number of cells for 0, 1, 10, and 50. Then, the cells were washed and infected with 1 MOI virus for additional 12 h. **(A)** Viral titers from wild type or MyD88 knockout BMMs were measured by plaque assay and **(B)** the production of IL-12p40 was measured by ELISA. Data are expressed as mean ± S.E.M. for 3 independent experiments. *, **, and *** indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

less susceptible to RSV infection and more importantly, anti-viral function of spore was markedly reduced upon depletion of AM, implying AM as a major target for *B. subtilis* spore as well as a key player for the protection.

How does then the spore influence on anti-viral function, differentiation, and/or migration of AM that would potentially benefit protective immunity to RSV? Our results showed that mice pre-treated spore through i.n. route rapidly up-regulated GM-CSF and M1 macrophage-related cytokines such as TNF-α, IFN-γ, IL-6, and IL-12p40. GM-CSF is a well-known essential factor for the differentiation (Guilliams et al., 2013), survival (Shibata et al., 2001), replenishment (Steinwede et al., 2011), and the ability of host defense (Ghoneim et al., 2013) of macrophages. Also, IFN-γ could lead monocytes differentiate into classically activated macrophages (M1-macrophages). This polarization is important in limiting tissue damage (Barros et al., 2013) and induces the increase of cytokines that enhance the ability of AM for the killing of intracellular pathogens (Martinez and Gordon, 2014). Thus, as a working mechanism for *B. subtilis* spore, it is conceivable that pre-treatment with *B. subtilis* spore might enhance the viability of macrophages, which promotes their antiviral functions. Alternatively, *B. subtilis* spore may facilitate AM proliferation and/or differentiation, skewing M1 vs. M2 balance toward favoring of M1 macrophage, which leads to an environment in which immune-stimulatory cytokines dominate for host protection against RSV. Supporting this idea, pre-treatment with spore indeed failed to induce TGF-β and PPAR-γ, which are known to induce differentiation of immune-suppressive M2 macrophages (Ginhoux, 2014; Schneider et al., 2014b). However, it is not always better to have M1 responses than M2 responses depending on how strong the immune response is and when the response was induced. It is important to induce appropriate and balanced immune responses because it has been reported that overwhelming pro-inflammatory responses with iNOS exacerbate the lung damage (Mgbemena et al., 2013). In addition, Th2 cytokines such as IL-4 and IL-13 induced after RSV infection could alleviate the damage of tissue (Shirey et al., 2010). Whether the preferences of M1/M2-type cytokines by spore indeed reflect AM proliferation/differentiation or selective enhancement of migration will need further investigation.

Besides the preference of M1 cytokines, mere increase of AM numbers we found after spore treatment would also have an advantage for protection against RSV infection, given that AM is known to play a role as reservoirs for inhaled antigens (Copenhaver et al., 2014) and thus the rapid increase of AM may effectively boost not only innate immunity but also prime adaptive immunity. Consistent with these, mice pre-treated with *B. subtilis* spore before RSV infection displayed a certain protective immunity as shown by significantly low level of viral titer and maintenance of body weight. Furthermore, *B. subtilis* spore treatment induced inflammatory monocytes with Ly6C high phenotype, known to regulate type I IFN signaling during acute viral pneumonia in mice (Seo et al., 2011) with an ability to differentiate into macrophages (Geissmann et al., 2010). Indeed, mice pre-treated with *B. subtilis* spore showed remarkably high IFN-β. Recent study showed that AM are the major source of type I IFNs during RSV infection and an underappreciated facet of type I IFNs-dependent resistance leads to a cell-extrinsic responses through rapid recruitment of antiviral inflammatory monocytes to the site of infection (Goritzka et al., 2015).

It would be important to postulate what are the mechanisms behind the modulatory ability of spore for AM. The most plausible explanation is that spore itself and/or its degraded components acts as a TLR ligand. TLR2 and TLR4 signaling pathways, in which MyD88 serves as a downstream molecule, are essential for the protection in RSV infection (Shirey et al., 2010). Consistent with those reports, in the present study, MyD88$^{-/-}$

mice showed persistence of high viral counts in parallel with impaired body weight gain after the infection suggesting that MyD88 is indispensable for the protection against RSV infection. Furthermore, it was evident that MyD88$^{-/-}$ mice failed to recruit AM into the lung region, irrespective of *B. subtilis* spore administration, suggesting that *B. subtilis* spore-induced increase of AM in the lung and BAL was dependent on MyD88.

MyD88$^{-/-}$ mice also displayed severe pathological signs in the lung compared to wild type mice. These results imply that MyD88-dependent signaling might be the key mechanism by which AM treated with *B. subtilis* spore contribute to the protection of mice against RSV infection. This requirement of MyD88 signaling may account for the ability of spore to promote AM capable of producing GM-CSF and M1-related cytokines, which enhance their viability and M1 signature cytokines including IFN-γ and TNF-α, as well as producing IFN-β and IL-12p40, which may serve as an alarm signal and facilitate IFN-γ production. Whether all these MyD88-dependent effects by spore are indeed mediated through direct binding on TLR and whether there are MyD88-independent TLR signaling such as NOD2 being involved will need to be addressed. Collectively, our results provided a key modulatory role of *B. subtilis* spore in the anti-viral function of AM and have useful implication for effective treatment against RSV infection.

Even after depletion of AM, to a lesser extent, spore treatment can reduce the viral load of RSV. There are other possibilities than macrophages which may contribute to the protective function of spore against RSV infection. For instance, components derived from commensal microbes have been reported to induce anti-microbial peptides from epithelial cells that act as a first line defense against viral infection (Gomez and Prince, 2008). Furthermore, TLR2 signal can up-regulate the tight junction proteins in epithelial cells (Ragupathy et al., 2014) which can enhance the tolerance to viral infection.

Several studies have been done with *B. subtilis* spore to protect against infections in mice and human (Colenutt and Cutting, 2014; Lefevre et al., 2015, 2017). Even though the spores have been reported to be safe in human trials, safety should be more elucidated further, for example, on administration of spores in the respiratory tracts or as subcutaneous injection. In addition, the status of spore as a live or heat-killed form should be studied more in terms of safety. Collectively, we proved a key function of *B. subtilis* spore in antiviral activity and protection of lung environment against RSV-induced damage in mice infected with RSV via MyD88.

MATERIALS AND METHODS

Mice and Ethics Statement

Female BALB/c mice, 6–8 week-old, were purchased from Orient Bio Inc., Korea. MyD88$^{-/-}$ mice were purchased from Jackson laboratory (Bar harbor, ME, United States). Animals were maintained and procedures were performed with approval of the IACUCs of Seoul National University (Approval No.: SNU-130527-5) and International Vaccine Institute (Approval No.: 2012-022) in accordance to Laboratory Animal Act of Korean Ministry of Food and Drug Safety for enhancing the ethics and reliability on animal testing through appropriate administration of laboratory animals and animal testing.

Preparation and Isolation of *B. subtilis* Spore

Bacillus subtilis was spread in an agar plate containing 3% Trypticase soy broth (TSB), 0.5% Yeast extract (YE), and 1.5% Bacto Agar (all from BD Biosciences, San Diego, CA, United States) and incubated at 37°C for 9 h. One colony was picked and inoculated in 25 ml of 3% TSB and 0.5% YE liquid media. Then, it was incubated for 5 h in the shaking incubator at 150 rpm at 37°C until the OD value reached between 0.45 and 0.6. For sporulation, culture was transferred to 500 ml of the autoclaved media containing 5 ml of 10% KCl, 5 ml of 1.2% $MgSO_4{\cdot}7H_2O$ (pH 7.6), 0.5 ml of 1 M $Ca(NO_2)_3$, 0.01 M $MnCl_2$, and 1 mM $FeSO_4$. The culture was incubated at 37°C for 48 h with shaking at 150 rpm. The cells were collected by centrifugation at 5516 × g for 10 min, re-suspended in distilled water, and incubated at 4°C for 48 h on the rocker. Then, the cells were sonicated at 35% amplitude (1 watt) for 90 s with 0.5 s pulse. Spore loaded on the layers of 35, 25, and 15% OptiPrep Density gradient (Sigma-Aldrich, Wt. Louis, MO, United States) was centrifuged at 10,000 × g for 40 min at 25°C without break for the purification. The spore was washed 3 times with distilled water and re-suspended in 1 ml of distilled water.

Preparation and Isolation of Respiratory Syncytial Virus A2

Respiratory syncytial virus A2 strain was amplified as follows: HEp-2 cells (ATCC, Manassas, VA, United States) were grown in MEM containing 10% of FBS and 1% of antibiotics (Jang et al., 2011). When the cells reached at approximately 80% confluence, the cells were washed and inoculated with 0.01–0.05 MOI of virus in MEM containing 1% of antibiotics and 25 mM HEPES (Gibco, Grand Island, NY, United States). The cells were incubated for 2 h at 37°C, added MEM containing 6% of FBS only, and then incubated for additional 72–96 h. The cells were scraped and combined into the conical tubes on ice and centrifuged at 1,400 rpm for 4 min at 4°C. The cell pellets were collected into conical tubes, re-suspended with cold conditioned media with 60% sorbitol, sonicated for 15 min in slurry ice and centrifuged at 4,000–5,000 rpm for 10 min at 4°C, and then the sonication and centrifugation step was repeated under the same condition. The supernatants were transferred into new tubes, centrifuged at 23,000 rpm for 1 h at 4°C, and then discarded. The resulting whitish virus pellet was re-suspended with 500 μl of cold MEM and the titer was determined by standard RSV plaque assay.

Isolation and Culture of Bone Marrow-Derived Macrophages and Macrophage Cell Line

Bone marrow was flushed from femurs and tibias of the mice using Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 2% FBS (Gibco). Red blood cells were lysed with ACK lysing buffer (Gibco) and whitish marrow cells were

seeded in 90 × 15 mm Petri dish with complete media containing 10% FBS, 1% antibiotics with 20% L-929 conditioned media (Zhang et al., 2008) and cultured at 37°C in humidified incubator with 5% CO_2 for 7 days. On day 3, another 5 ml of fresh complete media was added to each dish. On day 7, only adherent cells were collected using non-enzymatic cell dissociation solution (Gibco). MH-S cells (ATCC, Manassas, VA, United States), mouse AM cell line, were grown in RPMI-1640 GlutaMax medium containing 10% FBS, 1.5% antibiotics (all from Gibco) in a humidified incubator with 5% CO_2 at 37°C.

RNA Isolation and Quantitative Real Time-PCR

For quantitative analysis for the expression of GM-CSF, TNF-α, IFN-γ, IL-12p40, IL-6, PPAR-γ, and TGF-β1 at mRNA level, quantitative real time polymerase chain reaction (qRT-PCR) was conducted. RNA was extracted from perfused lungs using TRIZOL (Invitrogen, Carlsbad, CA, United States). Total RNA was isolated by adding chloroform followed by centrifugation at 4°C, 12,000 × g for 15 min and addition of isopropanol for 10 min at room temperature for RNA precipitation. RNA pellet obtained by washing with 75% ethanol was air dried for 10–15 min. Then it was re-suspended with DEPC distilled water (Sigma-Aldrich, Wt. Louis, MO, United States) and quantified with NanoDrop (Amersham Bioscience, United States) at A_{260}. One microgram of RNA was reverse transcribed into cDNA and amplified with murine primers specific for GM-CSF (forward primer: 5′-CTGCCTTAAAGGGACCAAGAGA-3′, reverse primer: 5′-TTCCGCTGTCCAAGCTGAGT-3′), TNF-α (forward primer: 5′-GCCAACGGCATGGATCTC-3′, reverse primer: 5-GTGGGTGAGGAGCACGTAGTC-3′), IFN-γ (forward primer: 5′-GCCATCGGCTGACCTAGAGA-3′, reverse primer: 5′-GCAGTGTGTAGCGTTCATTGTCT-3′), IL-12p40 (forward primer: 5′-GAAAGGTGCGTTCCTCGTAGA-3′, reverse primer: 5′-GGAACACATGCCCACTTGCT-3′), IL-6 (forward primer: 5′-CACAGAGGATACCACTCCCAACA-3′, reverse primer: 5′-TCAGAATTGCCATTGCACAAC-3′), PPAR-γ (forward primer: 5′-CAGGAGCCTGTGAGACCAACA-3′, reverse primer: 5′-ATCAGTGGTTCACCGCTTCTTT-3′), TGF-β1 (forward primer: 5′-TCGTCTGCATTGCACTTATGC-3′, reverse primer: 5′-GTGGTGCCCTCTGAAATGAAA-3′), and GAPDH (forward primer: 5′-CTCCACTCACGGCAAATTCA-3′, reverse primer: 5′-GCCTCACCCCATTTGATGTT-3′). Real-time PCR was performed using Power SYBR Green PCR master mix (Applied Biosystem, Waltham, MA, United States) and analysis of the data was performed by One-step RT PCR (Applied Biosystem). Target gene expression was normalized to GAPDH expression.

Measurement of Cytokine Production

Broncho-alveolar lavage (BAL) samples were collected via tracheotomy using 600 μl of PBS and the cells were separated from the BAL fluid by centrifugation at 1400 rpm for 5 min at 4°C. For *in vitro* experiments, supernatants from BMMs or MH-S after virus infection were carefully collected. Production of IFN-β (BioLegend, San Diego, CA, United States), IL-12p40, GM-CSF, and TNF-α (R&D System, Minneapolis, MN, United States) was examined using ELISA kit.

Phenotypic Characterization of the Cells

To examine the absolute number of AM and other innate immune cells, BAL cells were collected as above and perfused lungs were isolated and minced through 70 μm cell strainer using MEM. The cells were stained with FITC-conjugated anti-CD11c (HL3), PE-conjugated anti-Siglec-F (E50-2440), PerCP-conjugated anti-Ly6C (AL-21), PE-Cy7-conjugated anti-Ly6G (1A8), APC-conjugated anti-CD11b (M1-70) or APC-conjugated anti-F4/80 (BM8), and APC-Cy7-conjugated anti-CD45 (30-F11) (all from BD Biosciences except anti-F4/80 from BioLegend). The cells were acquired using FACS LSR II and flow cytometric data were analyzed by using FlowJo software (Tree Star, San Carlos, CA, United States). We defined $CD45^+Ly6C^-Ly6G^-CD11c^+Sigleg\text{-}F^+F4/80^+$ as AMs, $CD45^+CD11b^+Ly6C^+Ly6G^-$ for inflammatory monocytes, and $CD45^+CD11b^+Ly6G^+Ly6C^-$ for neutrophils.

Selective Depletion of Alveolar Macrophages

To selectively deplete AM, 350 mg per mouse of clodronate-encapsulated liposome (FormuMax Scientific Inc., CA, United States) was given i.t. in a volume of 50 μl at 1 and 3 days before the RSV challenge with or without spore treatment. To verify the depletion of AM, naïve mice were given control liposome via i.t.

Virus Titration in the Lung

To determine the viral titers, lungs from RSV-infected mice were isolated at day 4 post-infection. The lungs were minced through 70-μm cell strainer using cold MEM. Cell lysates were collected and RSV titers were determined by plaque assay using HEp-2 cells. The virus titers in the whole lung were normalized to weight of the lung tissue and indicated as PFU/g.

Lung Histology and Pathology Scoring

For histology studies, mice were administered with spore and/or clodronate encapsulated liposome prior to RSV infection. For control, mice were administered with PBS or control liposome. At 4 days post infection, BAL fluid was removed from lung and blood was perfused. Then, lungs were fixed with 4% paraformaldehyde and embedded in paraffin. Lung sections were produced and stained with Hematoxylin and Eosin to examine the abnormalities. Four inflammatory parameters were scored independently from 0 to 5 for each section: alveolitis (inflammatory cells within alveolar spaces), interstitial pneumonitis (increased thickness of alveolar walls associated with inflammatory cells), peribronchiolitis (inflammatory cells surrounding a bronchiole), and perivasculitis (inflammatory cells surrounding a blood vessel). Slides were randomized, read blindly, and scored for each parameter.

In vivo or *in vitro* Spore Administration and RSV Infection

In vivo mouse model, each mouse was administered with either 1×10^9 CFU of spore or, PBS as a control, i.n. in a volume of 20 μl at 5 days before the infection. Then, the mice were infected i.t. with 2×10^6 PFU of live RSV A2. BMMs or MH-S cell line were cultured in 12-well plate (1×10^6 cells/ml) with various ratio of spore for 24 h. Cells were washed with PBS and then infected with 1 MOI of live RSV A2 for additional 12 h.

Statistical Analysis

Results are presented as means ± SEM. Statistical differences between two means were evaluated using unpaired Student *t* test. For comparisons of multiple groups, one-way ANOVA was used. Statistical significance was set at a P value of < 0.05.

AUTHOR CONTRIBUTIONS

C-HY conceived the study. MS, SH, and C-HY designed the study. JH, Y-CK, S-MP, and IC performed all the experiments. JH and C-HY wrote the manuscript. HC, B-CP, Y-MP, JC, and J-HC analyzed and discussed the data.

FUNDING

This work was carried out with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01336401)" Rural Development Administration, South Korea. This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1A2B2006793 and NRF-2015R1D1A1A02061577). This work was also supported by the BK21 Plus Program of the Department of Agricultural Biotechnology, Seoul National University, Seoul, South Korea.

ACKNOWLEDGMENTS

We thank Dr. Cutting SM (Royal Holloway University of London) for kindly providing *Bacillus subtilis* PY79 strain spore.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00447/full#supplementary-material

REFERENCES

Barros, M. H., Hauck, F., Dreyer, J. H., Kempkes, B., and Niedobitek, G. (2013). Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS One* 8:e80908. doi: 10.1371/journal.pone.0080908

Colenutt, C., and Cutting, S. M. (2014). Use of *Bacillus subtilis* PXN21 spores for suppression of *Clostridium difficile* infection symptoms in a murine model. *FEMS Microbiol. Lett.* 358, 154–161. doi: 10.1111/1574-6968.12468

Copenhaver, A. M., Casson, C. N., Nguyen, H. T., Fung, T. C., Duda, M. M., Roy, C. R., et al. (2014). Alveolar macrophages and neutrophils are the primary reservoirs for *Legionella pneumophila* and mediate cytosolic surveillance of type IV secretion. *Infect. Immun.* 82, 4325–4336. doi: 10.1128/IAI.01891-14

Garcia-Crespo, K. E., Chan, C. C., Gabryszewski, S. J., Percopo, C. M., Rigaux, P., Dyer, K. D., et al. (2013). *Lactobacillus* priming of the respiratory tract: heterologous immunity and protection against lethal pneumovirus infection. *Antiv. Res.* 97, 270–279. doi: 10.1016/j.antiviral.2012.12.022

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., and Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science* 327, 656–661. doi: 10.1126/science.1178331

Ghoneim, H. E., Thomas, P. G., and Mccullers, J. A. (2013). Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *J. Immunol.* 191, 1250–1259. doi: 10.4049/jimmunol.1300014

Ginhoux, F. (2014). Fate PPAR-titioning: PPAR-gamma 'instructs' alveolar macrophage development. *Nat. Immunol.* 15, 1005–1007. doi: 10.1038/ni.3011

Gomez, M. I., and Prince, A. (2008). Airway epithelial cell signaling in response to bacterial pathogens. *Pediatr. Pulmonol.* 43, 11–19. doi: 10.1002/ppul.20735

Goritzka, M., Makris, S., Kausar, F., Durant, L. R., Pereira, C., Kumagai, Y., et al. (2015). Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *J. Exp. Med.* 212, 699–714. doi: 10.1084/jem.20140825

Guilliams, M., De Kleer, I., Henri, S., Post, S., Vanhoutte, L., De Prijck, S., et al. (2013). Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J. Exp. Med.* 210, 1977–1992. doi: 10.1084/jem.20131199

Hashimoto, D., Miller, J., and Merad, M. (2011). Dendritic cell and macrophage heterogeneity in vivo. *Immunity* 35, 323–335. doi: 10.1016/j.immuni.2011.09.007

Herold, S., Mayer, K., and Lohmeyer, J. (2011). Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. *Front. Immunol.* 2:65. doi: 10.3389/fimmu.2011.00065

Hong, H. A., Huang, J. M., Khaneja, R., Hiep, L. V., Urdaci, M. C., and Cutting, S. M. (2008). The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J. Appl. Microbiol.* 105, 510–520. doi: 10.1111/j.1365-2672.2008.03773.x

Jang, J. E., Lee, J. B., Kim, K. H., Park, S. M., Shim, B. S., Cheon, I. S., et al. (2011). Evaluation of protective efficacy of respiratory syncytial virus vaccine against A and B subgroup human isolates in Korea. *PLoS One* 6:e23797. doi: 10.1371/journal.pone.0023797

Kiso, M., Takano, R., Sakabe, S., Katsura, H., Shinya, K., Uraki, R., et al. (2013). Protective efficacy of orally administered, heat-killed *Lactobacillus* pentosus b240 against influenza A virus. *Sci. Rep.* 3:1563. doi: 10.1038/srep01563

Kolli, D., Gupta, M. R., Sbrana, E., Velayutham, T. S., Chao, H., Casola, A., et al. (2014). Alveolar macrophages contribute to the pathogenesis of human metapneumovirus infection while protecting against respiratory syncytial virus infection. *Am. J. Respir Cell Mol. Biol.* 51, 502–515. doi: 10.1165/rcmb.2013-0414OC

Kumagai, Y., Takeuchi, O., Kato, H., Kumar, H., Matsui, K., Morii, E., et al. (2007). Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 27, 240–252. doi: 10.1016/j.immuni.2007.07.013

Kurt-Jones, E. A., Popova, L., Kwinn, L., Haynes, L. M., Jones, L. P., Tripp, R. A., et al. (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immunol.* 1, 398–401. doi: 10.1038/80833

Lee, I. K., Kye, Y. C., Kim, G., Kim, H. W., Gu, M. J., Umboh, J., et al. (2016). Stress, nutrition, and intestinal immune responses in pigs – A review. *Asian-Australas J. Anim. Sci.* 29, 1075–1082. doi: 10.5713/ajas.16.0118

Lee, Y. N., Youn, H. N., Kwon, J. H., Lee, D. H., Park, J. K., Yuk, S. S., et al. (2013). Sublingual administration of *Lactobacillus rhamnosus* affects respiratory immune responses and facilitates protection against influenza virus infection in mice. *Antiv. Res.* 98, 284–290. doi: 10.1016/j.antiviral.2013.03.013

Lefevre, M., Racedo, S. M., Denayrolles, M., Ripert, G., Desfougeres, T., Lobach, A. R., et al. (2017). Safety assessment of *Bacillus subtilis* CU1 for use as a probiotic in humans. *Regul. Toxicol. Pharmacol.* 83, 54–65. doi: 10.1016/j.yrtph.2016.11.010

Lefevre, M., Racedo, S. M., Ripert, G., Housez, B., Cazaubiel, M., Maudet, C., et al. (2015). Probiotic strain *Bacillus subtilis* CU1 stimulates immune system of elderly during common infectious disease period: a randomized, double-blind placebo-controlled study. *Immun. Ageing* 12:24. doi: 10.1186/s12979-015-0051-y

Marr, N., Turvey, S. E., and Grandvaux, N. (2013). Pathogen recognition receptor crosstalk in respiratory syncytial virus sensing: a host and cell type perspective. *Trends Microbiol.* 21, 568–574. doi: 10.1016/j.tim.2013.08.006

Martinez, F. O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6:13. doi: 10.12703/P6-13

Maus, U. A., Koay, M. A., Delbeck, T., Mack, M., Ermert, M., Ermert, L., et al. (2002). Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 282, L1245–L1252. doi: 10.1152/ajplung.00453.2001

McGill, J., Heusel, J. W., and Legge, K. L. (2009). Innate immune control and regulation of influenza virus infections. *J. Leukoc. Biol.* 86, 803–812. doi: 10.1189/jlb.0509368

McKenney, P. T., Driks, A., and Eichenberger, P. (2013). The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat. Rev. Microbiol.* 11, 33–44. doi: 10.1038/nrmicro2921

Meng, J., Stobart, C. C., Hotard, A. L., and Moore, M. L. (2014). An overview of respiratory syncytial virus. *PLoS Pathog.* 10:e1004016. doi: 10.1371/journal.ppat.1004016

Mgbemena, V., Segovia, J., Chang, T. H., and Bose, S. (2013). KLF6 and iNOS regulates apoptosis during respiratory syncytial virus infection. *Cell Immunol.* 283, 1–7. doi: 10.1016/j.cellimm.2013.06.002

Murawski, M. R., Bowen, G. N., Cerny, A. M., Anderson, L. J., Haynes, L. M., Tripp, R. A., et al. (2009). Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. *J. Virol.* 83, 1492–1500. doi: 10.1128/JVI.00671-08

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., et al. (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 41, 14–20. doi: 10.1016/j.immuni.2014.06.008

Nair, H., Nokes, D. J., Gessner, B. D., Dherani, M., Madhi, S. A., Singleton, R. J., et al. (2010). Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet* 375, 1545–1555. doi: 10.1016/S0140-6736(10)60206-1

Park, M. K., Ngo, V., Kwon, Y. M., Lee, Y. T., Yoo, S., Cho, Y. H., et al. (2013). *Lactobacillus plantarum* DK119 as a probiotic confers protection against influenza virus by modulating innate immunity. *PLoS One* 8:e75368. doi: 10.1371/journal.pone.0075368

Pribul, P. K., Harker, J., Wang, B., Wang, H., Tregoning, J. S., Schwarze, J., et al. (2008). Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. *J. Virol.* 82, 4441–4448. doi: 10.1128/JVI.02541-07

Ragupathy, S., Esmaeili, F., Paschoud, S., Sublet, E., Citi, S., and Borchard, G. (2014). Toll-like receptor 2 regulates the barrier function of human bronchial epithelial monolayers through atypical protein kinase C zeta, and an increase in expression of claudin-1. *Tissue Barriers* 2, e29166. doi: 10.4161/tisb.29166

Schneider, C., Nobs, S. P., Heer, A. K., Kurrer, M., Klinke, G., Van Rooijen, N., et al. (2014a). Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLoS Pathog.* 10:e1004053. doi: 10.1371/journal.ppat.1004053

Schneider, C., Nobs, S. P., Kurrer, M., Rehrauer, H., Thiele, C., and Kopf, M. (2014b). Induction of the nuclear receptor PPAR-gamma by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages. *Nat. Immunol.* 15, 1026–1037. doi: 10.1038/ni.3005

Seo, S. U., Kwon, H. J., Ko, H. J., Byun, Y. H., Seong, B. L., Uematsu, S., et al. (2011). Type I interferon signaling regulates Ly6C(hi) monocytes and neutrophils during acute viral pneumonia in mice. *PLoS Pathog.* 7:e1001304. doi: 10.1371/journal.ppat.1001304

Shi, T., Mcallister, D. A., O'brien, K. L., Simoes, E. A. F., Madhi, S. A., Gessner, B. D., et al. (2017). Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study. *Lancet* 390, 946–958. doi: 10.1016/S0140-6736(17)30938-8

Shibata, Y., Berclaz, P. Y., Chroneos, Z. C., Yoshida, M., Whitsett, J. A., and Trapnell, B. C. (2001). GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* 15, 557–567. doi: 10.1016/S1074-7613(01)00218-7

Shim, Y. R., and Lee, H. K. (2015). Caspase-1 independent viral clearance and adaptive immunity against mucosal respiratory syncytial virus infection. *Immune Netw.* 15, 73–82. doi: 10.4110/in.2015.15.2.73

Shirey, K. A., Pletneva, L. M., Puche, A. C., Keegan, A. D., Prince, G. A., Blanco, J. C., et al. (2010). Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R alpha-TLR4-, and IFN-beta-dependent. *Mucosal Immunol.* 3, 291–300. doi: 10.1038/mi.2010.6

Snelgrove, R. J., Goulding, J., Didierlaurent, A. M., Lyonga, D., Vekaria, S., Edwards, L., et al. (2008). A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. *Nat. Immunol.* 9, 1074–1083. doi: 10.1038/ni.1637

Song, M., Hong, H. A., Huang, J. M., Colenutt, C., Khang, D. D., Nguyen, T. V., et al. (2012). Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. *Vaccine* 30, 3266–3277. doi: 10.1016/j.vaccine.2012.03.016

Steinwede, K., Tempelhof, O., Bolte, K., Maus, R., Bohling, J., Ueberberg, B., et al. (2011). Local delivery of GM-CSF protects mice from lethal pneumococcal pneumonia. *J. Immunol.* 187, 5346–5356. doi: 10.4049/jimmunol.1101413

Tan, I. S., and Ramamurthi, K. S. (2014). Spore formation in *Bacillus subtilis*. *Environ. Microbiol. Rep.* 6, 212–225. doi: 10.1111/1758-2229.12130

Tate, M. D., Pickett, D. L., Van Rooijen, N., Brooks, A. G., and Reading, P. C. (2010). Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice. *J. Virol.* 84, 7569–7580. doi: 10.1128/JVI.00291-10

Ugonna, K., Bingle, C. D., Plant, K., Wilson, K., and Everard, M. L. (2014). Macrophages are required for dendritic cell uptake of respiratory syncytial virus from an infected epithelium. *PLoS One* 9:e91855. doi: 10.1371/journal.pone.0091855

Yaqoob, P. (2014). Ageing, immunity and influenza: a role for probiotics? *Proc. Nutr. Soc.* 73, 309–317. doi: 10.1017/S0029665113003777

Zhang, X., Goncalves, R., and Mosser, D.M. (2008). The isolation and characterization of murine macrophages. *Curr. Protoc. Immunol.* 14:Unit 14.11.

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

frontiers in Microbiology

REVIEW
published: 26 March 2019
doi: 10.3389/fmicb.2019.00569

Recent Aspects on the Pathogenesis Mechanism, Animal Models and Novel Therapeutic Interventions for Middle East Respiratory Syndrome Coronavirus Infections

Sinosh Skariyachan, Sneha Basavaraj Challapilli, Swathi Packirisamy, Supreetha Toplar Kumargowda and Vaishnavi Sneha Sridhar*

R&D Centre, Department of Biotechnology, Dayananda Sagar College of Engineering, Bengaluru, India

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is an emerging zoonotic virus considered as one of the major public threat with a total number of 2 298 laboratory-confirmed cases and 811 associated deaths reported by World Health Organization as of January 2019. The transmission of the virus was expected to be from the camels found in Middle Eastern countries via the animal and human interaction. The genome structure provided information about the pathogenicity and associated virulent factors present in the virus. Recent studies suggested that there were limited insight available on the development of novel therapeutic strategies to induce immunity against the virus. The severities of MERS-CoV infection highlight the necessity of effective approaches for the development of various therapeutic remedies. Thus, the present review comprehensively and critically illustrates the recent aspects on the epidemiology of the virus, the structural and functional features of the viral genome, viral entry and transmission, major mechanisms of pathogenesis and associated virulent factors, current animal models, detection methods and novel strategies for the development of vaccines against MERS-CoV. The review further illustrates the molecular and computational virtual screening platforms which provide insights for the identification of putative drug targets and novel lead molecules toward the development of therapeutic remedies.

Keywords: MERS-CoV, emerging zoonotic virus, mechanisms of pathogenesis, animal models, probable drug targets, vaccine development

OPEN ACCESS

Edited by:
Kelvin To,
University of Hong Kong, Hong Kong

Reviewed by:
Man Lung Yeung,
University of Hong Kong, Hong Kong
Mahmoud Mohamed Shehata,
National Research Centre, Egypt

****Correspondence:***
Sinosh Skariyachan
sinoshskariya@gmail.com;
sinosh-bt@dayanandasagar.edu

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *28 October 2018*
Accepted: *05 March 2019*
Published: *26 March 2019*

Citation:
Skariyachan S, Challapilli SB, Packirisamy S, Kumargowda ST and Sridhar VS (2019) Recent Aspects on the Pathogenesis Mechanism, Animal Models and Novel Therapeutic Interventions for Middle East Respiratory Syndrome Coronavirus Infections. Front. Microbiol. 10:569. doi: 10.3389/fmicb.2019.00569

INTRODUCTION

The coronaviruses such as HCoV-229E, HCoV-NL63 (α-coronavirus) and HCoV-OC43 and HKU1 (β-coronavirus) circulated in the human population and caused mild respiratory infections (Hamre and Procknow, 1966; Fouchier et al., 2004; van der Hoek et al., 2004; Woo et al., 2005). Over the last two decades, there have been two zoonotic emergence of coronaviruses into the human population, both linked with Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

(Drosten et al., 2003; Kuiken et al., 2003) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Zaki et al., 2012; Chafekar and Fielding, 2018).

Middle East Respiratory Syndrome Coronavirus belonged to β-coronavirus, clade-c (van Boheemen et al., 2012; de Groot et al., 2013) and was initially known as Human Coronavirus Erasmus Medical Center/2012. or HCoV-EMC/2012 (Chan et al., 2012). MERS-CoV infections resulted high mortality rate in human, and till date, very limited therapeutic intervention or vaccine are available for the treatment of infections caused by the virus (Mustafa et al., 2017; Hui et al., 2018).

Middle East Respiratory Syndrome Coronavirus found in various natural hosts such as the dromedary calves (*Camelus dromedarius*), bats (*Vespertilio superans* and *Neoromicia capensis*), and European hedgehog (*Erinaceus europaeus*) (Zhang et al., 2016; Lau et al., 2017). The replication of the virus has observed in various other animals such as rabbit, goat, civet, pig, camelid, and horse (Muller et al., 2012; Chan et al., 2013a; Al-Tawfiq et al., 2014; Eckerle et al., 2014; Meyer et al., 2015). The prevalence MERS-CoV was recently reported in non-camelid domestic mammals such as sheep, goat, donkey, and cow that were in contact with camels (Kandeil et al., 2019). The origin of human MERS-CoV is considered to be from the bats. Several coronaviruses were isolated and found that which were genetically similar to human MERS-CoV. Phylogenetic analysis of MERS-CoV showed that a close relationship to Pipistrellus bat coronavirus HKU5 and Tylonycteris bat coronavirus HKU4 (Selinger et al., 2014). Studies have suggested that there were few evidences where the virus showed camel origin (Zhang et al., 2016). Studies revealed that the sero-prevalence of the virus was greater among the people those who were exhibited direct and indirect contact with dromedary camels when compared to those who were exhibited with the general population (Watson et al., 2014; Zhou et al., 2015; Conzade et al., 2018). Recent study revealed that the prevalence of MERS-CoV infection in camel workers was found to be very high in Saudi Arabia (Alshukairi et al., 2018). Thus, this review provide the recent perspectives on the epidemiology and genome composition of MERS-CoV with a special emphasis on the viral entry, clinical manifestations, mechanism of pathogenesis, animal models, detection, and development of vaccines with an insight on various computational biology perspectives.

EPIDEMIOLOGY

The first case of MERS-CoV infection was reported in Saudi Arabia in 2012 (Zaki et al., 2012). From the period of its emergence, the virus has infected and propagated across more than 20 countries, contributing to a wide number of laboratory-confirmed cases. In 2015, the MERS-CoV outbreak in South Korea involved 186 cases which included 38 fatalities. A total of 83% cases of transmission were due to five super spreaders, and 44% of 186 cases were in the patients those who were experienced nosocomial transmission at 16 hospitals. The epidemic lasted for 2 months and the government authorities quarantined 16,993 persons for 14 days to manage the outbreak. This outbreak provides an exclusive opportunity to seal the gap in our awareness of MERS-CoV infection (Oh et al., 2018). Recent statistics suggested that as of 31 December 2018, the total of 2,279 laboratory-confirmed cases of MERS-CoV with 806 associated deaths (case fatality rate: 35.4%) were reported in 27 countries [Harcourt et al., 2018; World Health Organization (WHO), 2018a]. Among various cases, 1901 confirmed cases with 732 related deaths (fatality rate 38.5%) were reported from Saudi Arabia [Harcourt et al., 2018; World Health Organization (WHO), 2018a].

In a comprehensive report by WHO, 1841 laboratory cases were confirmed between 2012 and 2016 in which 80% of them were reported from Kingdom of Saudi Arabia. The remaining cases were reported from 27 countries in Middle East, Asia, North Africa, United States of America, and Europe [World Health Organization (WHO), 2016a]. A total of 13 cases were reported between October to November 2017, amongst which 12 cases were reported in male and only one case was reported in female. Most of the infected individuals were hospitalized due to the contact with camels and consumption of camel milk with the virus [World Health Organization (WHO), 2017a]. Additionally, two laboratory cases were also reported in which the patients belonged to the countries such as Malaysia and United Arab Emirates [World Health Organization (WHO), 2018a]. The laboratory confirmed cases and deaths reported in several countries from the year 2012 to 2018 are shown in **Table 1**.

THE GENOME OF MERS-CoV

In the early years of 2000, coronaviruses were not acquired great importance in research and development. With the emergence of SARS in the year 2002 and MERS in 2012, the interest of many researchers was stimulated toward coronavirus (Perlman, 2015).

The morphology of coronavirus includes spherical or pleomorphic shapes with a diameter of 80–120 nm (Masters, 2006). The genome of the coronavirus consists of 6 and 7 open reading frames (ORFs). The ORF 1a and 1b encompass two-third of the viral genome which encodes the non-structural poly-proteins and the other four ORFs on the downstream side encode for the structural proteins such as envelope protein (E), Spike protein (S), nucleocapsid protein (N), and membrane protein (M). In some coronaviruses, hemagglutinin-esterase (HE) gene is present in the region between ORF 1b and S (Marra et al., 2003; Rota et al., 2003; Snijder et al., 2003). These structural proteins are folded and entered in to the endoplasmic reticulum (ER) and transported to the ER-Golgi transitional slot (Masters, 2006). During the replication of coronavirus, substantial amounts of structural proteins are synthesized in order to assemble the progeny virions (Fung and Liu, 2014). They occupy the RNA genome which encodes structural proteins such as S protein, M protein, and N protein (Snijder et al., 2003).

The genome size of the first imported MERS-CoV strain was identified to be 30,114 nucleotide (nt) long, including the 3′and 5′ UTRs. This strain showed the genome structural ornaisation of typical betacoronavirus such as a 5′-untranslated

TABLE 1 | Laboratory confirmed cases of MERS-CoV infections and deaths reported in several countries from the year 2012 to 2018.

Year	Region	MERS-CoV infections	Deaths due to MERS-CoV infections	Reference
2012	Saudi Arabia, Qatar, and Jordan	9	3	World Health Organization, 2012
2013	Saudi Arabia, United Kingdom, France, Tunisia, and Italy	167	71	World Health Organization (WHO), 2013a
2014	Saudi Arabia, United Arab Emirates, Jordan, Iran, and Egypt	765	273	World Health Organization, 2014
2015	Saudi Arabia, Republic of Korea, Jordan, Kuwait, The Philippines, Thailand, United Arab Emirates, Oman, Qatar, China, and Iran	680	237	World Health Organization (WHO), 2015a,b
2016	Saudi Arabia, United Arab Emirates, Qatar, Lebanon, and Oman	243	75	World Health Organization (WHO), 2016b
2017	Saudi Arabia, United Arab Emirates, Qatar, Lebanon, and Oman	258	81	World Health Organization (WHO), 2017b
2018	Saudi Arabia and Malaysia, United Arab Emirates	96	41	World Health Organization (WHO), 2018a

region (UTR) (nt 1 to 272), replicase complex ORF1ab (nt 273 to 21508), S gene (nt 21450 to 25511), ORF3 (nt 25526 to 25837), ORF4a (nt 25846 to 26175), ORF4b (nt 26087 to 26827), ORF5 (nt26834 to 27508), E gene (nt 27584 to 27832), M gene (nt 27847 to 28506), N gene (nt 28560 to 29801), ORF8b gene (nt 28756 to 29094), and 3′UTR (nt 29094 to 30114). The two main poly-proteins such as *pp1ab* and *pp1a* are cleaved into 15/16 non-structural proteins (nsp) by 3C-like protease (3CLpro) and papain-like protease (PLpro). These proteases are cleaved from *pp1ab* along with other ORFs encoding nsp which are essential for activating RNA dependent RNA polymerase, helicase, exo-ribonuclease, endo-ribonuclease, and methyltransferase. These nsp were identified to be nsp12, nsp13, nsp14, nsp15, and nsp16. The nsp14 is necessary for proofreading the ubiquitous mutation of RNA virus (Ziebuhr et al., 2000; Snijder et al., 2003; Gorbalenya et al., 2006; Smith et al., 2013; Raj et al., 2014; Durai et al., 2015). Studies revealed that the accessory ORF proteins play an important role in MERS-CoV infection and pathogenesis (Menachery et al., 2017). This study showed that the absence of major accessory ORFs such as deletion of ORF3, -4a, -4b, and -5 (dORF3-5) played major role in the viral replication and pathogenesis. Further, the attenuation of the mutant dORF3-5 was found to be responsible for the dysregulated host responses such as augmented interferon (IFN) pathway activation, disrupted cell processes, and robust inflammation. Thus, the disruption of accessory ORFs probably provide platform to the attenuation of future emergent strains of MERS-CoV accessory ORF mutants. The accessory ORF functions can be targeted for both therapeutic and vaccine treatments in response to MERS-CoV and related group 2C corona viruses (Menachery et al., 2017). The graphical representation of the genome organization of MERS-CoV is illustrated in **Figure 1**. Coronaviruses have demonstrated the establishment of double membrane vesicles (DMVs) in the infected cells. Based on immune-histochemistry and electron microscopic studies, the coronavirus replicase proteins are co-localized with the DMVs and are assumed to be the site of replication/transcription complex (Fung and Liu, 2014).

An interesting fact of coronaviruses is that they display recombination, high rates of mutation, and propensity to cross species. One such event was reported during the 2002–2003 epidemic, where the transmission of SARS-CoV has occurred from Chinese horseshoe bats to human populations (Perlman and Netland, 2009). MERS-CoV has the capability to adjust new environments and acquire various virulence factors and enhance their ability to transfer these factors from human-to-human due to continues outbreak (Zumla et al., 2015). The phylogenetic analysis revealed that all human and camel MERS-CoVs were identified to be homologous to each other. Additionally, the hedgehog and bat MERS-CoVs demonstrated para-phyletic group to human and camel MERS-CoV clade (Zhang et al., 2016).

Kim (2016) reported that the occurrence of a mutant strain of MERS-CoV toward the human CD26 receptor during the South Korean outbreak. Furthermore, they isolated 13 new viral genomes in which, 12 possessed point mutation in the receptor-binding domain (RBD) of S protein (Kim, 2016). The S protein is suggested to be essential for the host tropism through its interaction with the host CD26 receptor (van Doremalen et al., 2014; Wang et al., 2014).

