Viruses in the upper respiratory tract of individuals at risk of zoonotic infection and their animals in Vietnam: follow-up and virus discovery

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ACADEMIC DISSERTATION

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

i. **Nguyen Thi Kha Tu**, Ngo Tri Tue, Olli Vapalahti, Anna-Maija K. Virtala, Le Van Tan, Maia A. Rabaa, Juan Carrique-Mas, Guy E. Thwaites, Stephen Baker. Occupational Animal Contact in Southern and Central Vietnam. EcoHealth. 2019. doi:10.1007/s10393-019-01444-0.

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iii. **Nguyen Thi Kha Tu**, Nguyen Thi Thu Hong, Nguyen Thi Han Ny, Tran My Phuc, Pham Thi Thanh Tam, H. Rogier van Doorn, Ho Dang Trung Nghia, Dang Thao Huong, Duong An Han, Luu Thi Thu Ha, Xutao Deng, Guy Thwaites, Eric Delwart^, Stephen Baker^, Anna-Maija K Virtala^, Olli Vapalahti^ and Le Van Tan. The Virome of Acute Respiratory Diseases in Individuals at Risk of Zoonotic Infections. Viruses. 2020;12:960. doi:10.3390/v12090960.

iv. **Nguyen Thi Kha Tu**, Xutao Deng, Nguyen Thi Thu Hong, Nguyen Thi Han Ny, Tran My Phuc, Pham Thi Thanh Tam, Duong An Han, Luu Thi Thu Ha, Guy Thwaites, H. Rogier van Doorn, Anna-Maija K. Virtala^, Eric Delwart^, Stephen Baker^, Olli Vapalahti and Le Van Tan. Redondoviridae: High Prevalence and Possibly Chronic Shedding in Human Respiratory Tract, But No Zoonotic Transmission. Viruses. 2021; Vol. 13, Issue 4. doi.org/10.3390/v13040533.

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2. ABSTRACT

Historically, the majority of emerging infections of serious public health threat have been caused by respiratory viruses of zoonotic origin. Notably, Southeast Asia (including Vietnam) is considered as one of the hotspots of emerging infections. The ongoing coronavirus disease 2019 (COVID-19) pandemic emphasizes the importance of understanding the nature of human exposures to zoonotic reservoirs, conducting active surveillance for pathogens and novel viruses, especially at the human-animal interface, as well as having feasible laboratory testing available for quick identification of emerging viruses in future outbreaks. This thesis therefore aims to 1) to characterize the nature of exposures to potential zoonotic sources, 2) to determine the spectrum of respiratory viral viruses detected by PCR, 3) to gain insights into the human virome of acute respiratory infections by metagenomic analysis, 4) to assess zoonotic potential of novel viruses discovered by metagenomics in respiratory tract of individuals with sustained occupational contact with animals, and 5) to evaluate viral detection of metagenomic next generation sequencing (mNGS) and compare viral detection of mNGS versus PCR.

As a foundation for answering the proposed research questions, a three-year cohort of 581 individuals working with animals in Vietnam was established during 2013–2016. To establish the baseline data, each of the study participants had nasal-throat swabs collected at the beginning of each year when absence of any respiratory symptoms. During follow-up, whenever the participants reported having any respiratory symptom and fever, their nasal-throat swabs were collected. In parallel, nasal-throat swabs of their animals were also collected.

To characterize the nature of human exposures to animals, the baseline data from the three years were combined to analyze exposure as cross-sectional data. The data show that the participants were frequently exposed to zoonotic sources by both occupational activities (as animal raising farmers, animal health workers, slaughter-house workers and rat traders) and non-occupational activities (e.g. farming animals surrounding their house, consuming of raw animal blood or meat). The risks of exposure were increased by no or limited use of personal protective equipment, or by exposure to a large variety of animal species known as potential reservoirs of zoonotic pathogens.

To explore the spectrum of viruses associated with acute respiratory infections, multiplex RT-PCRs were used to screen for 15 common respiratory viral viruses in all swabs at baseline and disease episodes. Enteroviruses and human rhinoviruses were predominant viruses detected with higher frequency in disease-episode samples than in baseline samples throughout the study. This reinforces their role in respiratory infections and clinical significance of the *Enterovirus* genus besides causing life threatening infections such as poliomyelitis and brain stem encephalitis.

Yet, the majority (over 80%) of the tested specimens were RT-PCR negative. Additionally, to characterize the virome of acute respiratory infections and to evaluate our mNGS protocol in virus detection, mNGS was used to analyze 91 swabs at disease episodes collected in 2013. Of these, 15 samples were RT-PCR positive for at least one virus and included as controls. mNGS successfully detected and genotyped human rhinovirus, enterovirus, influenza A virus, coronavirus OC43, and RSV A in 13 of 15 (86.7%) RT-PCR positive samples. Additionally, rotavirus, torque teno virus, human papillomavirus and human betaherpesvirus 7 were also detected. Notably, a vientovirus of a recently discovered *Redondoviridae* family, a novel cyclovirus, a novel gemycircularvirus and a novel statovirus were also detected and genetically characterized. The results thus offer important insights into the virome of acute respiratory infections in individuals at high risk of zoonotic infections. The mNGS protocol used here is highly sensitive for sequence-independent detection of a wide range of viruses (including previously unrecognized viruses) in clinical samples.

To characterise the novel viruses, the recently discovered *Redondoviridae* family and to compare mNGS versus (RT-)PCR in virus detection, PCR was used to screen for their presence in all mNGS samples. Subsequently, while the virus detection of mNGS were successfully replicated by PCR, redondoviruses were additionally detected in 29, the novel cyclovirus in 5, the novel gemycircularvirus in 12 mNGS-negative samples. This result thus further expands the prevalence of these viruses in respiratory samples and emphasize that standard PCR remains a more sensitive diagnostic test for known viruses than mNGS, however, mNGS is an unbiased approach with the capability to detect both novel and unanticipated viruses in a single test.

To further characterize the novel viruses and redondoviruses, PCR was used to further screen the baseline samples. The prevalence of these viruses in baseline samples was comparable with or higher than that in disease-episode samples. This is in agreement with a previous redondovirus study. However, higher copy numbers of redondovirus DNA were previously reported in oropharyngeal samples of critically ill patients than those of healthy individuals. Therefore, copy numbers of redondovirus DNA in samples at disease episodes and baseline are suggested to be determined.

To assess zoonotic potential from co-carriage of the novel viruses and redondoviruses in animals, PCR screenings were also conducted on nasal-throat swabs from the animals that the virus-positive participants were exposed to. No evidence of redondoviruses was found in pigs, chicken, Muscovy ducks, ducks and dogs, suggesting these animals are less likely to be the hosts of redondoviruses. Similarly, the novel statovirus was not detected in animal samples. In contrast, sequences of the novel cyclovirus and gemycircularvirus were found in pig swabs. Notably, identical PCR amplicons (370bp) of the novel gemycircularvirus were found in both human participants and their pigs, suggesting zoonotic transmission probably happened. However, passive contamination in

respiratory tracts of both humans and their animals cannot be excluded since the natural hosts of gemycircularviruses and cycloviruses are undetermined yet.

Besides high prevalence and lack of zoonotic transmission as found above, additional tests showed that the same replication gene sequence of redondoviruses was detected in longitudinal nasal-throat swabs of several participants (window time 35–132 days). The data thus show likelihood of redondovirus persistence in the human nasopharynx. Phylogenetic analysis showed a close relatedness between the redondovirus strains detected in Vietnam, the US, Spain and China, suggesting a wide geographic distribution and diversity of redondoviruses. Collectively, these data provided new insights into the high prevalence, widespread, possible chronic shedding in human respiratory tracts and absence of evidence about the zoonotic origin of redondoviruses.

Collectively, this study reveals significant insights into the nature of exposures to animals among animal workers in Vietnam and into the virus spectrum in their respiratory samples collected at baseline and respiratory disease episodes. Additionally, the analysis has also led to the discovery and initial characterization of several novel viruses and their zoonotic potentials. This study also expands understanding of *Redondoviridae* family. The mNGS protocol herein allows for the detection of a variety of respiratory viruses, including novel ones, and therefore feasibly support for prompt detection of emerging viruses in future outbreaks.

3. ABBREVIATIONS

ADV	adenovirus
AIDS	acquired immunodeficiency syndrome
BoV	human bocavirus
CI	confidence interval
CoV	human coronavirus
COVID-19	coronavirus disease 2019
CRESS	circular Rep-encoding single-stranded
Ct	cycle threshold
DFA	direct fluorescent antibody
DNA	deoxyribonucleic acid
EIA	enzyme imunoassays
ELISA	enzyme-linked immunosorbent assay
EVs	enterovirus
HIV	human immunodeficiency virus
IgM	immunoglobulin M
IgG	immunoglobulin G
RV	rhinovirus
MERS-CoV	Middle East respiratory syndrome coronavirus
mNGS	metagenomic next-generation sequencing
MPV	human metapneumovirus
ORF	open reading frame
PCR	polymerase chain reaction
PEV	parechovirus
PIV	parainfluenza virus
PPE	personal protective equipment
RdRp	RNA-dependent RNA polymerase
RIAs	rapid immunoassays
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	(reverse transcription) real time polymerase chain reaction
SARS	severe acute respiratory syndrome
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
US	the United States
VIZIONS	Vietnam Initiative on Zoonotic Infections

4. LITERATURE REVIEW

4.1. Human respiratory tract

The human respiratory tract is made up of passages for transferring air from environment into the body and respiratory surface for gas exchange [1]. The airway epithelium is structured by four main histological layers, including respiratory mucosa, submucosa, cartilage and/or muscular layer and adventitia [2]. The airway epithelium from the nose to respiratory bronchioles is the main tropism of majority of respiratory viruses. For example, α2,6-linked sialic acid receptor of epithelial cells on the airway epithelium is known as the preferentially binding site of human influenza A viruses [3,4]. The function of the epithelium is also as a barrier to protect the host from infection of pathogens and foreign particles [2]. The protective mechanism of epithelial cells is via innate immunity and production of cytokines and chemokines to communicate with immune cells to activate and regulate antiviral responses [5]. Additionally, the tonsils also play as the first line to protect against ingested or inhaled pathogens since they consist a mass of lymphoid tissue [6].

The respiratory tract is divided into upper and lower parts. The upper respiratory tract is the airways from the nostrils to vocal cords, including nose, nasal cavity, paranasal sinus, pharynx (throat) and middle ear. The lower respiratory tract is all structures below the pharynx of respiratory tract, including the larynx, trachea, bronchi, bronchioles, and the alveoli of the lungs [1,7] (Figure 1).

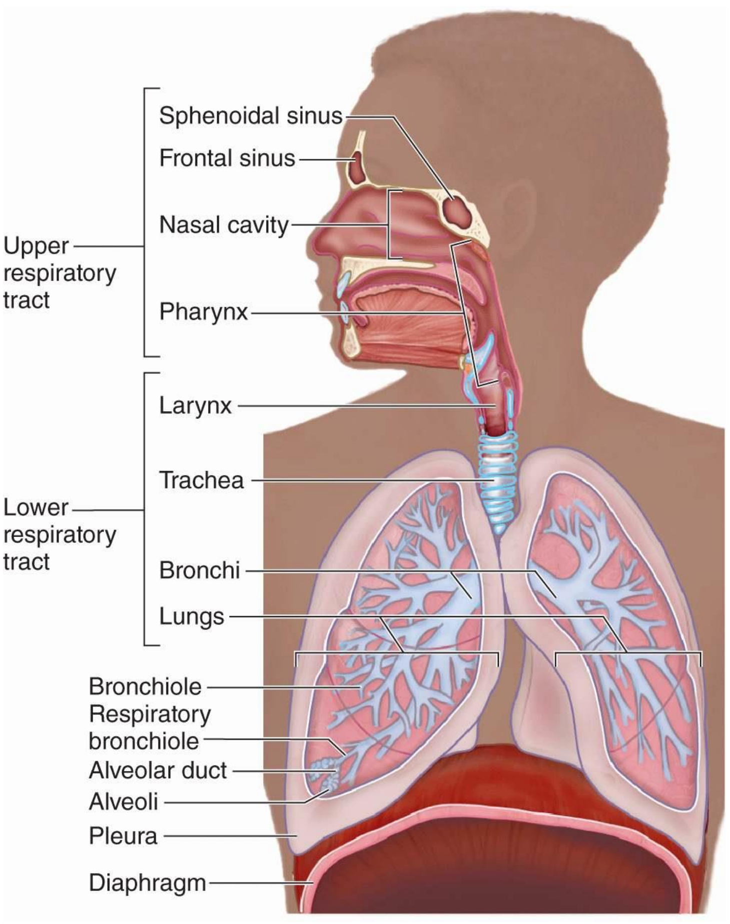


Figure 1: The anatomy of human respiratory system including upper and lower respiratory tracts. Copy from open-access sources, Basicmedical Key [8].

4.2. Acute respiratory infections

Acute respiratory infections are the most widespread infectious diseases in humans and the leading cause of morbidity and mortality worldwide. They are annually responsible for over 3 million deaths worldwide [9,10]. Acute lower respiratory tract infections include croup, tracheobronchitis, bronchiolitis and, commonly, pneumonia and can be severe and fatal. In contrast, acute upper respiratory tract infections include common cold, sinusitis, pharyngitis, epiglottitis and laryngotracheitis [11]. The demarcations between lower and upper infections are not always clear like the case of laryngotracheitis that larynx belongs to lower respiratory tract, while laryngotracheitis is classified as an acute upper respiratory infection [11]. An acute respiratory infection is classified as upper or lower respiratory tract infection based on symptomatology and anatomic involvement [11].