PATHOGENESIS

Viral Entry

The entry of MERS-CoV to the host is facilitated by its type-I transmembrane glycoprotein known as S protein (Xia et al., 2014). The RBD of MERS-CoV consists of S1 subunit which ranged from 367 to 606 amino acids and can be grouped into an external sub domain and core domain as shown in **Figures 1C,D**. The receptor-binding motif, V484 – L567 of RBD is situated at the external sub domain and the core sub domain consists of anti-parallel β-sheet (five-stranded) and six helices in the center. The two small β-strands make a globular fold. The core domain structure is balanced by three disulfide linkages. The MERS-CoV RBD external sub domain contains a β-sheet along with

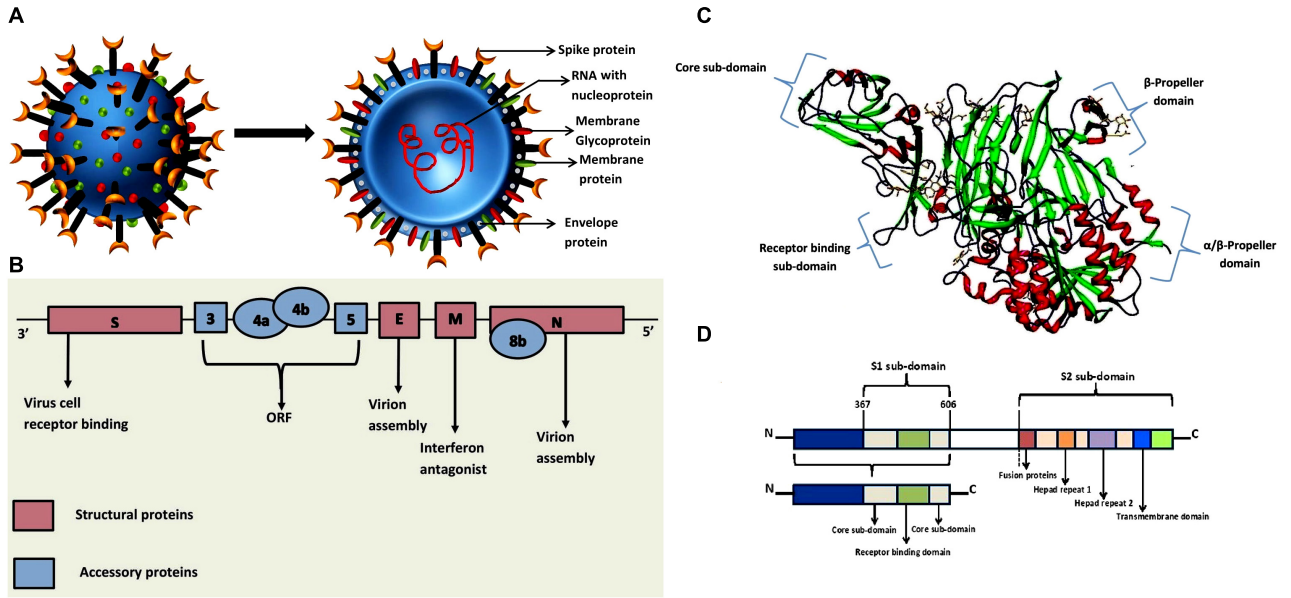


FIGURE 1 | (A) Graphical representation of the structure of the MERS-CoV. The schematic representation shows that the structure of Middle East Respiratory Syndrome Coronavirus, which contains the RNA enclosed in the nucleocapsid within the envelope that is embedded with spike proteins, glycoproteins and membrane proteins (van den Brand et al., 2015). **(B)** Genome organization of MERS CoV. The genomic organization of MERS-CoV displays the viral genes S, 3, 4a, 4b, 5, E, M, 8b, and N. The genome encodes for five unique accessory proteins such as 3, 4a, 4b, 5 and 8b and four major structural proteins which are illustrated in the genome scheme (Gao et al., 2016). **(C)** Experimental structure of MERS-CoV complexes with human DPP4 (PDB ID: 4L72). DPP4 is an extracellular RBD domain, which comprises of both N- and C-terminal. The N-terminal is the β-propeller domain and C-terminal is the α/β hydrolase domain (Wang et al., 2013). **(D)** Genome organization of receptor binding domain (RBD) that illustrates the S1 and S2 sub-domains (Zhang et al., 2015).

one small and three large strands which are organized in anti-parallel manner. The RBD core is attached to this region by special intervening loops and it binds to the core sub-domain by a clamp at the lower and upper regions. Most of the connecting loops and two small 3_{10} helices are located in the inner region of the sheet. The fourth disulfide linkage is stabilized among the residues starting from C503 to C526 and β6-strand linked with η3-helix. Three helices (HR1) at the center and three chains (HR2) near to the core promote the release of the viral particle into the cytoplasm and promote the progression of the infection (Durai et al., 2015).

Middle East Respiratory Syndrome Coronavirus can also enter the cell through an auxiliary pathway on the cell surface via transmembrane proteases. The binding of MERS-CoV into host cell and replication of the virus is shown in **Figure 2**. The host protease cleaves coronavirus S protein into two functional domains distinctive to each other at the N-terminal region (denoted as the S1 subunit) which comprised of RBD, and C-terminal portion (S2 subunit) comprised of a fusion peptide, two heptad repeats (HR2 and HR1) and the transmembrane (TM) domain (Weiss and Navas-Martin, 2005). The membrane fusion is conciliated by main conformational rearrangement which exposed the fusion peptide resulting in the development of six-helix bundle (6HB). The core of 6HB consists of a triple-stranded coiled like structure constructed by HR1s of the three spike subunits forming trimers; the HR2 elements are found to be packed within the grooves of the coiled coil in an anti-parallel direction. Owing to their central role in membrane fusion, it was reported that various antiviral peptides interfere the formation 6HB (Forni et al., 2015).

Transmission and Clinical Presentation

The clinical presentation of MERS-CoV compasses from severe respiratory diseases to subclinical infections (Drosten et al., 2013; Hui et al., 2018). Infected patients often indicate the presence of hemoptysis, sore throat, fever, cough, shortness of breath, and other gastrointestinal symptoms such as diarrhea and vomiting (Albarrak et al., 2012; Assiri et al., 2013a,b; Guery et al., 2013; Health Protection Agency, 2013; Omrani et al., 2013). A low range of lower pulmonary infiltrate associated with viral pneumonia was observed in the radiograph of patients infected with MERS-CoV (Memish et al., 2013a; Wang, 2014). It was observed that greater than 60% of the initially reported cases of MERS-CoV infection, the patients experienced severe disease which demanded intensive care treatments like extracorporeal membrane oxygenation and mechanical ventilation. Hematological aberrations delineated for the clinical cases of neutrophilia (8%), lymphopenia (34%), thrombocytopenia (36%), and lymphocytosis (11%). While the conditions of kidney failure necessitate renal replacement therapy for a considerable number of MERS-CoV cases, several studies detected MERS-CoV antigens and particles in the renal tissues *in vivo*, which is the direct indication of the virus replication in renal tissues and long term acute infection (Yeung et al., 2016; Alsaad et al., 2018).

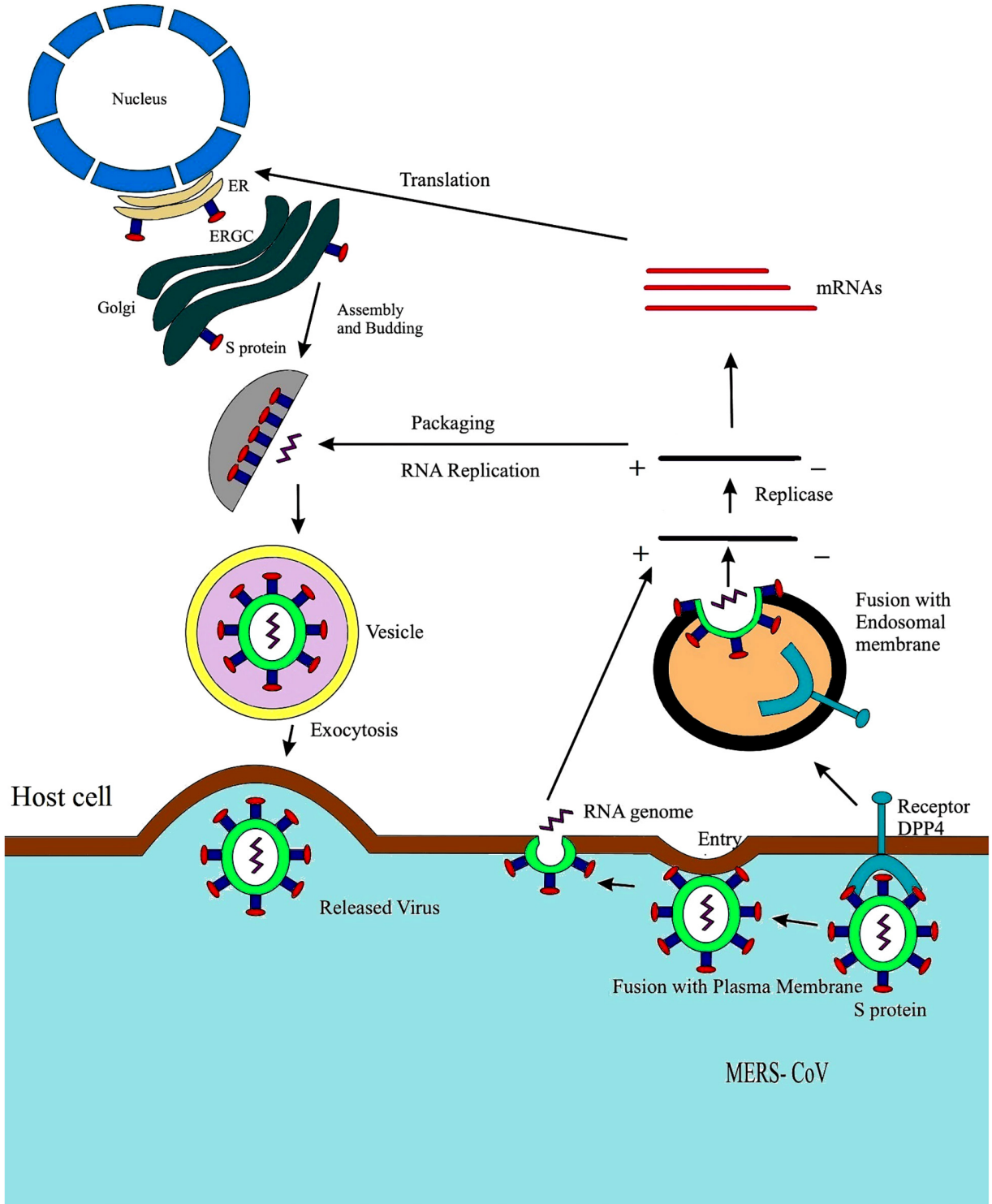


FIGURE 2 | Life cycle of MERS-CoV displaying fusion with plasma membrane. The fusion of S protein to the plasma membrane of host cell, formation of a double membrane vesicle in the host cell, eventually releasing the RNA enclosed in the nucleocapsid followed by genome transcription. The viral RNA undergoes replication and transcription followed by the 4, 5, and 6 RNA synthesis and translation; the endoplasmic reticulum aids the assembly and packaging of virus particle forming a complete double membrane vesicle and lastly through exocytosis and MERS-CoV is released out of the host cell (Du et al., 2009).

The first evidence of human-to-human transmission of MERS-CoV was reported in few cases in United Kingdom, when an adult male traveled to Saudi Arabia and transferred the infectious virus to two of his family members (Perlman and Netland, 2009). It was suggested that MERS-CoV infections were due to the introduction of the virus in humans and the close contact with camels was one of risk factors for the transmission of MERS as reported by World Health Organization [World Health Organization (WHO), 2016b].

The transmission of MERS-CoV is determined as spasmodic, often healthcare associated, intra-familial, and required prolonged and close contact (Memish et al., 2013a,b, 2014a,b; Puzelli et al., 2013; Drosten et al., 2014). In a domestic study conducted with MERS-CoV infected patients, 14 out of 280 contacts of 26 positively indexed patients (antibody positive or RNA) suggested that the frequency of the transmission even during outbreaks was approximately 3% (Drosten et al., 2014). Several studies suggested that the virus was not self-sustained, as during local epidemic of MERS-CoV, which has not been readily transmitted to more than one human and has been reported in majority of human related cases (Bauch and Oraby, 2013; Breban et al., 2013; Cauchemez et al., 2014; Chowell and Nishiura, 2014). The mode of transmission of the virus is illustrated in **Figure 3**.

The serological analysis and extensive investigation of those potentially exposed to the patients suggested that there were no secondary infections [World Health Organization, 2013b]. As a minimum of 18 cases of asymptomatic MERS-CoV infections were reported in health care personals, the function of these subclinical cases in the transfer of infection was found to be uncertain (de Sousa et al., 2014).

Molecular Mechanism of the Pathogenesis

While there were intensive studies on coronavirus research over the past 5 years, limited reports are available on the pathogenesis of MERS-CoV. Recent post-mortem histopathological studies revealed that the localization of viral particles in the pulmonary and extra pulmonary tissue of a 33-year-old male patient of T cell lymphoma, who acquired MERS-CoV infection (Ng et al., 2016; Alsaad et al., 2018). The histopathological analysis of the biopsies were collected from lung, brain, liver, heart, kidney, and skeletal muscle demonstrated that there were pulmonary diffuse alveolar damage, necrotizing pneumonia, acute kidney injury, portal and lobular hepatitis and myositis with muscle atrophic changes. Further, the viral particles were found to be localized in the pulmonary macrophages, pneumocytes, renal proximal

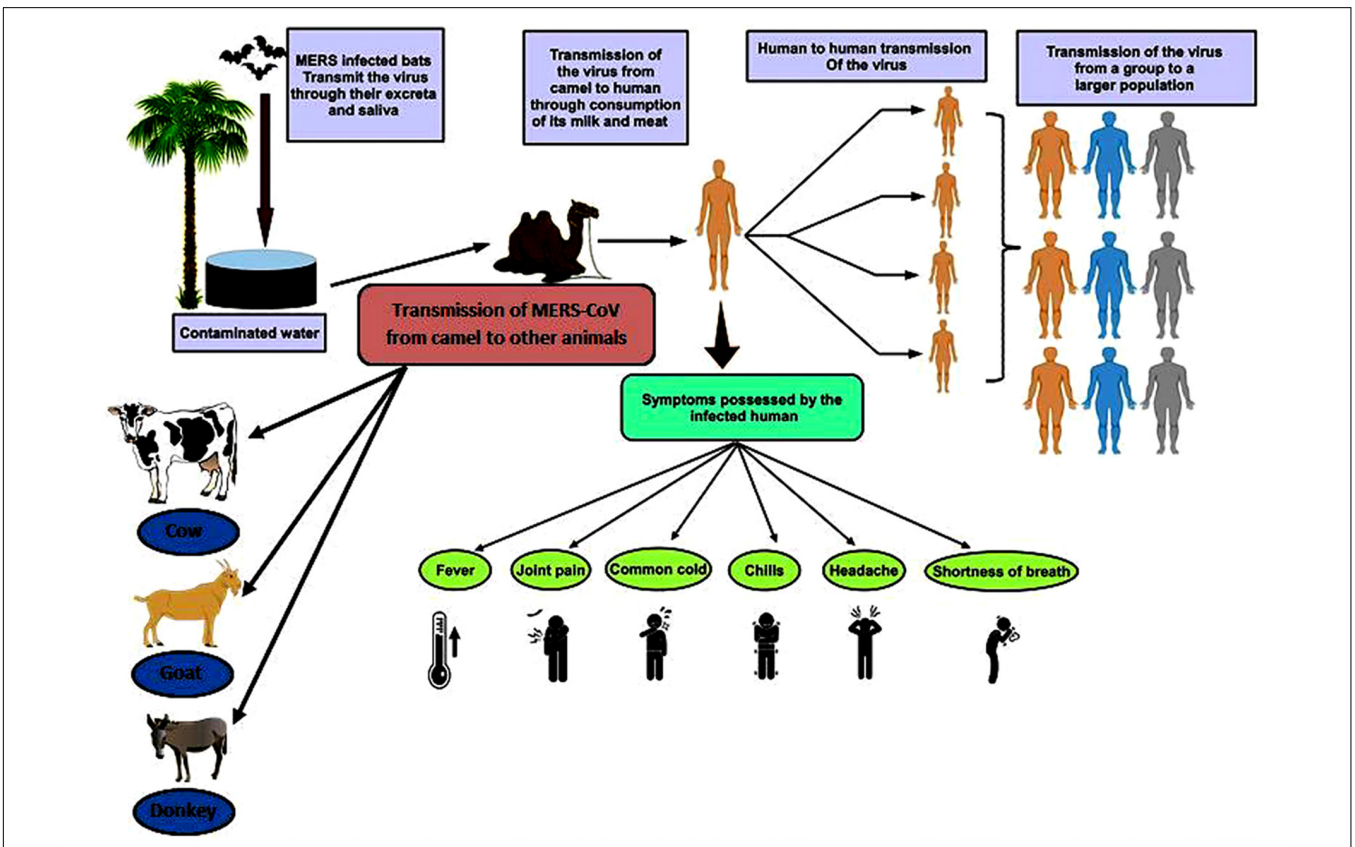


FIGURE 3 | Transmission of MERS-CoV and symptoms possessed by infected human. The figure shows the path by which the virus is transmitted from the infected camel to the human and camel to various animals (Kandeil et al., 2019); further, this virus is transmitted to other human population via human to human transmissions (Shehata et al., 2016).

tubular epithelial cells and macrophages infiltrating the skeletal muscles (Alsaad et al., 2018).

Studies suggested that the pathogenesis of MERS-CoV in human and animals are mainly due to three mechanisms such as DPP4 (dipeptidyl peptidase-4) mediated mechanism, papain like protease PLpro mediated mechanism and accessory proteins like p4a and membrane M protein mediated mechanism. Hence, these proteins are probably considered as one of the potential therapeutic targets for MERS-CoV infections (Durai et al., 2015). The molecular mechanisms exhibited by various modes of pathogenesis are illustrated in **Figure 4**.

DPP4 MEDIATED MECHANISM

DPP4 is a type-II transmembrane glycoprotein consists of approximately 766 amino acids and function as major receptor for MERS-CoV. The three dimensional structural analysis revealed that this receptor has α/β-hydrolase domain and a β-propeller domain with eight blades that helped in the binding of receptor binding domain in MERS-CoV (Raj et al., 2014). The crystal structure of DPP4 is illustrated in **Figure 1C**. A DPP4 binding protein, adenosine deaminase, is considered as one of the major inhibitors of the S protein of MERS-CoV (Drosten, 2013).

A number of cell lines such as human-derived HFL, Calu-3, Caco-2, Huh-7, HEK, His-1 cell lines, CD8+, and CD4+ that are susceptible to MERS-CoV were reported (Chan et al., 2013b; Shirato et al., 2013; Chu et al., 2016). Two S protein ecto-domains can be divided into fusion-catalyzing domains (FD) and RBD. The S RBDs bind to DPP4 after which the FDs are exposed through unfolding. Further, the cellular and viral membranes were joined together by refolding (Raj et al., 2013). The unfolding FD required the cleavage of S protein by host proteases. Out of various proteolytic cleavage sites between the FD and RBD, the cleavage at S1/S2 site is essential for the infection of Calu3 cells by MERS-CoV (Park et al., 2016).

It has been revealed that upon infection, monocyte chemo attractant protein-1 and IFN-γ-inducible protein-10 were induced and suppressed the proliferation of human myeloid progenitor cells (Zhou et al., 2014). MERS-CoV can infect T-cells from human lymphoid organs and causes the peripheral blood inducing apoptosis by intrinsic and extrinsic pathways

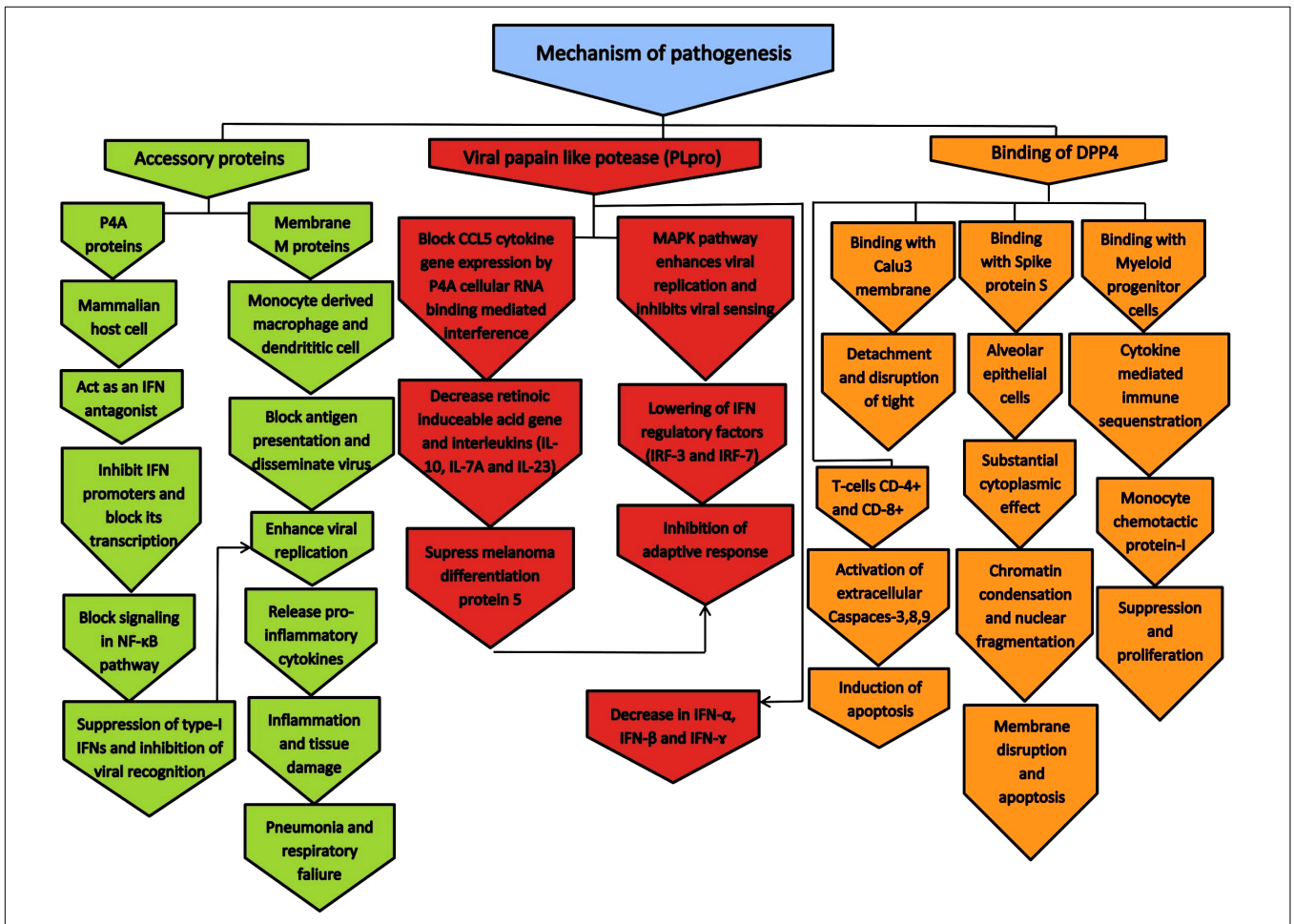


FIGURE 4 | The molecular mechanism of pathogenesis by MERS-CoV in human and animals. The pathogenesis mechanism is mainly occurred by DPP4, papian like protease PLpro, and accessory proteins such as p4a and membrane M protein (Durai et al., 2015).

(Chu et al., 2016). A study conducted by Al-Qahtani et al. (2017) suggested that the inhibition of DPP4 mitigated the induction of PPARγ (the transcriptional repressor) and IRAK-M (negative regulator of TLR signaling) which indicated that the immunosuppressive action of S glycoprotein regulated by DPP4.

PAPAIN LIKE PROTEASE PLpro MEDIATED MECHANISM

Upon entry into the cells, two ORFs at the 5′-end of the viral genome are translated into two poly-proteins. They are processed by two viral proteases, *PLpro* and 3C-like proteinase (3CLpro) and form 16 nonstructural proteins which are necessary for the membrane-associated replication complex. *PLpro* is located in nonstructural protein 3. *PLpro* was found to be multifunctional enzymes with deISGylating (removal of ISG15 conjugates from host cell factors) and de-ubiquitinating (cleavage of ubiquitin from host cell factors) properties in addition to protease activity in many coronaviruses (Clasman et al., 2017). This resulted in the host antiviral immune response being antagonized and promoting the viral replication. *PLpro* can inhibit the mitochondrial anti-viral signaling protein induced IFN-β reporter activity and reduce TNF-α induced NF-κB reporter activity. Hence, MERS-CoV *PLpro* is considered to be important antiviral target (Mielech et al., 2014; Harrigan et al., 2018).

The coronavirus genome is encoded by two ubiquitin-like domains (Ubl1 and Ubl2), out of which Ubl2 is considered to be an important component which influences the *PLpro* activity. Clasman et al. (2017) reported that *PLpro* has not showed the deISGylating, de-ubiquitinating or protease activities in the absence of Ubl2 domain. There were no variations in the process of inhibition, substrate specificity, catalytic efficiency and thermal stability (Clasman et al., 2017).

ACCESSORY PROTEINS MEDIATED MECHANISM

The viral genome is recognized by melanoma differentiation-associated protein-5 (MDA5), retinoic acid inducible gene-1 (RIG-1) and endosomal toll-like receptor 3 (TLR3) as pathogen-associated molecular patterns. This recognition resulted in the formation of type-1 interferon (IFN1). As an evasion mechanism, virus synthesize proteins that hinder the production IFN1 in the pathway (Zinzula and Tramontano, 2013).

In the absence of MDA5 and RIG-1, MERS-CoV used the protein such as 4a (p4a) which contain RNA-binding motif for binding (Rabouw et al., 2016). By a direct binding, this motif masks the dsRNA of MERS-CoV. The p4a possesses αββββα fold with the β1-β2 loop and α1 helix which bind to the minor groove of dsRNA. The mutational studies revealed that p4a-dsRNA complex stabilized by the amino acids such as K27, W45, K63, and K67 (Batool et al., 2017). In a study carried out by Canton et al. (2018) suggested that, NF-κB remained in the cytoplasm of MERS-CoV infected cells while 4b was found to be attached with the nucleus. In the absence of 4b, expression of pro-inflammatory cytokines was observed as NF-κB translocated to the nucleus. This was also observed in the case of 4b mutants that devoid of nuclear localization signal, indicating that nuclear localization signal mediated process of 4b is essential for NF-κB expression (Canton et al., 2018).

As a component of the viral envelope, M protein is involved in various functions such as viral assembly along with other proteins such as E, S, N. The M, E, and S proteins interact with N protein in the ER-Golgi complex. This interaction hinders the fusion of viral and cellular membranes (Mustafa et al., 2018). It was observed that the expression of M protein in MERS-CoV might suppressed the type-I IFN expression in response to poly (I: C) induction or Sendai virus infection. This reaction was detected to be specific for the activation of IFN regulatory factor 3 (IRF3), however, not activated the nuclear factor-κB. The interaction of MERS-CoV M protein with TRAF3 and the association of disrupted TRAF3–TBK1 lead to reduced activation of IRF3 (Lui et al., 2016).

HOST IMMUNE RESPONSE

Middle East Respiratory Syndrome Coronavirus can readily infect human respiratory epithelial cells, macrophages, T-cells, dendritic cells and can influence the production and induction of pro-inflammatory cytokines and chemokines (Zhou et al., 2015). Studies on *ex vivo* human lung tissue and respiratory epithelial cell lines showed that the induction of antiviral interferons followed by MERS-CoV infection by stimulated epithelial cells was found to be limited. A study by Menachery et al. (2014) described that the cells infected with MERS-CoV demonstrated distinctly altered chromatin structures such as repressive histone markers, that could limit the transcription factors from binding to the promoter regions of interferon-stimulated gene. The changes in DNA methylation found to be one of the reasons for down regulation of IFN-γ–associated antigen-presenting gene after the infection (Menachery et al., 2018). MERS-CoV proteins such as 4a, 4b, M, and PLpro were found to be suppressed the induction of interferons (Yang et al., 2013; Mielech et al., 2014; Canton et al., 2018). Further, the induction of pro-inflammatory cytokine response in these cells was found to be limited. A delayed induction of pro-inflammatory cytokines/chemokines such as IL-6, IL-8, and IL-1β were observed upon the viral infection (Lau et al., 2013; Canton et al., 2018).

Middle East Respiratory Syndrome Coronavirus could infect and replicate in human monocyte-derived macrophages and immature monocyte-derived dendritic cells, while it failed to perform similar functions in mature monocyte-derived dendritic cells. This indicate that the maturation restrict the viral replication. Immature dendritic cells were unable to stimulate T-cell even after the antigen uptake, delaying the activation of T-cells and permitting further viral replication (Cong et al., 2018). The infection of human macrophages induced the expression of pro-inflammatory cytokines/chemokines such as MCP-1/CCL-2, IFN-α2, IFN-γ, MIP-1α/CCL-3, IP-10/CXCL-10, RANTES/CCL-5, IL-8, TNF-α, IL-12p40, and IL-6

(Zhou et al., 2015; Cong et al., 2018). The infected dendritic cells induced the expression of IL-12, RANTES/CCL-5, IP-10/CXCL-10 and IFN-γ, however, showed low expression of IFN-α and no expression of IFN-β (Zhou et al., 2015).

T-cells can be directed to the infection site by type-I IFN stimulated secretion of IL-10 and CXCL10. Due to their uncontrolled and strong expression, the expression of IFN-γ and IL-12 were inhibited and restricted the activation of T-helper cells. With the added effect of down-regulated antigen-presentation pathways, the activation of T cells is inhibited. Hence, the sequestered T cells failed to target the virus (Ying et al., 2016). Previous studies revealed that when the serum samples and bronchoalveolar lavage were analyzed from two patients, it was observed that the patients with low level secretion of IFN-α were failed to survive and the patients displayed high level secretion of type-I IFN were survived (Faure et al., 2014).

ANIMAL MODELS

The animal models are one of the vital components for translation of the findings from drug discovery process from bench to bedside. These animal models are indispensable for the deeper understanding of any diseases and safe and protective vaccine development process (Lorenzen et al., 2015). The main criteria for the validation of animal models in preclinical studies are face validity, predictive validity and target validity. The face validity means that the proper similarity between the animal model and the human disease in terms of the biology and symptoms of the disease. However, assessing face validity is a tedious task due to the lack of understanding on the core biology of the disease symptoms. Predictive validity means the display of clinical interventions which exhibited similar effect in the animal models. This is also very difficult to achieve due to the incomplete correlation between animal models, the mechanism of human diseases and the incapability of standard drugs to active in many animal models. Target validity is another essential component for the validation of animal models in which the target under study must have an analogous function in the disease model as in the clinical condition (Denayer et al., 2014).

An ideal animal model is the one where an immunocompetent animal, on receiving the challenge virus at a realistic dose through a suitable inoculation route, is able to replicate the characteristics of a human disease as closely as possible (Doremalen and Munster, 2015). Ferrets, hamsters, mice and other small laboratory animals are not ideal animal models for MERS-CoV owing to the variations in DPP4 receptor. The rhesus macaque and the common marmoset are the first non-human primate species (NPS) to be used as the animal models for MERS-CoV. Glycosylation of mouse DPP4 (mDPP4) prevents its binding to S protein of MERS-CoV. Hence, transgenic hDPP4 (human DPP4) mice were developed as animal model (Peck et al., 2015). The major advantages and limitations of MERS-CoV animal models are listed in **Table 2**.

Mice

Studies suggested that the glycosylation of certain amino acids prevented the binding of mouse DPP4 (mDPP4) to S protein of MERS-CoV (Doremalen and Munster, 2015). The first transgenic MERS-CoV mouse model was established by Agrawal et al. (2015). The mice expressed hDPP4 and developed severe respiratory MERS-CoV infections. The transgenic mice infected with an intra-nasal dose of 10^6 TCID50 of MERS-CoV developed pneumonia, weight loss, acute pulmonary viral infection, ruffled fur, squinting, and death within few days (Agrawal et al., 2015). A transgenic mouse model developed by Zhao et al. (2015) showed that limited utility for pathogenic studies due to the systemic dissemination and development of severe neurological diseases. The other lung infection models were lack mortality and weight loss, however, developed lung pathology in response to 50% $TCID^{50}$ of MERS-CoV (Tao et al., 2015).

Recent studies suggested that the transgenic mice models were developed where the full-length mDPP4 gene replaced by hDPP4 (Coleman et al., 2017). The infection and replication of MERS-CoV was observed in the lungs, however, disseminated slow

TABLE 2 | Advantages and limitations of MERS-CoV experimental animal models.

Species	Advantages	Limitations	Reference
Rabbit	Readily available and easy to handle	Animal-to-animal transmission studies are not available No clinical disease, Low viral titers in tissues upon infection Develop mild pulmonary lesions	Haagmans et al., 2015; Vergara-Alert et al., 2017
Rhesus macaques	Human-specific reagents available for immunologic analysis Immune and respiratory systems similar to humans; clinical disease similar to humans Useful for confirming vaccine efficacy testing	Limited availability and expensive Expert husbandry requirements Animal-to-animal transmission studies are not available Transient disease Ethical concerns	Baseler et al., 2016; Yu et al., 2017
Common marmoset	Model severe, potentially fatal MERS-CoV infection Some human-specific immunological reagents cross-react Respiratory and immune systems similar to humans; clinical disease similar to humans Useful for confirming antiviral and vaccine efficacy testing	Limited availability and expensive Expert husbandry requirements Ethical concerns Animal-to-animal transmission studies are not available	Vergara-Alert et al., 2017; Yu et al., 2017
hDPP4-transgenic mice	Model severe, potentially fatal MERS-CoV infection Easy to handle Reagents available Useful for screening antivirals and vaccines	Global over expression of hDPP4	Baseler et al., 2016; Coleman et al., 2017

impact to the other organs during the post injection with dose range of 102–105 PFU. The pathology of infected mice showed pneumonia-like symptoms (Coleman et al., 2017).

The CRISPR-Cas9 gene editing tools were used to produce mice susceptible to MERS-CoV infection by modifying their genome sequence (positions 288 and 330) homologous to the sequence of hDPP4. In certain mice model the characteristic symptoms such as pulmonary hemorrhage, low survival, decreased pulmonary function and weight loss were observed. The 288-330+/+MERS-CoV mouse model demonstrated severe respiratory infection as observed in humans (Cockrell et al., 2016).

Rhesus Macaques and Marmosets

Upon infection with MERS-CoV, rhesus macaques and marmosets were developed moderate respiratory disease which are comparable with mild human MERS-CoV infection. As observed in human cases, they also showed complete blood count abnormalities. Rhesus macaque and marmoset models were found to be suitable for pathogenic studies of mild infections. Both the models can be used for the efficacy testing of prophylactic and therapeutic countermeasures (Baseler et al., 2016).

Yu et al. (2017) reported that when 2-3 old rhesus macaques and common marmosets were intra-tracheally injected with hCoV-EMC, hematoxylin and eosin stained tissues of the infected model demonstrated lesions primarily in the lungs with various degrees of inflammation, hemorrhaging, pulmonary oedema, interstitial pneumonia, eosinophil infiltration and necrosis in bronchial epithelial cells and pneumocytes. The lungs of the infected marmosets demonstrated widespread pulmonary oedema and hemorrhaging. The oedematous alveolar cavities showed fibrinous exudates and neutrophil infiltration. In both the cases, DPP4 was widely expressed in alveolar macrophages and type-I and II pneumocytes were also actively involved. The pathological changes as a result of viral infection were not observed in any other organs of the infected animal (Yu et al., 2017).

Rabbits

In most cases, MERS-CoV causes severe infection in the lower respiratory tract in humans. Nasal swabs collected from the infected rabbits demonstrated the presence of MERS-CoV. They generally develop respiratory diseases such as asymptomatic and mild human infections as sometimes observed in immune-competent patients (Haagmans et al., 2015).

Recently, Houser et al. (2017) revealed that the infected New Zealand rabbits were developed asymptomatic pulmonary infections with high levels of viral antigens and RNA. Multiple lung lobes showed peri-vascular inflammation. The antibodies developed by the rabbits lacked neutralizing activity, and as a result the animals were susceptible to re-infection. In fact, enhanced pulmonary inflammation was observed after re-infection. However, re-infection elicited the neutralizing antibodies (Nabs). Hence, it was suggested that upon re-infection, there might be greater risk of severe lung disease in those who do not develop neutralizing antibody response, or those whose neutralizing antibody titers have decreased after the initial infection (Houser et al., 2017). The clinical signs and lesions along with the occurrence of MERS-CoV RNA and antigens observed in MERS-CoV infected animal models are shown in **Table 3**.

DIAGNOSTIC APPROACHES

The primary diagnosis of MERS-CoV infection was performed by molecular techniques such as real-time reverse transcriptase PCR (RT-PCR) (Corman et al., 2012a), reverses transcription–loop-mediated isothermal amplification (RT-RTPA) (Shirato et al., 2014) and reverses transcription-recombinase polymerase amplification (RT-LAMP) (Abd et al., 2013). Numerous serological assays were used to detect MERS-CoV or closely related viruses in seropositive camels; these tests were protein microarrays like indirect enzyme-linked immunosorbent assay (ELISA), recombinant spike immunofluorescent assay, spike pseudoparticle neutralization and microneutralization assay (Buchholz et al., 2013; Perera et al., 2013). However, none of the serological assays showed evidence of the precise occurrence of MERS-CoV in camels (Song et al., 2015). Recent study reported that a rapid and specific assay for the detection of MERS-CoV such as nucleic acid visualization technique which combine the reverse transcription loop-mediated isothermal amplification technique and a vertical flow visualization strip (RT-LAMP-VF) to detect the N gene of MERS-CoV (Huang et al., 2018). The study suggested that in comparison with the

TABLE 3 | Clinical signs and lesions along with the occurrence of MERS-CoV RNA and antigens as seen in MERS-CoV infected animal models.

Animal model	Clinical signs	Lesions	Occurrence of MERS-Cov RNA	Occurrence of MERS-antigen	Reference
Rabbit	Asymptotic	Rhinitis with necrosis	Lung, upper respiratory tract, lymph nodes	Type I and II pneumocytes	Haagmans et al., 2015
Rhesus Macaques	Mild to moderate respiratory disease	Interstitial pneumonia	Lung, lymph nodes, upper respiratory tract	Type I and II pneumocytes, alveolar macrophages	de Wit et al., 2013a,b; Yao et al., 2014; Yu et al., 2017
Common Marmoset	Mild to severe respiratory disease	Broncho-interstitial pneumonia	Lung, blood, lymph nodes, visceral organs, upper respiratory tract	Type I pneumocytes, alveolar macrophages	Falzarano et al., 2014; Yu et al., 2017
hDPP4- transgenic mice	Severe fatal respiratory disease	Broncho-interstitial pneumonia	Lung, brain, visceralorgans	Type I and type II pneumocytes	Agrawal et al., 2015

real-time RT-PCR (rRT-PCR) method recommended by the World Health Organization, the RT-LAMP-VF assay is easy to perform and the rapid detection of MERS-CoV within 35 min was achieve by this approach (Huang et al., 2018).

An assay was developed based on the detection of N protein of MERS-CoV (Song et al., 2015). The NP protein preferred as better target than the S protein. In this type of assay, firstly, the antigen binds to gold labeled monoclonal antibodies which lead to the formation of antigen-antibody complex. The complex further passed through nitrocellulose membrane and bind to monoclonal antibody at the test region and formed band which could be conveniently analyzed (Tang, 2014).

Currently, multiple purified monoclonal antibodies are available in clinical and preclinical studies for the development of antimicrobials (Rockx et al., 2010). Studies suggested that two human antibodies (REGN3048 and REGN3051) are known to bind with MERS-CoV RBD and prevent the interaction of cellular receptor DPP4 with S protein and effectively neutralize MERS-CoV infection (Pascal et al., 2015). The antibodies such as REGN3048 and REGN3051 showed no binding with S protein, thus, employed as effective combination for inhibiting MERS-CoV (Aderem et al., 2011). REGN3048 and REGN3051 co-interact with MERS-CoV RBD, which suggested that these antibodies bind to discrete epitopes as they bind to regions of MERS-CoV S protein that are conserved during the natural evolution of the virus. These two antibodies were blocked entry of the virus into susceptible cell lines and neutralize the infection (Pascal et al., 2015).

The diagnostically relevant variations in the neutralization activity have not been detected in several isolates of MERS-CoV. In order to detect the serological response to a specific type of single MERS-CoV serotype, specific protein based sero-assays are essential to be performed (Muth et al., 2015). The development of potential serological assays demand well delineated human or animal sera and antibodies specific to MERS-CoV (Meyer et al., 2014).

VACCINES AND THERAPEUTICS

Previous studies revealed that both cellular and humoral responses are required for the complete protection against MERS-CoV infections (Faure et al., 2014; Park et al., 2015; Corman et al., 2016). Nabs and T-cell responses are the main agents in providing protective immunity against coronaviruses. In a study by Zhao et al. (2014) reported that MERS-CoV cleared by mice which lack B-cells while those deficient in T-cells failed to perform similar functions. Further, since virus-specific humoral responses were decreased over time, developing a vaccine which could induce long-lived memory T-cell response considered to be beneficial. However, any vaccine compounds which are able to induce both T and B-cell responses are required for immune protection (Okba et al., 2017).

Current Vaccine and Antiviral Candidates

MERS-CoV vaccine candidates are still under development with DNA based, protein based, live attenuated and vectored-virus based vaccines (Arabi et al., 2017). Out of the four structural proteins and sixteen non structural proteins encoded by the viral genome, S protein showed the highest immunogenicity which induced T-cell responses. In most of the MERS-CoV, Nabs target the RBD of the S protein. The variations in amino acid sequences of the S protein detected amongst MERS-CoV is limited, thus, designing a vaccine against one strain most likely to be protected against all the other circulating strains (Okba et al., 2017). The S1 protein located outside the RBD probably considered as potential drug target to develop therapeutic strategies against MERS-CoV. RBD-specific mAbs are considered to be displayed greater neutralizing activity than those were targeted the S1 or S2 region outside RBD (Du et al., 2013).

DPP4-targeting therapeutic agents are capable of blocking the interaction between MERS-CoV RBD and DPP4, and therefore inhibit MERS-CoV infection. The anti-DPP4 (CD26) antibodies such as 1F7, 2F9, and YS110 prevent the entry of MERS-CoV into susceptible cells, obstructing the virus infection (Ohnuma et al., 2013). Additionally, DPP4-binding protein adenosine deaminase (ADA) competes the binding with MERS-CoV RBD to DPP4, specifically at crucial residues such as Q286 and L294, defining their role as a naturally occurring antagonist (Raj et al., 2014).

Recent studies revealed that the DNA vaccines are the safest vaccines for immune-compromised patients. They possessed comparatively greater amounts of DNA (e.g., 0.5–2 mg) thus requires multiple inoculations to induce potent immune responses in rhesus macaques and mice (Wirblich et al., 2017). Chi et al. (2017) developed a DNA vaccine which encoded the first 725 amino acids of S protein in MERS-CoV which induced the cell mediated and antigen-specific humoral immune responses in mice. Post three immunizations, it was observed that high titers of Nabs (up to 1: 104) were produced without any adjuvant. A prophylactic DNA-plasmid vaccine known as GLS-5300, which encodes for S protein, is the first MERS-CoV vaccine to be considered for the human trials developed by GeneOne Life Inc., which has been enlisted in the on-going list of vaccines by WHO [Rabaan et al., 2017; World Health Organization (WHO), 2018b].

The vaccines that imitate the natural infection, such as recombinant viral vectors or live-attenuated virus present the antigens in natural ways which further stimulate the activation of humoral and cell-mediated immunity to the native protein conformation. As the live attenuated viruses have been the most immunogenic platforms available, they exhibited the capability to present numerous antigens across the viral life cycle in their native conformations (Henderson and Moss, 1999). Agrawal et al. (2016) vaccinated the mice with an inactivated MERS-CoV vaccine and which demonstrated hypersensitivity risk factors in MERS-CoV infection. Although there were extensive research in the last few years, limited anti-MERS-CoV therapeutics have been approved for human use. Originally, conventional IFAs were employed in the human sero-surveys and these relied on the MERS-CoV infected cell cultures. These cell cultures were the primary antigen sources for detecting the presence of human anti-MERS-CoV Nabs, IgM, and IgG in human samples (Corman et al., 2012b). Further, it was reported that human monoclonal

antibody m336 demonstrated significant neutralizing properties against MERS-CoV *in vitro* conditions (Houser et al., 2016).

Recent studies revealed that there were few antiviral agents in the developmental stage. It was reported that the antiviral drug chlorpromazine demonstrated strong anti-MERS-CoV potential; however, the high cytotoxicity of the drug limiting the potential window for drug utilization. Similarly, another drug Toremifene showed moderate activity when tested in antigen presenting cells, however, the high cytotoxicity of this drug also narrowing its application as a therapeutic remedy against MERS-CoV (Cong et al., 2018). Further, studies suggested that a nucleoside analog GS-5734 known as Remdesivir, which showed potential antiviral activity against human and zoonotic CoVs *in vitro* and the drug inhibited MERS-CoV at 50% effective concentration values (EC50) (Agostini et al., 2018).