Acute upper respiratory tract diseases are usually not serious, most of the cases are benign, transitory and self-limited [11]. However, their complications are more crucial than the infection themselves since the acute viral infections can lead to bacterial infections causing the sinuses and middle ear or lower respiratory tract diseases [7]. Therefore, identification of the etiology of acute respiratory infections is crucial for disease management.

4.3. Etiology of acute respiratory infections

Upper respiratory tract infections

Viruses are predominant agents in upper respiratory infections [7,11,12]. Bacteria often cause the diseases in upper respiratory tract as secondary infections [11]. The most prevalent type of all respiratory infections is the common cold, of which rhinoviruses (RV) and influenza viruses are the most common pathogens, followed by human coronaviruses (CoV) [11,13,14]. Parainfluenza viruses (PIV), respiratory syncytial virus (RSV), adenoviruses (ADV), human metapneumovirus (MPV), enteroviruses (EVs, not including RV), human bocavirus (BoV), SARS-CoV-2 and parechoviruses (PEV) are also frequently detected pathogens [11,15–17].

For pharyngitis, influenza virus is the most common pathogen, followed by EVs A. *Streptococcus pyogenes* is the most important bacterial agent of acute pharyngitis and tonsillitis infection, followed by *Mycoplasma pneumoniae*. *Chlamydophila pneumoniae* is associated with pharyngitis in adult [11] while *Moraxella catarrhalis* is more commonly detected in children with pharyngitis [18].

For laryngotracheitis, PIV is the most common agent, followed by RSV, ADV, influenza viruses, EVs. However, the laryngotracheitis becomes more severe with bacterial infections including *Haemophilus influenzae* type b, *S. pyogenes* and *Corynebacterium diphtheria* [11].

Lower respiratory tract infections

For acute diseases of lower respiratory tract, apart from viruses, mycoplasmata, rickettsiae and fungi as etiological agents, bacteria are the more prominent pathogens [11]. Overall, *Streptococcus pneumoniae*, *H. influenzae*, *C. pneumoniae* and *M. pneumoniae* are most common bacterial agents, while influenza virus and RSV are the most common viral pathogen detected [11,19].

For pneumonia, *S. pneumoniae* is considered the most common bacterial pathogens of community-acquired acute pneumonia [11], followed by *H. influenzae*, *M. pneumoniae*, *Legionella pneumophila*

and *C. pneumoniae* [13,20,21]. *Klebsiella pneumoniae* was identified as an important community acquired pathogen in Vietnam [22]. *C. pneumonia* induces pneumonia commonly in neonates and young infants [11]. *H. influenzae* is also common pathogen in children under age 5 [11]. *M. pneumoniae* causes atypical pneumonia mostly in young people (5–19 years old). *L. pneumophila* is also known to cause atypical pneumonia, including in large outbreaks [11]. *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter* spp., *K. pneumoniae*, *Acinetobacter* spp., *P. aeruginosa* and *Staphylococcus aureus* are the most common agents for nosocomial pneumonias [11,13].

The major cause of viral pneumonia, especially in infants and children at younger age, is RSV which results in hundreds of thousands of death of children worldwide [13,23,24]. For older children, rhinovirus is the predominant agent [24]. Pneumonia caused by influenza viruses and SARS-CoV-2 is deadly in the elderly and in patients with underlying diseases [17,25]. Viral pneumonia is more deadly with bacterial pneumonia as secondary infections [25]. *S. aureus* is the most common bacterial agent causing secondary pneumonia [26]. ADV is found to be associated with severe, fatal pneumonia in infants [27]. Measles virus may cause pneumonia in adults [7,28], while *Cytomegalovirus* infections can lead to pneumonia in neonates and immunosuppressed individuals [29].

Bronchitis often develops from an upper respiratory tract infection. In infants, bronchitis is a viral causality, respiratory syncytial virus (RSV) is the primary agent. PIV, influenza viruses, RV and ADV are also associated with bronchitis [11,13]. *H. influenzae* and *S. pneumoniae* and occasionally *M. pneumoniae* are known to cause bronchitis [11].

Viruses of acute respiratory infections

The most, if not all, pathogens of acute respiratory infections are bacteria and viruses. For acute respiratory infections in general, viral pathogens are more frequent agents than bacterial ones. Viral pathogens also have highest potential to cause outbreaks and pandemics [30–33]. Pandemics of SARS-CoV-2 [30], and MERS-CoV in 2014 [32,34] or avian influenza virus A/H5N1 in 2003 [35], influenza virus A/H1N1pdm09 in 2009 [31] or SARS-CoV in 2003 [33] are typical examples.

Overall, RSV A, RSV B, influenza A virus, influenza B virus, ADV, EVs, RV, MPV, PIV 1, PIV 2, PIV 3, PIV 4, CoV, BoV and PEV are currently the most common viral agents of acute respiratory infections (Table 1). They are also most common viruses screened in routine laboratory diagnosis of acute respiratory infections. Besides causing the acute diseases, these pathogens were frequently detected in asymptomatic respiratory infections [36–38].

Influenza A virus, influenza B virus and CoV infections have been most frequently detected in people over five years old, while RV, RSV and PIV infections have been found mostly in children

under five years in South East Asia since 2000 [7,16,38–43]. Additionally, many viruses infecting humans with suspected pathogenicity to cause acute respiratory infections was frequently identified in respiratory samples, including herpesviruses, anelloviruses, papillomaviruses, polyomaviruses (Table 1).

Table 1: Common viruses detected in samples of human respiratory tract

Virus family or genus	**Species or Types**
Picornaviruses	Rhinovirus A/B/C [44,45], Enterovirus A/B/D [44], Parechovirus [46]
Paramyxoviridae	RSV A, B [47], Parainfluenzavirus 1–4 [44,47], Metapneumovirus [48], Measles virus [48,49]
Orthomyxoviridae	Influenzavirus A/B/C [44,49]
Coronaviridae	Coronavirus HKU1, OC43, 229E, NL63 [47], SARS-CoV-2 [30]
Adenoviruses	Adenovirus (C or untyped) [47]
Parvoviruses [32]	Bocavirus [47], Parvovirus B19 [50] or untyped [48]
Herpesviruses	Epstein-Barr virus [52], Human herpesvirus 8 (Kaposi sarcomavirus) [44,48,52,53], Herpes simplex viruses 1/2 [54–56] Cytomegalovirus (human betaherpesvirus 5) [29,34,57], Roseolavirus (human betaherpesvirus 6A/6B/7) [44,48,52,53]
Anelloviruses	Torque teno virus [44,47,49,52,58], Torque teno midi virus [44,49,58], Torque teno mini virus [44,47,49,52,58] or untyped [44,47,49]
Papillomaviruses	Papillomavirus 1/4/8/13/B19 [44,48]
Polyomaviruses	Polyomavirus 4 [44], KI [49], WU [52] or untyped [48]

Fungi (*Aspergillus* [59–61], *Cryptococcus* [62–64], *Pneumocystis* [65,66] and endemic fungi [65,67,68] (e.g. *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Talaromyces marneffei*, *Histoplasma duboisii*

or *Paracoccidioides brasiliensis*)) are considered as opportunistic pathogens infecting immunocompromised populations (e.g. HIV patients and transplantation recipients), rather than immunocompetent individuals. Mycoplasmata and rickettsiae are rare agents of acute respiratory infections

However, the majority of etiological studies on acute respiratory infections have been conducted in hospitalized patients, while those in general community are not well characterized. Moreover, despite intensive laboratory investigations, a substantial proportion of acute respiratory infections are of unknown etiology [69–72]. Therefore, pathogen discovery is crucial for better control of acute respiratory infections.

4.4. Viruses detected in human respiratory tract with known human tropism or unknown tropism

Beside common etiological agents, a wide range of viruses have been frequently identified in samples of individuals with acute respiratory infections, such as saffold virus, aichi virus (human kobuvirus), cosavirus, Rubella virus, rotavirus, vientovirus, brisavirus, cyclovirus and gemycircularvirus (Table 2). However, pathogenicity and human tropism of these viruses are unidentified yet or remain suspected.

Table 2: Other viruses with known or unknown human tropism and previously detected in samples of human respiratory tract

Virus family or genus	Species or Types
Picornaviruses	Saffold virus [73,74]
	Aichi virus (human kobuvirus) [75]
	Cosavirus [76]
Togaviridae	Rubella virus [48]
Reoviruses [77]	Rotavirus [78–80]
Redondoviridae	Vientovirus [81]
	Brisavirus [81]
Cycloviruses	Cyclovirus [82,83]
Gemycircularviruses	Gemycircularvirus [84]

Redondoviridae family

Redondoviridae is a novel circular single stranded DNA virus family, of which the viruses of *Circovirus* genus are globally well-known fatal pathogens in swine (e.g., porcine circovirus-2), birds (e.g., beak and feather disease virus) and chicken (e.g., chicken anemia virus) [85]. The first reported redondovirus genome named human PoSCV5-like circular virus was detected from the respiratory secretions of a febrile patient [86,87]. Redondoviruses were found to be the second most prevalent eukaryotic DNA virus family in human respiratory tract, proposed to colonize human oro-respiratory sites [81,88]. The detection rates of redondoviruses were about 15% in the oropharynx of healthy Americans [81], 11% in Italians [89] and 2% in Spanish subjects [88].

There is no evidence of presence of virus redondovirus sequences detected in animals, freshwater, marine, air, soil samples or in laboratory reagents by screening the virus genome in the mNGS databases [81] (Figure 2). The respiratory samples of animals were from bats (n=85), poultry (n=16), pigs (n=16 and 2), cattle (n=1), wild rodents and small mammals (n=104), captive wild giant pandas (n=1) [81]. Additionally, the animals samples were from intestinal tract of a shrew (n=1), neonatal piglets (n=29), bats (n=14), pigs (n=14), a wild pigeon (droppings, n=1), a dromedary camel (n=1), red foxes (n=8), a domestic duck (*Anas platyrhynchos*) (n=1), fur seals (n=2), bovine rumen (n=61), post weaning multisystemic wasting syndrome-affected pigs (n=8), dogs (n=16), gorilla (n=23), a red-crowned crane (n=1), a golden monkey (n=1), domestic caprids (n=2), simian bushmeat (n=2), and chicken and turkey (n=3); plasma of cattle (n=1), and skin swabs from wild rats (n=13) [81]. However, the methods of mNGS analysis used in above studies were not fully presented and mNGS analysis is not currently the standard method in detection of viruses. Notably viruses of the group of CRESS (circular Rep-encoding single-stranded) DNA viruses have been widely found in animals. Additionally deltaviruses which were previously thought be confined to the human host have recently been detected in birds, snakes, fish, amphibians, and invertebrates [90,91]. It seems plausible that redondoviruses will be identified in animal samples in the future. Therefore, virus surveillance in various animal species is recommended.

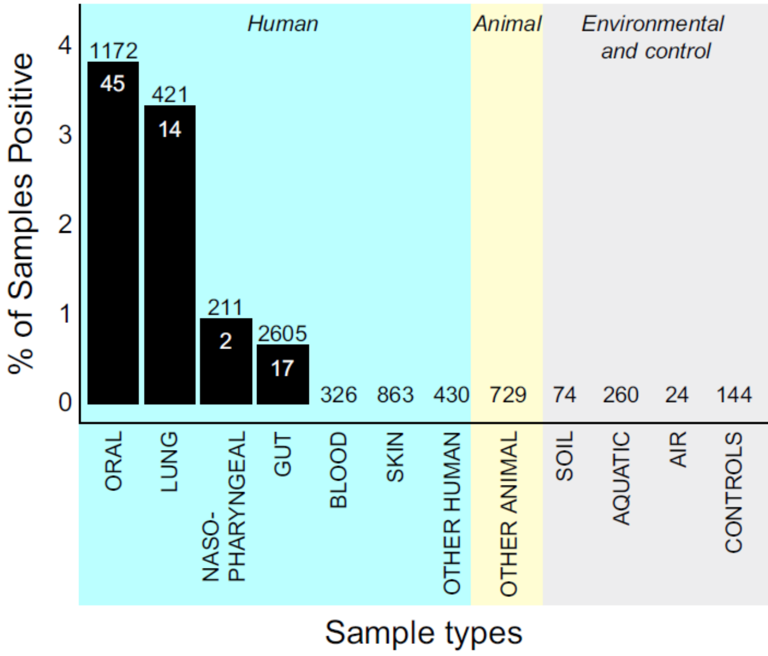


Figure 2: Frequency of redondovirus sequences detected in different types of samples. The data from 173 metagenomic datasets were aligned to redondovirus genomes. A positive hit was defined to have ≥25% coverage of redondovirus genome by short-read alignment. The number above the bar is the number of total samples analyzed. The number of positive samples are shown within the bar. Copy from Abbas AA, Taylor LJ, Dothard MI, et al. Redondoviridae, a Family of Small, Circular DNA Viruses of the Human Oro-Respiratory Tract Associated with Periodontitis and Critical Illness. *Cell Host and Microbe*. 2019;25(5):719-729.e4 [81]. Reprinted with permission from Elsevier.

Redondoviruses were suggested to be potentially associated with several human disorders. Abbas et al.[81] found an association of higher whole genome quantity of the viruses in critically ill than in healthy individuals by selective whole genome amplification (SWGA) technique. This family was also associated with periodontal disease and abundances decreased with standard periodontal treatment [81].