NOVEL THERAPEUTIC APPROACHES

The conserved nature of the N protein was used to develop vaccines that can induce an adaptive immune response against these proteins. In a study by Zhao et al. (2016) reported that a vaccine candidate induced airway memory CD4+ T cell response against N-specific epitopes of MERS-CoV. In another study suggested that vaccination with recombinant N-terminal domain (rNTD) triggered greater T-cell response than the mice vaccinated with rRBD. The neutralizing activity was also observed in the sera (Jiaming et al., 2017). Recent study revealed that the combination of fusion inhibitory peptide which targets the protein HR1 domain of MERS-CoV S2 and a neutralizing antibody specific for the S1 protein RBD showed synergistic therapeutic activities against MERS-CoV (Wang et al., 2019). Further, the researchers have developed novel neutralizing nanobodies (Nb) which specifically bind to the RBD of MERS-CoV S protein. The Nb was found to be interacted with the conserved domain of MERS-CoV RBD with high affinity and blocked binding of RBD to the receptor of MERS-CoV (Zhao et al., 2018).

Some immunodominant epitopes within the protein structure may contribute negatively to the neutralization activity of the vaccine. For example, a short region of RBD was able to induce higher titer of IgG when compared to other longer regions (Ma et al., 2014). The negative effects of these epitopes can be surmounted by immunofocusing; mapping of the most neutralizing RBD fragment and eliminating unnecessary fragments. This can help in focusing the immune response to important epitopes (Ma et al., 2014). Pallesen et al. (2017) suggested that the structure-based design of S proteins improved their immunogenicity where the high titers of Nabs were observed against MERS-CoV. The major categories of vaccines developed and tested against various animal models are shown in **Table 4**.

Therapeutic Screening by Computational Biology

The technological progression is reflected upon a series of genome wide molecular screening platforms and computational biology approaches that provided novel insights to prompt response against emerging viral diseases. The prototype of these approaches included the *in vivo* animal model, tissue culture model, human challenge model, and vaccine studies. These model systems are anxious by challenges, preferably yielding a spectra of the severity of the diseases like lethal versus sub-lethal in order to increase divergence for downstream data mining and modeling (McDermott et al., 2011; Drosten et al., 2013; Law et al., 2013). A diversity of computational methodologies and network approach were used to detect the regulatory networks of various viral systems by *de novo* studies (Hou and Zhao, 2013; Mitchell et al., 2013).

Recently, studies have shown that peptides can be utilized as highly potent signal transduction agents for various viral infections. It has been suggested that antimicrobial peptides (AMPs) can be used as potential therapeutic option against MERS-CoV infections (Mustafa et al., 2018). Computational biology approach in combination with virology can be employed to design novel peptide based therapeutics against various strains of MERS-CoV with high efficacy. Recent studies have suggested that repurposing of existing and clinically approved anti-viral peptides can be used as promising lead molecules for the development of novel anti-MERS-CoV agents (Mustafa et al., 2018).

The RBD and S protein based vector vaccines are considered to be one of the efficient targets for designing MERS-CoV vaccine, as it comprised of multiple neutralizing epitopes which offer higher potential to prompt Nabs against the virus (Shi et al., 2015). The vast developments in computational biology have led to the new line of immunoinformatics databases and tools, which are other operative ways for vaccine development (Terry et al., 2015).

TABLE 4 | Categories of vaccine developed against MERS-CoV, their potential candidate, and their target long with their immunological response and efficacy.

Vaccine categories	Vaccine candidate	Target antigen	Animal model	Immunological response	Efficacy	Reference
Protein based	S1	S1	Mice	Nab	Not tested	Wang et al., 2015
	rRBD-Fc	S377-588	Ad/hDPP4-mice	T-cell, Nab	Protective	Ma et al., 2014
	rNTD	S18-353	Ad/hDPP4-mice	T-cell, Nab	Protective	Jiaming et al., 2017
Recombinant viral vectors	BNSP333-S1	S1	Ad/hDPP4-mice	Nab	Protective	Wirblich et al., 2017
	S377-588-Fc	S	Ad/hDPP4-mice	T-cell, Nab	Not tested	Tang et al., 2015
	VRP-S	S	288/330+/+mice	Nab	Protective	Cockrell et al., 2016
DNA vaccine	pcDNA3.1-S1	S1	Ad5/hDPP4-mice	T-cell, Nab	Protective	Chi et al., 2017
	pVax1-S	S	Non human primates	T-cell, Nab	Protective	Muthumani et al., 2015

The screening of dominant immunogens based on the availability of genome sequence data is crucial for designing novel vaccines, which is assisted by immunoinformatics (Firbas et al., 2006). The epitope directed lead design was one of the critical steps involved in the lead identification by immunoinformatics approaches (Shi et al., 2015). MHC-I restrained CD8+ cytotoxic T-lymphocytes (CTLs) identification provides profound insight in designing novel antiviral agents (Terry et al., 2015).

In conclusion, MERS-CoV infection continued to be fatal disease and has emerged as one of the epidemic concern. Irrespective of the various studies conducted to determine the distribution, the exact intermediates remain unidentified. There are various animal models available which provided profound insight in understanding the transmission and pathogenecity of the virus in human. The common pathogenesis mechanisms of the virus such as DPP4, PLpro, accessory proteins like p4a and membrane M protein provided significant insight for the screening of novel drug targets for vaccine development. The detailed understanding of the binding mechanism of various inhibitors toward the structural, non-structural, and accessory proteins of the virus probably provide profound insight for lead development. The integration of genome analysis, proteomics studies, immunoinformatics, and systems biology approaches on various animal models have made the recognition of new targets and lead molecules much easier in than the approaches available during earlier time. By considering MERS-CoV infections as one of the great public threats, there is high demand for undertaking the coronavirus research at the deeper molecular level to understand the mechanism of viral infection, development of advanced and rapid detection methods and futuristic therapeutic strategies to combat MERS-CoV infections.

AUTHOR CONTRIBUTIONS

SS, SC, SP, SK, and VS collected data and prepared the complete manuscript. Further, SS reviewed, revised, and edited the manuscript.

REFERENCES

Abd, E. W. A., Patel, P., Heidenreich, D., Hufert, F. T., and Weidmann, M. (2013). Reverse transcription recombinase polymerase amplification assay for the detection of Middle East respiratory syndrome coronavirus. *PLoS Curr.* 5:ecurrents.outbreaks.62df1c7c75ffc96cd59034531e2e8364. doi: 10.1371/currents.outbreaks.62df1c7c75ffc96cd59034531e2e8364

Aderem, A., Adkins, J. N., Ansong, C., Galagan, J., Kaiser, S., Korth, M. J., et al. (2011). A systems biology approach to infectious disease research: innovating the pathogen-host research paradigm. *mBio* 2:e00325-10. doi: 10.1128/mBio.00325-10 e00325-10,PMID:21285433

Agostini, M. L., Andres, E. L., Sims, A. C., Graham, R. L., Sheahan, T. P., Lu, X., et al. (2018). Coronavirus susceptibility to the antiviral Remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *mBio* 9:e00221-18. doi: 10.1128/mBio.00221-18e00221-18

Agrawal, A. S., Garron, T., Tao, X., Peng, B. H., Wakamiya, M., Chan, T. S., et al. (2015). Generation of a transgenic mouse model of middle east respiratory syndrome coronavirus infection and disease. *J. Virol.* 89, 3659–3670. doi: 10.1128/JVI.03427-14

Agrawal, A. S., Tao, X., Algaissi, A., Garron, T., Narayanan, K., Peng, B. H., et al. (2016). Immunization with inactivated Middle East Respiratory Syndrome coronavirus vaccine leads to lung immunopathology on challenge with live virus. *Hum. Vaccin. Immunother.* 12, 2351–2356. doi: 10.1080/21645515.2016.1177688

Albarrak, A., Stephens, G., Hewson, R., and Memish, Z. (2012). Recovery from severe novel coronavirus infection. *Saudi Med. J.* 33, 1265–1269.

Al-Qahtani, A. A., Lyroni, K., Aznaourova, M., Tseliou, M., Al-Anazi, M. R., Al-Ahdal, M. N., et al. (2017). Middle east respiratory syndrome corona virus spike glycoprotein suppresses macrophage responses via DPP4-mediated induction of IRAK-M and PPARγ. *Oncotarget* 8, 9053–9066. doi: 10.18632/oncotarget.14754

Alsaad, K. O., Hajeer, A. H., Al Balwi, M., Al Moaiqel, M., Al Oudah, N., Al Ajlan, A., et al. (2018). Histopathology of Middle East respiratory syndrome corona virus (MERS-CoV) infection - clinicopathological and ultrastructural study. *Histopathology* 72, 516–524. doi: 10.1111/his.13379

Alshukairi, A. N., Zheng, J., Zhao, J., Nehdi, A., Baharoon, S. A., Layqah, L., et al. (2018). High prevalence of MERS-CoV infection in camel workers in Saudi Arabia. *mBio* 9:e01985-18. doi: 10.1128/mBio.01985-18e01985-18

Al-Tawfiq, J. A., Momattin, H., Dib, J., and Memish, Z. A. (2014). Ribavirin and interferon therapy in patients infected with the Middle East respiratory syndrome coronavirus: an observational study. *Int. J. Infect. Dis.* 20, 42–46. doi: 10.1016/j.ijid.2013.12.003

Arabi, Y. M., Balkhy, H. H., Hayden, F. G., Bouchama, A., Luke, T., Baillie, J. K., et al. (2017). Middle East Respiratory Syndrome. *N. Engl. J. Med.* 376, 584–594. doi: 10.1056/NEJMsr1408795

Assiri, A., Al-Tawfiq, J., and Al-Rabeeah, A. (2013a). Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome corona virus disease from Saudi Arabia: a descriptive study. *Lancet Infect. Dis.* 13, 752–761. doi: 10.1016/S1473-3099(13)70204-4

Assiri, A., McGeer, A., Perl, T. M., Price, C. S., Al Rabeeah, A. A., Cummings, D. A., et al. (2013b). KSA MERS-CoV investigation team. hospital outbreak of middle east respiratory syndrome coronavirus. *N. Engl. J. Med.* 369:886. doi: 10.1056/NEJMoa1306742

Baseler, L., de Wit, E., and Feldmann, H. (2016). A comparative review of animal models of middle east respiratory syndrome coronavirus infection. *Vet. Pathol.* 53, 521–531. doi: 10.1177/0300985815620845

Batool, M., Shah, M., Patra, M. C., Yesudhas, D., and Choi, S. (2017). Structural insights into the Middle East respiratory syndrome coronavirus 4a protein and its dsRNA binding mechanism. *Sci. Rep.* 7:11362. doi: 10.1038/s41598-017-11736-6

Bauch, C. T., and Oraby, T. (2013). Assessing the pandemic potential of MERS-CoV. *Lancet* 382, 662–664. doi: 10.1016/S0140-6736(13)61504-4

Breban, R., Riou, J., and Fontanet, A. (2013). Inter-human transmissibility of Middle East respiratory syndrome corona virus: estimation of pandemic risk. *Lancet* 382, 694–699. doi: 10.1016/S0140-6736(13)61492-0

Buchholz, U., Müller, M., Nitsche, A., Sanewski, A., Wevering, N., Bauer-Balci, T., et al. (2013). Contact investigation of a case of human novel corona virus infection treated in a German hospital, October-November 2012. *Euro. Surveill* 18:20406.

Canton, J., Fehr, A. R., Fernandez-Delgado, R., Gutierrez-Alvarez, F. J., Sanchez-Aparicio, M. T., García-Sastre, A., et al. (2018). MERS-CoV 4b protein interferes with the NF-κB-dependent innate immune response during infection. *PLoS Pathog.* 14:e1006838. doi: 10.1371/journal.ppat.1006838

Cauchemez, S., Fraser, C., Van, K. M. D., Donnelly, C. A., Riley, S., and Rambaut, A. (2014). Middle East respiratory syndrome corona virus: quantification of the extent of the epidemic, surveillance biases, and transmissibility. *Lancet Infect. Dis.* 14, 50–56. doi: 10.1016/S1473-3099(13)70304-9

Chafekar, A., and Fielding, B. C. (2018). MERS-CoV: Understanding the latest human Corona virus threat. *Viruses* 10:E93. doi: 10.3390/v10020093

Chan, J. F., Chan, K. H., Choi, G. K., To, K. K., Tse, H., Cai, J. P., et al. (2013a). Differential cell line susceptibility to the emerging novel human betacoronavirus 2c EMC/2012: implications for disease pathogenesis and clinical manifestation. *J. Infect. Dis.* 207, 1743–1752. doi: 10.1093/infdis/jit123

Chan, J. F., Lau, S. K., and Woo, P. C. (2013b). The emerging novel Middle East respiratory syndrome corona virus: the 'knowns' and 'unknowns'. *J. Formos. Med. Assoc.* 112, 372–381. doi: 10.1016/j.jfma.2013.05.010

Chan, J. F., Li, K. S., To, K. K., Cheng, V. C., Chen, H., and Yuen, K. Y. (2012). Is the discovery of the novel human betacoronavirus 2c EMC/2012 (HCoV-EMC) the beginning of another SARS-like pandemic? *J. Infect.* 65, 477–489. doi: 10.1016/j.jinf.2012.10.002

Chi, H., Zheng, X., Wang, X., Wang, C., Wang, H., Gai, W., et al. (2017). DNA vaccine encoding Middle East respiratory syndrome coronavirus S1 protein induces protective immune responses in mice. *Vaccine* 35, 2069–2075. doi: 10.1016/j.vaccine.2017.02.063

Chowell, G., and Nishiura, H. (2014). Transmission dynamics and control of Ebola virus disease (EVD): a review. *BMC Med.* 12:196. doi: 10.1186/s12916-014-0196-0

Chu, H., Zhou, J., Wong, B. H., Li, C., Chan, J. F., Cheng, Z. S., et al. (2016). Middle east respiratory syndrome coronavirus efficiently infects human primary T lymphocytes and activates the extrinsic and intrinsic apoptosis pathways. *J. Infect. Dis.* 213, 904–914. doi: 10.1093/infdis/jiv380

Clasman, J. R., Báez-Santos, Y. M., Mettelman, R. C., O'Brien, A., Baker, S. C., and Mesecar, A. D. (2017). X-ray structure and enzymatic activity profile of a core papain-like protease of MERS coronavirus with utility for structure-based drug design. *Sci. Rep.* 7:40292. doi: 10.1038/srep40292

Cockrell, A. S., Yount, B. L., Scobey, T., Jensen, K., Douglas, M., Beall, A., et al. (2016). A mouse model for MERS coronavirus-induced acute respiratory distress syndrome. *Nat. Microbiol.* 2:16226. doi: 10.1038/nmicrobiol.2016.226

Coleman, C. M., Sisk, J. M., Halasz, G., Zhong, J., Beck, S. E., Matthews, K. J., et al. (2017). CD8+ T Cells and macrophages regulate pathogenesis in a mouse model of middle east respiratory syndrome. *J. Virol.* 91:e01825-16.

Cong, Y., Hart, B. J., Gross, R., Zhou, H., Frieman, M., Bollinger, L., et al. (2018). MERS-CoV pathogenesis and antiviral efficacy of licensed drugs in human monocyte-derived antigen-presenting cells. *PLoS One* 13:e0194868. doi: 10.1371/journal.pone.0194868

Conzade, R., Grant, R., Malik, M. R., Elkholy, A., Elhakim, M., Samhouri, D., et al. (2018). Reported direct and indirect contact with dromedary camels among laboratory-confirmed MERS-CoV cases. *Viruses* 10:E425. doi: 10.3390/v10080425

Corman, V. M., Albarrak, A. M., Omrani, A. S., Albarrak, M. M., Farah, M. E., Almasri, M., et al. (2016). Viral shedding and antibody response in 37 patients with Middle East Respiratory Syndrome Coronavirus infection. *Clin. Infect. Dis.* 62, 477–483. doi: 10.1093/cid/civ951

Corman, V. M., Eckerle, I., Bleicker, T., Zaki, A., Landt, O., Eschbach-Bludau, M., et al. (2012a). Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. *Euro. Surveill.* 17:20285. doi: 10.2807/ese.17.39.20285-en

Corman, V. M., Muller, M. A., Costabel, U., Timm, J., Binger, T., and Meyer, B. (2012b). Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. *Euro. Surveill.* 17:1.

de Groot, R. J., Baker, S. C., Baric, R. S., Brown, C. S., Drosten, C., Enjuanes, L., et al. (2013). Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. *J. Virol.* 87, 7790–7792. doi: 10.1128/JVI.01244-13

de Sousa, R., Reusken, C., and Koopmans, M. (2014). MERS corona virus: Data gaps for laboratory preparedness. *J. Clin. Virol.* 59, 4–11. doi: 10.1016/j.jcv.2013.10.030

de Wit, E., Prescott, J., Baseler, L., Bushmaker, T., Thomas, T., Lackemeyer, M. G., et al. (2013a). The Middle East respiratory syndrome coronavirus (MERS-CoV) does not replicate in Syrian hamsters. *PLoS One* 8:e69127. doi: 10.1371/journal.pone.0069127

de Wit, E., Rasmussen, A. L., Falzarano, D., Bushmaker, T., Feldmann, F., and Brining, D. L. (2013b). Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16598–16603. doi: 10.1073/pnas.1310744110

Denayer, T., Stöhr, T., and Roy, M. V. (2014). Animal models in translational medicine: Validation and prediction. *New Horiz. Transl. Med.* 2, 5–11. doi: 10.1016/j.nhtm.2014.08.001

Doremalen, N. V., and Munster, V. J. (2015). Animal models of middle east respiratory syndrome coronavirus infection. *Antiviral Res.* 22, 28–38. doi: 10.1016/j.antiviral.2015.07.005

Drosten, C. (2013). Is MERS another SARS? *Lancet Infect. Dis.* 13, 727–728.

Drosten, C., Günther, S., Preiser, W., van der Werf, S., Brodt, H. R., Becker, S., et al. (2003). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* 348, 1967–1976. doi: 10.1056/NEJMoa030747

Drosten, C., Meyer, B., Müller, M. A., Corman, V. M., Al-Masri, M., Hossain, R., et al. (2014). Transmission of MERS-coronavirus in household contacts. *N Engl J Med.* 371, 828–835. doi: 10.1056/NEJMoa1405858

Drosten, C., Seilmaier, M., and Corman, V. (2013). Clinical features and virological analysis of a case of Middle East respiratory syndrome corona virus infection. *Lancet Infect. Dis.* 13, 745–751. doi: 10.1016/S1473-3099(13)70154-3

Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B. J., and Jiang, S. (2009). The spike protein of SARS-CoV–a target for vaccine and therapeutic development. *Nat. Rev. Microbiol.* 7, 226–236. doi: 10.1038/nrmicro2090

Du, L., Zhao, G., Kou, Z., Ma, C., Sun, S., and Poon, V. K. (2013). Identification of a receptor-binding domain in the S protein of the novel human corona virus Middle East respiratory syndrome corona virus as an essential target for vaccine development. *J. Virol.* 87, 9939–9942. doi: 10.1128/JVI.01048-13

Durai, P., Batool, M., Shah, M., and Choi, S. (2015). Middle East respiratory syndrome corona virus: transmission, virology and therapeutic targeting to aid in outbreak control. *Exp. Mol. Med.* 47:e181. doi: 10.1038/emm.2015.76

Eckerle, I., Corman, V. M., Muller, M. A., Lenk, M., Ulrich, R. G., and Drosten, C. (2014). Replicative capacity of MERS coronavirus in livestock cell lines. *Emerg. Infect. Dis.* 20, 276–279. doi: 10.3201/eid2002.131182

Falzarano, D., de Wit, E., Feldmann, F., Rasmussen, A. L., Okumura, A., and Peng, X. (2014). Infection with MERS-CoV causes lethal pneumonia in the common marmoset. *PLoS Pathog.* 10:e1004250. doi: 10.1371/journal.ppat.1004250

Faure, E., Poissy, J., Goffard, A., Fournier, C., Kipnis, E., Titecat, M., et al. (2014). Distinct immune response in two MERS-CoV-infected patients: can we go from bench to bedside? *PLoS One* 9:e88716. doi: 10.1371/journal.pone.0088716

Firbas, C., Jilma, B., Tauber, E., Buerger, V., Jelovcan, S., and Lingnau, K. (2006). Immunogenicity and safety of a novel therapeutic hepatitis C virus (HCV) peptide vaccine: a randomized, placebo controlled trial for dose optimization in 128 healthy subjects. *Vaccine* 24, 4343–4353. doi: 10.1016/j.vaccine.2006.03.009

Forni, D., Giulia, F., Rachele, C., Gioia, D. L., Pozzoli, P., and Al-Daghri, N. (2015). The heptad repeat region is a major selection target in MERS-CoV and related corona viruses. *Sci. Rep.* 25:14480. doi: 10.1038/srep14480

Fouchier, R. A., Schneeberger, P. M., Rozendaal, F. W., Broekman, J. M., Kemink, S. A., Munster, V., et al. (2004). Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1356–1361. doi: 10.1073/pnas.0308352100

Fung, T. S., and Liu, D. X. (2014). Corona virus infection, ER stresses apoptosis and innate immunity. *Front. Microbiol.* 5:296. doi: 10.3389/fmicb.2014.00296

Gao, H., Yao, H., Yang, S., and Li, L. (2016). From SARS to MERS: evidence and speculation. *Front Med.* 10, 377–382. doi: 10.1007/s11684-016-0466-7

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J., and Snijder, E. J. (2006). Nidovirales: evolving the largest RNA virus genome. *Virus Res.* 117, 17–37. doi: 10.1016/j.virusres.2006.01.017

Guery, B., Poissy, J., and el Mansouf, L. (2013). Clinical features and viral diagnosis of two cases of infection with Middle East Respiratory Syndrome corona virus: a report of nosocomial transmission. *Lancet* 381, 2265–2272. doi: 10.1016/S0140-6736(13)60982-4

Haagmans, B. L., van den Branda, J. M. A., Provaciaa, L. B., Raja, V. S., Stittelaarb, K. J., Getua, S., et al. (2015). Asymptomatic middle east respiratory syndrome coronavirus infection in rabbits. *J. Virol.* 89, 6131–6135. doi: 10.1128/JVI.00661-15

Hamre, D., and Procknow, J. J. (1966). A new virus isolated from the human respiratory tract. *Proc Soc Exp Biol Med.* 121, 190–193. doi: 10.3181/00379727-121-30734

Harcourt, J. L., Rudoler, N., Tamin, A., Leshemm, E., Rasis, M., Giladi, M., et al. (2018). The prevalence of Middle East respiratory syndrome coronavirus (MERS-CoV) antibodies in dromedary camels in Israel. *Zoonoses Public Health* doi: 10.1111/zph.12482 [Epub ahead of print].

Harrigan, J. A., Jacq, X., Martin, N. M., and Jackson, S. P. (2018). Deubiquitylating enzymes and drug discovery: emerging opportunities. *Nat. Rev. Drug Discov.* 17, 57–78. doi: 10.1038/nrd.2017.152

Health Protection Agency. (2013). Evidence of person-to-person transmission within a family cluster of novel corona virus infections, United Kingdom, February. *Euro Surveill.* 18:20427.

Henderson, D. A., and Moss, B. (1999). Recombinant Vaccinia Virus Vaccines. *Vaccines* 6, 43–46.

Hou, L., and Zhao, H. (2013). A review of post-GWAS prioritization approaches. *Front. Genet.* 4:280. doi: 10.3389/fgene.2013.00280

Houser, K. V., Broadbent, A. J., Gretebeck, L., Vogel, L., Lamirande, E. W., Sutton, T., et al. (2017). Enhanced inflammation in New Zealand white rabbits when MERS-CoV reinfection occurs in the absence of neutralizing antibody. *PLoS Pathog.* 13:e1006565. doi: 10.1371/journal.ppat.1006565

Houser, K. V., Gretebeck, L., Ying, T., Wang, Y., Vogel, L., Lamirande, E. W., et al. (2016). Prophylaxis with a Middle East respiratory syndrome coronavirus (MERS-CoV)-specific human monoclonal antibody protects rabbits from MERS-CoV infection. *J. Infect. Dis.* 213, 1557–1561. doi: 10.1093/infdis/jiw080

Huang, P., Wang, H., Cao, Z., Jin, H., Chi, H., Zhao, J., et al. (2018). A rapid and specific assay for the detection of MERS-CoV. *Front. Microbiol.* 9:1101. doi: 10.3389/fmicb.2018.01101

Hui, D. S., Azhar, E. I., Kim, Y. J., Memish, Z. A., Oh, M. D., and Zumla, A. (2018). Middle East respiratory syndrome corona virus: risk factors and determinants of primary, household, and nosocomial transmission. *Lancet Infect. Dis.* 18, e217–e227. doi: 10.1016/S1473-3099(18)30127-0

Jiaming, L., Yanfeng, Y., Yao, D., Yawei, H., Linlin, B., Baoying, H., et al. (2017). The recombinant N-terminal domain of spike proteins is a potential vaccine against Middle East respiratory syndrome coronavirus (MERS-CoV) infection. *Vaccine* 35, 10–18. doi: 10.1016/j.vaccine.2016.11.064

Kandeil, A., Gomaa, M., Shehata, M., El-Taweel, A., Kayed, A., Abiadh, A., et al. (2019). Middle East respiratory syndrome coronavirus infection in non-camelid domestic mammals. *Emerg. Microbes Infect.* 8, 103–108. doi: 10.1080/22221751.2018.1560235

Kim, T. (2016). *The Korean Ministry of Health & Welfare. Press release: MERS statistics. Sejong: Ministry of Health & Welfare*. Available at: http://english.mw.go.kr/front_eng/sg/ssg0111vw.jsp?PAR_MENU_ID1001&MENU_ID100111&page1&CONT_SEQ324470 [accessed July 15, 2016].

Kuiken, T., Fouchier, R. A., Schutten, M., Rimmelzwaan, G. F., van Amerongen, G., van Riel, D., et al. (2003). Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* 362, 263–270. doi: 10.1016/S0140-6736(03)13967-0

Lau, S. K., Lau, C. C., Chan, K. H., Li, C. P., Chen, H., Jin, D. Y., et al. (2013). Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East respiratory syndrome coronavirus: implications for pathogenesis and treatment. *J. Gen. Virol.* 94(Pt 12), 2679–2690. doi: 10.1099/vir.0.055533-0

Lau, S. K. P., Wong, A. C. P., Lau, T. C. K., and Woo, P. C. Y. (2017). Molecular Evolution of MERS Corona virus: dromedaries as a Recent Intermediate Host or Long-Time Animal Reservoir? *Int. J. Mol. Sci.* 18:E2138. doi: 10.3390/ijms18102138

Law, G. L., Korth, M. J., Benecke, A. G., and Katze, M. G. (2013). Systems virology: host-directed approaches to viral pathogenesis and drug targeting. *Nat. Rev. Microbiol.* 11, 455–466. doi: 10.1038/nrmicro3036

Lorenzen, E., Follmann, F., Jungersen, G., and Agerholm, J. S. (2015). A review of the human vs. porcine female genital tract and associated immune system in the perspective of using minipigs as a model of human genital Chlamydia infection. *Vet. Res.* 46:116. doi: 10.1186/s13567-015-0241-9

Lui, P. L., Wong, L. Y. R., Fung, C. L., Siu, K. L., Yeung, M. L., Yuen, K. S., et al. (2016). Middle East respiratory syndrome coronavirus M protein suppresses type I interferon expression through the inhibition of TBK1-dependent phosphorylation of IRF3. *Emerg. Microbes Infect.* 5:e39. doi: 10.1038/emi.2016.33

Ma, C., Wang, L., Tao, X., Zhang, N., Yang, Y., Tseng, C. K., et al. (2014). Searching for an ideal vaccine candidate among different MERS coronavirus receptor-binding fragments—The importance of immunofocusing in subunit vaccine design. *Vaccine* 32, 6170–6176. doi: 10.1016/j.vaccine.2014.08.086

Marra, M. A., Jones, S. J., Astell, C. R., Holt, R. A., Brooks-Wilson, A., Butterfield, Y. S., et al. (2003). The genome sequence of the SARS-associated coronavirus. *Science* 300, 1399–1404. doi: 10.1126/science.1085953

Masters, P. (2006). The molecular biology of corona viruses. *Adv. Virus Res.* 66, 193–292. doi: 10.1016/S0065-3527(06)66005-3

McDermott, J. E., Shankaran, H., Eisfeld, A. J., Belisle, S. E., Neuman, G., and Li, C. (2011). Conserved host response to highly pathogenic avian-influenza virus infection in human cell culture, mouse and macaque model systems. *BMC Syst. Biol.* 5:190. doi: 10.1186/1752-0509-5-190

Memish, Z., Mishra, N., and Olival, K. (2013a). Middle East respiratory syndrome corona virus in bats, Saudi Arabia. *Emerg. Infect. Dis.* 19, 1819–1823. doi: 10.3201/eid1911.131172

Memish, Z., Zumla, A., and Assiri, A. (2013b). Middle East respiratory syndrome corona virus infections in health care workers. *N. Engl. J. Med.* 2013, 884–886. doi: 10.1016/j.ajic.2017.08.010

Memish, Z. A., Assiri, A., Alhakeem, R., Yezli, S., Almasri, M., Zumla, A., et al. (2014a). Middle East Respiratory Syndrome Corona virus, MERS-CoV. Conclusions from the 2nd scientific advisory board meeting of the WHO collaborating center for mass gathering medicine, Riyadh. *Int. J. Infect. Dis.* 24, 51–53. doi: 10.1016/j.ijid.2014.05.001

Memish, Z. A., Assiri, A. M., and Al-Tawfiq, J. A. (2014b). Middle East respiratory syndrome corona virus (MERS-CoV) viral shedding in the respiratory tract: an observational analysis with infection control implications. *Int. J. Infect. Dis.* 29, 307–308. doi: 10.1016/j.ijid.2014.10.002

Menachery, V. D., Eisfeld, A. J., Schäfer, A., Josset, L., Sims, A. C., Proll, S., et al. (2014). Pathogenic influenza viruses and coronaviruses utilize similar and contrasting approaches to control interferon-stimulated gene responses. *mBio* 5:e01174-14. doi: 10.1128/mBio.01174-14 e01174-14,PMID:24846384

Menachery, V. D., Mitchell, H. D., Cockrell, A. S., Gralinski, L. E., Yount, B. L. Jr., Graham, R. L., et al. (2017). MERS-CoV Accessory ORFs play key role for infection and pathogenesis. *mBio* 8:e00665-17. doi: 10.1128/mBio.00665-17e00665-17

Menachery, V. D., Schäfer, A., Burnum-Johnson, K. E., Mitchell, H. D., Eisfeld, A. J., Walters, K. B., et al. (2018). MERS-CoV and H5N1 influenza virus antagonize antigen presentation by altering the epigenetic landscape. *Proc. Natl. Acad. Sci. U.S.A.* 115, E1012–E1021. doi: 10.1073/pnas.1706928115

Meyer, B., Drosten, C., and Muller, M. A. (2014). Serological assays for emerging corona viruses: challenges and pitfalls. *Virus Res.* 194, 175–183. doi: 10.1016/j.virusres.2014.03.018

Meyer, B., García-Bocanegra, I., Wernery, U., Wernery, R., Sieberg, A., Müller, M. A., et al. (2015). Serologic assessment of possibility for MERS-CoV infection in equids. *Emerg. Infect. Dis.* 21, 181–182. doi: 10.3201/eid2101.141342

Mielech, A. M., Kilianski, A., Baez-Santos, Y. M., Mesecar, A. D., and Baker, S. C. (2014). MERS-CoV papain-like protease has deISGylating and deubiquitinating activities. *Virology* 45, 64–70. doi: 10.1016/j.virol.2013.11.040

Mitchell, H. D., Eisfeld, A. J., Sims, A. C., McDermott, J. E., Matzke, M. M., and Webb-Robertson, B. J. (2013). A network integration approach to predict conserved regulators related to pathogenicity of influenza and SARS-CoV respiratory viruses. *PLoS One* 8:e69374. doi: 10.1371/journal.pone.0069374

Muller, M. A., Raj, V. S., Muth, D., Meyer, B., Kallies, S., Smits, S. L., et al. (2012). Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. *mBio* 3:e00515-12. doi: 10.1128/mBio.00515-12

Mustafa, S., Balkhy, H., and Gabere, M. N. (2017). Current treatment options and the role of peptides as potential therapeutic components for Middle East Respiratory Syndrome (MERS): a review. *J. Infect. Public Health* 11, 9–17. doi: 10.1016/j.jiph.2017.08.009

Mustafa, S., Balkhy, H., and Gabere, M. N. (2018). Current treatment options and the role of peptides as potential therapeutic components for middle east respiratory syndrome (MERS): a review. *J. Infect. Public Health* 11, 9–17. doi: 10.1016/j.jiph.2017.08.009

Muth, D., Corman, V. M., Meyer, B., Assiri, A., Al-Masri, M., and Farah, M. (2015). Infectious Middle East respiratory Syndrome Corona virus excretion and serotype variability based on live virus isolates from patients in Saudi Arabia. *J. Clin. Microbiol.* 53, 2951–2955. doi: 10.1128/JCM.01368-15

Muthumani, K., Falzarano, D., Reuschel, E. L., Tingey, C., Flingai, S., Villarreal, D. O., et al. (2015). A synthetic consensus anti-spike protein DNA vaccine

induces protective immunity against Middle East respiratory syndrome coronavirus in nonhuman primates. *Sci. Transl. Med.* 7:301ra132. doi: 10.1126/scitranslmed.aac7462

Ng, D. L., Al Hosani, F., Keating, M. K., Gerber, S. I., Jones, T. L., Metcalfe, M. G., et al. (2016). Clinicopathologic, Immunohistochemical, and Ultra structural Findings of a Fatal Case of Middle East Respiratory Syndrome Corona virus Infection in the United Arab Emirates, April 2014. *Am. J. Pathol.* 186, 652–658. doi: 10.1016/j.ajpath.2015.10.024

Oh, M. D., Park, W. B., Park, S. W., Choe, P. G., Bang, J. H., Song, K. H., et al. (2018). Middle East respiratory syndrome: what we learned from the 2015 outbreak in the Republic of Korea. *Korean J. Intern. Med.* 33, 233–246. doi: 10.3904/kjim.2018.031

Ohnuma, K., Haagmans, B. L., Hatano, R., Raj, V. S., Mou, H., Iwata, S., et al. (2013). Inhibition of Middle East respiratory syndrome coronavirus infection by anti-CD26 monoclonal antibody. *J. Virol.* 87, 13892–13899. doi: 10.1128/JVI.02448-13

Okba, N. M., Raj, V. S., and Haagmans, B. L. (2017). Middle East respiratory syndrome coronavirus vaccines: current status and novel approaches. *Curr. Opin. Virol.* 23, 49–58. doi: 10.1016/j.coviro.2017.03.007

Omrani, A., Matin, M., Haddad, Q., Al-Nakhli, D., Memish, Z., and Albarrak, A. (2013). A family cluster of Middle East Respiratory Syndrome Corona virus infections related to a likely unrecognized asymptomatic or mild case. *Int. J. Infect. Dis.* 17:72. doi: 10.1016/j.ijid.2013.07.001

Pallesen, J., Wang, N., Corbett, K. S., Wrapp, D., Kirchdoerfer, R. N., Turner, H. L., et al. (2017). Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. *Proc. Natl. Acad. Sci. U.S.A.* 114, E7348–E7357. doi: 10.1073/pnas.1707304114

Park, J. E., Li, K., Barlan, A., Fehr, A. R., Perlman, S., McCray, P. B., et al. (2016). Proteolytic processing of middle east respiratory syndrome coronavirus spikes expands virus tropism. *Proc. Natl. Acad. Sci. U.S.A.* 113, 12262–12267. doi: 10.1073/pnas.1608147113

Park, W. B., Perera, R. A., Choe, P. G., Lau, E. H., Choi, S. J., Chun, J. Y., et al. (2015). Kinetics of serologic responses to MERS Coronavirus Infection in Humans. South Korea. *Emerg. Infect. Dis.* 21, 2186–2189. doi: 10.3201/eid2112.151421

Pascal, K. E., Colemanb, C. M., Mujicaa, A. O., Kamata, V., Badithea, A., Fairhursta, J., et al. (2015). Pre- and postexposure efficacy of fully human antibodies againt Spike protein in a novel humanized mouse model of MERS-CoV infection. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8738–8743. doi: 10.1073/pnas.1510830112

Peck, K. M., Cockrell, A. S., Yount, B. L., Scobey, T., Baric, R. S., and Heise, M. T. (2015). Glycosylation of mouse DPP4 plays a role in inhibiting Middle East Respiratory Syndrome Coronavirus infection. *J. Virol.* 89, 4696–4699. doi: 10.1128/JVI.03445-14

Perera, R. A., Wang, P., Gomaa, M. R., El-Shesheny, R., Kandeil, A., Bagato, O., et al. (2013). Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt. *Euro. Surveill.* 18:20574.

Perlman, S. (2015). Research Driven by Curiosity: the Journey from basic molecular biology and virology to studies of human pathogenic corona viruses. *PLoS Pathog.* 11:e1005023. doi: 10.1371/journal.ppat.1005023

Perlman, S., and Netland, J. (2009). Coronaviruses post-SARS: update on replication and pathogenesis. *Nat. Rev. Microbiol.* 7, 439–450. doi: 10.1038/nrmicro2147

Puzelli, S., Azzi, A., Santini, M. G., Di, M. A., Facchini, M., and Castrucci, M. R. (2013). Investigation of an imported case of Middle East Respiratory Syndrome Corona virus (MERS-CoV) infection in Florence, Italy, May to June 2013. *Euro. Surveill.* 18:1.

Rabaan, A. A., Bazzi, A. M., Al-Ahmed, S. H., and Al-Tawfiq, J. A. (2017). Molecular aspects of MERS-CoV. *Front. Med.* 11, 365–377. doi: 10.1007/s11684-017-0521-z

Rabouw, H. H., Langereis, M. A., Knaap, R. C. M., Dalebout, T. J., Canton, J., Sola, I., et al. (2016). Middle east respiratory coronavirus accessory protein 4a inhibits PKR-mediated antiviral stress responses. *PLoS Pathog.* 12:e1005982. doi: 10.1371/journal.ppat.1005982

Raj, V. S., Mou, H., Smits, S. L., Dekkers, D. H., Müller, M. A., Dijkman, R., et al. (2013). Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* 495, 251–254. doi: 10.1038/nature12005

Raj, V. S., Osterhaus, A. D., and Fouchier, R. A. (2014). MERS: emergence of a novel human coronavirus. *Curr. Opin. Virol.* 5, 58–62. doi: 10.1016/j.coviro.2014.01.010

Rockx, B., Donaldson, E., Frieman, M., Sheahan, T., Corti, D., Lanzavecchia, A., et al. (2010). Escape from human monoclonal antibody neutralization affects in vitro and in vivo fitness of severe acute respiratory syndrome corona virus. *J. Infect. Dis.* 201, 946–955. doi: 10.1086/651022

Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., et al. (2003). Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300, 1394–1399. doi: 10.1126/science.1085952

Selinger, C., Tisoncik-Go, J., Menachery, V. D., Agnihothram, S., Law, G. L., Chang, J., et al. (2014). Cytokine systems approach demonstrates differences in innate and pro-inflammatory host responses between genetically distinct MERS-CoV isolates. *BMC Genomics* 15:1161. doi: 10.1186/1471-2164-15-1161

Shehata, M. M., Gomaa, M. R., Ali, M. A., and Kayali, G. (2016). Middle East respiratory syndrome coronavirus: a comprehensive review. *Front Med.* 10, 120–136. doi: 10.1007/s11684-016-0430-6

Shi, J., Zhang, J., Li, S., Sun, J., Teng, Y., and Wu, M. (2015). Epitope-based vaccine target screening against highly pathogenic MERS-CoV: An *in silico* approach applied to emerging infectious diseases. *PLoS One* 10:e0144475. doi: 10.1371/journal.pone.0144475

Shirato, K., Kawase, M., and Matsuyama, S. (2013). Middle East Respiratory Syndrome Coronavirus Infection Mediated by the Transmembrane Serine Protease TMPRSS2. *J. Virol.* 87, 12552–12561. doi: 10.1128/JVI.01890-13

Shirato, K., Yano, T., Senba, S., Akachi, S., Kobayashi, T., Nishinaka, T., et al. (2014). Detection of Middle East respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Virol J.* 11, 139. doi: 10.1186/1743-422X-11-139

Smith, E. C., Blanc, H., Surdel, M. C., Vignuzzi, M., and Denison, M. R. (2013). Corona viruses lacking exoribonuclease activity are susceptible to lethal mutagenesis: evidence for proofreading and potential therapeutics. *PLoS Pathog.* 9:e1003565. doi: 10.1371/journal.ppat.1003565

Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., et al. (2003). Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. *J. Mol. Biol.* 331, 991–1004. doi: 10.1016/S0022-2836(03)00865-9

Song, D., Ha, G., Serhan, W., Eltahir, Y., Yusof, M., and Hashem, F. (2015). Development and validation of a rapid immunochromatographic assay for detection of Middle East respiratory syndrome coronavirus antigen in dromedary camels. *J. Clin. Microbiol.* 53, 1178–1182. doi: 10.1128/JCM.03096-14

Tang, J., Zhang, N., Tao, X., Zhao, G., Guo, Y., Tseng, C. T., et al. (2015). Optimization of antigen dose for a receptor-binding domain-based subunit vaccine against MERS coronavirus. *Hum. Vaccin. Immunother.* 11, 1244–1250. doi: 10.1080/21645515.2015.1021527

Tang, X. C. (2014). Identification of human neutralizing antibodies against MERS-CoV and their role in virus adaptive evolution. *Proc. Natl. Acad. Sci. U.S.A.* 2014, E2018–E2026. doi: 10.1073/pnas.1402074111

Tao, X., Garron, T., Agrawal, A. S., Algaissi, A., Peng, B. H., Wakamiya, M., et al. (2015). Characterization and demonstration of the value of a lethal mouse model of middle east respiratory syndrome coronavirus infection and disease. *J. Virol.* 90, 57–67. doi: 10.1128/JVI.02009-15

Terry, F. E., Moise, L., Martin, R. F., Torres, M., Pilotte, N., and Williams, S. A. (2015). Time for T? Immunoinformatics addresses vaccine design for neglected tropical and emerging infectious diseases. *Expert Rev. Vaccin.* 14, 21–35. doi: 10.1586/14760584.2015.955478

van Boheemen, S., de Graaf, M., Lauber, C., Bestebroer, T. M., Raj, V. S., Zaki, A. M., et al. (2012). Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *mBio* 3:e00473-12. doi: 10.1128/mBio.00473-12

van den Brand, J. M., Smits, S. L., and Haagmans, B. L. (2015). Pathogenesis of Middle East respiratory syndrome coronavirus. *J. Pathol.* 235, 175–184. doi: 10.1002/path.4458

van der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-Oost, W., Berkhout, R. J., Wolthers, K. C., et al. (2004). Identification of a new human coronavirus. *Nat. Med.* 10, 368–373. doi: 10.1038/nm1024

van Doremalen, N., Miazgowicz, K. L., Milne-Price, S., Bushmaker, T., Robertson, S., Scott, D., et al. (2014). Host species restriction of Middle East respiratory syndrome coronavirus through its receptor, dipeptidyl peptidase 4. *J. Virol.* 88, 9220–9232. doi: 10.1128/JVI.00676-14

Vergara-Alert, J., Vidal, E., Bensaid, A., and Segalés, J. (2017). Searching for animal models and potential target species for emerging pathogens: experience gained from Middle East respiratory syndrome (MERS) coronavirus. *One Health* 3, 34–40. doi: 10.1016/j.onehlt.2017.03.001

Wang, C., Hua, C., Xia, S., Li, W., Lu, L., and Jiang, S. (2019). Combining a fusion inhibitory peptide targeting the MERS-CoV S2 protein HR1 domain and a neutralizing antibody specific for the S1 protein receptor-binding domain (RBD) showed potent synergism against pseudotyped MERS-CoV with or without mutations in RBD. *Viruses* 11:E31. doi: 10.3390/v11010031

Wang, L., Shi, W., Joyce, M. G., Modjarrad, K., Zhang, Y., Leung, K., et al. (2015). Evaluation of candidate vaccine approaches for MERS-CoV. *Nat. Commun.* 6:7712. doi: 10.1038/ncomms8712

Wang, N., Shi, X., Jiang, L., Zhang, S., Wang, D., Tong, P., et al. (2013). Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. *Cell Res.* 23, 986–993. doi: 10.1038/cr.2013.92

Wang, Q. (2014). Bat origins of MERS-CoV supported by bat corona virus HKU4 usage of human receptor CD26. *Cell Host Microbe* 16, 328–337. doi: 10.1016/j.chom.2014.08.009

Wang, Q., Qi, J., Yuan, Y., Xuan, Y., Han, P., Wan, Y., et al. (2014). Bat origins of MERS-CoV supported by bat coronavirus HKU4 usage of human receptor CD26. *Cell Host Microbe* 16, 328–337. doi: 10.1016/j.chom.2014.08.009

Watson, J. T., Hall, A. J., Erdman, D. D., Swerdlow, D. L., and Gerber, S. I. (2014). Unraveling the mysteries of Middle East respiratory syndrome corona virus. *Emerg. Infect. Dis.* 20, 1054–1056. doi: 10.3201/eid2006.140322

Weiss, S. R., and Navas-Martin, S. (2005). Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol. Mol. Biol. Rev.* 69, 635–664. doi: 10.1128/MMBR.69.4.635-664.2005

Wirblich, C., Coleman, C. M., Kurup, D., Abraham, T. S., Bernbaum, J. G., Jahrling, P. B., et al. (2017). One-Health: a Safe, efficient, dual-use vaccine for humans and animals against Middle East Respiratory Syndrome Corona virus and Rabies Virus. *J. Virol* 91:e02040-16.e02040-16 doi: 10.1128/JVI.02040-16

Woo, P. C., Lau, S. K., Chu, C. M., Chan, K. H., Tsoi, H. W., Huang, Y., et al. (2005). Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J. Virol.* 79, 884–895. doi: 10.1128/JVI.79.2.884-895.2005

World Health Organization (2012). *Novel Corona virus Infection – Update 2012*. Available at: http://www.who.int/csr/don/2012_11_30/en/ [accessed February 9, 2018].

World Health Organization (WHO) (2013a). *Middle East Respiratory Syndrome Corona Virus Joint Kingdom of Saudi Arabia/ WHO mission*. Available at: http://www.who.int/csr/disease/coronavirus_infections/MERSCov_WHO_KSA_Mission_Jun13_.pdf [accessed January 30, 2018].

World Health Organization (2013b). *Middle East Respiratory Syndrome Coronavirus (MERS-CoV) – update 2013*. Available at: http://www.who.int/csr/don/2013_12_31/en/ [accessed February 9, 2018].

World Health Organization (2014). *Middle East Respiratory Syndrome Coronavirus (MERS-CoV) – Saudi Arabia*. Available at: http://www.who.int/csr/don/26-december-2014-mers/en/ [accessed February 9, 2018].

World Health Organization (WHO) (2015a). *Middle East Respiratory Syndrome Coronavirus (MERS-CoV) – Saudi Arabia*. Available at: http://www.who.int/csr/don/4-december-2015-mers-saudi-arabia/en/ [accessed February 9, 2018].

World Health Organization (WHO) (2015b). *Summary and Risk Assessment of Current Situation in Republic of Korea and China*. Available at: http://www.who.int/csr/disease/coronavirus_infections/risk-assessment-19june2015/en/

World Health Organization (WHO) (2016a). *Middle East Respiratory Syndrome Coronavirus (MERS-CoV) – Saudi Arabia*. Available at: http://www.who.int/csr/don/19-december-2016-2-mers-saudi-arabia/en/ [accessed February 9, 2018].

World Health Organization (WHO) (2016b). *Middle East Respiratory Syndrome Corona Virus (MERS-CoV) WHO MERS-CoV Global Summary and risk assessment 5 December 2016 WHO/MERS/RA/16.1*. Available at: https://www.who.int/emergencies/mers-cov/en/

World health Organization (WHO) (2017a). *Middle East Respiratory Syndrome Corona Virus (MERS-CoV) – Oman*. Available at: http://www.who.int/csr/don/10-november-2017-mers-oman/en/ [accessed December 1, 2017].

World Health Organization (WHO) (2017b). *Middle East Respiratory Syndrome Coronavirus (MERS-CoV) – United Arab Emirates*. Available at: http://www.who.int/csr/don/22-december-2017-mers-cov-united-arab-emirates/en/ [accessed February 9, 2018].

World Health Organization (WHO) (2018a). *Middle East Respiratory Syndrome Coronavirus (MERS-CoV)*. Available at: https://www.who.int/emergencies/mers-cov/en/

World Health Organization (WHO) (2018b). *Middle East respiratory syndrome coronavirus (MERS-CoV) – Saudi Arabia*. Available at: http://www.who.int/csr/don/31-december-2018-mers-saudi-arabia/en/ [accessed December 31, 2018].

Xia, S., Liu, Q., Wang, Q., Sun, Z., Su, S., and Du, L. (2014). Middle East respiratory syndrome corona virus (MERS-CoV) entry inhibitors targeting spike protein. *Virus Res.* 194, 200–210. doi: 10.1016/j.virusres.2014.10.007

Yang, Y., Zhang, L., Geng, H., Deng, Y., Huang, B., Guo, Y., et al. (2013). The structural and accessory proteins M, ORF 4a, ORF 4b, and ORF 5 of Middle East respiratory syndrome coronavirus (MERS-CoV) are potent interferon antagonists. *Protein Cell* 4, 951–961. doi: 10.1007/s13238-013-3096-8

Yao, Y., Bao, L., Deng, W., Xu, L., Li, F., and Lv, Q. (2014). An animal model of MERS produced by infection of rhesus macaques with MERS coronavirus. *J. Infect. Dis.* 209, 236–242. doi: 10.1093/infdis/jit590

Yeung, M. L., Yao, Y., Jia, L., Chan, J. F., Chan, K. H., Cheung, K. F., et al. (2016). MERS corona virus induces apoptosis in kidney and lung by upregulating Smad7 and FGF2. *Nat. Microbiol.* 1:16004. doi: 10.1038/nmicrobiol.2016.4

Ying, T., Li, W., and Dimitrov, D. S. (2016). Discovery of T-Cell infection and apoptosis by Middle East Respiratory Syndrome Coronavirus. *J. Infect. Dis.* 213, 877–879. doi: 10.1093/infdis/jiv381

Yu, P., Xu, Y., Deng, W., Bao, L., Huang, L., Xu, Y., et al. (2017). Comparative pathology of rhesus macaque and common marmoset animal models with middle east respiratory syndrome coronavirus. *PLoS ONE.* 12:e0172093. doi: 10.1371/journal.pone.0172093

Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D., and Fouchier, R. A. (2012). Isolation of a novel corona virus from a man with pneumonia in Saudi Arabia. *N. Engl. J. Med.* 367, 1814–1820. doi: 10.1056/NEJMoa1211721

Zhang, N., Tang, J., Lu, L., Jiang, S., and Du, L. (2015). Receptor-binding domain-based subunit vaccines against MERS-CoV. *Virus Res.* 202, 151–159. doi: 10.1016/j.virusres.2014.11.013

Zhang, Z., Shen, L., and Gu, X. (2016). Evolutionary Dynamics of MERS-CoV: Potential Recombination, Positive Selection and Transmission. *Sci. Rep.* 6:25049. doi: 10.1038/srep25049

Zhao, G., He, L., Sun, S., Qiu, H., Tai, W., Chen, J., et al. (2018). A novel nanobody targeting Middle East respiratory syndrome corona virus (MERS-CoV) receptor-binding domain has potent cross-neutralizing activity and protective efficacy against MERS-CoV. *J. Virol.* 92:e00837-18. e00837-18,PMID:NOPMID doi: 10.1128/JVI.00837-18

Zhao, G., Jiang, Y., Qiu, H., Gao, T., Zeng, Y., Guo, Y., et al. (2015). Multi-organ damage in human dipeptidyl peptidase 4 transgenic mice infected with middle east respiratory syndrome-coronavirus. *PLoS One* 10:e0145561. doi: 10.1371/journal.pone.0145561

Zhao, J., Li, K., Wohlford-Lenane, C., Agnihothram, S. S., Fett, C., Zhao, J., et al. (2014). Rapid generation of a mouse model for Middle East respiratory syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4970–4975. doi: 10.1073/pnas.1323279111

Zhao, J., Mangalam, A. K., Channappanavar, R., Fett, C., Meyerholz, D. K., Agnihothram, S., et al. (2016). Airway memory CD4(+) T cells mediate protective immunity against emerging respiratory coronaviruses. *Immunity* 44, 1379–1391. doi: 10.1016/j.immuni.2016.05.006

Zhou, J., Chu, H., Chan, J. F. W., and Yuen, K. Y. (2015). Middle East respiratory syndrome corona virus infection: virus-host cell interactions and implications on pathogenesis. *Virol. J.* 12:218. doi: 10.1186/s12985-015-0446-6

Zhou, J., Chu, H., Li, C., Wong, B. H., Cheng, Z. S., Poon, V. K., et al. (2014). Active replication of Middle East respiratory syndrome coronavirus and aberrant induction of inflammatory cytokines and chemokines in human macrophages:

implications for pathogenesis. *J. Infect. Dis.* 209, 1331–1342. doi: 10.1093/infdis/jit504

Ziebuhr, J., Snijder, E. J., and Gorbalenya, A. E. (2000). Virus-encoded proteinases and proteolytic processing in the *Nidovirales*. *J. Gen. Virol.* 81(Pt 4), 853–879. doi: 10.1099/0022-1317-81-4-853

Zinzula, L., and Tramontano, E. (2013). Strategies of highly pathogenic RNA viruses to block dsRNA detection by RIG-I-like receptors: hide, mask, hit. *Antiviral Res.* 100, 615–635. doi: 10.1016/j.antiviral.2013.10.002

Zumla, A., Hui, D. S., and Perlman, S. (2015). Middle East respiratory syndrome. *Lancet* 38, 995–1007. doi: 10.1016/S0140-6736(15)60454-8

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

frontiers in Medicine

REVIEW
published: 28 May 2019
doi: 10.3389/fmed.2019.00109

Drugs for Influenza Treatment: Is There Significant News?

Nicola Principi[1], *Barbara Camilloni*[2], *Anna Alunno*[2], *Ilaria Polinori*[3], *Alberto Argentiero*[3] *and Susanna Esposito*[3]*

[1] Università degli Studi di Milan, Milan, Italy, [2] Department of Medicine, Università degli Studi di Perugia, Perugia, Italy, [3] Department of Surgical and Biomedical Sciences, Pediatric Clinic, Università degli Studi di Perugia, Perugia, Italy

Vaccines remain the best measure to reduce total influenza burden. However, presently available influenza vaccines have some limitations that cause a reduced efficacy compared to immunization practices with other respiratory pathogens. This paper shows the clinical roles of antiviral drugs against influenza that have been licensed in at least one country and the potential roles of compounds that are in development. Several attempts have been made to develop new agents against influenza viruses to overcome the supposed or demonstrated limitations of neuraminidase inhibitors (NAIs). Antibodies against the highly conserved stem region of the haemagglutinin molecule of influenza A viruses and drugs that target different stages of the influenza virus life cycle than NAIs in human cells have been developed and tested. Among these preparations, baloxavir marboxil (BAM), and favipiravir (FP) (i.e., polymerase inhibitors) are the only drugs that have reached the market (the first in Japan and the USA, and the second only in Japan). Other antiviral compounds and monoclonal antibodies are in advanced stage of development, but none of these new drugs and monoclonal antibodies in development have adequate characteristics to substitute for NAIs at present. However, although NAIs remain the drug of choice for influenza treatment, their overuse has to be avoided. Accurate selection of patients for whom treatment is truly needed is required.

Keywords: antiviral drugs, baloxavir marboxil, favipiravir, influenza, monoclonal antibodies, neuraminidase inhibitors

OPEN ACCESS

Edited by:
Ville Peltola,
Turku University Hospital, Finland

Reviewed by:
Elena A. Govorkova,
St. Jude Children's Research Hospital, United States
Esteban A. Hernandez-Vargas,
Frankfurt Institute for Advanced Studies, Germany

***Correspondence:**
Susanna Esposito
susanna.esposito@unimi.it

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *14 February 2019*
Accepted: *02 May 2019*
Published: *28 May 2019*

Citation:
Principi N, Camilloni B, Alunno A, Polinori I, Argentiero A and Esposito S (2019) Drugs for Influenza Treatment: Is There Significant News? Front. Med. 6:109. doi: 10.3389/fmed.2019.00109

INTRODUCTION

Vaccines represent the best way to reduce the influenza impact (1–3). However, influenza viruses vary continuously through antigenic drifts and occasional antigenic shifts that help the virus evade pre-existing immunity (4). Therefore, continuous reformulation of vaccine compositions and annual immunization are needed. In the case of viral shifts, licensed vaccines are completely ineffective, and *ad hoc* vaccine preparations are generally available only several weeks after the emergence and spread of a pandemic influenza virus (5). Finally, the immune responses induced by the influenza vaccines are suboptimal in a number of subjects, especially in younger children and the elderly, who are at risk of severe influenza, which further reduces the protection offered by influenza vaccination (6).

In addition to the intrinsic limitations of influenza vaccines, a second problem limits the vaccine-induced prevention of influenza. Universal immunization against influenza in pediatric age is recommended only in a minority of countries (2). Healthy children and adults frequently are not included in the list of patients for whom official health authorities strongly suggest influenza

immunization (7). Moreover, even when vaccines are recommended worldwide, for example, in the elderly, influenza vaccination coverage remains suboptimal (8, 9). The World Health Organization estimates that 5–10% of the global population suffers from influenza every year, 3–5 million people develop severe influenza and 290,000–650,000 people die (10); thus, developing safe and effective alternatives for prophylaxis and treatment is critical.

In this paper, the clinical roles of antiviral drugs against influenza that have been licensed in at least one country will be discussed. Additionally, the potential roles of the anti-influenza compounds in development are evaluated.

CURRENTLY LICENSED ANTI-INFLUENZA DRUGS

Traditional Anti-influenza Virus Drugs

Antiviral drugs have been developed for a long time in an attempt to overcome the abovementioned problems and reduce the influenza-related risks. For years, the adamantane derivatives rimantadine and amantadine and the neuraminidase inhibitors (NAIs) oseltamivir, zanamivir (used worldwide) and, more recently, laninamivir and peramivir (used first in Japan and subsequently in China, Japan, South Korea, and the USA) have been the only drugs licensed for influenza prevention and control. However, these drugs have differences in their pharmacokinetic characteristics, routes of administration and ages of the targeted patients (11).

Starting from the 2004–2005 influenza season, use of adamantane derivatives was no longer recommended, mainly due to the emergence of resistance in most circulating influenza viruses. However, their activity was limited to influenza A viruses, and they showed poor tolerability, which could be considered sufficient reasons *per se* to avoid prescription of these drugs (12). In practice, only NAIs have been prescribed for influenza prevention and treatment since that time period. The emergence of influenza virus strains resistant to NAIs has been reported. Resistance to oseltamivir emerged only during the 2007–2008 and 2008–2009 influenza seasons, with up to 90% of circulating strains exhibiting resistance to this NAI (13–15). Fortunately, the influenza virus strains circulating during the 2009 pandemic and in the following years rarely contained the mutations in the neuraminidase viral surface glycoprotein that conferred resistance to oseltamivir. Localized clusters of oseltamivir-resistant influenza virus have been reported (16) and resistance to NAIs is increasing (17).

However, generally, an influenza virus resistant to oseltamivir is sensitive to the other NAIs, because cross resistance among oseltamivir and other NAIs has not been observed (18, 19). Patients with influenza due to an oseltamivir-resistant virus can be successfully treated with other NAIs, such as zanamivir (15). In patients undergoing treatment, the NAI-resistant viruses are found to be NA subtype–specific and drug-specific (16, 19, 20). These clinically-derived NAI-resistant variants of influenza A viruses of N1 NA subtype most frequently carry H274Y and N294S NA amino acid substitutions. Viruses of N2 NA subtype carry E119Vand R292K NA mutations, and NAI-resistant variants of influenza B viruses harbor R152K and D198N NA mutations. Another important point is that NAI-resistant viruses can emerge either under drug-selection pressure or naturally in the course of influenza virus evolution (without drug intervention). The high prevalence of oseltamivir-resistant A/Brisbane/59/2007(H1N1)-like viruses (subclade 2B) carrying the NA H274Y–resistance marker was reported worldwide during the 2007–2009 influenza seasons (16, 19, 20). Epidemiologic studies did not show evidence of an association between the development of resistance and oseltamivir use. Thus, H274Y NA amino acid substitution occurred naturally, and viruses with this mutation acquired remarkable transmissibility and superior fitness compared to their drug-sensitive counterparts (19, 20).

Moreover, all NAIs have been found to be safe and well tolerated. In particular, oseltamivir, which is the most frequently used NAI, can be administered not only to otherwise healthy adults and subjects with severe underlying disease but also to neonates, younger infants (18) and pregnant women (21) with age- and weight-appropriate doses without significant risks of severe adverse events.

Based on the results of several clinical trials, NAIs have been considered effective for the prevention and control of influenza infection (22, 23). Most available studies in this regard were carried out with oseltamivir and zanamivir, which were marketed before the other NAIs (24–28). Moreover, all of the data collected in younger children were derived from studies in which oseltamivir was used instead of zanamivir, which was licensed for use only in school-age patients. The effectiveness of NAIs has been documented in some meta-analyses, including those by Jefferson et al. (24) and Dobson et al. (25). A global evaluation of all of the studies analyzed in these meta-analyses suggests the conclusion that NAIs can limit the severity and duration of influenza in patients of any age with uncomplicated disease. However, the efficacy is time-dependent, because it is seen mainly in subjects who receive these drugs within the first 48 h of symptom onset. Jefferson et al. (24) and Dobson (25) calculated that administration of oseltamivir in adults with uncomplicated influenza decreased the time to first alleviation of symptoms of influenza-like illness (ILI) by 16.8 h [95% confidence interval [CI], 8.4–25.1] and 17.8 h (95% CI, 27.1 to −9.3), respectively. When only laboratory-confirmed influenza cases were considered, the advantage was slightly higher, because the time to perceived benefits was decreased by 25.2 h (95% CI, 16.0–36.2) (25). Similar results were reported for zanamivir, with the difference in time to alleviation of ILI symptoms reported as 14.4 h (95% CI, 9.36–19.44). No substantial differences were noted in the results obtained in children treated with oseltamivir (24). Regarding prophylaxis, it was calculated that oseltamivir could reduce the absolute risk for laboratory-confirmed influenza among community members and nursing home residents by 3.05% [relative risk [RR] 0.45; 95% CI, 0.30–0.67] and in a household setting by 13.6% (RR 0.20; 95% CI, 0.09–0.44) (21).

However, the available data do not permit firm conclusions regarding the effect of NAIs on severe cases (26–28). Although administration of NAIs was associated with a reduction

of mortality during the recent 2009 pandemic (29), the impact on the development of serious influenza complications, hospitalization and mortality was not definitively demonstrated. The results of studies specifically planned to evaluate these problems were conflicting (30). A good example in this regard is given by studies that have measured the prevention of acute otitis media (AOM), which is a common complication of influenza in children. Jefferson et al. (24) did not find any statistically significant effect of oseltamivir on this disease (RR 0.8; 95% CI, 0.62–1.02), whereas Wang et al. (31) reported a statistically significant reduction in the AOM incidence with oseltamivir treatment in children 1–12 years of age (risk difference [RD] −0.09; 95% CI, −0.16 to −0.03). Moreover, in some cases, such as the meta-analysis by Muthuri et al. (32), NAI treatment was shown to be associated with an increased risk of severe outcomes, including pneumonia (OR 2.29; 95% CI, 1.16–4.53). Because laninamivir (33) and peramivir (34) do not seem to have substantially different efficacies compared to that of oseltamivir, these findings explain why the present possibility of treating influenza with NAIs cannot be considered completely satisfactory and the development of new drugs against influenza viruses has been deemed mandatory.

Several new approaches have been attempted to achieve influenza prevention and control. Antibodies against the highly conserved stem region of the haemagglutinin (HA) molecule of influenza A viruses and drugs that target different stages of the influenza virus life cycle than NAIs in human cells have been developed and tested (35). Among these preparations, baloxavir marboxil (BAM) and favipiravir (FP) are the only drugs that have reached the market (the first in Japan and the USA and the second only in Japan).

RECENTLY-LICENSED ANTI-INFLUENZA VIRUS DRUGS

Baloxavir Marboxil (BAM)

BAM was licensed in 2018 in Japan and the USA for the treatment of uncomplicated influenza in subjects aged ≥ 12 years with influenza clinical manifestations for ≤48 h (36). BAM is a prodrug that is given by mouth and is hydrolysed in the intestine, blood, and liver to form baloxavir acid (BXA), which is the active compound (37). The drug acts by inhibiting the cap-dependent endonuclease activity of the influenza A and B virus polymerase acidic protein (PA) to prevent the so-called cap snatching (i.e., the mechanism used by viruses to deviate the host mRNA transcription system and allow synthesis of viral RNAs) (37). In practice, BMA, in contrast to NAIs that reduce viral release from infected cells (38), inhibits viral replication. *In vitro* studies have shown that this activity is exerted without cytotoxicity even in cells infected with NAI-resistant influenza viruses. Moreover, BMA was shown to be effective not only against influenza viruses that usually infected humans but also against avian subtypes (39).

Phase 1 clinical trials were carried out in a total of 55 healthy subjects aged 20–59 years and were mainly directed to evaluate the safety, tolerability, pharmacokinetics and food effects (40). BAM was well tolerated, as no serious adverse events or deaths were reported. Moreover, treatment-emergent adverse events, such as headache, stomatitis, an increased eosinophil count, and elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), emerged in a very small number of subjects, were mild and resolved spontaneously within a few days. Only one subject had an increased lactate dehydrogenase (LDH) serum concentration that did not disappear during the follow-up period (22 days). Pharmacokinetic data confirmed that the drug could be administered as a single dose because its half-life was very long (more than 70 h). Moreover, even the lowest dose of the drug used in these trials (6 mg of BAM) was associated with high BXA serum concentrations 24 h after the single dose. Finally, food could influence drug metabolism, since BXA concentrations were lower in subjects who were fed or when measured before meal states. However, the levels remained high enough to ensure significant viral inhibition (40).

Phase 2 and 3 studies involving subjects aged 12–64 years confirmed the efficacy and safety of BAM (41). Adverse events, such as diarrhea, bronchitis, nausea, common cold symptoms (nasopharyngitis), and headache, were reported in ∼2% of patients and were not considered severe. Moreover, in otherwise healthy subjects with fever and mild to moderate uncomplicated respiratory disease for <48 h, administration of the drug could lead to a reduction of the mean time to alleviation of symptoms of ∼50–65 h compared to the 77.7–80 h required for patients who received a placebo. However, the clinical efficacy was not different from that evidenced in patients receiving oseltamivir, although BAM administration was associated with a more rapid decline in the viral load and a shorter duration of infectious virus detection (41). Similar results were obtained with a double-blind, randomized, placebo-, and oseltamivir-controlled trial carried out in patients >12 years old suffering from influenza for <48 h with an underlying disease that was considered a risk factor for influenza-related complications. In this study, a shorter time to improvement of symptoms was also evidenced in the BAM group than in the placebo group (median 73.2 h vs. 102.3 h, $p < 0.0001$), whereas the efficacy of oseltamivir was quite similar (81.0 h, $p = 0.8347$). However, when the efficacy against the different types of influenza viruses was tested, BAM and oseltamivir demonstrated similar efficacies against the A/H3N2 virus, but the new drug was superior to oseltamivir against B viruses (time to symptom improvement 74.6 h vs. 101.6 h; $p = 0.0251$) (41). Moreover, despite the presence of severe underlying diseases, adverse events remained uncommon and did not differ from those found in patients receiving placebo or oseltamivir.

BAM has at least two important advantages over other available anti-influenza virus drugs. It is administered as a single dose by mouth, and it can overcome the problem of influenza viruses resistant to NAIs (39). Oseltamivir is also given by mouth, but to be effective it needs 5 days of administration, which can reduce compliance. Peramivir is given as a single dose by the intravenous route, requires administration by a nurse or a doctor and is more expensive than BAM. Zanamivir and laninamivir are administered by inhalation and are difficult to use in younger children.

However, despite obtaining a Food and Drug Administration (FDA) license, some unsolved problems seem to indicate that the use of BAM in clinical practice as an alternative to NAIs, particularly oseltamivir, for treatment of uncomplicated influenza cases can be debated. First, BAM is more expensive than oseltamivir. The cost of treatment with BAM has been calculated to be ∼3 times higher than that of treatment with oseltamivir (42). Moreover, the epidemiological and clinical importance of resistance to BAM is not precisely defined. Before obtaining the license, almost all isolated influenza virus strains were susceptible to BAM. A study carried out in the USA showed that the frequency of genetic mutations (amino acid 38 substitutions for isoleucine in the endonuclease domain of the viral RNA polymerase PA subunit) that caused reduced susceptibility of influenza viruses to BAM was very low during the 2016/2017 and 2017/2018 seasons (43). Moreover, no mutated virus was detected in 2018/2019 before BAM was licensed (43). However, treatment was associated with a rapid and substantial emergence of resistant strains. Some of the studies used to obtain the license evidenced that mutated viruses could be identified in 2.2–9.7% of cases (41). Resistance to BAM was also evidenced in Japan after its introduction in clinical practice. A pediatric study showed that ∼20% of treated children developed mutations (44). Recently, this problem was confirmed in strains isolated from adult patients (45). Although the mutated viruses remained sensitive to NAIs and had an impaired replicative capacity *in vitro* (44), patients with mutations shed the viruses and remained symptomatic for a longer period than those without the mutations (41), suggesting a potential negative effect of resistance to BAM.

These findings seem to suggest that emergence of resistance to BAM should be strictly monitored and that the clinical effects of BAM should be more carefully evaluated. Because definitive data in this regard are lacking, the routine use of BAM for treatment of uncomplicated influenza in adults seems premature. On the other hand, development of BAM is not complete. BAM is not licensed for complicated influenza cases. Moreover, data from children, pregnant women and patients at risk are rare or completely lacking, as are data concerning influenza prophylaxis.

Favipiravir (FP)

FP is a prodrug. FP is a nucleoside analog that after oral ingestion requires intracellular phosphoribosylation to be transformed into its active form, FP ribofuranosyl-5′-triphosphate (FRTP). Similar to BAM, FRTP acts by reducing viral replication through inhibition of the RNA-dependent RNA polymerase (RdRp) of RNA viruses (46). However, its activity is not limited to influenza viruses but instead is extended to several other RNA viruses, including arenaviruses, phleboviruses, hantaviruses, flaviviruses, enteroviruses, respiratory syncytial virus, and noroviruses (47). *In vitro* studies have shown that FP is effective against all of the influenza virus subtypes, including the avian viruses and those that are poorly sensitive or insensitive to NAIs (48–50). In experimental animals, FP was found to be significantly more effective than oseltamivir in preventing influenza or reducing its severity in mice infected with lethal doses of influenza viruses, even when the treatment was started 72 h after infection (51). Moreover, when used in combination with oseltamivir, protection due to the NAI was increased and the treatment efficacy window was extended to 96 h after symptom onset (52).

The safety and tolerability of FP were found to be good, although some data regarding the potential teratogenicity of FP were collected in all animal species assessed (53). Interestingly, the exposure causing teratogenicity was quite similar to that found in humans receiving the dosage of the drug that was useful for influenza treatment. Finally, although *in vitro* studies have shown that viral mutations associated with non-viable viral phenotypes rapidly develop in influenza viruses exposed to this drug, *in vivo* use of FP is only rarely associated with influenza virus mutations (54).

Some phase 2 and 3 studies in adults have been carried out in Japan, the USA and Europe (NCT01068912, NCT02026349, NCT02008344, NCT01728753, and NCT03394209). Generally, the drug was administered by mouth two times per day for 5 days even when different dosages were used. Most studies were completed several months ago, but the results are not available with the exception of those from NCT01068912, which aimed to identify the most effective and best tolerated drug dosage. The reasons for the unavailability of the study results are unknown. However, FP is licensed for use in humans in Japan. Its use is authorized only in patients infected by a novel or re-emerging influenza virus (i.e., a pandemic virus?) that is resistant to all other available influenza drugs. To avoid irrational prescriptions, FP is distributed only upon request by the Minister of Health, Labor and Welfare of Japan. Moreover, the guidelines clearly establish that pregnant women are excluded from use of FP, and females of childbearing potential have to avoid pregnancy in the 7 days following drug use to obtain the complete elimination of the FP concentration from the plasma (55).

ANTIVIRAL PREPARATIONS IN ADVANCED STAGES OF DEVELOPMENT

Pimodivir

Pimodivir is an oral drug that inhibits the polymerase basic protein 2 (PB2) subunit of the influenza A virus polymerase complex. Consequently, it inhibits viral replication (56). *In vitro* and experimental animal studies have shown that pimodivir is effective against influenza A virus, even when strains resistant to NAIs are tested. Compared to oseltamivir, it was more effective in improving body weight and reducing the severity of lung infection. However, its activity can significantly vary according to the influenza A virus subtype, probably as a consequence of differences in the PB2 structure among various A viruses (57). Positive results were also obtained when the drug was tested in humans with uncomplicated influenza, for whom pimodivir reduced viral shedding and influenza symptoms. Generally, adverse events (mainly diarrhea) were mild and spontaneously resolved within a few days (58). These findings were confirmed by a double-blinded phase 2b study involving adults with uncomplicated influenza A in whom different pimodivir dosages were given 2 times per day for 5 days alone or in combination with oseltamivir (59). This study showed that 600 mg of the drug per dose was adequate to obtain the best results. In the

TABLE 1 | Antiviral drugs against influenza available on the market.

Antiviral drugs	Characteristics
Adamantane derivatives	
Rimantadine Amantadine	Prevention of the uncoating of the virus's protective shells, which are the envelope and capsid of influenza virus Activity limited on influenza A virus Poor tolerability Emergence of resistance No longer recommended starting from 2004 to 2005
Neuraminidase inhibitors (NAIs)	
Oseltamivir Zanamivir Laninamavir Peramivir	Reduction of viral release from the infected cells Activity on influenza A and B viruses Good tolerability Emergence of resistant strains without cross-resistance between the drugs Laninamavir and peramivir are used only in Japan, China, South Korea, and the USA
Polymerase inhibitors	
Baloxavir marboxil Favipiravir	Inhibition of viral replication Activity on influenza A and B viruses Efficacy on NAIs-resistant strains Effective on avian influenza subtypes Good safety and tolerability More expensive Problem of resistance still unknown

treated groups, both the viral load titer and the time to symptom resolution were reduced compared to those of the placebo group. Moreover, combined therapy was more effective than administration of pimodivir alone (59, 60).

However, the use of pimodivir has been associated with the emergence of mutations leading to viruses with a several fold decrease in susceptibility to the drug. In particular, emergence of PB2 substitutions or phenotypic resistance to pimodivir was evidenced in 11 of 172 patients (6.4%) (59, 60). The mutations included S324K/N/R, F325L, S337P, K376N/R, T378S, and N510K (59, 60). Although the mutated viruses were not associated with a deterioration of clinical conditions and the patients did not shed the virus after treatment, this finding deserves attention.

Monoclonal Antibodies

Studies of natural immune responses to influenza virus infection evidenced that N-linked glycosylation sites in the haemagglutinin stem region of influenza A viruses were relatively well conserved and that antibodies against these sites were effective against a large number of influenza viruses (61–64). Starting from this evidence, a number of monoclonal antibodies targeting the HA conserved regions was developed and tested for the treatment of influenza A.

Preparations identified as MHAA4549A, MEDI8852, and VIS 410 have reached the phase II stage of development. All of these preparations were found to be safe and well tolerated and were able to reduce the peak viral load, duration of viral shedding, and influenza symptom scores compared to those of the placebo group (65–67). Moreover, no mutated virus was evidenced after monoclonal antibody administration (65–67). Compared to those of oseltamivir, all of these preparations have superior pharmacokinetic properties and a longer therapeutic window. The half-life is ∼3 weeks, one dose can be sufficient to control infection and efficacy can be obtained even if administration occurs after 48 h from symptom onset (65, 68, 69). However, two clinical trials (NCT02293863 and NCT02603952) in which the efficacy of oseltamivir alone was compared to that of the combination oseltamivir/MHAA4549A or MEDI8852 in adults with influenza A infection revealed that addition of the monoclonal antibody did not add any significant clinical advantage to those offered by the old NAI. When these results are considered together with the fact that the activity is limited to influenza A viruses, this finding seems to be a relevant limitation that may preclude extensive use of monoclonal antibodies. However, a definitive conclusion in this regard can only be drawn when studies in patients with severe influenza illnesses and those at high risk, including children and pregnant women, are performed.

CONCLUSIONS

Table 1 summarizes antiviral drugs against influenza available on the market. Several attempts have been made to develop new agents against influenza viruses that are able to overcome the supposed or demonstrated limitations of NAIs. These drugs, mainly oseltamivir, are discussed in terms of the risk of emergence of resistant strains and doubts regarding their efficacy in severe influenza cases and prevention of bacterial complications or death. Some of the new drugs have been licensed or are in the advanced stages of development. However, none of them has been completely developed, and the lack of data regarding patients for whom oseltamivir is recommended is discussed. Moreover, in some cases, use of the new drugs has been associated with emergence of resistance. In practice, none of these new drugs and monoclonal antibodies that are in development has adequate characteristics to substitute for NAIs at present. However, although NAIs (especially oseltamivir due to its greater ease of administration) remain the drug of choice for influenza treatment, their overuse has to be avoided. Accurate selection of patients for whom treatment is truly needed is necessary. Finally, the limitations of antivirals can be overcome with more extensive use of influenza vaccines.

AUTHOR CONTRIBUTIONS

NP wrote the first draft of the manuscript. BC and AAl gave scientific contributions. IP and AAr performed the literature review. SE co-wrote the manuscript and supervised all activities. All authors approved the final submitted version of the manuscript.

FUNDING

This work has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 701088.

REFERENCES

1. Paules C, Subbarao K. Influenza. *Lancet*. (2017) 390:697–708. doi: 10.1016/S0140-6736(17)30129-0
2. Principi N, Senatore L, Esposito S. Protection of young children from influenza through universal vaccination. *Hum Vaccin Immunother*. (2015) 11:2350–8. doi: 10.1080/21645515.2015.1055428
3. Principi N, Esposito S, Marchisio P, Gasparini R, Crovari P. Socioeconomic impact of influenza on healthy children and their families. *Pediatr Infect Dis J*. (2003) 22(10 Suppl):S207–10. doi: 10.1097/01.inf.0000092188.48726.e4
4. Kim H, Webster RG, Webby RJ. Influenza virus: dealing with a drifting and shifting pathogen. *Viral Immunol.* (2018) 31:174–83. doi: 10.1089/vim.2017.0141
5. Chua JV, Chen WH. Bench-to-bedside review: vaccine protection strategies during pandemic flu outbreaks. *Crit Care.* (2010) 14:218. doi: 10.1186/cc8891
6. Sano K, Ainai A, Suzuki T, Hasegawa H. The road to a more effective influenza vaccine: up to date studies and future prospects. *Vaccine*. (2017) 35:5388–95. doi: 10.1016/j.vaccine.2017.08.034
7. Principi N, Camilloni B, Esposito S. Influenza immunization policies: which could be the main reasons for differences among countries? *Hum Vaccin Immunother*. (2018) 14:684–92. doi: 10.1080/21645515.2017.1405188
8. European Center for Disease prevention and Control. *Seasonal Influenza Vaccination in Europe Vaccination Recommendations and Coverage Rates in the EU Member States for Eight Influenza Seasons 2007–2008 to 2014–2015.* Available online at: https://ecdc.europa.eu/sites/portal/files/documents/seasonal-influenza-antiviral-use-2018.pdf (accessed May 15, 2019).
9. Chiu APY, Dushoff J, Yu D, He D. Patterns of influenza vaccination coverage in the United States from 2009 to 2015. *Int J Infect Dis.* (2017) 65:122–7. doi: 10.1016/j.ijid.2017.10.004
10. World Health Organization. *Influenza (Seasonal).* Available online at: https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal) (accessed January 25, 2019).
11. Ison MG. Antiviral treatments. *Clin Chest Med*. (2017) 38:139–53. doi: 10.1016/j.ccm.2016.11.008
12. Duwe S. Influenza viruses - antiviral therapy and resistance. *GMS Infect Dis.* (2017) 5:Doc04. doi: 10.3205/id000030y
13. Dharan NJ, Gubareva LV, Meyer JJ, Okomo-Adhiambo M, McClinton RC, Marshall SA, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA*. (2009) 301:1034–41. doi: 10.1001/jama.2009.294
14. Hurt AC, Ernest J, Deng YM, Iannello P, Besselaar TG, Birch C, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res.* (2009) 83:90–3. doi: 10.1016/j.antiviral.2009.03.003
15. Esposito S, Molteni CG, Colombo C, Daleno C, Daccò V, Lackenby A, et al. Oseltamivir-induced resistant pandemic A/H1N1 influenza virus in a child with cystic fibrosis and *Pseudomonas aeruginosa* infection. *J Clin Virol.* (2010) 48:62–5. doi: 10.1016/j.jcv.2010.02.019
16. Webster RG, Govorkova EA. Continuing challenges in influenza. *Ann N Y Acad Sci*. (2014) 1323:115–39. doi: 10.1111/nyas.12462
17. Hurt AC, Hardie K, Wilson NJ, Deng YM, Osbourn M, Gehrig N, et al. Community transmission of oseltamivir-resistant A(H1N1)pdm09 influenza. *N Engl J Med*. (2011) 365:2541–2. doi: 10.1056/NEJMc1111078
18. McKimm-Breschkin JL. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza Other Respir Viruses.* (2013) 7 (Suppl 1):25–36. doi: 10.1111/irv.12047
19. Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza, Bautista E, Chotpitayasunondh T, Gao Z, Harper SA, Shaw M, et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. *N Engl J Med*. (2010) 362:1708–19. doi: 10.1056/NEJMra1000449
20. Fraaij PL, Heikkinen T. Seasonal influenza: the burden of disease in children. *Vaccine*. (2011) 29:7524–8. doi: 10.1016/j.vaccine.2011.08.010
21. Reece PA. Neuraminidase inhibitor resistance in influenza viruses. *J Med Virol.* (2007) 79:1577–86. doi: 10.1002/jmv.20951
22. Esposito S, Principi N. Oseltamivir for influenza infection in children: risks and benefits. *Expert Rev Respir Med.* (2016) 10:79–87. doi: 10.1586/17476348.2016.1126182
23. Oboho IK, Reed C, Gargiullo P, Leon M, Aragon D, Meek J, et al. Benefit of early initiation of influenza antiviral treatment to pregnant women hospitalized with laboratory-confirmed influenza. *J Infect Dis.* (2016) 214:507–15. doi: 10.1093/infdis/jiw033
24. Jefferson T, Jones MA, Doshi P, Del Mar CB, Hama R, Thompson MJ, et al. Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. *Cochrane Database Syst Rev.* (2014) 4:CD008965. doi: 10.1002/14651858.CD008965.pub4
25. Dobson J, Whitley RJ, Pocock S, Monto AS. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. *Lancet*. (2015) 385:1729–37. doi: 10.1016/S0140-6736(14)62449-1
26. Parra-Rojas C, Nguyen VK, Hernandez-Mejia G, Hernandez-Vargas EA. Neuraminidase inhibitors in influenza treatment and prevention. Is it time to call it a day? *Viruses*. (2018) 10:E454. doi: 10.3390/v10090454
27. Kmietowicz Z. WHO downgrades oseltamivir on drugs list after reviewing evidence. *BMJ*. (2017) 357:j2841. doi: 10.1136/bmj.j2841
28. Doll MK, Winters N, Boikos C, Kraicer-Melamed H, Gore G, Quach C. Safety and effectiveness of neuraminidase inhibitors for influenza treatment, prophylaxis, and outbreak control: a systematic review of systematic reviews and/or meta-analyses. *J Antimicrob Chemother.* (2017) 72:2990–3007. doi: 10.1093/jac/dkx271
29. Muthuri SG, Venkatesan S, Myles PR, Leonardi-Bee J, Al Khuwaitir TS, Al Mamun A, et al. Effectiveness of neuraminidase inhibitors in reducing mortality in patients admitted to hospital with influenza A H1N1pdm09 virus infection: a meta-analysis of individual participant data. *Lancet Resp Med*. (2014) 2:395–404. doi: 10.1016/S2213-2600(14)70041-4
30. European Center for Disease Prevention and Control. *Expert Opinion on Neuraminidase Inhibitors for the Prevention and Treatment of Influenza. Review of Recent Systematic Reviews and Meta-Analyses*. Available online at: https://ecdc.europa.eu/sites/portal/files/documents/Scientific-advice-neuraminidase-inhibitors-2017.pdf (accessed January 23, 2019).
31. Wang K, Shun-Shin M, Gill P, Perera R, Harnden A. Neuraminidase inhibitors for preventing and treating influenza in children (published trials only). *Cochrane Database Syst Rev*. (2012) 4:CD002744. doi: 10.1002/14651858.CD002744.pub4
32. Muthuri SG, Myles PR, Venkatesan S, Leonardi-Bee J, Nguyen-Van-Tam JS. Impact of neuraminidase inhibitor treatment on outcomes of public health importance during the 2009-2010 influenza A(H1N1) pandemic: a systematic review and meta-analysis in hospitalized patients. *J Infect Dis.* (2013) 207:553–63. doi: 10.1093/infdis/jis726
33. Higashiguchi M, Matsumoto T, Fujii T. A meta-analysis of laninamivir octanoate for treatment and prophylaxis of influenza. *Antivir Ther.* (2018) 23:157–65. doi: 10.3851/IMP3189
34. Wester A, Shetty AK. Peramivir injection in the treatment of acute influenza: a review of the literature. *Infect Drug Resist.* (2016) 9:201–14. doi: 10.2147/IDR.S86460
35. Davidson S. Treating Influenza Infection, from now and into the future. *Front Immunol.* (2018) 9:1946. doi: 10.3389/fimmu.2018.01946
36. *Roche Announces FDA Approval of Xofluza (baloxavir marboxil) for Influenza*. Available online at: https://www.roche.com/media/releases/med-cor-2018-10-24.htm. (accessed March 27, 2019).
37. Tomassini J, Selnick H, Davies ME, Armstrong ME, Baldwin J, Bourgeois M, et al. Inhibition of cap (m7GpppXm)-dependent endonuclease of influenza virus by 4-substituted 2,4-dioxobutanoic acid compounds. *Antimicrob Agents Chemother*. (1994) 38:2827–37. doi: 10.1128/AAC.38.12.2827
38. Shtyrya YA, Mochalova LV, Bovin NV. Influenza virus neuraminidase: structure and function. *Acta Naturae.* (2009) 1:26–32.
39. Noshi T, Kitano M, Taniguchi K, Yamamoto A, Omoto S, Baba K, et al. *In vitro* characterization of baloxavir acid, a first-in-class cap-dependent endonuclease inhibitor of the influenza virus polymerase PA subunit. *Antiviral Res.* (2018) 160:109–17. doi: 10.1016/j.antiviral.2018.10.008
40. Koshimichi H, Ishibashi T, Kawaguchi N, Sato C, Kawasaki A, Wajima T. Safety, tolerability, and pharmacokinetics of the novel anti-influenza agent baloxavir marboxil in healthy adults: phase I study findings. *Clin Drug Investig.* (2018) 38:1189–96. doi: 10.1007/s40261-018-0710-9

41. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, et al. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. *N Engl J Med*. (2018) 379:913–23. doi: 10.1056/NEJMoa1716197
42. Yang T. Baloxavir marboxil: the first cap-dependent endonuclease inhibitor for the treatment of influenza. *Ann Pharmacother*. (2019) 53:754–9. doi: 10.1177/1060028019826565
43. Gubareva LV, Mishin VP, Patel MC, Chesnokov A, Nguyen HT, De La Cruz J, et al. Assessing baloxavir susceptibility of influenza viruses circulating in the United States during the 2016/17 and 2017/18 seasons. *Euro Surveill.* (2019) 24:1800666. doi: 10.2807/1560-7917.ES.2019.24.3.1800666
44. Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, Kawai M, et al. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci Rep.* (2018) 8:9633. doi: 10.1038/s41598-018-27890-4
45. Takashita E, Kawakami C, Morita H, Ogawa R, Fujisaki S, Shirakura M, et al. Detection of influenza A(H3N2) viruses exhibiting reduced susceptibility to the novel cap-dependent endonuclease inhibitor baloxavir in Japan, December 2018. *Euro Surveill.* (2019) 24:1800698. doi: 10.2807/1560-7917.ES.2019.24.3.1800698
46. Furuta Y, Komeno T, Nakamura T. Favipiravir (T-705), a broad spectrum inhibitor of viral RNA polymerase. *Proc Jpn Acad Ser B Phys Biol Sci*. (2017) 93:449–63. doi: 10.2183/pjab.93.027
47. Furuta Y, Gowen BB, Takahashi K, Shiraki K, Smee DF, Barnard DL. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. *Antiviral Res.* (2013) 100:446–54. doi: 10.1016/j.antiviral.2013.09.015
48. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet*. (2004) 364:759–65. doi: 10.1016/S0140-6736(04)16934-1
49. Stephenson I, Democratis J, Lackenby A, McNally T, Smith J, Pareek M, et al. Neuraminidase inhibitor resistance after oseltamivir treatment of acute influenza A and B in children. *Clin Infect Dis.* (2009) 48:389–96. doi: 10.1086/596311
50. Sleeman K, Mishin VP, Deyde VM, Furuta Y, Klimov AI, Gubareva LV. *In vitro* antiviral activity of favipiravir (T-705) against drug-resistant influenza and 2009 A(H1N1) viruses. *Antimicrob Agents Chemother*. (2010) 54:2517–24. doi: 10.1128/AAC.01739-09
51. Sidwell RW, Barnard DL, Day CW, Smee DF, Bailey KW, Wong MH, et al. Efficacy of orally administered T-705 on lethal avian influenza A (H5N1) virus infections in mice. *Antimicrob Agents Chemother*. (2007) 51:845–51. doi: 10.1128/AAC.01051-06
52. .Marathe BM, Wong SS, Vogel P, Garcia-Alcalde F, Webster RG, Webby RJ, et al. Combinations of oseltamivir and T-705 extend the treatment window for highly pathogenic influenza A(H5N1) virus infection in mice. *Sci Rep*. (2016) 6:26742. doi: 10.1038/srep26742
53. Nagata T, Lefor AK, Hasegawa M, Ishii M. Favipiravir: a new medication for the Ebola virus disease pandemic. *Disaster Med Public Health Prep*. (2015) 9:79-81. doi: 10.1017/dmp.2014.151
54. Furuta Y, Takahashi K, Shiraki K, Sakamoto K, Smee DF, Barnard DL, et al. T-705 (favipiravir) and related compounds: novel broad-spectrum inhibitors of RNA viral infections. *Antiviral Res.* (2009) 82:95–102. doi: 10.1016/j.antiviral.2009.02.198
55. Delang L, Abdelnabi R, Neyts J. Favipiravir as a potential countermeasure against neglected and emerging RNA viruses. *Antiviral Res*. (2018) 153:85–94. doi: 10.1016/j.antiviral.2018.03.003
56. Clark MP, Ledeboer MW, Davies I, Byrn RA, Jones SM, Perola E, et al. Discovery of a novel, first-in-class, orally bioavailable azaindole inhibitor (VX-787) of influenza PB2. *J Med Chem*. (2014) 57:6668–78. doi: 10.1021/jm5007275
57. Smee DF, Barnard DL, Jones SM. Activities of JNJ63623872 and oseltamivir against influenza A H1N1pdm and H3N2 virus infections in mice. *Antiviral Res*. (2016) 136:45–50. doi: 10.1016/j.antiviral.2016.10.009
58. Trevejo JM, Asmal M, Vingerhoets J, Polo R, Robertson S, Jiang Y, et al. Pimodivir treatment in adult volunteers experimentally inoculated with live influenza virus: a Phase IIa, randomized, double-blind, placebo-controlled study. *Antivir Ther*. (2018) 23:335–44. doi: 10.3851/IMP3212
59. Finberg RW, Lanno R, Anderson D, Fleischhackl R, van Duijnhoven W, Kauffman RS, et al. Phase 2b Study of pimodivir (JNJ-63623872) as monotherapy or in combination with oseltamivir for treatment of acute uncomplicated seasonal iInfluenza A: TOPAZ Trial. *J Infect Dis*. (2018) 219:1026–34. doi: 10.1093/infdis/jiy547
60. Medina RA, Stertz S, Manicassamy B, Zimmermann P, Sun X, Albrecht RA, et al. Glycosylations in the globular head of the hemagglutinin protein modulate the virulence and antigenic properties of the H1N1 influenza viruses. *Sci Transl Med*. (2013) 5:187ra170. doi: 10.1126/scitranslmed.3005996
61. Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*. (2011) 333:850–6. doi: 10.1126/science.1205669
62. Dilillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus *in vivo*. *Nat Med*. (2014) 20:143–51. doi: 10.1038/nm.3443
63. Dilillo DJ, Palese P, Wilson PC, Ravetch JV. Broadly neutralizing antiinfluenza antibodies require Fc receptor engagement for *in vivo* protection. *J Clin Invest*. (2016) 126:605–10. doi: 10.1172/JCI84428
64. McBride JM, Lim JJ, Burgess T, Deng R, Derby MA, Maia M, et al. Phase 2 randomized trial of the safety and efficacy of MHAA4549A, a broadly neutralizing monoclonal antibody, in a human influenza A virus challenge model. *Antimicrob Agents Chemother*. (2017) 61:e01154–17. doi: 10.1128/AAC.01154-17
65. Ali O, Takas T, Andrew BS, Nyborg C, KathrynD, Jensen N, et al. A Phase 2a study to evaluate the safety of MEDI8852 in outpatient adults with acute, uncomplicated influenza A. *Open Forum Infect Dis*. (2017) 4 (Suppl. 1):S519. doi: 10.1093/ofid/ofx163.1352
66. Hershberger E, Sloan S, Narayan K, Hay CA, Smith P, Engler F, et al. Safety and efficacy of monoclonal antibody VIS410 in adults with uncomplicated influenza A infection: results from a randomized, double-blind, phase-2, placebo-controlled study. *EBioMedicine*. (2019). 40:574–82.doi: 10.1016/j.ebiom.2018.12.051
67. Gupta P, Kamath AV, Park S, Chiu H, Lutman J, Maia M, et al. Preclinical pharmacokinetics of MHAA4549A, a human monoclonal antibody to influenza A virus, and the prediction of its efficacious clinical dose for the treatment of patients hospitalized with influenza A. *MAbs*. (2016) 8:991–7. doi: 10.1080/19420862.2016.1167294
68. Kallewaard NL, Corti D, Collins PJ, Neu U, Mcauliffe JM, Benjamin E, et al. Structure and function analysis of an antibody recognizing all influenza A subtypes. *Cell*. (2016) 166:596–608. doi: 10.1016/j.cell.2016.05.073
69. Baranovich T, Jones JC, Russier M, Vogel P, Szretter KJ, Sloan SE, et al. The hemagglutinin stem-binding monoclonal antibody VIS410 controls influenza virus-induced acute respiratory distress syndrome. *Antimicrob Agents Chemother*. (2016) 60:2118–31. doi: 10.1128/AAC.02457-15

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 June 2019
doi: 10.3389/fmicb.2019.01328

Adaptive Evolution of Human-Isolated H5Nx Avian Influenza A Viruses

*Fucheng Guo[1], Yiliang Li[1], Shu Yu[1], Lu Liu[2], Tingting Luo[1], Zhiqing Pu[1], Dan Xiang[2], Xuejuan Shen[1], David M. Irwin[3,4], Ming Liao[1,5] and Yongyi Shen[1,2,5]**

[1] College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [2] Joint Influenza Research Centre (SUMC/HKU), Shantou University Medical College, Shantou, China, [3] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, [4] Banting and Best Diabetes Centre, University of Toronto, Toronto, ON, Canada, [5] Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, Guangzhou, China

Avian influenza A viruses (AIVs) H5N1, first identified in 1996, are highly pathogenic in domestic poultry and continue to occasionally infect humans. In this study, we sought to identify genetic changes that occurred during their multiple invasions to humans. We evaluated all available H5Nx AIV genomes. Significant signals of positive selection were detected in 29 host-shift branches. 126 parallel evolution sites were detected on these branches, including 17 well-known sites (such as T271A, A274T, T339M, Q591K, E627K, and D701N in PB2; A134V, D154N, S223N, and R497K in HA) that play roles in allowing AIVs to cross species barriers. Our study suggests that during human infections, H5Nx viruses have experienced adaptive evolution (positive selection and convergent evolution) that allowed them to adapt to their new host environments. Analyses of adaptive evolution should be useful in identifying candidate sites that play roles in human infections, which can be tested by functional experiments.

Keywords: H5Nx, Avian influenza A virus, convergent evolution, human infection, host shift

OPEN ACCESS

Edited by:
Ville Peltola,
Turku University Hospital, Finland

Reviewed by:
Ming Xiu Peng,
Zhejiang University, China
Ahmed Mostafa,
National Research Centre, Egypt

****Correspondence:***
Yongyi Shen
shenyy@scau.edu.cn

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *01 February 2019*
Accepted: *28 May 2019*
Published: *12 June 2019*

Citation:
Guo F, Li Y, Yu S, Liu L, Luo T, Pu Z, Xiang D, Shen X, Irwin DM, Liao M and Shen Y (2019) Adaptive Evolution of Human-Isolated H5Nx Avian Influenza A Viruses. Front. Microbiol. 10:1328. doi: 10.3389/fmicb.2019.01328

INTRODUCTION

Avian influenza viruses (AIVs) pose a continuous threat to public health due to their pandemic potential (Widdowson et al., 2017). The H5N1 subtype of highly pathogenic avian virus (HPAIV) is highly pathogenic in domestic poultry, since its initial detection in China in 1996 (Peiris et al., 2007). Since its first discovery in humans during 1997 in Hong Kong (Yuen et al., 1998), H5N1 HPAIVs continue to result in occasional human infections with an high fatality rate of more than 60% (Yu et al., 2008; Cowling et al., 2013). H5 viruses evolved into different clades, and have reassorted with different NA subtypes, including N1, N2, N3, N5, N6, and N8, resulting in outbreaks in poultry and lethal human infections (Smith and Donis, 2015). H5 is the predominate AIV subtype that infect human populations, and thus, pose great threat to public health (Peiris et al., 2007).

Human infections of H5Nx are mainly through direct avian-to-human transmission. The human-isolated H5Nx viruses are distributed into different phylogenetic clades, that is, these host-shift events occurred independently multiple times. During the adaptation of an organism to a new environment, adaptive evolution occurs. When similar morphological or physiological changes

are observed on multiple evolutionary lineages, convergent or parallel amino acid changes in key genes occur (Zhang, 2006; Shen et al., 2012). Viruses face great challenges when they emerge in new hosts. Our previous study showed that during the multiple invasions of humans by H7N9 AIVs, convergent evolution occurred to allow these human-isolated viruses to adapt their new hosts (Xiang et al., 2018).

Although H5Nx viruses do not have the ability to be transmitted efficiently in a sustained manner from person-to-person, these HPAIVs, which are panzootic in poultry, continue to spread, and their interspecific transmission poses a major challenge to human health. In this study, we conducted a comprehensive evolutionary analysis of H5Nx viruses by collecting all available sequence data to examine molecular mechanisms used by H5Nx viruses to frequently infect humans.

MATERIALS AND METHODS

Data Source and Preliminary Treatment

All available sequences of H5Nx viruses were downloaded from three databases: the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI)[1], the Global Initiative on Sharing Avian Influenza Data[2], and the Influenza Research Database (IRD)[3]. Redundant sequences, laboratory strains and short (<80% of the corresponding gene) sequences were removed. Sequences from egg isolations from human hosts were excluded, as these sequences might carry additional *in vitro* adaptive mutations (Bush et al., 2000). Our final dataset contains 9945, 6719, 6845, 7966, 6454, 6401, 6466, and 6423 HA, NS, M, NA, NP, PA, PB1, and PB2 sequences, respectively (**Supplementary Table 1**). The sequences in each dataset were aligned by MAFFT v7.221, separately (Katoh and Toh, 2010). Initial phylogenetic trees for the eight genes were constructed separately, using the maximum likelihood method RAxML v.8.0.14 (Stamatakis, 2006). Best-fit evolutionary models for the sequences in each datasets were identified using ModelTest (Posada and Crandall, 1998).

Selection Analyses

The CODEML program in the PAML package (Yang, 2007) was used to identify signals of potential positive selection. The branch-site model, which was used to determine whether a gene had undergone positive selection on a foreground branch, was used to assess selective pressure. Bayes Empirical Bayes (BEB) analysis was used to calculate the Bayesian posterior probability of any positively selected site or branch. Finally, LRT statistics were calculated between the branch-site model and the branch-site model with fixed $\omega_0 = 1$. The significance of the difference between the models was determined using twice the difference in the log-likelihood values of LRTs (2ΔlnL) between the two models, which follows a chi-squared ($\chi 2$) distribution with degrees of freedom equaling the difference in the number of parameter estimated (Zhang et al., 2005).

Convergent Evolution Analyses

Ancestral amino acid sequences for target nodes of each dataset were inferred using PAML4.0 (Yang, 2007). The statistical significance of the number of convergent/parallel evolutionary substitutions between pairs of branches was tested using the method of Zhang and Kumar (1997). Candidate substitutions were defined if (i) the topology of each lineage consisting of human isolate and its genetically related isolates had high bootstrap support values ($\geq$90), and (ii) the posterior probabilities of the character states at each ancestral node was $\geq$0.90. The corresponding sites in HA protein were mapped onto a published three-dimensional (3-D) structure of A/duck/Egypt/10185SS/2010 (H5N1) virus (Protein Data Bank code: 5E2Y) using PyMOL (Molecular Graphics System, version 2.0.7.0 Schrödinger, LLC, accessed on 19-Jan-2018)[4] (Delano, 2002).

RESULTS

Phylogenetic Analyses

The HA phylogeny reconstructed using RAxML v.8.0.14 (Stamatakis, 2006) revealed that the H5 sequences are grouped into 10 clades (clades 0–9), and that the human-isolated sequences distribute to clades 0 (16 human-isolated sequences), 1 (101 human-isolated sequences), 2 (360 human-isolated sequences), 3 (one human-isolated sequences), and 7 (two human-isolated sequences) (**Figure 1**, **Supplementary Figures 1**, **2**, and **Supplementary Table 2**). Similarly, phylogenetic trees were reconstructed, separately, for other genes.

In order to simplify the calculations that focused on the human-isolated viruses, we divided the HA, NS, M, NA, NP, PA, PB1, and PB2 sequences into 132, 101, 98, 114, 87, 80, 90, and 92 datasets, respectively, based on the initial phylogenetic trees. Each dataset contains the human isolates and their closely related avian isolates (**Supplementary Figures 3–10**). These HA, NS, M, NA, NP, PA, PB1, and PB2 gene datasets contained 266, 147, 206, 260, 186, 155, 164, and 171 host-shift branches, respectively.

Positive Selection on Host-Shift Branches

We used the CODEML program from the PAML package (Yang, 2007) to identify signals of positive selection on host-shift (avian-to-human) branches. In total, 29 branches with 38 sites (H5 numbering) were identified as having experienced significant positive selection, including branches HA-107b, HA-18c (473R), HA-6a, HA-64b (11N, 15Q, 20M, 314K, 315T, 522T, 529L, 546L, 547Q, and 548C), HA-68a (212R and 500R), HA-72b, HA-74a, HA-75a, HA-76a, HA-77a, HA-83a, and HA-107b in HA; PB2-14d and PB2-74b in PB2; MP-46e, MP-50a (5T, 6E, 7V, 8E, 257T, 258E, 259V, and 260E), and MP-85a (277P, 279V, 282A, 283N, 284I, 285I, 287I, 292L, 328Y, 330Q, 336V, 339D, 340D,

[1] www.ncbi.nlm.nih.gov/genomes/FLU

[2] www.gisaid.org

[3] www.fludb.org/brc/home.spg?decorator=influenza

[4] http://www.pymol.org/

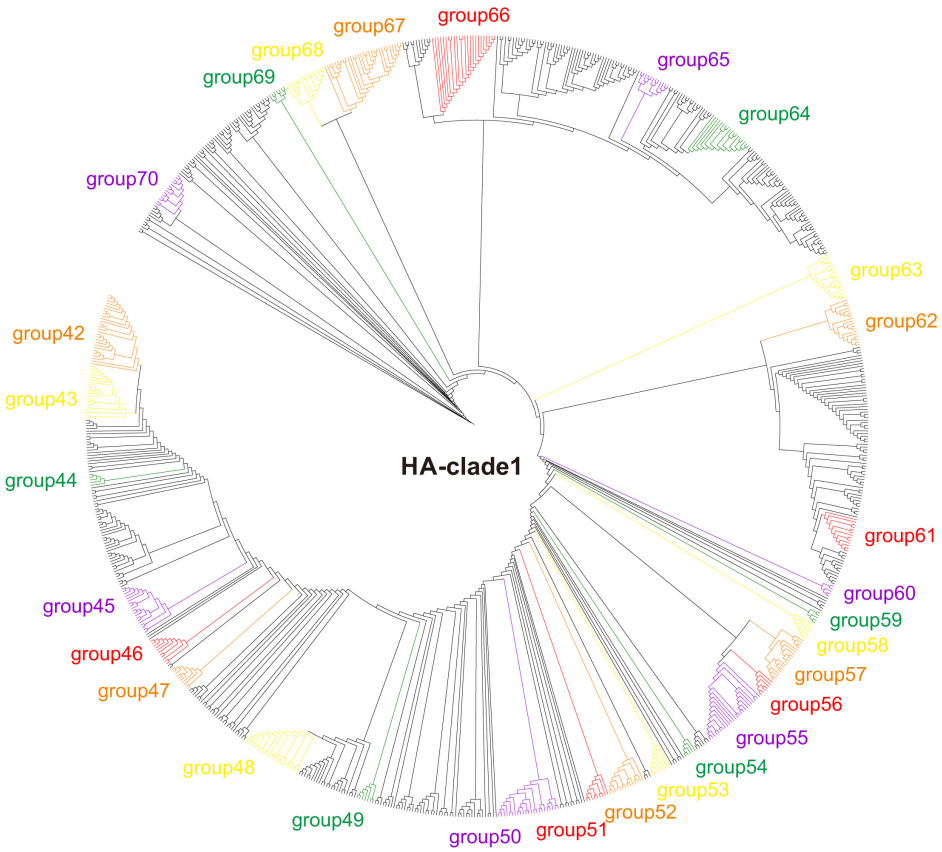


FIGURE 1 | Phylogenetic tree of H5 clade 1 of H5Nx viruses. Maximum likelihood tree of H5Nx viral sequences generated using RAxML v.8.0.14 with the best fitting sequence evolutionary model identified by ModelTest.

and 344V) in MP; NA1-15b (188N) in NA1, NA6-2b in NA6; NP-32a, NP-65a (486S and 487Y) and NP-66c in NP; PA-11a, PA-25d, and PA-72b in PA; NS-31a, NS-72c, and NS-89a in NS, and PB1-31b in PB1 (**Supplementary Table 3**). Other host-shift branches did not show significant signals of positive selection (**Supplementary Table 4**).

Convergent/Parallel Evolution of Human-Isolated H5Nx Viruses

To determine whether convergent/parallel evolution occurred during the multiple avian-to-human transmissions of H5Nx HPAIVs, ancestral amino acid sequences for the target nodes that lead to host-shift branches were reconstructed for convergent evolutionary analyses. In total, we identified 126 parallel evolution substitutions (34 in HA, 20 sites in PB2, seven in PB1, 13 in PA, eight in NP, 20 in NA, six in MP, and 18 in NS) that occurred on the host-shift branches (**Table 1**).

For the HA gene, the parallel amino acid substitutions S223N (H5 numbering) occurred on seven host-shift branches, A134V on four branches, and six mutations (D31N, D154N, T/N195I, V219I, I375M, and E502G, H5 numbering) were each discovered on three branches. The remaining 26 parallel mutations occurred each on pairs of branches. Of these convergent/parallel amino acid mutations, four substitutions (A134V, D154N, S223N, and R497K) had been identified in earlier studies as having functional roles and were mapped to the three-dimensional (3-D) structure of the HA protein (**Figure 2**).

For the PB2 gene, the well-known mutations E627K and D701N (Hatta et al., 2001; Li et al., 2005; Song et al., 2014; Zhu et al., 2015) were observed on 30 and 10 host-shift branches, respectively. N456D and G727R were detected on five and four branches, respectively. An additional four substitutions (T339M, I451V, S471F, and R369K) occur in parallel on three sets of branches and the remaining 12 parallel substitutions were shared by pairs of host-shift branches.

For the PB1 gene, there were seven parallel-evolved variations (I57T, E172D, M179I, S361G, N375S, K387R, and L598P) that occur on pairs of host-shift branches.

For the PA gene, three mutations K142E, N321K, and K615R were each detected on three host-shift branches and ten mutations (F4C, M12I, M86V, T97I, F105L, L226F, E237K, P275L, T369A, and V387I) parallelly occurred on pairs of host-shift branches.

For the NA gene, 20 parallel-evolved variations in the NA1 subtype, including three mutations I/V16A, V33I, and I243V occurred on each of three host-shift branches, and 17 mutations (V16I, N39S, P45T, K55R, A58T, K241R, N305T, G318S, P323S, S364N, G365E, I380V, V404I, N430S, N430D,

TABLE 1 | Convergent/parallel evolution sites for eight genes in H5 human isolates.

Gene	Convergent/ Parallel sites	*P* values	Phenotype
PB2	I64M	$P < 0.05$*	Human host marker
	V108A	$P < 0.05$*	
	I147M	$P < 0.05$*	
	E192K	$P < 0.05$*	
	T271A	$P < 0.05$*	Human host marker (host-specific polymerase activity)
	A274T	$P < 0.05$*	Increased polymerase activity, increased virulence in mammals and birds
	T339M	$P < 0.05$*	Enhanced polymerase activity, increased virulence in mice
	R369K	$P < 0.05$*	
	V444L	$P > 0.05$	
	V451I	$P < 0.05$*	
	N456D	$P < 0.05$*	
	I461V	$P < 0.05$*	
	S489P	$P > 0.05$	
	M570V	$P < 0.05$*	
	Q591K	$P < 0.05$*	Increased virulence in mammals
	I615V	$P < 0.05$*	
	E627K	$P < 0.05$*	Human host marker, enhanced polymerase activity, increased virulence in mammals
	V649I	$P < 0.05$*	
	D701N	$P < 0.05$*	Increased polymerase activity, increased virulence in mammals, mammalian host marker
	G727R	$P < 0.05$*	
	D740N	$P < 0.05$*	
	S741F	$P < 0.05$*	
HA	Q30P	$P > 0.05$	
	D31N	$P < 0.05$*	
	K35R	$P < 0.05$*	
	D45N	$P < 0.05$*	
	A86V	$P > 0.05$	
	D88G	$P < 0.05$*	
	A127T	$P < 0.05$*	
	A/T127S	$P < 0.05$*	
	A134V	$P < 0.05$*	Increased virus binding to 2,6
	R140K	$P < 0.05$*	
	M140T	$P < 0.05$*	
	S141P	$P < 0.05$*	
	D154N	$P < 0.05$*	Airborne transmissibility in mammals
	R162I	$P < 0.05$*	
	V174I	$P < 0.05$*	
	N182S	$P < 0.05$*	Increased virus binding to 2,6
	A184E	$P > 0.05$	
	E184G	$P < 0.05$*	Increased virulence in mammals
	T195A	$P < 0.05$*	
	T/N195I	$P < 0.05$*	
	K189R	$P < 0.05$*	
	V210A	$P > 0.05$	
	V219I	$P < 0.05$*	
	S223N	$P < 0.05$*	Increased virus binding to 2,6
	R310K	$P < 0.05$*	
	R323K	$P < 0.05$*	
	R326K	$P < 0.05$*	
	I375M	$P < 0.05$*	
	D376N	$P < 0.05$*	Increased virulence in mammals
	D387N	$P < 0.05$*	
	E433G	$P < 0.05$*	
	N476D	$P < 0.05$*	
	E477K	$P < 0.05$*	
	M479I	$P < 0.05$*	
	R497K	$P < 0.05$*	Increased virus binding to 2,6
	E502G	$P < 0.05$*	
	M532I	$P < 0.05$*	
	V533I	$P < 0.05$*	
PB1	I57T	$P < 0.05$*	
	E172D	$P < 0.05$*	
	M179I	$P < 0.05$*	
	S361G	$P < 0.05$*	
	N375S	$P < 0.05$*	Increased polymerase activity, increased virulence in mammals, human host marker
	K387R	$P < 0.05$*	
	L598P	$P < 0.05$*	Increased polymerase activity and replication efficiency
PA	F4C	$P < 0.05$*	
	M12I	$P < 0.05$*	
	M86V	$P < 0.05$*	
	T97I	$P < 0.05$*	Enhanced polymerase activity, increased virulence in mice
	F105L	$P < 0.05$*	
	K142E	$P > 0.05$	
	L226F	$P < 0.05$*	
	E237K	$P < 0.05$*	
	C241Y	$P > 0.05$	Enhance the replicative ability of an H5N1 virus in A549 cells and enhance its pathogenicity in mice
	P275L	$P < 0.05$*	
	N321K	$P < 0.05$*	Increased polymerase activity
	T369A	$P < 0.05$*	
	V387I	$P < 0.05$*	
	K615R	$P < 0.05$*	Increased polymerase activity, increased virulence in mammals, mammalian host marker
NP	R100I	$P < 0.05$*	
	A284T	$P < 0.05$*	Increased virulence in mice
	V343I	$P < 0.05$*	Highly BNP-sensitive to moderately BNP-resistant
	R384K	$P < 0.05$*	
	S413L	$P < 0.05$*	

(Continued)

TABLE 1 | Continued

Gene	Convergent/ Parallel sites	*P* values	Phenotype
	P419S	$P < 0.05$*	
	R452K	$P < 0.05$*	
	P453S	$P < 0.05$*	
NA1	I8T	$P < 0.05$*	
	I/V16A	$P < 0.05$*	
	V16I	$P < 0.05$*	
	V33I	$P < 0.05$*	
	N39S	$P < 0.05$*	
	P45T	$P < 0.05$*	
	K55R	$P < 0.05$*	
	A58T	$P < 0.05$*	
	K241R	$P < 0.05$*	
	I243V	$P < 0.05$*	
	N305T	$P < 0.05$*	
	G318S	$P < 0.05$*	
	P323S	$P < 0.05$*	
	S364N	$P < 0.05$*	
	G365E	$P < 0.05$*	
	I380V	$P < 0.05$*	
	V404I	$P < 0.05$*	
	N430S	$P < 0.05$*	
	N430D	$P < 0.05$*	
	G435S	$P < 0.05$*	
	T441D	$P < 0.05$*	
NA2, NA3, NA4, NA5, NA6, NA7, NA8, NA9	NA		
MP	T137A	$P < 0.05$*	Human host marker
	A239T	$P < 0.05$*	
	C269Y	$P < 0.05$*	
	V280I	$P < 0.05$*	
	S283N	$P < 0.05$*	
	D340N	$P < 0.05$*	
NS1	G47S	$P < 0.05$*	
	N48S	$P < 0.05$*	
	R59H	$P < 0.05$*	
	R67Q	$P < 0.05$*	
	E70K	$P < 0.05$*	
	T81I	$P < 0.05$*	
	R88C	$P < 0.05$*	
	V136A	$P < 0.05$*	
	I137V	$P < 0.05$*	
	D139N	$P < 0.05$*	
	L185F	$P < 0.05$*	
	S205N	$P < 0.05$*	Decreased IFN antagonism, conferred enhanced in-contact transmissibility in guinea pigs
	D209N	$P < 0.05$*	
	V209I	$P < 0.05$*	
	L212F	$P < 0.05$*	
NEP/NS2	M/A14V	$P < 0.05$*	
	A48T	$P < 0.05$*	
	T/V115A	$P < 0.05$*	

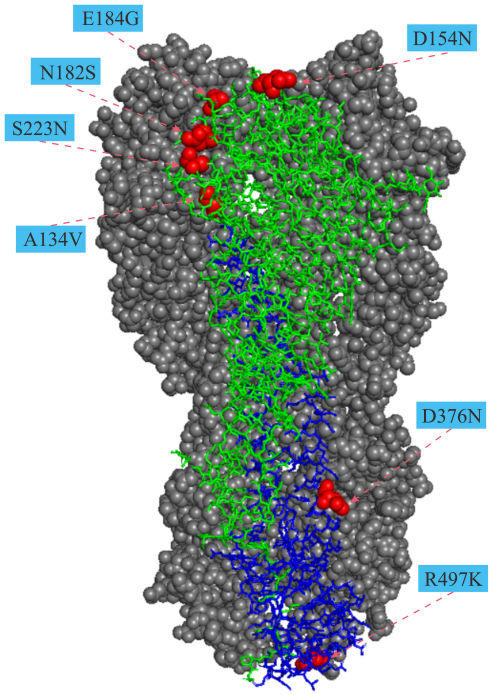


FIGURE 2 | Mapping of parallel evolution sites on the structure of HA protein. Parallel evolution sites were mapped to know HA structure for the A/duck/Egypt/10185SS/2010 (H5N1) virus (Protein Data Bank code: 5e2y). The monomer shows the HA1 (A134V, D154N, N182S, and E184G) and the HA2 (D376N and R497K) subunits. Numbers in colored rectangle represents codon alignment number (H5 numbering) and their locations in the three-dimensional structure are shown with red spheres.

G435S, and T441D) were shared by pairs of branches. No parallel substitutions were detected in the other NA subtypes.

For the NP gene, two mutations A284T and V343I were shared by four and three human-isolated branches, respectively, and five mutations (R100I, R384K, S413L, P419S, R452K, and P453S) were shared by pairs of human-isolated branches.

For the MP gene, six mutations were detected as parallel-evolved variations, including T137A, A239T, C269Y, V280I, S283N, and D340N. Each of these was separately shared by two human-isolated branches.

For the NS gene, 18 parallel-evolved variations were detected, 15 of which were in NS1 and three in NS2. Of the 15 NS1 mutations, four (R67Q, T81I, L212F, and L185F) were each shared by three branches, and 11 (G47S, N48S, R59H, E70K, R88C, V136A, I137V, D139N, S205N, D209N, and V209I) were identified in pairs of human-isolated branches. For the NEP/NS2 genes, three mutations M/A14V, A48T, and T/V115A were shared by pairs of branches.

DISCUSSION

Since their first detection in 1996, H5 AIVs have had a substantial impact on veterinary and human health (Harfoot and Webby, 2017). Although the direct transmission of H5Nx viruses from

avian species to humans remains a relatively rare event, they still pose a serious pandemic threat due to their high virulence and mortality, and their increasingly expanding host range, as well as the significant ongoing evolution toward efficient transmission in mammals (Guan and Smith, 2013). Sporadic human infections continue to occur in countries where H5Nx have become endemic in birds, providing a persistent threat to global health due to the possibility of virus adaptation to humans. Thus, the study of their genetic mechanisms of human adaptation remains essential.

Adaptive evolution of functional important genes is essential for the invasion of new niches (Zhang and Kumar, 1997; Zhang, 2006; Shen et al., 2012). AIVs should face great challenges when they emerge in humans from their avian sources. During the host-shift process of the multiple human H7N9 AIV invasions, convergent evolution occurred for the human-isolated viruses to adapt to their new hosts (Xiang et al., 2018). Similar to H7N9, human-isolated H5 sequences are distributed in multiple phylogenetic positions (**Figure 1** and **Supplementary Figures 1**, **2**), suggesting that there were multiple independent invasions of H5Nx AIVs into humans. Here, we found that adaptive evolution (positive selection, and convergent/parallel evolution) occurred on the independent host-shift branches in H5Nx AIVs, and that some of the adaptive sites have functional importance (**Table 1**).

Avian influenza viruses preferentially bind α2-3 sialic acid receptors. In contrast, human-adapted influenza viruses preferentially bind α2-6 sialic acid receptors. The switch of preference, from avian to human type sialic acid receptors, is considered to be a key element necessary for AIVs to cause human pandemics (Matrosovich et al., 2000; Parrish and Kawaoka, 2005). The receptor-binding domain (RBD) of HA is formed by four loops and one helix in the RBS, which contact the base and potentially the extension region of the human receptor. Among the 34 parallel amino acid substitutions detected in the HA gene (**Table 1**), two of them A134V and S223N, have previously been shown to associate with the switch of preference from avian to human type sialic acid receptors (Yamada et al., 2006; Auewarakul et al., 2007; Imai et al., 2010; Chen et al., 2012). In addition, three others, S141P (130-loop), R162I (150-loop), and T/N195A/I(190-loop), are in or around the RBD and may dramatically alter the receptor binding preference of the H5Nx influenza viruses. Furthermore, S223N has previously been detected by *in silico* prediction and experimentally confirmed to enhance human receptor specificity of H5N1 influenza A viruses (Schmier et al., 2015), indicating that mutations detected by several methods might have greater potential functional effects and that the combination of multiple methods is recommended for selecting potential functional mutations for experimental studies. S223N is located close to the sialic-acid-binding site (**Figure 2**), and this mutation in H5N1 AIVs has weakened affinity toward α-2, 3 and an increased affinity toward α-2, 6 sialic acid receptors (Yamada et al., 2006; Chen et al., 2012). Alanine at position 134 (A134) is in the 130-loop of the receptor binding domain (**Figure 2**). This site is highly conserved in avian H5N1 viruses. The Ala to Val substitution at position 134 could change the receptor-binding preference of H5-HA from α-2,3 to both α-2,3 and α-2,6-sialic acid binding (Auewarakul et al., 2007; Imai et al., 2010). To initiate influenza virus infection, hemagglutinin (HA), which is the major surface glycoprotein of influenza viruses, binds to the host cell surface complex glycans via a terminal sialic acid. The preference of HA for particular sialic acid moieties on host cells is a key determinant of host range and tissue tropism (Matrosovich et al., 2000). Parallel evolution of amino acid sites that play important roles in the change of receptor-binding preference in host-shift branches suggests that during human invasion by AIVs that they have adapted to the common challenge of a difference in the surface glycoprotein between birds and humans.

After binding and entering human cells, efficient replication (efficient production of new viruses) is a critical factor that influences viral infection. A series of parallel evolution sites with roles in increasing polymerase activity and replication efficiency in mammals were identified, such as T271A (Finkelstein et al., 2007), A274T (Leung et al., 2010), Q591K (Yamada et al., 2010), E627K (Hatta et al., 2001; Song et al., 2014), and D701N (Li et al., 2005; Zhu et al., 2015), in PB2 (**Table 1**). The E627K and D701N mutations might be especially important as 17.5% (30 of 171) and 5.85% (10 of 171) of the host-shift lineages in the PB2 gene share these two mutations. In addition, some parallel sites in other genes are also suggested to be associated with enhanced polymerase activity and/or increased virulence in mammals (**Table 1**), such as L598P in PB1 (Xu et al., 2012), T97I and N321K in PA (Cheng et al., 2014; Yu et al., 2014; Wu et al., 2016; Zhao et al., 2016; Nam et al., 2017), and A284T in NP (Zhao et al., 2016). Parallel evolution at these sites suggests that during the host-shift of H5Nx AIVs, these changes have allowed more efficient replication in human cells. Compared with the adaptive evolution of human-isolated H7N9 AIVs (Xiang et al., 2018), human-isolated H5Nx also have the E627K and D701N mutations in PB2 genes in the host-shift branches. This suggests that these two mutations play important roles in human adaptation of AIVs in both subtypes. Other adaptive sites are not shared by these two viruses, suggesting that there are some differences in the adaptation of H7N9 and H5Nx to humans.

Positive selection is the force that drives an increase in the prevalence of advantageous new mutations. In this study, 29 host-shift branches had significant signals of positive selection, with 12 in HA, two in PB2, three in MP, one in NA1, one in NA6, three in NP, three in PA, three in NS, and one in PB1. The frequency of positive selection in host-shift branches is quite low, indeed, only a few adaptive events were detected by the PAML package. This is an unavoidable limitation of the current approaches for positive selection analyses. A total of 38 positively selected amino acid sites were identified. The 340D mutation in the MP gene had significant signals in both the positive selection and the convergent evolution analyses, implying that this site might be important for adaptation to new host environments. More attention on this positive selection site, and its functional roles, is needed.

To determine whether the human-adaptive sites were actually part of human adaptation following zoonosis, we calculated the prevalence of the adaptive mutations (positive selection and convergent/parallel evolution sites) in the

human- and avian-isolated strains (**Supplementary Table 5**). It is expected that the adaptive-evolution substitutions should be more prevalent in the human viruses than in the avian viruses (Arai et al., 2016). For most of the adaptive mutations, their proportions in the human isolates are higher, with many tending to be fixed, than in the avian isolates, suggesting that these mutations have higher fitness in humans (**Supplementary Table 5**).

Host barriers restrict interspecies transmission of AIVs. Factors that contribute to AIVs infection and transmission in humans are complex. Although some amino acid changes associated with receptor affinity, temperature tolerance, viral replication, and mammalian adaptation have been found to play a role (Widdowson et al., 2017), the genetic basis for host shifts is not fully understood. In this study, we identified a series of adaptive changes at sites during the multiple human invasions by H5Nx viruses. Some of the adaptive mutations are known to have functional importance in cross-species transmission from avian to humans, while others are useful candidates for further experimental studies, especially those located in critical domains. Analyses of adaptive evolution should identify useful additional candidate sites that might play roles in human infections for functional studies.

AUTHOR CONTRIBUTIONS

YS conceived, designed, and supervised the study. FG, YL, SY, LL, TL, ZP, DX, and XS collected and analyzed the data. YS and DI wrote the drafts of the manuscript. ML commented on and revised the drafts of the manuscript. All authors read and approved the final draft of the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (31822056) and Guangdong Natural Science Funds for Distinguished Young Scholar (2014A030306046).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01328/full#supplementary-material

REFERENCES

Arai, Y., Kawashita, N., Daidoji, T., Ibrahim, M. S., El-Gendy, E. M., Takagi, T., et al. (2016). Novel polymerase gene mutations for human adaptation in clinical isolates of avian H5N1 influenza viruses. *PLoS Pathog.* 12:e1005583. doi: 10.1371/journal.ppat.1005583

Auewarakul, P., Suptawiwat, O., Kongchanagul, A., Sangma, C., Suzuki, Y., Ungchusak, K., et al. (2007). An avian influenza H5N1 virus that binds to a human-type receptor. *J. Virol.* 81, 9950–9955. doi: 10.1128/jvi.00468-07

Bush, R. M., Smith, C. B., Cox, N. J., and Fitch, W. M. (2000). Effects of passage history and sampling bias on phylogenetic reconstruction of human influenza a evolution. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6974–6980. doi: 10.1073/pnas.97.13.6974

Chen, L. M., Blixt, O., Stevens, J., Lipatov, A. S., Davis, C. T., Collins, B. E., et al. (2012). In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity. *Virology* 422, 105–113. doi: 10.1016/j.virol.2011.10.006

Cheng, K., Yu, Z., Chai, H., Sun, W., Xin, Y., Zhang, Q., et al. (2014). PB2-E627K and PA-T97I substitutions enhance polymerase activity and confer a virulent phenotype to an H6N1 avian influenza virus in mice. *Virology* 46, 207–213. doi: 10.1016/j.virol.2014.08.010

Cowling, B. J., Jin, L., Lau, E. H. Y., Liao, Q., Wu, P., Jiang, H., et al. (2013). Comparative epidemiology of human infections with avian influenza a H7N9 and H5N1 viruses in China: a population-based study of laboratory-confirmed cases. *Lancet* 382, 129–137. doi: 10.1016/s0140-6736(13)61171-x

Delano, W. L. (2002). *Pymol Molecular Graphics System*. http://pymol.org/ (accessed January 19, 2018).