Genome of Redondoviruses, 3.0–3.1 kb, encodes a capsid and replication-associated protein in opposite directions, as well as a third protein (ORF 3) with unknown functions which is not homological with other virus or cellular proteins. Redondovirus replication and capsid proteins are phylogenetically distinct from other members of CRESS DNA virus families. A conserved stem loop structure is located immediately upstream of the replication-protein ORF, likely representing the viral origin of replication.

The capsid gene is highly conserved, sharing 67.5%–99.6% (median 82.3%) pairwise amino acid identities within the family of viruses, while the more variable site (36.6%–99.7% amino acid identity within the viruses of the family) is the replication gene used for identification[81]. The *Redondoviridae* family consists of one genus *Torbeviruses* with two species, named vientovirus and brisavirus, with the demarcation proposed as 50% identity of the replication protein [81,87]. A redondovirus-similar virus named porcine stool-associated circular virus-5 (accession number: KJ433989) detected in porcine stool samples has a replication protein similar to that of the redondoviruses, but has a distinct capsid protein and lacks an ORF3 protein [87]. Therefore, the sequence of capsid gene is a target to confirm detection of redondoviruses, while the replication gene is for genus or species identification of viruses in the *Redondoviridae* family.

The viruses were supposed to less likely to represent bacteriophages since no prokaryotic ribosome binding site was detected proximally to any protein coding sequence. Persistence of redondoviruses for almost 3 weeks was found in endotracheal aspirates of four critically ill subjects [81]. However, the knowledge of host, prevalence and characteristics of this recently discovered virus family remains very patchy.

Cycloviruses

Cycloviruses belong to the *Circoviridae* family. The closely related circoviruses are well known globally to cause fatal diseases in swine, birds and chicken [85,92]. Cycloviruses have small circular, single-stranded DNA genomes, 1.7 to 1.9 kb, containing two major ORFs encoding the replication and capsid proteins in two different directions [85]. Both replication and capsid proteins of cycloviruses have similar features compared to these proteins of circoviruses [51,93]. The cyclovirus replication protein contains motifs similar to circovirus RCR and SF3 helicase motifs [51]. A cyclovirus with genome sharing < 80% genome-wide pairwise identity with other viruses of the *Cyclovirus* genus is classified as new cyclovirus species [85,94].

In contrast to circoviruses, the host and pathogenic potential of the *Cyclovirus* genus has been under debate since they were spontaneously found in both vertebrates and invertebrates [95], such as humans, other mammals (bats, cats, cows, goats, horses, squirrels, sheep) [96–104], birds (chickens) [97,98] and insects (cockroaches and dragonflies) [105–108]. In human, cyclovirus sequences have been detected in blood [109,110], cerebrospinal fluid [110,111], respiratory tract [83,111], faeces [111,112], and with persistent detection of identical sequences in serum of immunodeficient patients [113]. However, no consistent association between cycloviruses and any human-health disorder have been identified, and the detection were only by mNGS or PCRs, while no specific immunity or virus isolation has

been reported. Therefore, further investigations of cycloviruses are in need to better understand the role of this genus in human health.

Gemycircularviruses

Gemycircularviruses are ssDNA circular viruses of *Genomoviridae* family in group of CRESS DNA viruses. Whether gemycircularviruses can infect humans is unknown. Gemycircularvirus genome sequences were identified in a wide range of hosts, including faeces of different animals, plants, the body of insects, sewage, human respiratory tract, and were recently reported in blood from a patient with multiple sclerosis, and in cerebrospinal fluid of encephalitis patients [84,114–117]. However, no association of this virus genus with human diseases was found and no virus specific humoral antibody response has been detected. In addition, virus has not yet been cultivated from tissues.

Like CRESS DNA viruses, gemycircularviruses have a small genome, 2,089–2,290 nucleotides. The 78% pairwise identity is species demarcation of gemycircularviruses and all the species of *Gemycircularvirus* genus share 56–77% genome-wide sequence similarity with each other [118]. More studies are required to better understand the roles of this virus genus in human health.

Statoviruses

Statoviruses belong to a novel taxon of RNA viruses and have been detected in stool samples from a variety of mammals, including human, macaque, mouse, cow, but not in sequencing datasets of bacteria, fungi, plants, unicellular eukaryotic organisms or of analysis on environmental samples or human respiratory tract [119]. The whole genome of these viruses is ~4 kb with phylogenetic similarity to members of the RNA-dependent RNA polymerase (RdRp) Superfamily II domain. Viruses infect both animals and plants, though statovirus is phylogenetically closer to plant than animal viruses [119]. Currently, the understanding of this novel taxon is limited.

4.5. Interactions of pathogens

Mixed-infections of pathogens in respiratory tract are commonly detected. Up to 27% of all cases of community-acquired pneumonia are found to be mix-infections by pathogens [120]. Although the pathogens in mix-infections can simply be independent with each other, it is widely recognised that the mixed-infections can result in positive or negative interactions between microbes that can

affect the course and severity of the infections [14,121]. Positive interactions is microbes creating an advantaging conditions for the others, while the negative interactions result from competitions of microbes or from triggering host immune responses that disproportionally affect infections of the competing microbes [14].

Bacteria-bacteria interactions

A well-recognised negative interaction is production of hydrogen peroxidase (H_2O_2) of bacterium to kill most other one. *S. pneumoniae* is known to tolerate and produce H_2O_2 that is lethal even for catalase-producing bacteria (H_2O_2-neutralizing enzyme), such as *S. aureus* [122] and *H. influenzae* [123]. Another mechanism of negative interaction is targeting structures of the competing microbes to inhibit their attachment to the host epithelium. *S. pneumoniae* was known to produce neuraminidase that can cut off the sialic acids on cell surface of *H. influenzae* and *Neisseria meningitidis*, thereby inhibiting their adherence to the surface of host airway cells [124]. Similarly, a well-described mechanism of the negative interactions relates to phosphorylcholine, a cell-surface molecule mediating adherence to host cell receptors. *S. pneumoniae* [125] and *H. influenzae* [126] are bacteria expressing this molecule. Pre-infection of these bacteria activates production of antibodies against phosphorylcholine, so inducing clearance of other competing bacteria [127].

In contrast, a bacterial pathogen can enable the colonization of another one. *M. catarrhalis* is documented to release ubiquitous surface proteins on outer membrane vesicles in co-colonization with *H. influenzae*. The ubiquitous proteins lead to inactivate complement factor C3, which is a crucial component of the complement system, thereby, protecting *H. influenzae* from complement-mediated killing [128].

Viruses and bacteria interactions

The most highlighted positive interaction is between influenza virus and *S. pneumoniae*. Respiratory infections with influenza virus alone can be fatal. However, secondary infections with *S. pneumoniae* could further increase the risk of poor outcomes (including death). Most of the millions of deaths during the "Spanish flu" pandemic between 1918–1919 were found to be caused by secondary pneumococcal pneumonia (e.g. *S. pneumoniae*, *S. aureus*) rather than the primary infections of the influenza virus [129]. In animal experiments, all the mice infected with both two pathogens died within one day, while the single infection of influenza virus or *S. pneumoniae* resulted in fatality of just 35% and 15%, respectively [130]. Other synergistic effects of virus-bacteria interactions are also reported (Table 3).