Finkelstein, D. B., Mukatira, S., Mehta, P. K., Obenauer, J. C., Su, X., Webster, R. G., et al. (2007). Persistent host markers in pandemic and H5N1 influenza viruses. *J. Virol.* 81, 10292–10299. doi: 10.1128/jvi.00921-07

Guan, Y., and Smith, G. J. D. (2013). The emergence and diversification of panzootic H5N1 influenza viruses. *Virus Res.* 178, 35–43. doi: 10.1016/j.virusres.2013.05.012

Harfoot, R., and Webby, R. J. (2017). H5 influenza, a global update. *J. Microbiol.* 55, 196–203. doi: 10.1007/s12275-017-7062-7

Hatta, M., Gao, P., Halfmann, P., and Kawaoka, Y. (2001). Molecular basis for high virulence of Hong Kong H5N1 influenza a viruses. *Science* 293, 1840–1842. doi: 10.1126/science.1062882

Imai, H., Shinya, K., Takano, R., Kiso, M., Muramoto, Y., Sakabe, S., et al. (2010). The HA and NS genes of human H5N1 influenza a virus contribute to high virulence in ferrets. *PLoS Pathog.* 6:e1001106. doi: 10.1371/journal.ppat.1001106

Katoh, K., and Toh, H. (2010). Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics* 26, 1899–1900. doi: 10.1093/bioinformatics/btq224

Leung, B. W., Chen, H., and Brownlee, G. G. (2010). Correlation between polymerase activity and pathogenicity in two duck H5N1 influenza viruses suggests that the polymerase contributes to pathogenicity. *Virology* 401, 96–106. doi: 10.1016/j.virol.2010.01.036

Li, Z., Chen, H., Jiao, P., Deng, G., Tian, G., Li, Y., et al. (2005). Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. *J. Virol.* 79, 12058–12064. doi: 10.1128/jvi.79.18.12058-12064.2005

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M. R., et al. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J. Virol.* 74, 8502–8512. doi: 10.1128/jvi.74.18.8502-8512.2000

Nam, J. H., Shim, S. M., Song, E. J., Espano, E., Jeong, D. G., Song, D., et al. (2017). Rapid virulence shift of an H5N2 avian influenza virus during a single passage in mice. *Arch. Virol.* 162, 3017–3024. doi: 10.1007/s00705-017-3451-9

Parrish, C. R., and Kawaoka, Y. (2005). The origins of new pandemic viruses: the acquisition of new host ranges by canine parvovirus and influenza a viruses. *Annu. Rev. Microbiol.* 59, 553–586. doi: 10.1146/annurev.micro.59.030804.121059

Peiris, J. S., de Jong, M. D., and Guan, Y. (2007). Avian influenza virus (H5N1): a threat to human health. *Clin. Microbiol. Rev.* 20, 243–267. doi: 10.1128/cmr.00037-06

Posada, D., and Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818. doi: 10.1093/bioinformatics/14.9.817

Schmier, S., Mostafa, A., Haarmann, T., Bannert, N., Ziebuhr, J., Veljkovic, V., et al. (2015). In silico prediction and experimental confirmation of HA residues conferring enhanced human receptor specificity of H5N1 influenza a viruses. *Sci. Rep.* 5:11434. doi: 10.1038/srep11434

Shen, Y.-Y., Liang, L., Li, G.-S., Murphy, R. W., and Zhang, Y.-P. (2012). Parallel evolution of auditory genes for echolocation in bats and toothed whales. *PLoS Genet.* 8:e1002788. doi: 10.1371/journal.pgen.1002788

Smith, G. J., and Donis, R. O. (2015). Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013–2014. *Influenza Other Respir. Viruses* 9, 271–276. doi: 10.1111/irv.12324

Song, W., Wang, P., Mok, B. W., Lau, S. Y., Huang, X., Wu, W. L., et al. (2014). The K526R substitution in viral protein PB2 enhances the effects of E627K on influenza virus replication. *Nat. Commun.* 5:5509. doi: 10.1038/ncomms6509

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690. doi: 10.1093/bioinformatics/btl446

Widdowson, M. A., Bresee, J. S., and Jernigan, D. B. (2017). The global threat of animal influenza viruses of zoonotic concern: then and now. *J. Infect. Dis.* 216(Suppl. 4), S493–S498. doi: 10.1093/infdis/jix331

Wu, H., Peng, X., Peng, X., Cheng, L., Jin, C., Lu, X., et al. (2016). Multiple amino acid substitutions involved in the adaptation of avian-origin influenza a (H10N7) virus in mice. *Arch. Virol.* 161, 977–980. doi: 10.1007/s00705-015-2722-6

Xiang, D., Shen, X., Pu, Z., Irwin, D. M., Liao, M., and Shen, Y. (2018). Convergent evolution of human-isolated H7N9 avian influenza a viruses. *J. Infect. Dis.* 217, 1699–1707. doi: 10.1093/infdis/jiy082

Xu, C., Hu, W. B., Xu, K., He, Y. X., Wang, T. Y., Chen, Z., et al. (2012). Amino acids 473V and 598P of PB1 from an avian-origin influenza a virus contribute to polymerase activity, especially in mammalian cells. *J. Gen. Virol.* 93(Pt 3), 531–540. doi: 10.1099/vir.0.036434-0

Yamada, S., Hatta, M., Staker, B. L., Watanabe, S., Imai, M., Shinya, K., et al. (2010). Biological and structural characterization of a host-adapting amino acid in influenza virus. *PLoS Pathog.* 6:e1001034. doi: 10.1371/journal.ppat.1001034

Yamada, S., Suzuki, Y., Suzuki, T., Le, M. Q., Nidom, C. A., Sakai-Tagawa, Y., et al. (2006). Haemagglutinin mutations responsible for the binding of H5N1 influenza a viruses to human-type receptors. *Nature* 444, 378–382. doi: 10.1038/nature05264

Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24, 1586–1591. doi: 10.1093/molbev/msm088

Yu, H., Gao, Z., Feng, Z., Shu, Y., Xiang, N., Zhou, L., et al. (2008). Clinical characteristics of 26 human cases of highly pathogenic avian influenza a (H5N1) virus infection in China. *PLoS One* 3:e2985. doi: 10.1371/journal.pone.0002985

Yu, Z., Cheng, K., Xin, Y., Sun, W., Li, X., Huang, J., et al. (2014). Multiple amino acid substitutions involved in the adaptation of H6N1 avian influenza virus in mice. *Vet. Microbiol.* 174, 316–321. doi: 10.1016/j.vetmic.2014.09.032

Yuen, K. Y., Chan, P. K. S., Peiris, M., Tsang, D. N. C., Que, T. L., Shortridge, K. F., et al. (1998). Clinical features and rapid viral diagnosis of human disease associated with avian influenza a H5N1 virus. *Lancet* 351, 467–471. doi: 10.1016/s0140-6736(98)01182-9

Zhang, J. (2006). Parallel adaptive origins of digestive RNases in Asian and African leaf monkeys. *Nat. Genet.* 38, 819–823. doi: 10.1038/ng1812

Zhang, J., and Kumar, S. (1997). Detection of convergent and parallel evolution at the amino acid sequence level. *Mol. Biol. Evol.* 14, 527–536. doi: 10.1093/oxfordjournals.molbev.a025789

Zhang, J., Nielsen, R., and Yang, Z. (2005). Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Mol. Biol. Evol.* 22, 2472–2479. doi: 10.1093/molbev/msi237

Zhao, Y., Yu, Z., Liu, L., Wang, T., Sun, W., Wang, C., et al. (2016). Adaptive amino acid substitutions enhance the virulence of a novel human H7N9 influenza virus in mice. *Vet. Microbiol.* 187, 8–14. doi: 10.1016/j.vetmic.2016.02.027

Zhu, W., Li, L., Yan, Z., Gan, T., Li, L., Chen, R., et al. (2015). Dual E627K and D701N mutations in the PB2 protein of A(H7N9) influenza virus increased its virulence in mammalian models. *Sci. Rep.* 5:14170. doi: 10.1038/srep14170

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

frontiers in Public Health

ORIGINAL RESEARCH
published: 18 June 2019
doi: 10.3389/fpubh.2019.00150

Bigger and Better? Representativeness of the Influenza A Surveillance Using One Consolidated Clinical Microbiology Laboratory Data Set as Compared to the Belgian Sentinel Network of Laboratories

Sigi Van den Wijngaert[1], Nathalie Bossuyt[2], Bridget Ferns[3,4], Laurent Busson[1], Gabriela Serrano[5], Magali Wautier[1], Isabelle Thomas[6], Matthew Byott[7], Yves Dupont[2], Eleni Nastouli[3,8], Marie Hallin[1†], Zisis Kozlakidis[7,9†] and Olivier Vandenberg[5,7,10†]*

[1] Department of Microbiology, LHUB-ULB, Pole Hospitalier Universitaire de Bruxelles, Université Libre de Bruxelles, Brussels, Belgium, [2] Sciensano, SD Epidemiology and Surveillance, Service 'Epidemiology of Infectious Diseases', Brussels, Belgium, [3] Department of Clinical Virology, University College London Hospitals NHS Foundation Trust, London, United Kingdom, [4] UCLH/UCL Biomedical Research Centre, NIHR, London, United Kingdom, [5] Research Centre on Environmental and Occupational Health, School of Public Health, Université Libre de Bruxelles, Brussels, Belgium, [6] National Influenza Centre, Sciensano, Brussels, Belgium, [7] Division of Infection and Immunity, Faculty of Medical Sciences, University College London, London, United Kingdom, [8] Department of Population, Policy and Practice, Great Ormond Street Institute of Child Health, University College London, London, United Kingdom, [9] International Agency for Research on Cancer, World Health Organization, Lyon, France, [10] Innovation and Business Development Unit, LHUB-ULB, Pole Hospitalier Universitaire de Bruxelles, Université Libre de Bruxelles, Brussels, Belgium

OPEN ACCESS

Edited by:
Kelvin To,
The University of Hong Kong, Hong Kong

Reviewed by:
Pedro Xavier-Elsas,
Federal University of Rio de Janeiro, Brazil
Anthony Tam,
Queen Mary Hospital, Hong Kong

***Correspondence:**
Olivier Vandenberg
olivier.vandenberg@ulb.ac.be

†The last three authors act as equivalent co-senior authors

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *25 December 2018*
Accepted: *23 May 2019*
Published: *18 June 2019*

Citation:
Van den Wijngaert S, Bossuyt N, Ferns B, Busson L, Serrano G, Wautier M, Thomas I, Byott M, Dupont Y, Nastouli E, Hallin M, Kozlakidis Z and Vandenberg O (2019) Bigger and Better? Representativeness of the Influenza A Surveillance Using One Consolidated Clinical Microbiology Laboratory Data Set as Compared to the Belgian Sentinel Network of Laboratories. Front. Public Health 7:150. doi: 10.3389/fpubh.2019.00150

Infectious diseases remain a serious public health concern globally, while the need for reliable and representative surveillance systems remains as acute as ever. The public health surveillance of infectious diseases uses reported positive results from sentinel clinical laboratories or laboratory networks, to survey the presence of specific microbial agents known to constitute a threat to public health in a given population. This monitoring activity is commonly based on a representative fraction of the microbiology laboratories nationally reporting to a single central reference point. However, in recent years a number of clinical microbiology laboratories (CML) have undergone a process of consolidation involving a shift toward laboratory amalgamation and closer real-time informational linkage. This report aims to investigate whether such merging activities might have a potential impact on infectious diseases surveillance. Influenza data was used from Belgian public health surveillance 2014–2017, to evaluate whether national infection trends could be estimated equally as effectively from only just one centralized CML serving the wider Brussels area (LHUB-ULB). The overall comparison reveals that there is a close correlation and representativeness of the LHUB-ULB data to the national and international data for the same time periods, both on epidemiological and molecular grounds. Notably, the effectiveness of the LHUB-ULB surveillance remains partially subject to local regional variations. A subset of the Influenza samples had their whole genome sequenced so that the observed epidemiological trends could be correlated to molecular observations from the same period, as an added-value

proposition. These results illustrate that the real-time integration of high-throughput whole genome sequencing platforms available in consolidated CMLs into the public health surveillance system is not only credible but also advantageous to use for future surveillance and prediction purposes. This can be most effective when implemented for automatic detection systems that might include multiple layers of information and timely implementation of control strategies.

Keywords: Belgium, influenza A, surveillance, clinical microbiology laboratory, sequencing

INTRODUCTION

Infectious diseases remain a serious public health concern in industrialized and low- and middle-income countries. Due to their considerable effect on global human demographics (1, 2) and the economy (3, 4), the public health community has developed many surveillance strategies and systems to improve infectious disease surveillance around the world. Even if diagnostic and computer resources have expanded considerably, infectious disease surveillance remains challenging. The 2009 H1N1 influenza pandemic and the recent Ebola outbreak in West Africa are few examples showing that infectious diseases cannot easily be predicted and modeled reliably in real-time (5, 6). Therefore, the need for reliable and representative surveillance systems remains as acute as ever. One of the more established surveillance strategies, known as traditional public health surveillance of infectious diseases, is the use of reported positive results from sentinel clinical laboratories or laboratory networks to survey the presence of specific microbial agents known to constitute a threat to public health in a given population (7).

According to Colson et al. a total of 31 laboratory-based surveillance systems have been implemented in Europe for the purpose of global surveillance (8). Two such characteristic examples of surveillance systems implemented successfully using the above reporting strategy are the Health Protection Agency in England and Wales which counts infectious pathogens detected by hospital and specialist laboratories (9), and the surveillance systems of the Netherlands Reference Laboratory for Bacterial Meningitis (10).

In Belgium, such a strategy has been launched and implemented in 1983 by Sciensano, formerly known as Scientific Institute of Public Health (WIV-ISP), with the establishment of the sentinel laboratory network collecting information on the epidemiology of infectious diseases (11, 12). With 79–88 participating clinical microbiology laboratories (CMLs) across different geographical areas, the main objective of the Belgian Sentinel Network of Laboratories (BSNL) is to monitor the emergence and evolution of different infectious diseases over time, based on a representative fraction of the microbiology laboratories nationally (13, 14). With a ratio of 60% of participating laboratories to the total number of laboratories, the BSNL is able to describe trends and monitor changes in 12 groups of pathogens both at national and regional levels (15). However, in most European countries such laboratory surveillance is made on a voluntary base and is often not financially covered.

In parallel, the rationalization of public health costs has led to the development of novel strategies for laboratories' cost containment. In this perspective, a number of CMLs have undergone a process of consolidation involving a shift toward laboratory amalgamation and closer real-time informational linkage. Through this consolidation activity, an operational model has emerged with large centralized clinical laboratories performing on one central platform and one or several distal laboratories dealing locally only with urgent analyses. The increasing centralization of diagnostic services over a large geographical region has given rise to the concept of "microbiology laboratories network" (16). The reduction in the number of small clinical laboratories and the aggregation of the remaining ones, may condition the ability to detect epidemiological changes. The sensitivity and representativeness of national surveillance systems should be therefore carefully monitored using coverage measures which indicate the proportion of the target population included within the surveillance system (15).

It is conceivable that the consolidated CMLs could become a cornerstone of public health models in the near future akin to the regional healthcare hospital networks with interactive surveillance for AMR control in France and cross-border regions (17). Due to the adoption of a 24/7 working scheme and improved automation, consolidated CMLs are also able to analyse a large influx of samples in the context of an outbreak investigation. In addition to the volume capacities and the large range of diagnostic tools, the ability of consolidated CMLs to access multiple different partners, geographies and clinical specialities can enhance their capabilities to provide advanced systems for disease surveillance and early recognition.

As there is the potential that such CMLs merging might have a consequence on infectious diseases surveillance, we used the availability of Influenza data to evaluate whether influenza infection trends could be estimated effectively from only one CML serving the Brussels area. The obtained data was compared to the available laboratory surveillance data provided by the directorate Epidemiology and public health of Sciensano in Belgium for the same time period.

MATERIALS AND METHODS

Location

The study took place in the Department of Microbiology of the Laboratoire Hospitalier Universitaire de Bruxelles-University Laboratory of Brussels (LHUB-ULB) serving 5 University

Hospitals located on three geographical poles i.e., Center, North and West of Brussels and representing close to 3,000 beds. Their catchment population covers a population of 700,000 inhabitants according to the Belgian statistical office, where the inhabitants of Brussels area are described for the year 2017 (18). Altogether, the LHUB-ULB performs annually more than 1,200,000 microbiology analysis including viral culture (12,000) and molecular diagnosis (26,000).

Data Collected

In our routine surveillance perspective, all patients diagnosed with flu infection by either ImmunoChromatographic Test (ICT), Lateral Flow Chromatography (LFC), molecular diagnostic tests or by cell culture methods are considered as notifiable cases of flu infection. These laboratory diagnosed cases of flu infection are transferred on a weekly basis to the BSNL by mail. The encoded variables include the diagnosed infectious disease, some patients demographic data allowing the identification of duplicates i.e., date of birth (or previously age), gender, and postal code. In addition the specimen and its sample identification number, the diagnostic method and the date of diagnosis are recorded as well. To protect the patients' identity, all data transfers are anonymous.

To assess the representativeness of the LHUB-ULB flu surveillance, LHUB-UB's data collected from 1st January 2014 to 31st December 2017, were compared with all flu cases notification of the BSNL according to the geographical coverage and time distribution. In addition to BSNL epidemiological data's, weekly reports on the incidence of clinical influenza-like illness (ILI) and virological data collected by sentinel network of general practitioners (SNGPs) were also considered to better describe the epidemiological trends of flu at the regional and national level.

The SNGPs comprises about 120 general practices spread throughout Belgium who weekly report data about eight different health problems (infectious and non-infectious diseases). The coverage of the network is estimated at 1.1–1.5% of the Belgian population. Since 2007, the SNGPs has continuously recorded the general medicine consultations for ILI and acute respiratory tract infections. For each episode age group, vaccination status, outcome and hospitalization are recorded. In a subset of these patients, a clinical sample is collected and virologically tested by the NRC Influenza (https://www.sciensano.be/en/projects/network-general-practitioners).

According to Flu News Europe, the influenza season spent from week 40 to 20 of the following year. The yearly epidemic threshold was derived from historical surveillance and laboratory data using the moving epidemic method (19).

Data from both sources were collected retrospectively and anonymized before analysis in a routine surveillance perspective. Ethics approval was granted by the Ethics Committee of the Saint-Pierre University Hospital. No written informed consent was collected.

To test the validity of LHUB-ULB data as a source for flu surveillance, the flu notification trends of the LHUB-ULB were compared with the trends of the overall BSLN network, BSLN network minus LHUB-ULB notification (BSLN-) and ILI consultation rate by Spearman's rank correlation coefficient within each epidemic season and for the corresponding data over 4 years investigated at the national and regional level. All statistical tests were two-sided with the alpha set at 0.05. Analyses were done with the STATA/IC, version 14.

TABLE 1 | Coverage of the LHUB-ULB notification by municipalities, surface and inhabitants.

Year	Number of municipalities with ≥50% coverage	Covered surface (in km^2) of these municipalities	Number of inhabitants in these municipalities
2014	22	7,655	965,715
2015	29	9,082	1,137,722
2016	32	10,983	1,210,077
2017	21	6,579	950,857

Belgium area: 30,528 km^2.
Belgian population: 11,776,158 inhabitants.

Microbiology Methods

To better assess the input of the LHUB-ULB as early warning lab for molecular surveillance, the genomes of 39 randomly selected Influenza A(H1N1)pdm09 isolates obtained from patients attending the emergency room of Saint-Pierre University Hospital, located in Brussels (01/02/2016 to 15/03/2016), were whole genome sequenced and compared to equivalent Influenza A(H1N1)pdm09 sequences deposited at the Epiflu database for the same location and period and against France, Germany, Netherland and UK-derived sequences from the same time period (Table of all isolate IDs used in this study are included in the **Supplemental Digital Content 1**).

All samples were initially screened for influenza A virus by reverse transcription-PCR targeting the matrix gene. A total of 39 influenza A virus-positive samples had their whole genome sequenced. RNA was amplified using a modified eight-segment method. Library preparations were generated as previously described (20) and short reads were assembled *de novo* (21). A neighbor joining phylogenetic tree was constructed using the Multiple Sequence Alignment Software v.7 (MAFFT) (22).

RESULTS

From 2014 to 2017, 31,809 flu cases were declared by the sentinel laboratories to the BSNL. Among them, 3,186 infections were reported by the LHUB-ULB representing 38.75% (2,746/7,088) and 10.1% (3,186/31,809) of all influenza cases reported by the BSNL in the Brussels region and at the national level, respectively. During the study period, the number of municipalities in which the LHUB-ULB represents at least 50% of coverage ranges from 21 (2017) to 32 (2016) representing 28.1% (21.6–36%) of the Belgium territory and 9.1% (8.1–10.4%) of the Belgium population (**Table 1**). Even if most of the municipalities are located in the Brussels region area; we note that each year the LHUB-ULB represents 100% of the notification of 5 to 10 municipalities located outside of the Brussels area either in Flanders or in Wallonia underscoring the variation in BSNL coverage at provincial level. In another hand, the LHUB-ULB's

coverage was globally lower in municipalities located in Flanders, and for Walloon region in the provinces of Namur, Liege and Luxembourg. **Supplemental Digital Content 2** describes the geographical representativeness of the flu infection's notification by the LHUB-ULB in the BSNL notification for the year 2016.

The representativeness of the LHUB-ULB to describe the trends of the flu notification at the regional and national level is described in **Figure 1**. In order to better describe the flu trends, the incidence ILI based on data's provided by the network of SGPs is also represented.

Overall for the 4 years, the notifications provided by the BSLN closely follow the trends of the incidence of consultations by the SGP for ILI per 100,000 inhabitants with coinciding start, peak and end of the epidemics. In addition, the three sources of data show a comparable course over years and a high correlation within each season, i.e., Spearman's rank correlation for each epidemic season ranged from 0.70 to 0.98 for the Brussels region and at the national level. However, LHUB-ULB's data only were not able to reflect the trends of flu infection for the Flanders and Walloon regions, i.e., the lowest correlation (although still statistically significant) was observed in w40/14 to w39/15 for the Walloon Region (**Supplemental Digital Content 3**).

The representativeness of the LHUB-ULB at the microbiological level was also assessed by comparing the microbiological data's with those of National Influenza Centre for the three consecutive flu seasons (2014–2017). Among the 2,309 respiratory samples sent by the network of SGPs during the study period and analyzed at the National Influenza Centre (NIC), 1,197 (51.8%) were positive for influenza (70.0% influenza A, 23.1% influenza B). Thirty samples were co-infected with influenza A and B. Among the influenza A samples that were subtyped, 25.6% (236/922) were A(H1N1)pdm2009 and 70.4% (649/922) were A(H3N2). Thirty-six samples (7.6%) could not be subtyped due to their low viral load. Of the 277 influenza B samples analyzed, 70.8% (196/277) belonged to the Victoria lineage and 28.8% (80/277) to the Yamagata lineage. During the same period, 72.5% (1,553/2,141) and 28.1% (601/2,141) of influenza viruses detected by the LHUB-ULB overall were type A and type B, respectively. Difference in proportion with the NIC globally and over time were not significant ($p > 0.05$) (**Supplemental Digital Content 4**).

The representativeness of the LHUB-ULB in terms of molecular epidemiology was assessed by the phylogenetic analysis of the sequenced isolates (**Figure 2**). The LHUB-ULB derived Hemagglutinin (HA) sequences ($n = 39$) were compared to all sequences available from GISAID from Belgium for the same time period ($n = 17$) and an equivalent number of sequences from the United Kingdom ($n = 56$), France ($n = 126$), and the Netherland ($n = 36$). Most of the LHUB-ULB samples constitute distinct phylogenetic clusters, co-located with other samples with Belgium as a place of origin, within a background of seasonal Influenza A phylogeny. Preliminary results show that no significant genomic differences were observed within the LHUB-ULB samples or against deposited genomes from the same location and time period in Belgium, the Netherlands and France. However, most LHUB-ULB derived sequences form clusters distinct to the UK-derived samples. This observation would require larger sample numbers than the ones currently available and to be repeated for a number of seasons in order to be further validated. **Figure 2** shows HA sequence analyses, though the LHUB-ULB samples did have the Neuraminidase (NA) genomic information available due to the whole genome sequencing, the availability of NA sequences in the public databases from the same samples was almost entirely unavailable.

DISCUSSION

In the twenty-first century, laboratory-based surveillance benefits from increased use of rapid diagnostic testing including ICT or LFT and multiplex PCR assay and increasingly rapid pathogen identification. The use of such advanced detection tools have dramatically cut the time to accurate diagnosis of infected patient but also increased the knowledge of epidemiological trends in comparison with older diagnostic methods based on culture and serology. The gradual integration of new detection and typing data into the European surveillance and alert systems represents one of the most exciting and challenging developments that could revolutionize the understanding of communicable diseases in the coming years (23).

It is clear that both individual contributors (the ultimate source of payment in fully privatized settings) as well as collective contributors (e.g., governmental interfaces) show a keen interest to such information on infectious disease occurrence. Individual payments (directly or through health insurance coverage) relate to an individual's health status and well-being; collective payments relate to the population-level health and the strategic knowledge of whether a pandemic is beginning. Hence, the interests of both individual and collective contributors are linked as an infection may be indicative of global trends of strategic importance. Therefore, "bigger" might indeed be "better" in the view that the more one knows about an epidemiological threat (of which influenza is the prototype), the better one can take care of the population, even though one may not be able to do much for the individual (as in Ebola outbreaks). If geographical coverage or microbiological tools are limited, detection of these threats may fail, thus CMLs which cover a large area and respond to many microbiological questions can represent an efficient solution (16). However, it is important to stress that one may be big yet in the wrong place or looking at the wrong threat, demonstrating volume in itself is not enough. LHUB-ULB is both centralized and well-located within highly connected and populated areas. As such it is effective in detecting Influenza, spreading very fast due to its high contagiousness and aided by the high population density and fast mobility of large numbers of people. It also concentrates well-focused subject expertise. Therefore, positioning and focus complement the "bigger" aspect.

However, such laboratory surveillance activity is often not financially covered and/or mandatory. Therefore, the cost containment leading to privatization of laboratory medicine may have a significant impact on this laboratory activity which is not directly centered on patient care. For example, in Belgium, the consolidation process leads to a decrease of clinical laboratories from 496 in 1996 to 148 in 2017 with a shift toward large

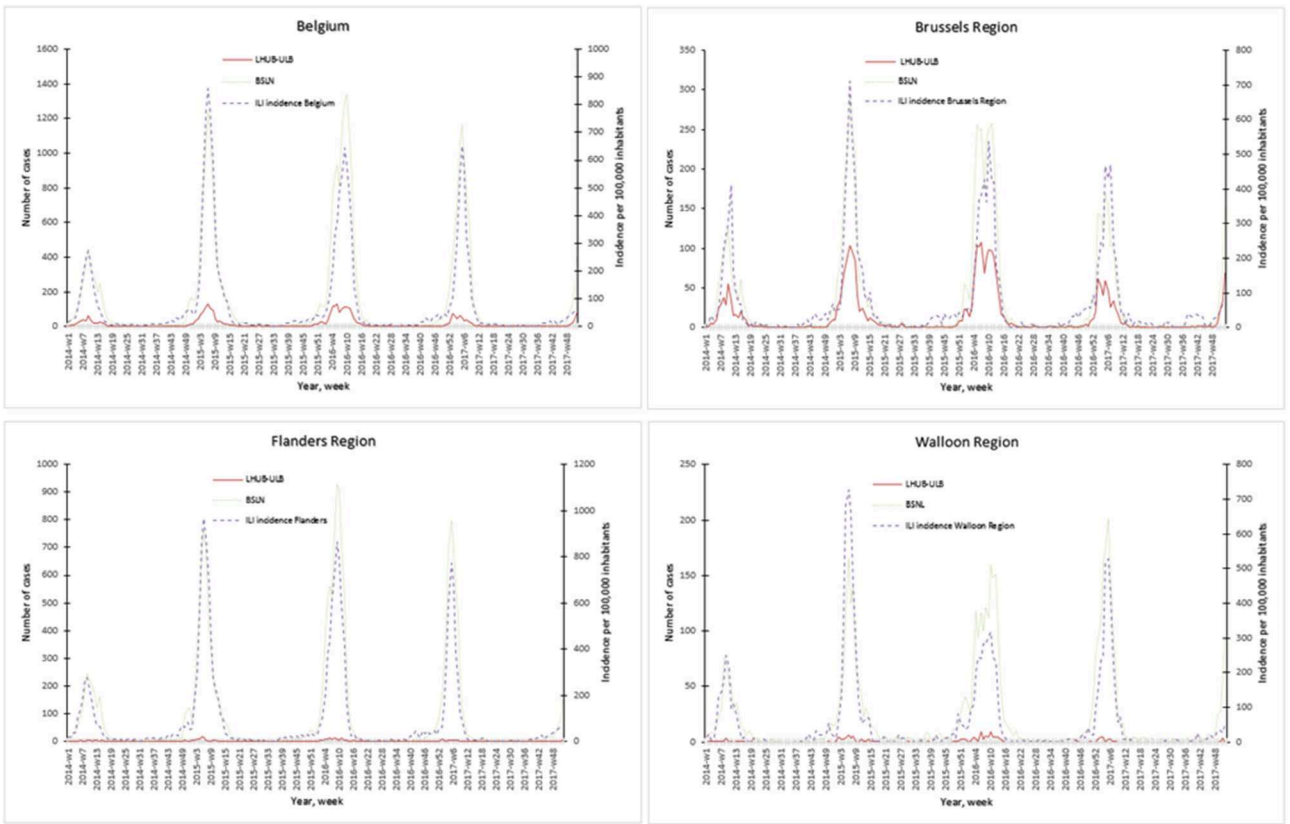


FIGURE 1 | Representativeness of the flu surveillance in the Belgian Sentinel Network of Laboratories using one clinical microbiology laboratory data's.

private laboratory structure. According to data on reimbursed microbiology tests obtained from the Belgian National Institute for Health and Disability Insurance (INAMI-RIZIV) for the period 2010–2015, on average 23.4% (range 22.5–24.3%) of all microbiological analysis (10,616,599 out of 45,355,005) performed were processed by private laboratories in regards to the 76.5% (range 77.4–75.7%) (34,696,723 out of 45,355,005) performed by university or hospital CMLs (Muyldermans G, unpublished data). The latter are more traditionally involved in the non-profit activities, such as research and development and/or public health surveillance.

The use of data from large CMLs may fill this gap that the privatization of the laboratory medicine may beget. One of the CMLs advantage is that more data are obtained from the processing of fewer samples, due to the higher analytical capacity of the consolidated lab, and since the data can be very representative, an increase in information can be achieved without necessitating a proportional increase in costs, even in cost-restricted settings (24). In this study, the handling of the flu data from the LHUB-ULB reveals its attractive features that can facilitate an early detection of seasonal influenza epidemics. These results illustrate that data are not only credible but also advantageous to use for surveillance and prediction purposes, especially for an automatic detection system. Despite, the LHUB-ULB catchment area represents a small geographical area; its representativeness for the nation-wide data is striking. In the future, the extent of representation will be further improved when data are collected from more consolidated laboratories or for a larger microbiological diversity.

In addition, this study confirms the lack of covering by BSLN in some municipalities located in Flanders or in Wallonia (15). In our study, the LHUB-ULB represented each year 100% of the notification in several municipalities located in these regions. The use of laboratory information's provided by one consolidated CML thanks to new molecular tools show to be a good complement of the information's provided by the SGPs and Hospital network and use by the NRC influenza (25, 26). The real-time integration of consolidated CMLs into the public health surveillance system would help the monitoring of influenza activity (intensity, duration, severity, …) all over the year, the determination of type and subtypes of circulating strains and their antigenic and genetic characterization. As such integration could also contribute to the annual determination of the influenza vaccine content, the monitoring of resistance to antivirals and the detection of new potentially pathogenic influenza viruses (27).

In this frame, the use of high-throughput whole genome sequencing platforms available in large CMLs network, such as met in the LHUB-ULB demonstrates its potential for molecular epidemiological surveillance. Because, early detection

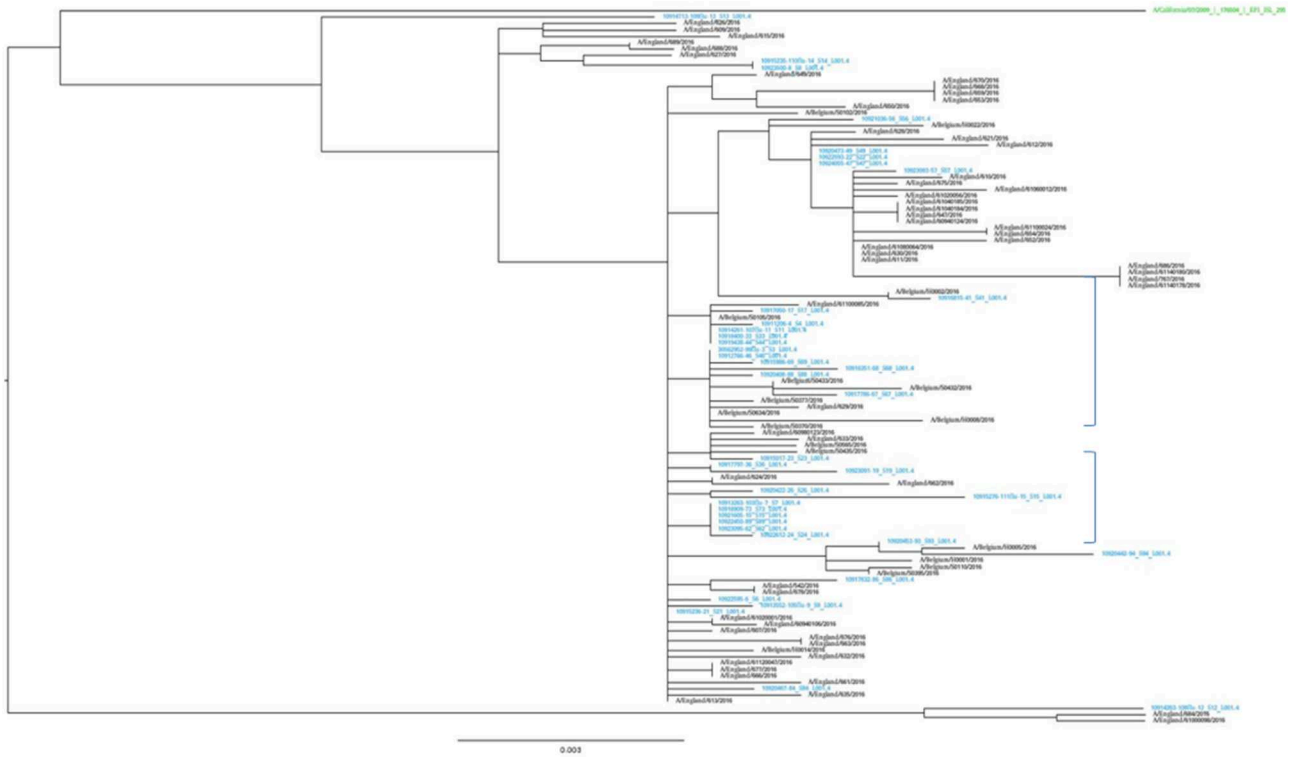


FIGURE 2 | Phylogenetic trees of the HA gene sequences of the 39 Influenza A A(H1N1)pdm09 isolates from LHUB-ULB (shown in Blue), tested in this study compared to the reference strain (shown in green); all 17 sequences with Belgium as a place of origin for the same time period and equivalent number (56) of sequences deposited at the Epiflu database from the UK and same time period. The authors gratefully acknowledge the originating and submitting laboratories who contributed sequences used in the phylogenetic analysis to GISAID (http://www.gisaid.org). In the phylogenetic graph, the two areas containing only samples derived from Belgium (including most of the LHUB-LUB samples) as place of origin are highlighted.

of epidemics is a key element to prevent loss of (quality of) life and its economic and material impact, such molecular surveillance would gain in efficiency through automated real-time monitoring and reporting to public health authorities from the regional to the European levels. Thus, governmental support of such CMLs seems a necessity, in view of the depth of real-time population-level information that can be obtained. Furthermore, other European countries have started demonstrating a clinical benefit from such data collected initiatives, as is the case in the UK at the National Mycobacterial Reference Service in Birmingham (28) and at UCLH with the integration of near real-time, whole genome sequencing utilized for the purposes of HIV and Influenza surveillance (20, 29). In the latter example, such integrated real-time surveillance was not only able to detect the emergence of novel subclade of influenza A(H3N2) virus in London, but also demonstrated its powerful to supplement traditional infection control procedures in the investigation and management of nosocomial outbreaks (19, 30).

However the availability of new microbial typing and detection techniques and culture-independent diagnostic methods, brings about a fundamental change in the way data has to be handled. These approaches are high-throughput and data-rich and create systematic stresses in the collection, analyses and safe handling of the generated data (31). For example one current obstacle toward clinical translation is that most algorithms in use need some programming expertise, together with specialized servers to handle and store all of the data. The generation of user-friendly informatics tools to effectively analyse high-throughput genomic data will be essential to the successful clinical application of genomic technology. In addition there are systemic aspects that also need to be addressed, such as the number of additional molecular or genomic testing parameters, regardless of the method, which can be supported routinely by the existing electronic health records that provide the architectural framework. These aspects can include the ordering of the test, the receiving of a document that summarizes the clinical interpretation, and storage of the interpretation (32). The integration of molecular genetic data to clinical and/or epidemiological data creates a challenge that requires interactive, information-sharing workspaces rather than uni-directional centralized reporting, such as those deployed in the TYPENED approach (33).

CONCLUSION

Our results suggest that consolidated CMLs represent a wealth of information, including data usable for public health surveillance. The advent of real-time sequencing of organisms, their direct

integration in surveillance tools at the regional, national and European levels would lead to real-time detection and alert, allowing the rapid prioritization of public health threats and the timely implementation of control strategies

AUTHOR CONTRIBUTIONS

SV wrote the initial draft and supervised the manuscript revisions. NB and MW analyzed data and contributed to writing. BF performed the next generation sequencing experiments in the laboratory. LB and IT supervised the validity of virological results at the clinical level and National Reference Centre, respectively. GS, YD, and MB analyzed data and co-wrote the manuscript. EN designed the study, analyzed data, and co-wrote the manuscript. MH coordinates the reliability of all laboratory results provided by LHUB-ULB, wrote substantial paragraphs, and provided critical review and commentary. ZK coordinated the whole genome sequencing pipeline for ICONIC. OV had the rationale for this work. All three co-senior authors contributed constructively in the writing of the manuscript and offered equally valuable advice for the discussion part of the manuscript.

ACKNOWLEDGMENTS

We wish to thank the personnel of the Department of Microbiology of LHUB-ULB for its daily technical assistance. This work has been supported in part by a UCL Global Engagement Fund award to OV and ZK, which is gratefully acknowledged. The authors would like to thank Gaetan Muyldermans for his help in the preparation of the manuscript. This work was partly presented at the 28th European Congress of Clinical Microbiology and Infectious Diseases, 2018 April 21–24, Madrid, Spain. p.112: P0001.

Where authors are identified as personnel of the International Agency for Research on Cancer/WHO, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/WHO.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2019.00150/full#supplementary-material

REFERENCES

1. Simonsen L, Spreeuwenberg P, Lustig R, Taylor RJ, Fleming DM, Kroneman M, et al. Global mortality estimates for the 2009 influenza pandemic from the GLaMOR project: a modelling study. *PLoS Med.* (2013) 10:e1001558. doi: 10.1371/journal.pmed.1001558
2. Dawood FS, Iuliano AD, Reed C, Meltzer MI, Shay DK, Cheng P-Y, et al. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *Lancet Infect Dis.* (2012) 12:687–95. doi: 10.1016/S1473-3099(12)70121-4
3. Hotez PJ, Alvarado M, Basáñez MG, Bolliger I, Bourne R, Boussinesq M, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. *PLoS Negl Trop Dis.* (2014) 8:e2865. doi: 10.1371/journal.pntd.0002865
4. Keech M, Beardsworth P. The impact of influenza on working days lost: a review of the literature. *Pharmacoeconomics.* (2008) 26:911–24. doi: 10.2165/00019053-200826110-00004
5. King AA, Domenech de Cellès M, Magpantay FM, Rohani P. Avoidable errors in the modelling of outbreaks of emerging pathogens, with special reference to Ebola. *Proc Biol Sci.* (2015) 282:20150347. doi: 10.1098/rspb.2015.0347
6. Heesterbeek H, Anderson RM, Andreasen V, Bansal S, De Angelis D, Dye C, et al. Modeling infectious disease dynamics in the complex landscape of global health. *Science.* (2015) 347:aaa4339. doi: 10.1126/science.aaa4339
7. Zeng D, Chen H, Castillo-Chavez C, Thurmond M. Clinical laboratory data for biosurveillance. In: Zeng D, Chen H, Castillo-Chavez C, Lober WB, Thurmond M, editors. *Infectious Disease Informatics and Biosurveillance*. New York, NY: Springer (2011). p. 67–87. doi: 10.1007/978-1-4419-6892-0
8. Colson P, Rolain JM, Abat C, Charrel R, Fournier PE, Raoult D. EPIMIC: a simple homemade computer program for real-time epidemiological surveillance and alert based on microbiological data. *PLoS ONE.* (2015) 10:e0144178. doi: 10.1371/journal.pone.0144178
9. Enki DG, Noufaily A, Garthwaite PH, Andrews NJ, Charlett A, Lane C, et al. Automated biosurveillance data from England and Wales, 1991–2011. *Emerg Infect Dis.* (2013) 19:35–42. doi: 10.3201/eid1901.120493
10. Bijlsma MW, Bekker V, Brouwer MC, Spanjaard L, van de Beek D, van der Ende A. Epidemiology of invasive meningococcal disease in the Netherlands, 1960–2012: an analysis of national surveillance data. *Lancet Infect Dis.* (2014) 14:805–12. doi: 10.1016/S1473-3099(14)70806-0
11. Grammens T, Schirvel C, Leenen S, Shodu N, Hutse V, Mendes da Costa E, et al. Ongoing measles outbreak in Wallonia, Belgium, December 2016 to March 2017: characteristics and challenges. *Euro Surveill.* (2017) 22:30524. doi: 10.2807/1560-7917.ES.2017.22.17.30524
12. Verhaegen J, Flamaing J, De Backer W, Delaere B, Van Herck K, Surmont F, et al. Epidemiology and outcome of invasive pneumococcal disease among adults in Belgium, 2009–2011. *Euro Surveill.* (2014) 19:14–22. doi: 10.2807/1560-7917.ES2014.19.31.20869
13. Muyldermans G, Ducoffre G, Leroy M, Dupont Y, Quolin S, Participating Sentinel Laboratories. Surveillance of infectious diseases by the Sentinel Laboratory Network in Belgium: 30 years of continuous improvement. *PLoS ONE.* (2016) 11:e0160429. doi: 10.1371/journal.pone.0160429
14. Walckiers D, Stroobant A, Yourassowsky E, Lion J, Cornelis R. A sentinel network of microbiological laboratories as a tool for surveillance of infectious diseases in Belgium. *Epidemiol Infect.* (1991) 106:297–303. doi: 10.1017/S0950268800048445
15. Berger N, Muyldermans G, Dupont Y, Quoilin S. Assessing the sensitivity and representativeness of the Belgian Sentinel Network of Laboratories using test reimbursement data. *Arch Public Health.* (2016) 74:29. doi: 10.1186/s13690-016-0145-9
16. Vandenberg O, Kozlakidis Z, Schrenzel J, Struelens MJ, Breuer J. Control of infectious diseases in the era of European Clinical Microbiology Laboratory consolidation: new challenges and opportunities for the patient and for public health surveillance. *Front Med (Lausanne).* (2018) 5:15. doi: 10.3389/fmed.2018.00015
17. Zhou K, Lokate M, Deurenberg RH, Tepper M, Arends JP, Raangs EG, et al. Use of whole-genome sequencing to trace, control and characterize the regional expansion of extended-spectrum β-lactamase producing ST15 *Klebsiella pneumoniae*. *Sci Rep.* (2016) 6:20840. doi: 10.1038/srep20840
18. Statbel. *The Belgian Statistical Office.* Available online at: https://statbel.fgov.be/fr (accessed April 24, 2018).
19. Vega T, Lozano JE, Meerhoff T, Snacken R, Beauté J, Jorgensen P, et al. Influenza surveillance in Europe: comparing intensity levels calculated using the moving epidemic method. *Influenza Other Respir Viruses.* (2015) 9:234–46. doi: 10.1111/irv.12330
20. Harvala H, Frampton D, Grant P, Raffle J, Ferns RB, Kozlakidis Z, et al. Emergence of a novel subclade of influenza A(H3N2) virus in

London, December 2016 to January 2017. *Euro Surveill.* (2017) 22:30466. doi: 10.2807/1560-7917.ES.2017.22.8.30466
21. Hunt M, Gall A, Ong SH, Brener J, Ferns B, Goulder P, et al. IVA: accurate de novo assembly of RNA virus genomes. *Bioinformatics.* (2015) 31:2374–6. doi: 10.1093/bioinformatics/btv120
22. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol.* (2013) 30:772–80. doi: 10.1093/molbev/mst010
23. Struelens MJ, Brisse S. From molecular to genomic epidemiology: transforming surveillance and control of infectious diseases. *Euro Surveill.* (2013) 18:20386. doi: 10.2807/ese.18.04.20386-en
24. Kiyaga C, Sendagire H, Joseph E, Grosz J, McConnell I, Narayan V, et al. Consolidating HIV testing in a public health laboratory for efficient and sustainable early infant diagnosis (EID) in Uganda. *J Public Health Policy.* (2015) 36:153–69. doi: 10.1057/jphp.2015.7
25. Van Casteren V, Mertens K, Antoine J, Wanyama S, Thomas I, Bossuyt N. Clinical surveillance of Influenza A(H1N1)2009 pandemic through the network of Sentinel General Practitioners. *Arch Public Health.* (2010) 68:62–7. doi: 10.1186/0778-7367-68-2-62
26. Gérard C, Brochier B, Quoilin S, Wuillaume F, Van Casteren V, Thomas I. Virological surveillance of the influenza A (H1N1) 2009 pandemic: the role of the Belgian National Influenza Centre. *Arch Public Health.* (2010) 68:68–75. doi: 10.1186/0778-7367-68-2-68
27. Broberg E, Hungnes O, Schweiger B, Prosenc K, Daniels R, Guiomar R, et al. Improving influenza virological surveillance in Europe: strain-based reporting of antigenic and genetic characterisation data, 11 European countries, influenza season 2013/14. *Euro Surveill.* (2016) 21:30370 doi: 10.2807/1560-7917.ES.2016.21.41.30370
28. Quan TP, Bawa Z, Foster D, Walker T, Del Ojo Elias C, Rathod P, et al. Evaluation of whole-genome sequencing for mycobacterial species identification and drug susceptibility testing in a clinical setting: a large-scale prospective assessment of performance against line probe assays and phenotyping. *J Clin Microbiol.* (2018) 56:e01480-17. doi: 10.1128/JCM.01480-17
29. Yebra G, Frampton D, Gallo Cassarino T, Raffle J, Hubb J, Ferns RB, et al. A high HIV-1 strain variability in London, UK, revealed by full-genome analysis: results from the ICONIC project. *PLoS ONE.* (2018) 13:e0192081. doi: 10.1371/journal.pone.0192081
30. Houlihan CF, Frampton D, Ferns RB, Raffle J, Grant P, Reidy M, et al. Use of whole-genome sequencing in the investigation of a nosocomial influenza virus outbreak. *J Infect Dis.* (2018) 218:1485–9. doi: 10.1093/infdis/jiy335
31. Goldberg B, Sichtig H, Geyer C, Ledeboer N, Weinstock GM. Making the leap from research laboratory to clinic: challenges and opportunities for next-generation sequencing in infectious disease diagnostics. *MBio.* (2015) 6:e01888-15. doi: 10.1128/mBio.01888-15
32. Masys DR, Jarvik GP, Abernethy NF, Anderson NR, Papanicolaou GJ, Paltoo DN, et al. Technical desiderata for the integration of genomic data into Electronic Health Records. *J Biomed Inform.* (2012) 45:419–22. doi: 10.1016/j.jbi.2011.12.005
33. Niesters HG, Rossen JW, van der Avoort H, Baas D, Benschop K, Claas EC, et al. Laboratory-based surveillance in the molecular era: the TYPENED model, a joint data-sharing platform for clinical and public health laboratories. *Euro Surveill.* (2013) 18:20387. doi: 10.2807/ese.18.04.20387-en

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 September 2019
doi: 10.3389/fmicb.2019.02031

Soluble Recombinant Hemagglutinin Protein of H1N1pdm09 Influenza Virus Elicits Cross-Protection Against a Lethal H5N1 Challenge in Mice

*Shinya Yamada[1], Atsuhiro Yasuhara[1] and Yoshihiro Kawaoka[1,2,3]**

[1]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [2]Department of Pathobiological Sciences, School of Veterinary Sciences, Influenza Research Institute, University of Wisconsin-Madison, Madison, WI, United States, [3]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Currently, influenza vaccines are produced using embryonated chicken eggs. Recently, recombinant influenza vaccines have been developed as a potential alternative to egg-grown vaccines. In this study, we evaluated the efficacy of soluble recombinant hemagglutinin (HA) protein produced in human cell culture (Expi293F cells) as an influenza vaccine against homosubtypic and heterosubtypic influenza virus challenges in mice. Mice were immunized intramuscularly with purified soluble HA protein of H1N1pdm09 virus and then challenged with a lethal dose of H1N1pdm09, seasonal H3N2, or highly pathogenic avian influenza (HPAI) H5N1 virus. Vaccinated mice showed better morbidity than mock-vaccinated mice following H1N1pdm09 challenge. By contrast, all mice died following H3N2 challenge. Interestingly, all vaccinated mice survived challenge with H5N1 virus, whereas all mock-vaccinated mice died. These results suggest that intramuscular immunization with recombinant HA proteins produced in Expi 293F cells could be of value in influenza vaccine strategies.