Table 3: Synergistic effects of virus-bacteria interactions.

Influenza viruses	*S. pneumonia* [131], *H. influenzae* [132], *S. aureus* [133,134]
Parainfluenza virus	*S. pneumoniae* [132], *M. catarrhalis* [135]
Adenovirus	*S. pneumoniae* [136], *H. influenzae* [134,137], *M. catarrhalis* [137]
Coronavirus	*H. influenzae* [138]
Rhinovirus	*S. pneumoniae* [139], *H. influenzae* [139], *S. aureus* [139], *M. catarrhalis* [137]
MPV	*S. pneumoniae* [140]
RSV	*S. pneumoniae* [132], *H. influenzae* [132]

Several mechanisms of the positive interactions between viral and bacterial pathogens have been documented. The first line of defence against a bacterial infection is the epithelial layer of the respiratory tract mucosa, therefore, the mechanism of the positive interactions between viral and bacterial pathogens occurs on this layer. A viral infection can change the defence of the host epithelium rendering it more susceptible to bacterial colonization and infection [141]. Another way is disruption of the epithelium by virus infection, leading to loss of barrier function, thereby giving entry to the bacterial pathogens [142]. Moreover, viral infections may down-regulate the expression of antimicrobial peptides or up-regulate the expression adhesion proteins (e.g. ICAM-1, PAFr, CAECAM) in infected cells, including epithelial cells, that several bacteria utilize as receptors to enter the host cells [125,132,139,143,144]. Another mechanism is production of neuraminidase by viruses. Influenza and para-influenza viruses produce neuraminidase that cleaves sialic acids residues, thereby increasing bacterial adherence to host cells after viral infections [145,146]. Additionally, viral infections may cause immunosuppression in the host to cause bacterial superinfections [147].

Since the clinical symptoms of acute respiratory infections between etiological agents are undistinguishable, laboratory diagnostics for etiology plays a vital role for better management of the acute respiratory infections.

4.6. Laboratory diagnosis of respiratory viruses

4.6.1. Electron microscopy

Electron microscopy is one of oldest methods for both viral diagnostics and studying viral ultrastructure and pathogenesis [148]. It is also applied to detect novel or unknown viruses in outbreaks. Electron microscopy and culture were used in detection of the coronavirus causing SARS in 2002–2003 [149,150]. Electron microscopy is usually used to provide morphological clues for diagnosis to family level and subsequently for choosing further methods (cell line for culture or (consensus) primers for nucleic acid amplification) for virus detections and identification, especially for novel viruses in outbreaks [148]. However, the use of electron microscopy is limited in routine diagnostics since it is expensive, insensitive, complicated to operate and impossible for vius typing [151].

4.6.2. Cultivation

Culture was the "gold standard" for diagnostics of respiratory viral pathogens for decades [148]. Common respiratory viruses (e.g. ADV, influenza A/B virus, RSV, and PIV) can be cultivated [148,152]. Over the years, modified cell culture methods were introduced with advances. Centrifugation-enhanced shell-vial methods allow to reduce the turnaround time from 5 to 10 days to even 24 h in comparison to conventional culture [153]. Shell-vial culture combining different cell lines enables to simultaneously detect multiple respiratory viruses [154]. Many novel viruses were originally detected by culture, such as MPV [155] and SARS-CoV-1 [150]. Nowadays, virus culture is no longer a method of choice for viral diagnosis because of its low sensitivity and long turnaround time. Moreover, difficulty of many viruses to grow in cultured cells and the requirements of some viruses for specialized culture conditions also hinder applications of culture in diagnostics [156–158].

4.6.3. Serodiagnosis

Following an acute infection, IgM antibodies develop, and then IgG antibody level increase [159,160]. After the acute phase, IgM antibody level decreases quickly, while. IgG antibodies remain detectable for many years or even for life [161]. The serology tests are assays targeting the virus-specific antibodies for diagnosis. A seroconversion or a ≥ four fold increase in antibody titer is required to establish a serological diagnosis of an acute infection. Otherwise, detection of respiratory virus specific antibodies is more relevant for sero-survey or sero-surveillance study because it merely reflects a past or a recent exposure to a virus under investigation.

Serological methods that could detect virus specific antibodies include neutralizing assays, complement fixation, hemagglutination inhibition, immunofluorescence methods EIA and ELISA. Neutralizing antibodies remain the gold standard of immunoassays as the assays are most specific

and determining antibody efficacy [162]. However, it is not routinely used in diagnostic laboratory, because they are laborious methods [162]. Moreover, the specificity of serological assays have been significantly improved in recent years. For example, the specificity of COVID-19 serological assays can be up to >98% [163].

Enzyme immunoassays (EIA) and enzyme-linked immunosorbent assay (ELISA) are immunoassays, but different in assay design. Both of them use enzymes as the reporter label for detection and quantification of substances including antibodies [164,165]. EIA and especially ELISA are the major workhorse globally as they are easy to use and more sensitive than other traditional serodiagnostic methods such as complement fixation, hemagglutination inhibition and immunofluorescence methods. Moreover, they provide quick and reliable test results and require almost no resource setting [164,165].

The reservation of the serology tests for routine diagnosis is the turnaround time, which is attributable to the requirement of obtaining second blood to demonstrate a seroconversion. Therefore, they are not a good option for diagnosis of acute infections. Moreover, in newborns and in infants, IgG level may merely reflect mother's antibody level, because IgG is transferred through placenta [166], thus interfering the result interpretation.

4.6.4. Viral antigen detection

Antigen is the specific protein of the pathogens and induce antibody production within the infected hosts. Antigen-based diagnostics in acute respiratory infections are approaches using antigen-specific antibody to detect the antigen in the sample [167]. Therefore, antigen detection tests detect virus infections earlier than serology. Enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassays (EIA) as well as direct and indirect fluorescent antibody (DFA, IFA) tests are most commonly used and they have replaced radioimmunoassays (RIA). They are easy to use, highly sensitive and specific for diagnostics of multiple respiratory viral pathogens, including influenza A virus, influenza B virus, RSV, MPV, ADV and PIV [168–172].

The antigen detection assays are rapid, easy to perform, convenient and feasible to detect infectious viruses before appearance of clinical symptoms without requirement of costly investments. They can quickly provide test results and thus guiding proper treatment and coordination of patients [173,174]. Therefore, antigen tests remain valuable diagnostic methods for outpatient clinics, primary care, emergency department and in low resource settings [175]. However, poorer sensitivity compared to nucleic acid tests, especially in adults, is a major disadvantage of antigen-based diagnostics [175], while the interpretation of IFA and DFA results need special expertise [156].