Keywords: influenza, vaccines, recombinant hemagglutinin protein, intramuscular immunization, cross-protection

OPEN ACCESS

Edited by:
Ville Peltola,
Turku University Hospital, Finland

Reviewed by:
Masatoshi Okamatsu,
Hokkaido University, Japan
Rabeh El Shesheny,
St. Jude Children's Research Hospital, United States

***Correspondence:**
Yoshihiro Kawaoka
yoshihiro.kawaoka@wisc.edu

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *11 January 2019*
Accepted: *19 August 2019*
Published: *04 September 2019*

Citation:
Yamada S, Yasuhara A and Kawaoka Y (2019) Soluble Recombinant Hemagglutinin Protein of H1N1pdm09 Influenza Virus Elicits Cross-Protection Against a Lethal H5N1 Challenge in Mice. Front. Microbiol. 10:2031. doi: 10.3389/fmicb.2019.02031

INTRODUCTION

Vaccination is the main public health strategy used to prevent and control influenza. Influenza vaccines are currently produced by propagating selected vaccine seed viruses in embryonated chicken eggs. However, recent vaccine seed viruses have changed their antigenicity through egg adaptation, causing reduced vaccine effectiveness (Katz and Webster, 1989; Wang et al., 1989; Rajakumar et al., 1990; Skowronski et al., 2014). Cell-based vaccine production may offer a solution to this problem. Recently, recombinant proteins have been produced in mammalian cells, insect cells, plant cells, and *E. coli* as an alternative strategy for vaccine development (Lin et al., 2008; Wei et al., 2008; Biesova et al., 2009; Chiu et al., 2009; Shoji et al., 2009; Song et al., 2009; Cornelissen et al., 2010; Kalthoff et al., 2010; Du et al., 2011; Khurana et al., 2011; Chen et al., 2013; Prabakaran et al., 2013; Wohlbold et al., 2015; Ge et al., 2016; Sim et al., 2016; Dunkle et al., 2017). As an added advantage, the absence of egg proteins in cell-based vaccines eliminates the potential for egg-related allergic reactions. In addition, cell-based vaccines can be stably produced with a shorter lead time than the 6 months required

for egg-based vaccine production, and production would not be affected by the supply of eggs (Milian and Kamen, 2015). A recent report showed that a recombinant influenza vaccine provided 30% greater efficacy than a standard inactivated influenza vaccine against influenza H3N2 in a clinical trial with a total of 9,003 recipients 50 years of age or older (Dunkle et al., 2017). However, in many of these studies, the recombinant proteins were produced by using a baculovirus expression system. A previous report showed that hemagglutinins (HAs) produced in 293T cells induce higher hemagglutination inhibition (HI) antibody titers compared with those produced in insect cells (de Vries et al., 2012). Expi293F cells, which were developed to produce a large amount of recombinant protein, are derived from the 293 cell line (Jain et al., 2017). We, therefore, used this cell line for our study to produce a recombinant HA protein. However, the vaccine efficacy of a recombinant HA produced in Expi293F cells has not been fully explored.

In many previous protection studies involving immunization with recombinant proteins, only the efficacy against the homologous challenge was examined. Although some studies showed cross-protection in mice against heterologous H5 virus challenge, the results were not obtained through intramuscular immunization of the recombinant HA protein but *via* intranasal infection with cold-adapted H1N1pdm09 influenza virus or baculoviruses that possessed influenza HA proteins (H1N1pdm09) (Jang et al., 2012; Sim et al., 2016). In addition, attenuated H5 viruses (not highly pathogenic H5 viruses) were used in those studies. One study has shown that intramuscular immunization with recombinant headless HA protein protects mice from heterologous virus challenge with H5N1 virus, but, again, attenuated H5N1 virus was used in the challenge experiment (Wohlbold et al., 2015). Therefore, the efficacy of the recombinant HA protein of seasonal influenza virus against heterologous highly pathogenic H5N1 virus challenges as a vaccine has not been fully studied. Accordingly, in this study, we sought to evaluate the efficacy of a soluble form of the recombinant HA protein of seasonal H1N1pdm09 virus, expressed in mammalian Expi293F cells, in terms of its cross-protection of intramuscularly immunized mice against heterologous virus challenges with highly pathogenic H5N1 virus and seasonal H3N2 virus.

MATERIALS AND METHODS

Ethics and Biosafety Statements

Human blood was collected in accordance with protocols that were approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo. Written informed consent was obtained from all participants. All experiments with H5N1 viruses were performed in biosafety level 3 (BSL3) laboratories at the University of Tokyo, which are approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan. All experiments with mice were performed in accordance with the University of Tokyo's Regulations for Animal Care and Use and were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo.

Cells

Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (MEM) containing 5% newborn calf serum (NCS). Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. Expi293F cells (Thermo Fisher Scientific), maintained in Expi293 expression medium (Thermo Fisher Scientific), were incubated on an orbital shaker platform rotating at 125 rpm at 37°C under 8% CO_2.

Viruses

Mouse-adapted A/California/04/2009 (MA-CA04; H1N1pdm09) (Sakabe et al., 2011), mouse-adapted A/Aichi/2/1968 (MA-Aichi; H3N2), and A/Vietnam/1203/2004 (VN1203; H5N1) were propagated in MDCK cells or eggs and titrated in MDCK cells.

Cloning, Expression, and Purification of Recombinant Hemagglutinin Protein

A gene fragment encoding the HA protein of MA-Ca04 without the transmembrane or cytoplasmic tail domain was amplified by PCR and cloned into the pCAGGS vector with a six-His tag coding sequence at the C-terminus. Primer sequences are available upon request. The plasmid was transfected into Expi293F cells by using ExpiFectamine 293 (Thermo Fisher Scientific) according to the manufacturer's protocol. At 4–6 days post-transfection, supernatants were cleared by low-speed centrifugation (900 rpm, 4°C, 5 min) and incubated with Ni-nitrilotriacetic acid (NTA) resin (Invitrogen) according to the manufacturer's instructions. Subsequently, SDS-PAGE and western blot analysis with an anti-His-tag mouse monoclonal antibody (MBL, Medical & Biological Laboratories) were performed. The concentration of the purified soluble MA-Ca04 HA protein (sMA-Ca04HA) was measured by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Immunofluorescence Assay

Twenty-four hours after transfection with plasmids, 293 cells were washed with PBS and fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X100. Cells were reacted with a mixture of anti-H1N1pdm09 HA mouse monoclonal antibodies (3E11C8, 5G10F, 7C2G7, 9C4C11), which were prepared in our laboratory, and with an anti-His-tag monoclonal antibody (MBL, Medical & Biological Laboratories). The cells were then incubated with Alexa Fluor 546 goat anti-mouse immunoglobulin G (Invitrogen).

Enzyme-Linked Immunosorbent Assay

Ninety-six-well microtiter plates were coated with 2 μg/ml purified sMA-Ca04HA and then incubated with serially 5-fold diluted human or rabbit antibodies (starting at 5 μg/ml). We used two pdmH1-specific human monoclonal antibodies (10-5-64/6 and R4-5-72/1); an anti-H3HA human monoclonal antibody (1429D88/2), which was screened and established from human peripheral blood mononuclear cells (PBMCs) in our laboratory; CR9114, which broadly recognizes a conserved epitope in the

stalk region; and an H5HA-specific rabbit monoclonal antibody (clone ID89, Sino Biological Inc.). After a 2-h incubation at 4°C, the plates were washed three times with ice-cold PBS, and then incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG or mouse anti-rabbit IgG (Jackson Immuno Research) (1:2,000 dilution) at 4°C for 1 h. The plates were then incubated with OPD (*O*-phenylenediamine, Sigma) in PBS containing 0.01% H_2O_2 for 10 min at room temperature, and the reaction was stopped with 0.05 ml of 1 N HCl. Absorbance was determined at 490 nm.

Enzyme-Linked Immunosorbent Assay for Mouse Sera

Serum samples were collected from vaccinated mice and mock-vaccinated mice 2 weeks after the second and third immunizations. Ninety-six-well microtiter plates were coated with 2 μg/ml purified sMA-Ca04HA or recombinant HA derived from either A/Perth/16/2009 (H3N2) or A/Vietnam/1203/2004 (H5N1) (Sino Biological). After being blocked with 5-fold-diluted Blocking One (Nakarai), the plates were incubated with serially 4-fold diluted serum samples. After a 2-h incubation at 4°C, the plates were washed three times with ice-cold PBS, and then incubated with HRP-conjugated rabbit anti-mouse IgG (1:2,000 dilution) at 4°C for 1 h. The plates were then incubated with OPD in PBS containing 0.01% H_2O_2 for 10 min at room temperature, and the reaction was stopped with 0.05 ml of 1 N HCl. Absorbance was determined at 490 nm.

Immunization and Virus Challenge

Five 6-week-old female BALB/c mice (Japan SLC) per group were intramuscularly immunized with 10 μg of purified HA protein adjuvanted with 50 μl of AddaVax (InvivoGen) three times with a 10- to 14-day interval between vaccinations. As a control, mice in the non-immunized group were injected with the same volume of PBS. Serum samples were collected 2 weeks after the second and third immunizations. To assess the protective efficacy of vaccination with sMA-Ca04HA, mice were intranasally infected with 10 mouse median lethal doses (10 MLD_{50}) of mouse-adapted A/California/04/2009 (MA-CA04; H1N1pdm09), mouse-adapted A/Aichi/2/1968 (MA-Aichi; H3N2), or A/Vietnam/1203/2004 (VN1203; H5N1) under anesthesia 2 weeks after the final boost immunization. Morbidity and mortality were monitored for 13 days after challenge. Mice with body weight loss of more than 25% of their baseline body weight were euthanized.

Virus Neutralization Assay

Serum samples collected from three vaccinated mice or three mock-vaccinated mice, which were pretreated with a receptor-destroying enzyme (RED II; Denka Seiken, Tokyo, Japan), in duplicate, were serially two-fold diluted with MEM containing 0.3% bovine serum albumin (BSA-MEM) prior to being mixed with 100 $TCID_{50}$ (50% tissue culture infectious doses) of mouse-adapted A/California/04/2009 (H1N1pdm09), mouse-adapted A/Aichi/2/1968 (H3N2), or A/Vietnam/1203/2004 (H5N1) at 37°C for 30 min. The mixtures were then inoculated into MDCK cells and incubated for 1 h at 37°C. BSA-MEM containing TPCK-treated trypsin was added to each well and the cells were incubated for 3 days at 37°C. The cytopathic effect (CPE) was examined, and the neutralization titer was determined as the reciprocal of the highest serum dilution.

RESULTS

Generation and Characterization of Soluble Recombinant H1N1pdm09 Hemagglutinin Protein

To express soluble recombinant H1N1pdm2009 HA (pdmH1HA) in mammalian cells, the ectodomain of mouse-adapted A/California/04/2009HA (MA-Ca04HA) (Sakabe et al., 2011) and a His-tag were cloned into the pCAGGS expression vector (**Figure 1A**), which was then transfected into Expi293F cells. The secreted HA was purified using Ni-NTA agarose chromatography. The expression and secretion of the soluble MA-Ca04HA protein with the His tag (sMA-Ca04HA) was examined by means of gel electrophoresis followed by western blotting using an antibody against the His-tag. The results showed that sMA-Ca04HA was successfully secreted into the supernatant with an expected molecular mass of 75 kDa (**Figure 1B**). Immunofluorescence analysis showed anti-pdmH1HA monoclonal antibody binding to the expressed HA protein, suggesting that the HA protein had folded properly (**Figure 1C**). We also examined the binding properties of several other antibodies to the soluble MA-CA04HA by using an ELISA (**Figure 1D**). We used two pdmH1HA-specific human monoclonal antibodies (10-5-64/6 and R4-5-72/1), which were screened and established from human PBMCs in our laboratory (Kubota-Koketsu et al., 2009); CR9114, which broadly recognizes a conserved epitope in the stalk region (Dreyfus et al., 2012); an anti-H3HA human monoclonal antibody (1429D88/2), which was also screened and established from human PBMCs in our laboratory (Kubota-Koketsu et al., 2009); and an H5HA-specific rabbit monoclonal antibody (clone ID89, Sino Biological Inc.). The two pdmH1HA-specific human monoclonal antibodies and CR9114 bound to sMA-Ca04HA, but the anti-H3HA and anti-H5HA monoclonal antibodies did not. These results suggest that sMA-Ca04HA retained the conformation and antigenicity of the HA protein of A/California/04/2009.

Efficacy of sMA-Ca04HA as a Vaccine Against Lethal Infection in Mice

To evaluate the immunogenicity of sMA-Ca04HA, naïve 6-week-old BALB/c mice were vaccinated intramuscularly with 10 μg of purified sMA-Ca04HA protein adjuvanted with 50 μl of AddaVax (InvivoGen) (Goff et al., 2013) three times with a 10- to 14-day interval between vaccinations. As a control, mice in the non-immunized group were injected with the same volume of PBS. Serum samples were collected 2 weeks after the second and third immunizations. We examined the seroreactivity induced by sMA-Ca04HA by using an ELISA against purified HA proteins (**Figures 2A–C**). Mice vaccinated with sMA-Ca04HA elicited

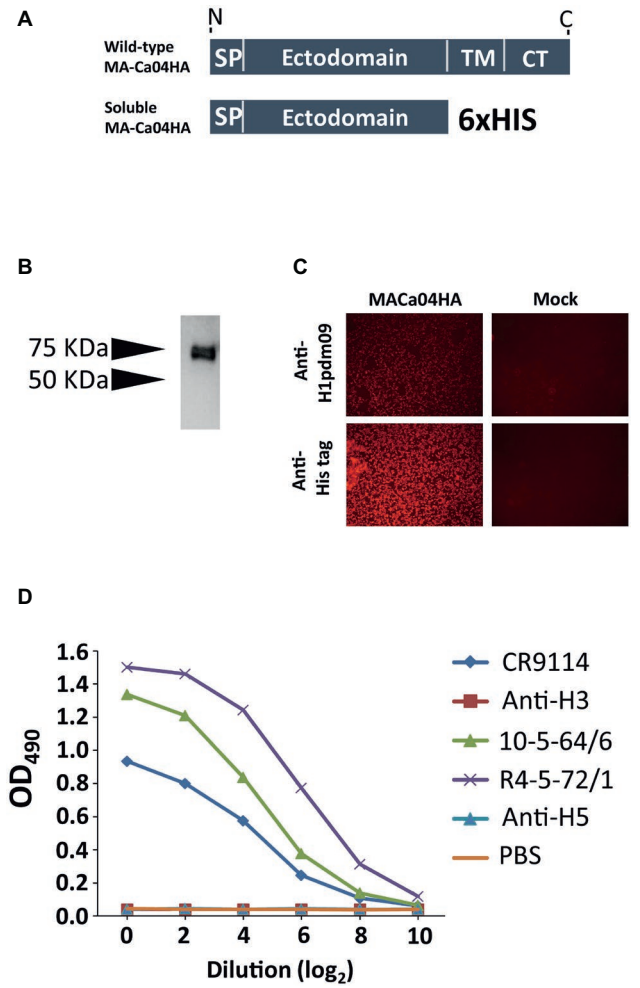


FIGURE 1 | Characterization and purification of soluble recombinant HA protein. **(A)** Schematic of wild-type HA and the constructed soluble HA (SP, signal peptide domain; TM, transmembrane domain; CT, cytoplasmic domain). **(B)** The ectodomain of the HA from H1N1pdm09 virus with a His tag was expressed in Expi293F cells; purified soluble HA protein was obtained by means of SDS-PAGE under denaturing and reducing conditions. The expression of HA was analyzed by western blotting with an anti-His-tag mouse monoclonal antibody. **(C)** 24 h after plasmid transfection, 293 cells were fixed and stained with anti-H1N1pdm09HA monoclonal (3E11C8, 5G10F, 7C2G7, 9C4C11) and anti-His-tag monoclonal antibodies. **(D)** Binding properties of antibodies to soluble MA-CA04HA, as measured by ELISA. Two pdmH1-specific human monoclonal antibodies (10-5-64/6 and R4-5-72/1), an anti-H3HA human monoclonal antibody (1429D88/2), an HA-stalk-specific antibody (CR9114), and an H5HA-specific rabbit monoclonal antibody (clone ID89) were examined.

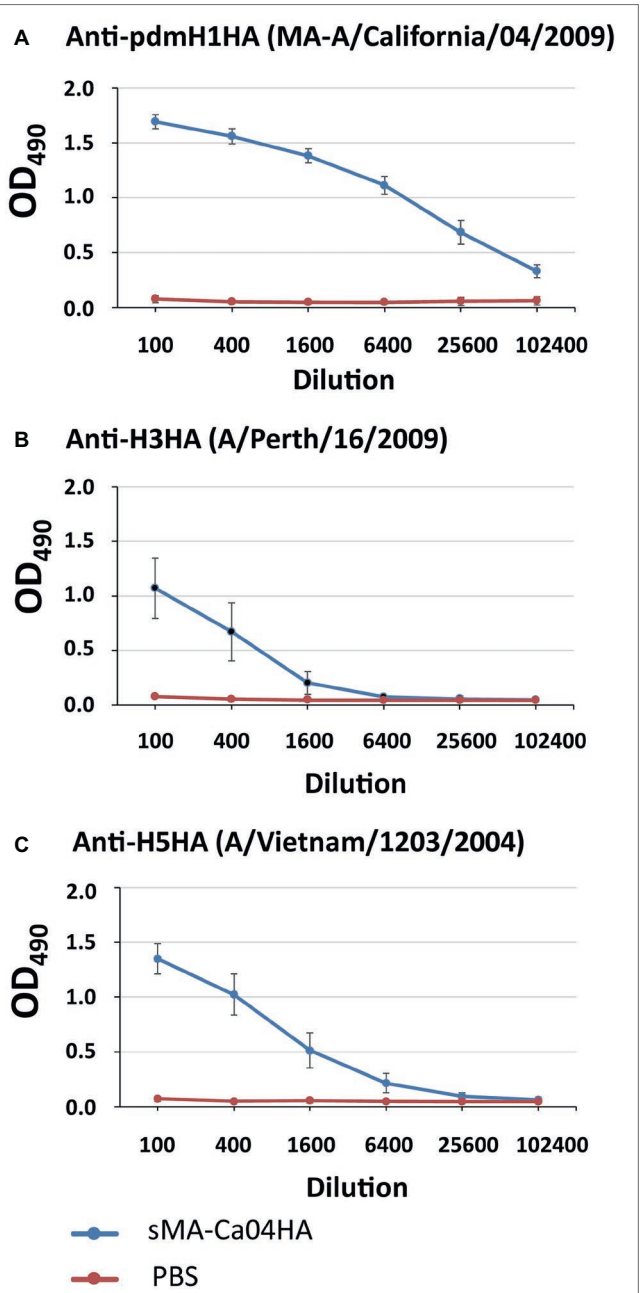


FIGURE 2 | Antibody responses against influenza HA proteins in immunized mice. Sera from mice vaccinated with sMA-Ca04HA were analyzed by means of an ELISA against the purified HA proteins of H1N1pdm09 **(A)**, H3N2 **(B)**, and H5N1 **(C)** virus.

high antibody titers against sMA-Ca04HA (**Figure 2A**). To determine whether immunization with sMA-Ca04HA could increase the breadth of reactivity, we analyzed heterologous seroreactivity against HA proteins from H5N1 and H3N2 viruses. The sera from sMA-Ca04HA-vaccinated mice showed reactivity in the ELISA to both H3HA and H5HA protein (**Figures 2B,C**). In contrast, mice injected with PBS elicited no antibodies reactive to the test HA proteins. To determine whether immunization with sMA-Ca04HA could induce neutralizing antibodies against homosubtypic H1N1pdm09 virus and heterosubtypic H3N2 and H5N1 viruses, we performed neutralization assays using sera obtained from three mice immunized with the recombinant H1N1pdm09 HA (**Table 1**). In the assay, we detected neutralizing activity against homosubtypic H1N1pdm09 virus but not against H3N2 or H5N1 viruses. To assess the protective efficacy of sMA-Ca04HA as a vaccine, we performed a viral challenge study (**Figures 3A–C**). Mice were infected with 10 MLD_{50} of mouse-adapted A/California/04/2009 (MA-CA04; H1N1pdm09),

TABLE 1 | Virus neutralization by serum of mice immunized with sMA-Ca04HA.

	Microneutralization titer using post-immunization mouse antisera against		
Mice inoculated with	**MA-A/California/04/2009**	**MA-A/Aichi/2/1968**	**A/Vietnam/1203/2004**
sMA-Ca04HA	2,560, 5,120, 10,240	<10, <10, <10	<10, <10, <10
PBS	<10, <10, <10	<10, <10, <10	<10, <10, <10

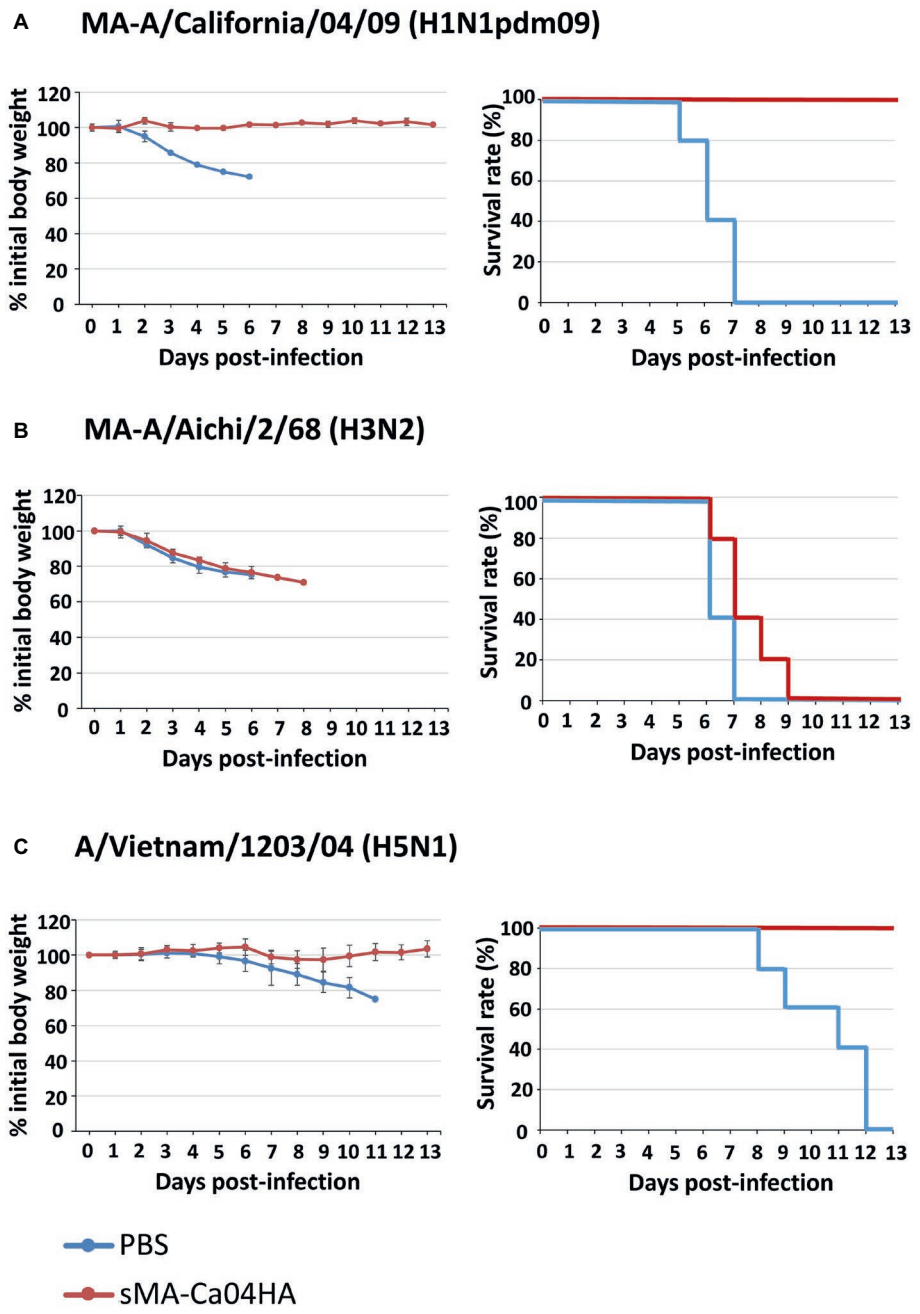


FIGURE 3 | Body weight changes and survival of mice after virus challenge. Five mice per group were mock-immunized with PBS or immunized with sMA-Ca04HA three times with a 10- to 14-day interval between vaccinations. Two weeks after the final vaccination, the mice were intranasally challenged with 10 MLD_{50} of MA-A/California/04/09 **(A)**, MA-A/Aichi/2/68 **(B)** or A/Vietnam/1203/04 **(C)**. Body weight (left) and survival (right) were monitored for 13 days after challenge. Values are expressed as mean changes in body weight ± SD (n = 5).

mouse-adapted A/Aichi/2/68 (MA-Aichi; H3N2), or A/Vietnam/1203/2004 (VN1203; H5N1) 2 weeks after their final boost vaccination. Upon challenge with MA-CA04, all mice vaccinated with sMA-Ca04HA showed no weight loss and survived, whereas mock-vaccinated mice lost more than 20% of their initial body weight and all of the mice died by day 7 post-infection (**Figure 3A**). In contrast, viral challenge with MA-Aichi/2/68 (H3N2) caused all vaccinated and mock-vaccinated mice to succumb to their infections within 9 days of challenge (**Figure 3B**). Interestingly, upon challenge with Vietnam/1203/04 (H5N1), all vaccinated mice survived and showed moderate weight loss, whereas the mock-vaccinated mice began to succumb to their infections at 8 days post-infection and all of these mice died within 12 days of challenge (**Figure 3C**). Our results indicate that this soluble recombinant HA protein mediates limited cross-protection against heterosubtypic influenza virus infection.

DISCUSSION

In this report, we showed that intramuscular inoculation of a recombinant Expi293F cell-based influenza vaccine candidate that comprised the ectodomain of H1N1pdm09 virus HA induced antibodies against the HAs of H1N1pdm09, H3N2, and highly pathogenic H5N1 viruses and provided protection against homosubtypic H1N1pdm09 and heterosubtypic H5N1 challenge in mice. Although sMA-Ca04HA immunization induced antibodies against H3HA protein, vaccinated mice were not protected from H3N2 challenge. H1 and H5 belong to group 1, whereas H3 belongs to group 2 of the phylogenetic groupings of hemagglutinin. Therefore, sMA-Ca04HA might induce antibodies against epitopes in the stalk region conserved among group 1 members that efficiently protect mice from challenge with viruses belonging to group 1. Interestingly, in neutralization assays using sera obtained from mice immunized with the recombinant H1N1pdm09 HA, we did not see any neutralizing activity against H5N1 virus in the sera, suggesting that the protection from the lethal H5N1 virus challenge afforded by the recombinant H1 HA is mediated by mechanisms other than virus neutralization.

Although our sMA-Ca04HA contained a His-tag, previous studies have shown that antibodies against His-tag are not generated in mice immunized with His-tagged recombinant proteins (Delaney et al., 2010; Shoji et al., 2011). We, therefore, assumed that antibodies against His-tag were not produced in our experiments and our results were not affected by the His-tag.

Recently, recombinant HA proteins have been produced on various platforms as an alternative strategy for influenza vaccine production. The glycosylation state of HA proteins differs depending on the cells in which they are expressed and this difference can affect the antigenicity of the proteins (de Vries et al., 2012). The report showed that HAs produced in 293T cells induce higher HI antibody titers compared with those produced in insect cells. In our study, we used Expi293F cells, which originated from the same 293 cell line as 293T cells. Therefore, we can assume that HAs expressed in Expi293F cells possess a similar glycosylation state and antigenicity to 293T cell-expressing HAs. Consequently, HAs produced in Expi293F cells may induce more effective antibodies compared with those produced in insect cells. The glycosylation state of the recombinant HA protein expressed in Expi293F might act advantageously to induce antibodies that target the conserved region of the HAs of H1N1pdm09 and H5N1 viruses and provide cross-protection mediated by mechanisms such as antibody-dependent cellular cytotoxicity. However, further studies are needed to better understand the influence of the glycosylation state of HA on its antigenicity and to understand which expression system is best suited for the development of a recombinant HA protein vaccine.

Previous reports have shown that intranasal infection of mice with cold-adapted H1N1pdm09 virus or baculovirus displaying the HA protein of H1N1pdm09 virus protected mice from H1N1pdm09 and H5 virus challenge (Jang et al., 2012; Sim et al., 2016). However, in these studies, attenuated viruses were used. By contrast, here we showed that intramuscular immunization with recombinant H1N1 pdm09 HA protein alone successfully protected mice from heterosubtypic highly pathogenic H5N1 challenge. Future mechanistic studies to understand how intramuscular immunization with recombinant HA could be sufficient to induce immunity to protect mice from heterosubtypic highly pathogenic H5N1 challenge will be important for vaccine development.

In conclusion, intramuscular immunization with Expi293F cell-based soluble recombinant HA protein induced protection against homosubtypic and heterosubtypic challenge in the same phylogenetic group. Our human Expi293F cell-based vaccine candidate thus offers a new strategy for influenza vaccine development. Further studies using other HA proteins from different subtypes would enhance the development of Expi293F cell-based vaccines. Further studies to understand the mechanistic basis of the immunity induced by the intramuscular administration of recombinant HA protein will aid in vaccine development.

AUTHOR CONTRIBUTIONS

SY designed the study and performed the experiments. SY, AY, and YK analyzed the data. SY and YK wrote the manuscript. All authors reviewed and approved the manuscript.

FUNDING

This research was supported by Strategic Basic Research Programs from the Japan Science and Technology Agency (JST); by Leading Advanced Projects for medical innovation (LEAP) from the Japan Agency for Medical Research and Development (AMED) (JP18am001007, JP18am001008); by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Science, Sports, and Technology (MEXT) of Japan (No. 16H06429, 16K21723, and 16H06434); by the Japan Initiative

for Global Research Network on Infectious Diseases (J-GRID) from the MEXT of Japan; by e-ASIA Joint Research Program from AMED (JP17jm0210042); by Research Program on Emerging and Re-emerging Infectious Diseases from AMED (JP19fk0108104, JP19fk0108066); by fund for the Promotion of Joint International Research [Fostering Joint International Research (B)] from Japan Society for the Promotion of Science (JSPS) (JP18KK0225); by MEXT KAKENHI Grant Number (15K08491, 18K07139); and by the NIAID-funded Center for Research on Influenza Pathogenesis (CRIP) (HHSN272201400008C).

ACKNOWLEDGMENTS

We would like to thank Dr. Susan Watson for editing the manuscript.

REFERENCES

Biesova, Z., Miller, M. A., Schneerson, R., Shiloach, J., Green, K. Y., Robbins, J. B., et al. (2009). Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. *Vaccine* 27, 6234–6238. doi: 10.1016/j.vaccine.2009.07.107

Chen, C. Y., Lin, S. Y., Cheng, M. C., Tsai, C. P., Hung, C. L., Lo, K. W., et al. (2013). Baculovirus vector as an avian influenza vaccine: hemagglutinin expression and presentation augment the vaccine immunogenicity. *J. Biotechnol.* 164, 143–150. doi: 10.1016/j.jbiotec.2012.12.013

Chiu, F. F., Venkatesan, N., Wu, C. R., Chou, A. H., Chen, H. W., Lian, S. P., et al. (2009). Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus. *Biochem. Biophys. Res. Commun.* 383, 27–31. doi: 10.1016/j.bbrc.2009.03.106

Cornelissen, L. A., De Vries, R. P., De Boer-Luijtze, E. A., Rigter, A., Rottier, P. J., and De Haan, C. A. (2010). A single immunization with soluble recombinant trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1. *PLoS One* 5:e10645. doi: 10.1371/journal.pone.0010645

de Vries, R. P., Smit, C. H., De Bruin, E., Rigter, A., De Vries, E., Cornelissen, L. A., et al. (2012). Glycan-dependent immunogenicity of recombinant soluble trimeric hemagglutinin. *J. Virol.* 86, 11735–11744. doi: 10.1128/JVI.01084-12

Delaney, K. N., Phipps, J. P., Johnson, J. B., and Mizel, S. B. (2010). A recombinant flagellin-poxvirus fusion protein vaccine elicits complement-dependent protection against respiratory challenge with vaccinia virus in mice. *Viral Immunol.* 23, 201–210. doi: 10.1089/vim.2009.0107

Dreyfus, C., Laursen, N. S., Kwaks, T., Zuijdgeest, D., Khayat, R., Ekiert, D. C., et al. (2012). Highly conserved protective epitopes on influenza B viruses. *Science* 337, 1343–1348. doi: 10.1126/science.1222908

Du, L., Leung, V. H., Zhang, X., Zhou, J., Chen, M., He, W., et al. (2011). A recombinant vaccine of H5N1 HA1 fused with foldon and human IgG Fc induced complete cross-clade protection against divergent H5N1 viruses. *PLoS One* 6:e16555. doi: 10.1371/journal.pone.0016555

Dunkle, L. M., Izikson, R., Patriarca, P., Goldenthal, K. L., Muse, D., Callahan, J., et al. (2017). Efficacy of recombinant influenza vaccine in adults 50 years of age or older. *N. Engl. J. Med.* 376, 2427–2436. doi: 10.1056/NEJMoa1608862

Ge, J., An, Q., Gao, D., Liu, Y., and Ping, W. (2016). Construction of recombinant baculoviruses expressing hemagglutinin of H5N1 avian influenza and research on the immunogenicity. *Sci. Rep.* 6:24290. doi: 10.1038/srep24290

Goff, P. H., Eggink, D., Seibert, C. W., Hai, R., Martinez-Gil, L., Krammer, F., et al. (2013). Adjuvants and immunization strategies to induce influenza virus hemagglutinin stalk antibodies. *PLoS One* 8:e79194. doi: 10.1371/journal.pone.0079194

Jain, N. K., Barkowski-Clark, S., Altman, R., Johnson, K., Sun, F., Zmuda, J., et al. (2017). A high density CHO-S transient transfection system: comparison of ExpiCHO and Expi293. *Protein Expr. Purif.* 134, 38–46. doi: 10.1016/j.pep.2017.03.018

Jang, Y. H., Byun, Y. H., Lee, Y. J., Lee, Y. H., Lee, K. H., and Seong, B. L. (2012). Cold-adapted pandemic 2009 H1N1 influenza virus live vaccine elicits cross-reactive immune responses against seasonal and H5 influenza A viruses. *J. Virol.* 86, 5953–5958. doi: 10.1128/JVI.07149-11

Kalthoff, D., Giritch, A., Geisler, K., Bettmann, U., Klimyuk, V., Hehnen, H. R., et al. (2010). Immunization with plant-expressed hemagglutinin protects chickens from lethal highly pathogenic avian influenza virus H5N1 challenge infection. *J. Virol.* 84, 12002–12010. doi: 10.1128/JVI.00940-10

Katz, J. M., and Webster, R. G. (1989). Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. *J. Infect. Dis.* 160, 191–198. doi: 10.1093/infdis/160.2.191

Khurana, S., Verma, S., Verma, N., Crevar, C. J., Carter, D. M., Manischewitz, J., et al. (2011). Bacterial HA1 vaccine against pandemic H5N1 influenza virus: evidence of oligomerization, hemagglutination, and cross-protective immunity in ferrets. *J. Virol.* 85, 1246–1256. doi: 10.1128/JVI.02107-10

Kubota-Koketsu, R., Mizuta, H., Oshita, M., Ideno, S., Yunoki, M., Kuhara, M., et al. (2009). Broad neutralizing human monoclonal antibodies against influenza virus from vaccinated healthy donors. *Biochem. Biophys. Res. Commun.* 387, 180–185. doi: 10.1016/j.bbrc.2009.06.151

Lin, Y. J., Deng, M. C., Wu, S. H., Chen, Y. L., Cheng, H. C., Chang, C. Y., et al. (2008). Baculovirus-derived hemagglutinin vaccine protects chickens from lethal homologous virus H5N1 challenge. *J. Vet. Med. Sci.* 70, 1147–1152. doi: 10.1292/jvms.70.1147

Milian, E., and Kamen, A. A. (2015). Current and emerging cell culture manufacturing technologies for influenza vaccines. *Biomed. Res. Int.* 2015:504831. doi: 10.1155/2015/504831

Prabakaran, M., Kolpe, A. B., He, F., and Kwang, J. (2013). Cross-protective efficacy of bivalent recombinant baculoviral vaccine against heterologous influenza H5N1 challenge. *Vaccine* 31, 1385–1392. doi: 10.1016/j.vaccine.2013.01.003

Rajakumar, A., Swierkosz, E. M., and Schulze, I. T. (1990). Sequence of an influenza virus hemagglutinin determined directly from a clinical sample. *Proc. Natl. Acad. Sci. USA* 87, 4154–4158.

Sakabe, S., Ozawa, M., Takano, R., Iwastuki-Horimoto, K., and Kawaoka, Y. (2011). Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. *Virus Res.* 158, 124–129. doi: 10.1016/j.virusres.2011.03.022

Shoji, Y., Bi, H., Musiychuk, K., Rhee, A., Horsey, A., Roy, G., et al. (2009). Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. *Vaccine* 27, 1087–1092. doi: 10.1016/j.vaccine.2008.11.108

Shoji, Y., Chichester, J. A., Jones, M., Manceva, S. D., Damon, E., Mett, V., et al. (2011). Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. *Hum. Vaccin.* 7(Suppl), 41–50. doi: 10.4161/hv.7.0.14561

Sim, S. H., Kim, J. Y., Seong, B. L., Nguyen, H. H., and Chang, J. (2016). Baculovirus displaying hemagglutinin elicits broad cross-protection against influenza in mice. *PLoS One* 11:e0152485. doi: 10.1371/journal.pone.0152485

Skowronski, D. M., Janjua, N. Z., De Serres, G., Sabaiduc, S., Eshaghi, A., Dickinson, J. A., et al. (2014). Low 2012-13 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not antigenic drift in circulating viruses. *PLoS One* 9:e92153. doi: 10.1371/journal.pone.0092153

Song, L., Zhang, Y., Yun, N. E., Poussard, A. L., Smith, J. N., Smith, J. K., et al. (2009). Superior efficacy of a recombinant flagellin:H5N1 HA globular head vaccine is determined by the placement of the globular head within flagellin. *Vaccine* 27, 5875–5884. doi: 10.1016/j.vaccine.2009.07.060

Wang, M. L., Katz, J. M., and Webster, R. G. (1989). Extensive heterogeneity in the hemagglutinin of egg-grown influenza viruses from different patients. *Virology* 171, 275–279. doi: 10.1016/0042-6822(89)90538-2

Wei, C. J., Xu, L., Kong, W. P., Shi, W., Canis, K., Stevens, J., et al. (2008). Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. *J. Virol.* 82, 6200–6208. doi: 10.1128/JVI.00187-08

Wohlbold, T. J., Nachbagauer, R., Margine, I., Tan, G. S., Hirsh, A., and Krammer, F. (2015). Vaccination with soluble headless hemagglutinin protects mice from challenge with divergent influenza viruses. *Vaccine* 33, 3314–3321. doi: 10.1016/j.vaccine.2015.05.038

Conflict of Interest Statement: YK has received speaker's honoraria from Toyama Chemical and Astellas Inc.; has received grant support from Chugai Pharmaceuticals, Daiichi Sankyo Pharmaceutical, Toyama Chemical, Tauns Laboratories, Inc., Otsuka Pharmaceutical Co., Ltd., and Denka Seiken Co., Ltd.; and is a co-founder of FluGen.

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

frontiers in Medicine

ORIGINAL RESEARCH
published: 22 October 2019
doi: 10.3389/fmed.2019.00235

Characteristics of Hospitalized Rhinovirus-Associated Community-Acquired Pneumonia in Children, Finland, 2003–2014

Maria Hartiala[1], Elina Lahti[1,2], Ville Forsström[1], Tytti Vuorinen[3], Olli Ruuskanen[1] and Ville Peltola[1]*

[1] Department of Pediatrics and Adolescent Medicine, Turku University Hospital, University of Turku, Turku, Finland, [2] Child and Adolescent Clinic, City of Turku Welfare Division, Turku, Finland, [3] Department of Virology and Clinical Virology, Turku University Hospital, University of Turku, Turku, Finland

Background: Rhinovirus (RV) is the most common cause of respiratory tract infections in children but, still, the clinical characteristics of RV-associated pneumonia have not been sufficiently investigated.

Methods: We identified children and adolescents younger than 18 years of age treated for community-acquired pneumonia as inpatients at the Turku University Hospital from 2003 to 2014 and analyzed for RV by PCR of a respiratory tract specimen. We collected the data from medical records and compared RV-positive children with RV-negative children.

Results: Of the study population of 313 children with pneumonia who were studied for RV, it was detected in 82 (26%). RV-positive children were younger (median age 2.6 years, interquartile range [IQR] 1.1–4.6 vs. 3.5 years, IQR 1.7–8.3, $p = 0.002$) and they had more often a history of preterm birth (16% vs. 5%, adjusted odds ratio 2.89, 95% confidence interval 1.21–6.92, $p = 0.017$) than RV-negative children. RV-positive children had a higher median white blood cell count than RV-negative children at presentation with pneumonia. The signs, symptoms, and severity of pneumonia were mostly similar in RV-positive and RV-negative children.

Conclusions: RV was frequently detected in young children hospitalized with community-acquired pneumonia. We identified premature birth as a factor associated with RV-positive pneumonia. The clinical features of pneumonia did not clearly differ between RV-positive and RV-negative children. Further studies are needed to clarify the clinical significance of detection of RV in children with pneumonia.

Keywords: children, pneumonia, respiratory tract infection, rhinovirus, white blood cell count

OPEN ACCESS

Edited by:
Olivier Vandenberg, LHUB-ULB, Belgium

Reviewed by:
Peng Yang, Capital Medical University, China
Ana Afonso, University of São Paulo, Brazil
Marijke Bernadette Reynders, AZ Sint-Jan Brugge-Oostende AV, Belgium

****Correspondence:***
Maria Hartiala
mkhonk@utu.fi

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *13 February 2019*
Accepted: *07 October 2019*
Published: *22 October 2019*

Citation:
Hartiala M, Lahti E, Forsström V, Vuorinen T, Ruuskanen O and Peltola V (2019) Characteristics of Hospitalized Rhinovirus-Associated Community-Acquired Pneumonia in Children, Finland, 2003–2014. Front. Med. 6:235. doi: 10.3389/fmed.2019.00235

INTRODUCTION

Pneumonia is a common cause of hospitalization in children. Rhinovirus (RV) is the most common cause of respiratory tract infections in children worldwide, and frequently detected in community-acquired pneumonia (CAP) (1–6). According to global estimates, 120 million episodes of pneumonia in children younger than 5 years of age are recorded annually (7). RV is detected in

up to 46% of children with CAP (5, 8, 9). RVs are small non-enveloped RNA viruses classified into three species (A, B, and C) with approximately 160 different types (10, 11). Simultaneous circulation of several RV types in populations year-round explains the high frequency of RV infections.