4.6.5. Diagnostics based on detection of viral nucleic acids

Amongst nucleic acid-based methods, e.g. nucleic acid amplification tests, (reverse transcription real time) polymerase chain reaction ((RT-) PCR) is widely used in routine diagnostics. Meanwhile, metagenomic next-generation sequencing (mNGS) is the most common method applied in research, although in recent years mNGS has been used with increased frequency as a tool for routine diagnostics, especially when all routine diagnostic tests return a negative result.

(Multiplex) (RT-) PCR assays

Molecular detection by nucleic acid amplification, e.g. (RT-)PCR, has been considered as the most sensitive and as standard for detection of most if not all respiratory viruses [12]. (RT-)PCR has shorter turnaround times and provides higher sensitivity, supporting detection of a wide panel of viruses [176–178] and mixed infections [71,179]. Compared to other methods, (RT-)PCR have greatly improved the diagnostics of microbes, including viral respiratory tract infections. (RT) PCR-based diagnostics have become more widely used, since their sensitivity and specificity have been greatly higher than conventional assays. Thereby, they remain mainstream in research and routine diagnosis of respiratory viruses [157].

In multiplex (RT-)PCR assays, several microbe specific primers simultaneously amplify genomic fragments of many pathogens within one reaction. Multiplex (RT-)PCR have been validated for rapid and precise detection of a wide panel of respiratory viruses [180–183]. Multiplex –PCR tests have shown to be 30% to 50% more sensitive than DFA and virus culture [184]. In comparison with singleplex (RT-)PCR, the crucial advantages of multiplex (RT-)PCRs are that they save time, nucleic acids and reagents, thus being cost effective. However, the multiplex assays were found be to slightly less sensitive compared to singleplex (RT-)PCR [185].

The major limitation of PCR methods in general is that target specific primers are needed. Therefore, it is not possible to identify viruses, whose genome sequence is not known.

Metagenomic next generation sequencing (mNGS)

Literature of mNGS was firstly presented in the mid-2000s. mNGS is a high-throughput sequencing method that can simultaneously sequence genomic material from all organisms. Unlike PCR methods relying on primers for detection, the abilities of sequence-independent and unbiased

sequencing make mNGS a powerful new platform to sequence the total DNA or RNA within the sample (so called as metagenomics). This therefore allows to characterize microbiome with greater taxonomic resolution in the sample that used to be impossible and high cost by the old techniques. Moreover, metagenomics allow for the detection of a wide range of pathogens in one test [186] as compared to the use of multiple separate PCR assays [72,187,188]. Therefore, mNGS has been widely applied for pathogen discovery [189,190], diagnosis of patients with severe infection of unknown origin [74,188], genetic characterization associated studies [191] and ecological studies [192]. Notably, mNGS was used to discover and genetically characterize SARS-CoV-2 in a cluster of patients presenting with community acquired infection of unknown origin in Wuhan China [190].This technique also represents a substantial advance of whole-genome sequencing that a complete bacteria genome is possibly sequenced in few days instead of taking several years in the past by traditional sequencing methods. Additionally, metagenomics has amazingly changed novel microbe discovery as it is dramatically easier and faster than previously. Detection by mNGS of SARS-CoV-2 causing ongoing COVID-19 pandemic is a typical example [190]. Other applications of mNGS include outbreak tracking, disease surveillance, variant/mutation detection and antimicrobial resistance detection, virulence profiling, and study of the microbiome and microecological factors in health effects [193,194].

Despite the great potential, mNGS application in routine laboratory diagnostics remains under consideration, while PCRs remain standard and most commonly applied in the routine screening. mNGS analysis has key reservations hindering it to become a mainstream laboratory method since it is complex and consists multistep processes. mNGS analysis includes four main steps: sample preparation, library preparation, sequencing, and data analysis where technical and methodological challenges remain, while there is no standard protocol for universal use [195].

Indeed, different methods in nucleic acid extraction or random amplification during mNGS procedure were found to induce substantially various results [196–198]. Moreover, mNGS results are strongly affected by the bioinformatics analysis used. The analysis steps consist processing of raw sequencing data (e.g. base calling, de-multiplexing, trimming and removal of reads (e.g., reads of low quality, low complexity, adapters and indexes, or of human origin)), read normalization, alignments, de-novo assembling and taxonomic assignment of reads or contigs, comparing the reads or contigs to databanks and annotate them to identify already known sequences or with further algorithms to identify new ones. There are multiple options among these steps and there remains ongoing debate about optimal methods. Therefore, a powerful and valid bioinformatic algorithm is in crucial need to remove the vast amounts of unwanted nucleic acid (e.g. host (e.g. human) or undesired microbes (e.g. fungi, algae), while sensitively and specifically detecting the

desired agents, especially novel ones of which the sequences are significantly different from previously known sequences [193].

Another barrier is complication of result interpretation due to difficulties in distinguishing between contamination (e.g. from reagents or cross-contamination in specimen collection, sequencing library preparation and assay run) and the microbes present in the sample. This therefore requires an additional step to verify the mNGS results. Additionally, a limitation of mNGS is the difficulty to detect wrong barcode indexes leading to false positives and further induces difficulty in analysis of mNGS outputs. All together, these reservations make mNGS remaining a high-complexity technology for routine laboratory diagnostics. Therefore, it is important to validate the protocols to have reliable mNGS methods for analysis of clinical samples since mNGS analysis remains a powerful platform for vast applications.

4.6.6. Diagnostics based on detection of biomarkers

A biomarker is any molecule or structure measured to detect normal or pathogenic biological processes or to measure responses to treatment in the body [199]. In acute respiratory infections, the biomarkers have been used as indicators to detect presence, type and severity of the infections. They therefore allow to achieve earlier recognition of severity from the infections (e.g. sepsis and pneumonia), for most appropriate treatment and antibiotics use [200].

Traditional biomarkers with commercially available tests for early diagnostics and prognostics of acute respiratory infections (especially pneumonia), sepsis and other infectious diseases are procalcitonin, C-reactive protein and proadrenomedullin [201–204]. However, no single biomarker has shown to be consistently accurate in diagnostics and prognosis of infections [201,205] since inflammatory response creating biomarkers in body is complex and poorly controlled. They can be affected by many factors such as age, antibiotic pretreatment, chronic hepatic disease, corticosteroids, renal impairment and viral coinfection [203]. The use of multiple biomarkers has shown to have more diagnostics' and prognostic's value compared with the use of a single biomarker alone. Therefore, a combination of microbiological methods with traditional biomarkers and emerging technologies (e.g., proteomics or metabolomics) is considered to improve diagnostics and therapy of infectious diseases, including acute respiratory infections [201,203].

4.6.7. Importance of having an accurate and sensitive diagnostic

Since metagenomic methods have been applied, discovery of novel viruses has been accelerated, indicating that many viruses were missed because of limitations of prior detection methods. Despite intensive laboratory investigations with a wide panel of respiratory viruses and analysis of metagenomics, a substantial proportion of acute respiratory infections are etiologically unknown, while non-specific clinical manifestations of acute respiratory infections causes difficulties for differential clinical diagnostics between the agents [69–72,74]. The unknown etiology is implied to partially be caused by limitations of current laboratory diagnostic methods that can hinder early detection and containment the emerging viruses.

An accurate diagnosis is crucial and beneficial in many aspects. It provides exact information of viruses circulating in the community to public health authorities for proper policies. The patient benefits from microbe specific diagnosis for directed treatment, such as oseltamivir for treatment of influenza virus infections or antibiotics for bacterial infections (e.g. *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*) [12].

Therefore, it is important to have a highly sensitive and specific diagnosis and to verify current laboratory diagnostic tools to be ready for feasible and quick detection and identification of the emerging viruses in future outbreaks. Recent epidemics or pandemics by zoonotic respiratory viruses like SARS, influenza A/H1N1-09 virus, MERS-CoV and ongoing pandemic of SARS-CoV-2 [206] emphasize particularly the importance of early identification of the emerging respiratory viruses for in-time intervention tracing the cases for containment of the emerging virus, preventing it from spreading globally, before it will be beyond our ability for containment.

4.7. Establishment of etiological causation of a microbe and a disease

The application of mNGS in the field of pathogen discovery has resulted in the identification of so many new viral species over the last decades. However, detection of a new virus represents the beginning of a new story. And it remains critical to determine the pathogenic potential of such newly discovered viruses. Therefore, several criteria to demonstrate the proof of causation have been proposed, and are outlined below.

4.7.1. Koch's postulates

In 1890, Robert Koch, based on his studies on communicable diseases such as anthrax and tuberculosis by staining and culture methods, and refined from earlier concepts described by Friedrich Jakob Henle, introduced standard for establishment of a given microbe as a causative agent of a given infectious disease [207], later named as Koch's postulates. The postulates include three conditions that a causal microbe needs to have:

(i) The microbes found in all disease cases and their pathological changes are corresponding with clinical courses of the disease.

(ii) No other disease was caused from the microbes spontaneously.

(iii) After being isolated from the body, the microbes can repeatedly grow in pure culture and can cause the disease in another host.

However, since the postulates were introduced, their limitations were soon exposed. *Vibrio cholerae* was also isolated from healthy individuals; *Mycobacterium leprae* was not isolated in pure culture until 1980s. Therefore, Koch then modified the postulates that etiological relationship between the microbe and the disease is validly established even if only two conditions of the guideline are fulfilled. Nevertheless, many pathogens remain out of the postulates such as *Plasmodium falciparum* or herpes simplex virus or other viruses unable to be cultured in cell-free culture, or viruses (human immunodeficiency virus (HIV)) causing untypical disease, or microbes (*M. tuberculosis*) with latent infections, or viruses (hepatitis D virus) requiring a helper virus (hepatitis B virus or hepadnaviruses) to supply essential structural components for reproduction in human tissue, or viruses causing chronic infections [91,208,209]. Therefore, a microbe failing to meet Koch's postulates may still be a causal agent of the disease.

4.7.2. Molecular guidelines for establishment of disease causation

By the limitations of the Koch's postulates and finding of increasing number of uncultivable microbes detected via molecular methods, Bradford Hill proposed in 1965 nine epidemiologic criteria for causal association between a microbe and a disease [210] that have been currently widely applied in epidemiology and emerging zoonoses [211,212]. The nine criteria include:

(i) Strength of association: a strong association between exposure and disease is more likely to be causal than a weak association.

(ii) Consistency of association: a more consistent or repetitive association is more likely to be causal than an inconsistent association.

(iii) Specificity of association: an association are more likely to be causal when exposure to microbe specifically result in only a disease.

(iv) Temporality: the exposure must occur prior the onset of the disease.

(v) Biological gradient: the association is more likely causal when increased exposure to the microbe causes increased incidence of disease.

(vi) Plausibility: the relationship between the microbe and the disease is likely causal when it has biological plausibility.

(vii) Coherence: the cause-and-effect association between the microbe and disease is likely to be causal when it agrees with present knowledge of the disease.

(viii) Experiment: the disease risk dropping down from the intervention or termination of the exposure to the microbe is strong evidence of a causal relationship between microbe and disease

(ix) Analogy: the causal relationship should be compatible with a previously described relationship of a similar microbe with a similar disease.

Over the time, many investigators such as Thomas Rivers [213], Heubner [214], Alfred Evans [215], Johnson and Gibbs [216] proposed their own postulates, suggestions or elements for establishment a causal relationship, however, they remains limited. Based on these postulates, suggestions, elements, the revised Koch's postulate and Hill's criteria, in 1996, David Relman and David Fredricks suggested a unified molecular guidelines for establishment of causation between a microbe and an infectious disease [217]. The guideline includes seven conditions:

(i) A nucleic acid sequence of a putative pathogen should be found in a majority of infectious disease cases. Microbial nucleic acids should be abundantly found in the diseased organs or gross anatomic sites and not in those organs without pathology.

(ii) Hosts or tissues without the disease should have few or no of pathogen-associated nucleic acid sequences.

(iii) The copy number of pathogen-associated nucleic acid sequences decrease from resolution of disease (for example, with clinically effective treatment) and the opposite occur when clinical relapse occurs.

(iv) Causal relationship between sequence-disease is more likely to be true when detection of pathogen-associated nucleic acid sequences predates disease, or the sequence copy number associates with disease severity.

(v) The putative nature of the microorganism from the achieved sequences should agree with the known biological characteristics of that organisms group. Sequence-based phylogenetic relationships enhance the certainty of the putative nature such as phenotypes (e.g., pathology, microbial morphology, and clinical features).

(vi) The correlation of diseased tissue and microbe sequence should be found at the cellular level by specific situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.

(vii) These sequence-based evidence for causal relationships of the microbe should be reproducible.

These conditions are not required to be all fulfilled for establishment of causality. More fulfilled conditions provide stronger evidence of etiology [217]. This molecular guideline remains currently applied in establishment of a causal relationship for uncultivated microbes. However, Bradford Hill's criteria remains the most common and valid framework for causal inference in epidemiologic investigations and emerging zoonoses [212,218]. Causal determination of Zika virus in microcephaly are the latest case of application of Bradford Hill's criteria [211].

4.8. Zoonotic diseases

4.8.1. Burden of zoonotic diseases

Any disease or infection that is naturally transmissible from animals to humans is defined as zoonosis [219]. Zoonotic diseases or zoonoses are also defined as caused by germs which spread from animals to people [220]. They are a global concern since a majority of known human diseases and 75% of newly emerging diseases associated with recent epidemics or pandemics are of zoonotic origin [219,221]. Zoonoses annually cause billions of cases of illness, millions of deaths and globally cost hundreds of