The scientific literature on asthma exacerbation and wheezing illnesses caused by RV is extensive (12), but the clinical characteristics of RV-associated pneumonia in children have not been thoroughly investigated. RV is commonly present in mixed viral-bacterial and viral-viral infections (13) and a substantial proportion of asymptomatic children are positive for RV by PCR of upper respiratory tract specimens (14). The role of RV as the causative agent of pneumonia is unclear.

The aim of this study was to assess the risk factors for, clinical characteristics and prevalence of RV-associated pneumonia in children. We compared the medical record data of RV-positive and RV-negative children hospitalized with CAP.

METHODS

Participants and Data Collection

This retrospective study involved children and adolescents younger than 18 years of age treated as inpatients at the Department of Pediatrics and Adolescent Medicine, Turku University Hospital (Turku, Finland), during a 12-year period from 2003 to 2014. To identify children who were hospitalized with CAP, we searched the Electronic Registry of the Turku University Hospital for International Classification of Diseases (ICD-10) codes related to pneumonia (J12–18, J10.0, J11.0, J85, J86, J90) with an age limit of 18 years. Of this patient population, we identified those with a diagnostic polymerase chain reaction (PCR) test for RV performed during the hospitalization for CAP. The medical records of these children were reviewed to collect the clinical and background data. Excluding preterm birth, only currently present underlying conditions were considered. Tachypnea was defined as respiratory rate >60/min in infants younger than 2 months of age, >50/min in infants from 2 to 12 months, >40/min in children from 1 to 5 years and >30/min in children 6 years of age or older.

The study was approved by the Institutional Review Board at the Clinical Research Centre of the Turku University Hospital.

Laboratory Detection

The diagnostic tests for RV were *in house* qualitative reverse transcription (RT) -PCR assays and commercial multiplex PCR tests for respiratory viruses including RV, which were in routine use in the diagnostic laboratory during the study period. The first *in house* PCR used detected RV and enterovirus (15). It was later replaced by a triplex test for RV, enterovirus and respiratory syncytial virus (16). The analytical procedures, sensitivities and specificities of the *in house* tests are described in the above-cited references. Since 2008 we also used commercial multiplex PCR kits, first a Seeplex RV12 Ace detection kit and since 2013 Anyplex RV16 detection kit (both from Seegene, Seoul, Korea). The commercial multiplex PCR methods may have slightly lower sensitivities for RV than the *in house* tests (17). A child was considered as a RV-positive case if RV was detected either by the in-house PCR or the multiplex PCR or both.

Data Analysis

RV-positive children were compared with RV-negative children. To test whether the results were affected by the presence of other viruses, we conducted a sensitivity analysis of children with a sole RV finding (no other viruses detected) compared with those who had no viruses detected. Data were presented as proportions, or medians with interquartile ranges (IQR). Univariate comparisons were performed for continuous data by use of the Wilcoxon rank-sum test and for categoric data by use of the χ^2 test or Fisher's exact test. All tests were two-sided. The significance level was $P < 0.05$. A multivariate logistic regression analysis was conducted to examine the independent risk factors for RV-positive CAP. The final model included age, sex and presence of the following prior diseases or conditions: asthma or reactive airway disease, premature birth, neurological condition, cardiovascular disease, and atopic eczema or sensitization to aeroallergen. Statistical analyses were performed using SAS system for Windows, version 9.4. (SAS Institute Inc., Cary, NC, USA) or SPSS version 23.0 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA).

RESULTS

Study Population, Characteristics and Underlying Conditions

Of a total of 2484 children with CAP, 1270 (51%) were treated as inpatients and 1214 (49%) as outpatients. Hospitalization was needed for 81 to 143 children with CAP per year (**Figure 1**). Inpatients were younger than outpatients (median age 2.88 [IQR 1.49–5.63] years vs. 3.38 [1.77–7.15] years, $p < 0.001$). Of 1270 inpatients 313 (25%) had PCR diagnostics for RV done during the hospitalization, and 82 (26% of 313) had RV detected. Children treated as outpatients for pneumonia were not tested for RV.

The final study population ($n = 313$) consisted of 171 males (55%) and 142 females (45%) with a median age of 3.09 (IQR 1.53–7.35) years (**Table 1**). All patients had radiologically confirmed pneumonia. The monthly peak occurrence of RV pneumonia was in October (**Figure 2**). RV-positive patients were younger (median age 2.59 [IQR 1.08–4.59] years) than RV-negative patients (median age 3.51 [IQR 1.68–8.26] years) ($p = 0.002$).

Preterm birth was more frequent in RV-positive (16%) compared to RV-negative children (5%, $p = 0.002$ in univariate analysis). This association remained significant in the multivariate logistic regression analysis (OR 2.89, 95% CI 1.21–6.92, $p = 0.017$). Other underlying conditions were not significantly associated with RV-positive CAP. Fifteen percent of RV-positive and 12% of RV-negative children had a diagnosis of asthma at the time of admission. Atopic eczema or sensitization to aeroallergen was present in 27% of RV-positive and in 19% of RV-negative children.

Clinical Findings

The clinical profiles of pneumonia were in general similar in children with or without RV, the most common symptoms

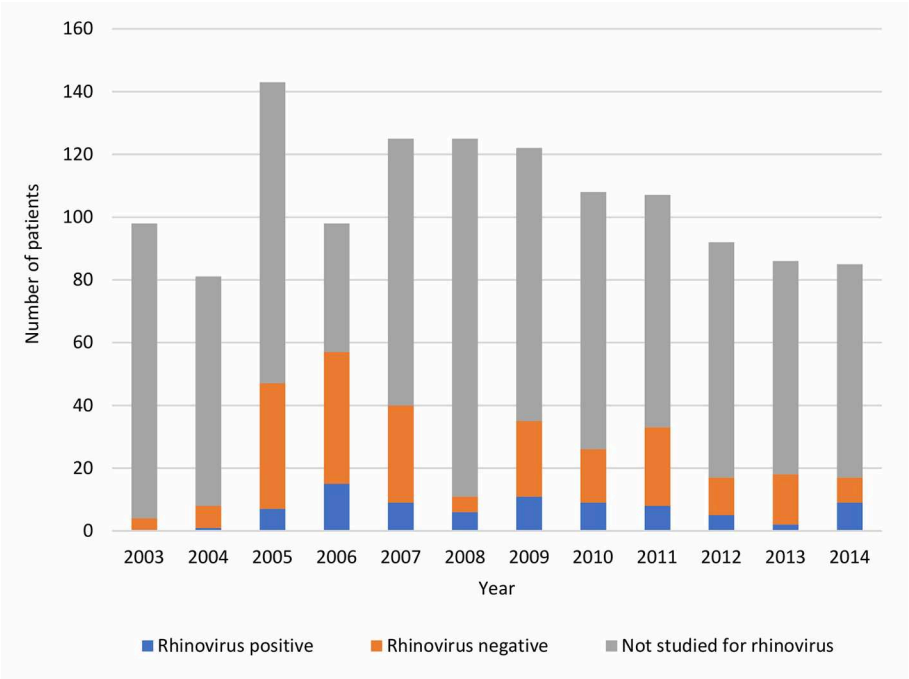


FIGURE 1 | Yearly numbers of pneumonia inpatients stratified by RV status during years 2003–2014.

TABLE 1 | Demographic characteristics and underlying conditions of children with community-acquired pneumonia requiring hospitalization; RV-positive patients compared to RV-negative patients.

Demographic characteristics and underlying conditions	RV-positive, $n = 82$	RV-negative, $n = 231$	Univariate analysis *P*-value	Logistic regression analysis	
				P-value	OR (95% CI)
Age, year – median (IQR)	2.59 (1.08–4.59)	3.51 (1.68–8.26)	0.002[a]	0.010	0.91 (0.86–0.98)
Age group – no. (%)					
<2 years	33 (40)	77 (33)			
2–4 years	32 (39)	55 (24)			
5–9 years	10 (12)	53 (23)			
10–17 years	7 (9)	46 (20)			
Males – no. (%)	49 (60)	122 (53)	0.278[b]	0.241	1.38 (0.81–2.35)
Underlying condition – no. (%)	39 (48)	94 (41)	0.280[b]		
Atopic eczema or sensitization to aeroallergen	22 (27)	43 (19)	0.115[b]	0.154	1.59 (0.84–3.01)
Preterm birth	13 (16)	12 (5)	0.002[b]	0.017	2.89 (1.21–6.92)
Asthma or reactive airway disease	12 (15)	28 (12)	0.558[b]	0.363	1.45 (0.65–3.20)
Cardiovascular disease	6 (7)	8 (3)	0.209[c]	0.366	1.76 (0.52–5.97)
Neurological condition	3 (4)	11 (5)	1.000[c]	0.526	0.61 (0.14–2.78)
Malignancy or immunosuppression	1 (1)	6 (3)	0.681[c]		

CI, confidence interval; IQR, interquartile range; OR, odds ratio; RV, rhinovirus.
[a] *Wilcoxon rank-sum test.*
[b] χ^2 *test.*
[c] *Fisher's exact test.*

being fever and cough in both groups (**Table 2**). However, the frequency of documented fever was lower in RV-positive than in RV-negative children (84% vs. 97%, $p < 0.001$). Dyspnea or shortness of breath was present in 35% of RV-positive patients and in 28% of RV-negative patients, and tachypnea in 30% and 29%, respectively. The duration of symptoms before referral

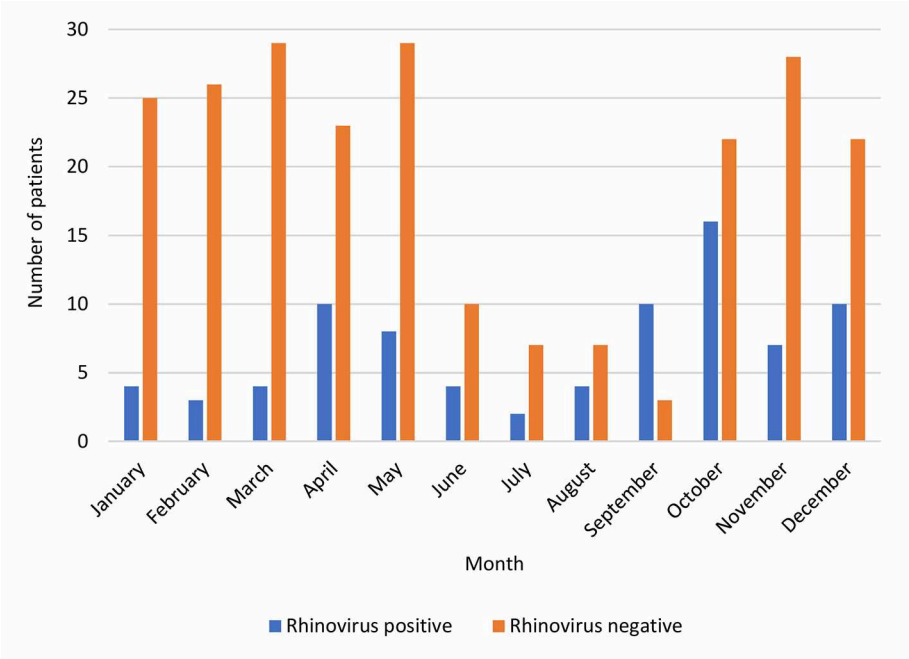


FIGURE 2 | The cumulative monthly numbers of RV-positive and RV-negative pneumonia inpatients during years 2003–2014.

was shorter in RV-positive (median 3 [IQR 1–7] days) than in RV-negative children (median 5 [IQR 2–8] days) ($p = 0.006$).

Laboratory Results

The white blood cell (WBC) count determined on admission was higher in RV-positive than in RV-negative children (median 16.4 [IQR 12.2–25.0] $\times$ 10^9/L vs. 14.1 [8.5–20.4] $\times$ 10^9/L, $p = 0.002$), whereas the C-reactive protein (CRP) concentrations were not significantly different (**Table 2**).

At least one virus other than RV was detected by PCR in 13 (16%) of 82 children positive for RV and in 45 (19%) of 231 children negative for RV. Five children had pathogenic bacteria in the blood culture: 1 RV-positive and 3 RV-negative children had *Streptococcus pneumoniae* and 1 RV-negative child had *Streptococcus pyogenes* bacteremia.

Treatment and Outcome

Thirteen percent of RV-positive and 21% of RV- negative patients received antimicrobial therapy before referral to the hospital, and all patients received antimicrobial therapy during hospitalization. Of RV-positive children, 34% received oxygen supplementation, 20% were admitted to the intensive care unit, and 10% required invasive mechanical ventilation, whereas the corresponding rates were, respectively, in RV-negative children 27, 15, and 5%. These differences were not statistically significant. The median duration of hospitalization was 2.0 days in both groups. Complicated pneumonia (defined as lung abscess, necrotising pneumonia, or empyema) was documented in 5 (6%) of RV-positive and in 20 (9%) of RV-negative patients. A 1-year-old boy with no underlying conditions, negative for RV but positive for influenza B and adenovirus, died of pneumonia after 5 days of hospital treatment. Thus, the mortality rate in the study population was 0.3%.

Sensitivity Analysis

As a test of sensitivity of our results to effects of other viruses, we compared children with a sole RV finding (no other virus detected) ($n = 69$) with those who had no virus detected ($n = 186$). The findings remained essentially similar. Children with RV only were younger than virus-negative children (median age 2.92 [IQR 1.11–4.67] vs. 4.52 [1.81–9.16] years, $p = 0.002$) and they had more often a history of preterm birth (14% vs. 5%, univariate $p = 0.009$, multivariate analysis OR 2.98 [95% CI 1.10–8.05], $p = 0.032$). Similar to results of all subjects, the duration of symptoms before referral was shorter, fever was documented less frequently, and the median WBC count was higher in sole RV-positive children compared to virus-negative children.

DISCUSSION

In this study covering a 12-year period, RV was detected in 26% of children hospitalized with radiologically-confirmed CAP in whom testing was performed. RV-associated CAP was particularly common in young children and in children born prematurely. Clinically, RV-associated pneumonia did not clearly differ from RV-negative pneumonia. CRP and WBC levels were in most cases high and patients were treated with antibiotics because of suspected bacterial pneumonia.

Hospitalization was required in about half of all children evaluated at the emergency department for CAP, which is similar

TABLE 2 | Clinical features, laboratory results and outcomes of children with community-acquired pneumonia requiring hospitalization; RV-positive patients compared to RV-negative patients.

Clinical features, laboratory results and outcomes	RV-positive, $n = 82$	RV-negative, $n = 231$	*P*-value
Clinical features – no. (%)			
Cough	58 (71)	185 (80)	0.081[a]
Fever	69 (84)	223 (97)	<0.001[a]
Tachypnea	25 (30)	67 (29)	0.800[a]
Dyspnea or shortness of breath	29 (35)	65 (28)	0.220[a]
Rhinitis or nasal congestion	36 (44)	83 (36)	0.201[a]
Sore throat or hoarse voice	7 (9)	13 (6)	0.355[a]
Headache	7 (9)	23 (10)	0.708[a]
Muscle pain	5 (6)	10 (4)	0.550[b]
Chest pain or abdominal pain	12 (15)	42 (18)	0.465[a]
Vomiting	24 (29)	69 (30)	0.918[a]
Otitis media	17 (21)	67 (29)	0.146[a]
Abnormal breath sounds	52 (63)	151 (65)	0.750[a]
Crackles	37 (45)	99 (43)	0.722[a]
Decreased breath sounds	17 (21)	58 (25)	0.425[a]
Wheezing	10 (12)	26 (11)	0.819[a]
Duration of symptoms before referral, days – median (IQR)	3.0 (1.0–7.0)	5.0 (2.0–8.0)	0.006
Antibiotic treatment received before referral – no. (%)	11 (13)	49 (21)	0.123[a]
Laboratory results			
WBC count on admission, $\times 10^9$/L – median (IQR)[d]	16.4 (12.2–25.0)	14.1 (8.5–20.4)	0.002[c]
Highest WBC count, $\times 10^9$/L – median (IQR)[e]	17.4 (13.4–25.1)	14.8 (9.5–20.6)	0.003[c]
0–3.9 $\times 10^9$/L – no. (%)	1 (1)	4 (2)	
4–14.9 $\times$ 10^9/L – no. (%)	28 (35)	112 (49)	
$\geq$15 $\times$ 10^9/L – no. (%)	52 (64)	113 (49)	
CRP on admission, mg/L – median (IQR)[f]	79 (20–192)	87 (25–181)	0.998[c]
Highest CRP, mg/L – median (IQR)[g]	86 (28–192)	100 (30–214)	0.582[c]
<20 mg/L – no. (%)	18 (22)	44 (19)	
20–39 mg/L – no. (%)	8 (10)	25 (11)	
40–79 mg/L – no. (%)	13 (16)	30 (13)	
$\geq$80 mg/L – no. (%)	42 (52)	131 (57)	
Outcomes			
Length of stay, days – median (IQR)	2.0 (1.0–4.0)	2.0 (1.0–3.0)	0.802[c]
Intensive care unit admission – no. (%)	16 (20)	35 (15)	0.358[a]
Oxygen supplementation – no. (%)	28 (34)	63 (27)	0.239[a]
Invasive mechanical ventilation – no. (%)	8 (10)	11 (5)	0.112[b]
Lung abscess, necrotising pneumonia or empyema – no. (%)	5 (6)	20 (9)	0.473[a]
Death in the hospital – no. (%)	0 (0)	1 (0.4)	

CRP, C-reactive protein; IQR, interquartile range; RV, rhinovirus; WBC, white blood cell.
[a] *χ^2 test.*
[b] *Fisher's exact test.*
[c] *Wilcoxon rank-sum test.*
[d] *Data available on 80 RV-positive and 228 RV-negative children.*
[e] *Data available on 81 RV-positive and 229 RV-negative children.*
[f] *Data available on 79 RV-positive and 228 RV-negative children.*
[g] *Data available on 81 RV-positive and 230 RV-negative children.*

to earlier data from Finland (18, 19). The yearly number of children hospitalized with CAP was variable and had a downward trend during the latter part of the study period (**Figure 1**). Worldwide, introduction of pneumococcal conjugate vaccines has had a substantial impact on children's hospitalizations with all-cause pneumonia and, also, on hospitalizations with virus-positive pneumonia (20). In Finland, a 10-valent pneumococcal conjugate vaccine was included in the national immunization program for all children in 2010. As pneumococcal pneumonia cases decrease due to vaccinations, viruses are presumably

becoming even more substantial cause of pneumonia. This was not seen for RV in this study, which did not include a long period of time after introduction of pneumococcal vaccinations; the yearly proportion of RV-positive CAP of those tested for RV did not systematically change. The RV prevalence documented in our study is in concordance with previous literature reporting the detection rate of RV from 14 to 46% in children hospitalized with pneumonia (2, 3, 5, 8, 9, 13). RV infections occur year-round but most commonly during autumn and spring (21), which was seen also in our study.

In our study, RV-positive pneumonia patients were younger than RV-negative patients. Children with RV-positive and RV-negative pneumonia were largely similar in respect of the severity of illness and the response to treatment. The need of oxygen supplementation, treatment at the intensive care unit, and invasive mechanical ventilation were non-significantly more common in those infected with RV. We found only a few earlier studies considering the clinical characteristics of RV pneumonia in children. Annamalay et al. compared clinical features as well as laboratory and microbiology findings of RV-positive and RV-negative children hospitalized with pneumonia in Mozambique, without finding any significant between-group differences (22). In the study of Ahn et al., RV-positive children hospitalized for acute lower respiratory tract infections were younger, had shorter fever duration, and higher frequencies of chest retraction and wheezing than RV-negative children (23). Other studies compared children with CAP or with an unspecified lower respiratory tract infection caused by different RV types and found only marginal differences (23–25).

Wheezing illnesses (bronchiolitis, recurrent wheezing, or exacerbation of asthma) often associate with RV (11, 12, 23, 26–28). Our study focused on pneumonia, which may be sometimes difficult to differentiate from wheezing illnesses. Furthermore, pneumonia and wheezing may have common risk factors. An association between childhood pneumonia and asthma has been noticed for long (29–31). Parental asthma, earlier wheezing, and exposure to tobacco smoke are associated with more severe clinical course of pneumonia (32). In our study, asthma was slightly more common in RV-positive patients than others, but, contrary to our expectation, wheezing in auscultation of lungs was not more prevalent among RV-positive than RV-negative CAP patients. The clinical picture of RV-associated pneumonia seems to be clearly different from RV-associated wheezing illness.

We found that RV-positive patients were considerably more often prematurely born than others. Accordingly, in the study of Miller et al., prematurely born children were found to be particularly susceptible to RV infections (33). Kennedy et al. have highlighted that it is the nature and extent of the immune response to the virus that determines the symptom profile (34). Numerous pathophysiological mechanisms, such as diminished immune responses and lung function but also inflammatory and airway re-modeling pathways activated by viruses, are proposed to influence the increased risk of respiratory disorders, including RV infections, following preterm birth (35).

The role of RV as a true pneumonia pathogen is unclear (36–41). Life-threatening cases of pneumonia caused by only RV have been reported (42), and RV has been detected directly from the lung tissue from a child with pneumonia (43). RV viremia is particularly common in CAP patients with RV-C (44, 45). In our study, as well as in most other studies concerning the etiology of pneumonia, RV was detected by PCR from upper respiratory tract specimens, and the presence of the virus at the site of infection, the lung, remains unknown. As RV is frequently (approximately in 15%) detected also in asymptomatic children (14), its role as a causative agent vs. a bystander in children with pneumonia can be questioned. It should be noted, however, that the mean virus shedding time after RV infection is as short as 11 days in immunocompetent children (46). Persistent shedding of RV is not known to occur in otherwise healthy subjects. The detection of RV in asymptomatic children may reflect previous infection, ongoing asymptomatic or mild infection (possibly by less virulent RV types), or an incubation period preceding the onset of symptoms.

The identification of RV directly from the lung is not feasible in common clinical settings. In severe pneumonia or in immunocompromised subjects, obtaining invasive samples like bronchoalveolar lavage for microbiological evaluation including RV PCR would be critically informative (47). RV loads in upper respiratory tract specimens do not clearly correlate with the clinical course of the infection. Children with wheezing or only rhinitis have been reported to have similar RV loads in their nasal washes (28). On the contrary, in a recent study, higher RV viral load was associated with more severe respiratory symptoms (48). Serologic assays for RV infection could help to prove acute infections but such tests are not in routine use. Recently, despite high phylogenetic diversity of RV, the development of RV species-specific antibody test has been successful (49). Integrating studies of host response, microbe detection, and airway microbiome is a modern approach in pneumonia diagnostics (50). Development of effective vaccines and drug treatments for RV would ultimately make it possible to show the impact of RV on childhood pneumonia. Recently, Toll-like receptor 3 blockage has been studied as one of the possible options for treatment (51).

RV has been associated with a severe course of pneumonia in children, similar to our findings (52). Children with RV-positive pneumonia may have a concomitant bacterial pneumonia, or a secondary bacterial pneumonia following a RV infection. In our children with RV-associated CAP, CRP and WBC levels were elevated, and the median WBC counts were even higher in the RV-positive than in the RV-negative group. Increased levels of WBC have been earlier reported in RV-related lower respiratory tract infections (53). CRP and WBC are not highly specific in differentiation between viral and bacterial infections and better biomarkers or microbiologic methods would be needed in order to confirm or exclude bacterial co-infection in RV-associated CAP. Mixed infections are increasingly recognized and possibly associated with a more severe course of pneumonia, particularly the combination of RV and *S. pneumoniae* (13, 54–57). *In vitro*, RV infection has been shown to stimulate adhesion of *S. pneumoniae* to airway epithelial cells via increases in the platelet-activating factor receptors (58). The impairment of immune response to bacterial products and phagocytosis of bacteria in human macrophages in response to RV exposure has been documented (59, 60). Moreover, the seasonality of pneumonia

coinciding with viral lower respiratory tract infections has been observed (61).

In the absence of effective antiviral drugs for other respiratory viruses than influenza A and B virus, the clinical significance of diagnosing viral etiology of pneumonia has not been firmly established. Avoidance of the unnecessary use of antibiotics and excessive laboratory or other tests, shortening of the length of stay in the hospital, prediction of the clinical course of illness, and prevention of transmission to other patients by isolation or other methods are potential benefits of virus detection, in addition to surveillance of local epidemiology of seasonal viruses. In our study, the characteristics of RV-associated pneumonia were closely similar to RV-negative pneumonia, and identification of RV did not result in the withholding of antibiotic treatment. The matter could be different in milder pneumonia cases not needing hospitalization.

Our study has notable limitations related to the retrospective setting. First, our study population was somewhat selected as RV tests were not routinely performed for all CAP patients. However, our study population can be considered to be a representative sample of overall CAP inpatients as key figures in patients undergoing RV detection in the present study and in CAP inpatients in our previous prospective CAP study are comparable (13). Second, data on the types of RV and viral loads were not available, and other viruses and bacteria were not comprehensively analyzed. In our previous CAP study with induced sputum as a diagnostic sample and patient population overlapping with our present study, 64% of RV findings belonged to RV A species and 36% to RV C species, and viral-bacterial co-infections were frequent (13). Similarly, other researchers have reported that RV A and C species are frequent and RV B species rare in children with pneumonia (23, 24). Third, the clinical data was collected from the medical records, which might be incomplete. Fourth, diagnostic methods and clinical practices varied during the 12-year study period. Fifth, our study included only hospitalized CAP patients. Sixth, the lack of a control group of healthy children is an obvious limitation in this study.

In conclusion, RV is frequently present in childhood pneumonia, particularly in vulnerable patients such as young children and those with a history of preterm birth. Among children hospitalized with CAP in this study, RV-positive pneumonia was a rather severe disease with high levels of inflammatory biomarkers and a clinical course that often necessitated intensive care, with no justification for withholding antibiotic treatment. Other studies are needed to establish the clinical characteristics of RV pneumonia in outpatients. Considering the large global burden of pneumonia and the high prevalence of RV in children with pneumonia, development of a diagnostic marker indicating RV as a true cause of the disease, and drugs and vaccines for RV-specific treatment and prevention could have major significance in the future.

DATA AVAILABILITY STATEMENT

The datasets for this manuscript are not publicly available because: Participant privacy prevents public sharing of individual-level data. Requests to access the datasets should be directed to Maria Hartiala, mkhonk@utu.fi.

ETHICS STATEMENT

This study involved only retrospective review of medical record data that had been collected during routine patient care. The study was approved by the Institutional Review Board at the Clinical Research Centre of the Turku University Hospital with a statement that an evaluation by the Ethics Committee was not needed.

AUTHOR CONTRIBUTIONS

MH and VF collected the clinical data. TV was responsible for virus diagnostics. MH analyzed data and wrote the manuscript with support of VP, EL, VF, TV, and OR. VP supervised the project.

FUNDING

This study was supported by the Turku University Foundation, the Väinö and Laina Kivi Foundation, the Research Foundation for the Pulmonary Diseases, and the Foundation for Pediatric Research.

ACKNOWLEDGMENTS

The authors thank M. Koivisto for help with data analyses.

REFERENCES

1. Wang M, Cai F, Wu X, Wu T, Su X, Shi Y. Incidence of viral infection detected by PCR and real-time PCR in childhood community-acquired pneumonia: a meta-analysis. *Respirology*. (2015) 20:405–12. doi: 10.1111/resp.12472
2. Jain S, Williams DJ, Arnold SR, Ampofo K, Bramley AM, Reed C, et al. Community-acquired pneumonia requiring hospitalization among U.S. children. *N Engl J Med*. (2015) 372:835–45. doi: 10.1056/NEJMoa1405870
3. Ruuskanen O, Lahti E, Jennings LC, Murdoch DR. Viral pneumonia. *Lancet*. (2011) 377:1264–75. doi: 10.1016/S0140-6736(10)61459-6
4. Don M, Canciani M, Korppi M. Community-acquired pneumonia in children: what's old? What's new? *Acta Paediatr*. (2010) 99:1602–8. doi: 10.1111/j.1651-2227.2010.01924.x
5. Nascimento-Carvalho AC, Vilas-Boas A-L, Fontoura M-SH, Vuorinen T, Nascimento-Carvalho CM. Respiratory viruses among children with non-severe community-acquired pneumonia: a prospective cohort study. *J Clin Virol*. (2018) 105:77–83. doi: 10.1016/j.jcv.2018.06.003
6. Toivonen L, Schuez-Havupalo L, Karppinen S, Teros-Jaakkola T, Rulli M, Mertsola J, et al. Rhinovirus infections in the first 2 years of life. *Pediatrics*. (2016) 138:e20161309. doi: 10.1542/peds.2016-1309
7. Walker CLF, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, et al. Global burden of childhood pneumonia and diarrhoea. *Lancet*. (2013) 381:1405–16. doi: 10.1016/S0140-6736(13)60222-6

8. Tsolia MN, Psarras S, Bossios A, Audi H, Paldanius M, Gourgiotis D, et al. Etiology of community-acquired pneumonia in hospitalized school-age children: evidence for high prevalence of viral infections. *Clin Infect Dis*. (2004) 39:681–6. doi: 10.1086/422996
9. O'Callaghan-Gordo C, Bassat Q, Morais L, Díez-Padrisa N, Machevo S, Nhampossa T, et al. Etiology and epidemiology of viral pneumonia among hospitalized children in rural Mozambique: a malaria endemic area with high prevalence of human immunodeficiency virus. *Pediatr Infect Dis J*. (2011) 30:39–44. doi: 10.1097/INF.0b013e3181f232fe
10. Daleno C, Piralla A, Scala A, Senatore L, Principi N, Esposito S. Phylogenetic analysis of human rhinovirus isolates collected from otherwise healthy children with community-acquired pneumonia during five successive years. *PLoS ONE*. (2013) 8:e80614. doi: 10.1371/journal.pone.0080614
11. Jacobs SE, Lamson DM, St George K, Walsh TJ. Human rhinoviruses. *Clin Microbiol Rev*. (2013) 26:135–62. doi: 10.1128/CMR.00077-12
12. Jartti T, Gern JE. Role of viral infections in the development and exacerbation of asthma in children. *J Allergy Clin Immunol*. (2017) 140:895–906. doi: 10.1016/j.jaci.2017.08.003
13. Honkinen M, Lahti E, Österback R, Ruuskanen O, Waris M. Viruses and bacteria in sputum samples of children with community-acquired pneumonia. *Clin Microbiol Infect*. (2012) 18:300–7. doi: 10.1111/j.1469-0691.2011.03603.x
14. Jartti T, Jartti L, Peltola V, Waris M, Ruuskanen O. Identification of respiratory viruses in asymptomatic subjects: asymptomatic respiratory viral infections. *Pediatr Infect Dis J*. (2008) 27:1103–7. doi: 10.1097/INF.0b013e31817e695d
15. Lönnrot M, Sjöroos M, Salminen K, Maaronen M, Hyypiä T, Hyöty H. Diagnosis of enterovirus and rhinovirus infections by RT-PCR and time-resoClved fluorometry with lanthanide chelate labeled probes. *J Med Virol*. (1999) 59:378–84. doi: 10.1002/(sici)1096-9071(199911)59:3<378::aid-jmv19>3.0.co;2-i
16. Österback R, Tevaluoto T, Ylinen T, Peltola V, Susi P, Hyypiä T, et al. Simultaneous detection and differentiation of human rhino- and enteroviruses in clinical specimens by real-time PCR with locked nucleic acid probes. *J Clin Microbiol*. (2013) 51:3960–7. doi: 10.1128/JCM.01646-13
17. Turunen R, Koistinen A, Vuorinen T, Arku B, Söderlund-Venermo M, Ruuskanen O, et al. The first wheezing episode: respiratory virus etiology, atopic characteristics, and illness severity. *Pediatr Allergy Immunol*. (2014) 25:796–803. doi: 10.1111/pai.12318
18. Jokinen C, Heiskanen L, Juvonen H, Kallinen S, Karkola K, Korppi M, et al. Incidence of community-acquired pneumonia in the population of four municipalities in eastern Finland. *Am J Epidemiol*. (1993) 137:977–88. doi: 10.1093/oxfordjournals.aje.a116770
19. Heiskanen-Kosma T, Korppi M, Jokinen C, Kurki S, Heiskanen L, Juvonen H, et al. Etiology of childhood pneumonia: serologic results of a prospective, population-based study. *Pediatr Infect Dis J*. (1998) 17:986–91. doi: 10.1097/00006454-199811000-00004
20. Fathima P, Blyth CC, Lehmann D, Lim FJ, Abdalla T, de Klerk N, et al. The impact of pneumococcal vaccination on bacterial and viral pneumonia in Western Australian children: record linkage cohort study of 469589 births, 1996-2012. *Clin Infect Dis*. (2018) 66:1075–85. doi: 10.1093/cid/cix923
21. Monto AS. The seasonality of rhinovirus infections and its implications for clinical recognition. *Clin Ther*. (2002) 24:1987–97. doi: 10.1016/S0149-2918(02)80093-5
22. Annamalay AA, Lanaspa M, Khoo S-K, Madrid L, Acácio S, Zhang G, et al. Rhinovirus species and clinical features in children hospitalised with pneumonia from Mozambique. *Trop Med Int Health*. (2016) 21:1171–80. doi: 10.1111/tmi.12743
23. Ahn JG, Kim DS, Kim KH. Clinical characteristics and cytokine profiles of children with acute lower respiratory tract infections caused by human rhinovirus. *PLoS ONE*. (2018) 13:e0198624. doi: 10.1371/journal.pone.0198624
24. Esposito S, Daleno C, Tagliabue C, Scala A, Tenconi R, Borzani I, et al. Impact of rhinoviruses on pediatric community-acquired pneumonia. *Eur J Clin Microbiol Infect Dis*. (2012) 31:1637–45. doi: 10.1007/s10096-011-1487-4
25. Xiang Z, Gonzalez R, Xie Z, Xiao Y, Chen L, Li Y, et al. Human rhinovirus group C infection in children with lower respiratory tract infection. *Emerging Infect Dis*. (2008) 14:1665–7. doi: 10.3201/eid1410.080545
26. Peltola V, Waris M, Osterback R, Susi P, Hyypiä T, Ruuskanen O. Clinical effects of rhinovirus infections. *J Clin Virol*. (2008) 43:411–4. doi: 10.1016/j.jcv.2008.08.014
27. Peltola V, Jartti T, Putto-Laurila A, Mertsola J, Vainionpää R, Waris M, et al. Rhinovirus infections in children: a retrospective and prospective hospital-based study. *J Med Virol*. (2009) 81:1831–8. doi: 10.1002/jmv.21590
28. Kennedy JL, Shaker M, McMeen V, Gern J, Carper H, Murphy D, et al. Comparison of viral load in individuals with and without asthma during infections with rhinovirus. *Am J Respir Crit Care Med*. (2014) 189:532–9. doi: 10.1164/rccm.201310-1767OC
29. Gold DR, Tager IB, Weiss ST, Tosteson TD, Speizer FE. Acute lower respiratory illness in childhood as a predictor of lung function and chronic respiratory symptoms. *Am Rev Respir Dis*. (1989) 140:877–84. doi: 10.1164/ajrccm/140.4.877
30. Castro-Rodríguez JA, Holberg CJ, Wright AL, Halonen M, Taussig LM, Morgan WJ, et al. Association of radiologically ascertained pneumonia before age 3 yr with asthmalike symptoms and pulmonary function during childhood: a prospective study. *Am J Respir Crit Care Med*. (1999) 159:1891–7. doi: 10.1164/ajrccm.159.6.9811035
31. Rantala AK, Jaakkola MS, Mäkikyrö EMS, Hugg TT, Jaakkola JJK. Early respiratory infections and the development of asthma in the first 27 years of life. *Am J Epidemiol*. (2015) 182:615–23. doi: 10.1093/aje/kwv093
32. Erdem SB, Can D, Girit S, Çatal F, Sen V, Pekcan S, et al. Does atopy affect the course of viral pneumonia? *Allergol Immunopathol (Madr)*. (2018) 46:119–26. doi: 10.1016/j.aller.2017.04.003
33. Miller EK, Bugna J, Libster R, Shepherd BE, Scalzo PM, Acosta PL, et al. Human rhinoviruses in severe respiratory disease in very low birth weight infants. *Pediatrics*. (2012) 129:e60–7. doi: 10.1542/peds.2011-0583
34. Kennedy JL, Turner RB, Braciale T, Heymann PW, Borish L. Pathogenesis of rhinovirus infection. *Curr Opin Virol*. (2012) 2:287–93. doi: 10.1016/j.coviro.2012.03.008
35. Townsi N, Laing IA, Hall GL, Simpson SJ. The impact of respiratory viruses on lung health after preterm birth. *Eur Clin Respir J*. (2018) 5:1487214. doi: 10.1080/20018525.2018.1487214
36. Spichak TV, Yatsyshina SB, Katosova LK, Kim SS, Korppi MO. Is the role of rhinoviruses as causative agents of pediatric community-acquired pneumonia over-estimated? *Eur J Pediatr*. (2016) 175:1951–8. doi: 10.1007/s00431-016-2791-x
37. Ruuskanen O, Järvinen A. What is the real role of respiratory viruses in severe community-acquired pneumonia? *Clin Infect Dis*. (2014) 59:71–3. doi: 10.1093/cid/ciu242
38. Shi T, McLean K, Campbell H, Nair H. Aetiological role of common respiratory viruses in acute lower respiratory infections in children under five years: a systematic review and meta-analysis. *J Glob Health*. (2015) 5:010408. doi: 10.7189/jogh.05.010408
39. Drysdale SB, Mejias A, Ramilo O. Rhinovirus - not just the common cold. *J Infect*. (2017) S41–6. doi: 10.1016/S0163-4453(17)30190-1
40. Baillie VL, Olwagen CP, Madhi SA. Review on clinical and molecular epidemiology of human rhinovirus-associated lower respiratory tract infections in African and Southeast Asian children. *Pediatr Infect Dis J*. (2018) 37:E185–94. doi: 10.1097/INF.0000000000001897
41. Korppi M. The role of rhinoviruses is overestimated in the aetiology of community-acquired pneumonia in children. *Acta Paediatr*. (2017) 106:363–5. doi: 10.1111/apa.13709
42. Broberg E, Niemelä J, Lahti E, Hyypiä T, Ruuskanen O, Waris M. Human rhinovirus C–associated severe pneumonia in a neonate. *J Clin Virol*. (2011) 51:79–82. doi: 10.1016/j.jcv.2011.01.018
43. Imakita M, Shiraki K, Yutani C, Ishibashi-Ueda H. Pneumonia caused by rhinovirus. *Clin Infect Dis*. (2000) 30:611–2. doi: 10.1086/313723
44. Lu X, Schneider E, Jain S, Bramley AM, Hymas W, Stockmann C, et al. Rhinovirus viremia in patients hospitalized with community-acquired pneumonia. *J Infect Dis*. (2017) 216:1104–11. doi: 10.1093/infdis/jix455
45. Fuji N, Suzuki A, Lupisan S, Sombrero L, Galang H, Kamigaki T, et al. Detection of human rhinovirus C viral genome in blood among children with severe respiratory infections in the Philippines. *PLoS ONE*. (2011) 6:e27247. doi: 10.1371/journal.pone.0027247
46. Peltola V, Waris M, Kainulainen L, Kero J, Ruuskanen O. Virus shedding after human rhinovirus infection in children, adults and patients

with hypogammaglobulinaemia. *Clin Microbiol Infect*. (2013) 19:E322–7. doi: 10.1111/1469-0691.12193
47. Karhu J, Ala-Kokko TI, Vuorinen T, Ohtonen P, Syrjälä H. Lower respiratory tract virus findings in mechanically ventilated patients with severe community-acquired pneumonia. *Clin Infect Dis*. (2014) 59:62–70. doi: 10.1093/cid/ciu237
48. Ng KT, Oong XY, Lim SH, Chook JB, Takebe Y, Chan YF, et al. Viral load and sequence analysis reveal the symptom severity, diversity, and transmission clusters of rhinovirus infections. *Clin Infect Dis*. (2018) 67:261–8. doi: 10.1093/cid/ciy063
49. Megremis S, Niespodziana K, Cabauatan C, Xepapadaki P, Kowalski ML, Jartti T, et al. Rhinovirus species-specific antibodies differentially reflect clinical outcomes in health and asthma. *Am J Respir Crit Care Med*. (2018) 198:1490–9. doi: 10.1164/rccm.201803-0575OC
50. Langelier C, Kalantar KL, Moazed F, Wilson MR, Crawford ED, Deiss T, et al. Integrating host response and unbiased microbe detection for lower respiratory tract infection diagnosis in critically ill adults. *Proc Natl Acad Sci U S A*. (2018) 115:E12353–62. doi: 10.1073/pnas.1809700115
51. Silkoff PE, Flavin S, Gordon R, Loza MJ, Sterk PJ, Lutter R, et al. Toll-like receptor 3 blockade in rhinovirus-induced experimental asthma exacerbations: a randomized controlled study. *J Allergy Clin Immunol*. (2018) 141:1220–30. doi: 10.1016/j.jaci.2017.06.027
52. Louie JK, Roy-Burman A, Guardia-Labar L, Boston EJ, Kiang D, Padilla T, et al. Rhinovirus associated with severe lower respiratory tract infections in children. *Pediatr Infect Dis J*. (2009) 28:337–9. doi: 10.1097/INF.0b013e31818ffc1b
53. Berce V, Unuk S, Duh D, Homšak M, Vičič M. Clinical and laboratory characteristics of viral lower respiratory tract infections in preschool children. *Wien Klin Wochenschr*. (2015) S255–62. doi: 10.1007/s00508-015-0843-2
54. Brealey JC, Sly PD, Young PR, Chappell KJ. Viral bacterial co-infection of the respiratory tract during early childhood. *FEMS Microbiol Lett*. (2015) 362:fnv062. doi: 10.1093/femsle/fnv062
55. Cawcutt K, Kalil AC. Pneumonia with bacterial and viral coinfection. *Curr Opin Crit Care*. (2017) 23:385–90. doi: 10.1097/MCC.0000000000000435
56. Jennings LC, Anderson TP, Beynon KA, Chua A, Laing RTR, Werno AM, et al. Incidence and characteristics of viral community-acquired pneumonia in adults. *Thorax*. (2008) 63:42–8. doi: 10.1136/thx.2006.075077
57. Costa LF, Queiróz DAO, Lopes da Silveira H, Bernardino Neto M, de Paula NT, Oliveira TFMS, et al. Human rhinovirus and disease severity in children. *Pediatrics*. (2014) 133:e312–21. doi: 10.1542/peds.2013-2216
58. Ishizuka S, Yamaya M, Suzuki T, Takahashi H, Ida S, Sasaki T, et al. Effects of rhinovirus infection on the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells. *J Infect Dis*. (2003) 188:1928–39. doi: 10.1086/379833
59. Oliver BGG, Lim S, Wark P, Laza-Stanca V, King N, Black JL, et al. Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. *Thorax*. (2008) 63:519–25. doi: 10.1136/thx.2007.081752
60. Finney LJ, Belchamber KBR, Fenwick PS, Kemp SV, Edwards MR, Mallia P, et al. Human rhinovirus impairs the innate immune response to bacteria in alveolar macrophages in COPD. *Am J Respir Crit Care Med*. (2019) 199:1496–1507. doi: 10.1183/13993003.congress-2018.PA5310
61. Ben-Shimol S, Greenberg D, Hazan G, Shemer-Avni Y, Givon-Lavi N, Dagan R. Seasonality of both bacteremic and nonbacteremic pneumonia coincides with viral lower respiratory tract infections in early childhood, in contrast to nonpneumonia invasive pneumococcal disease, in the pre-pneumococcal conjugate vaccine era. *Clin Infect Dis*. (2015) 60:1384–7. doi: 10.1093/cid/civ023

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

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership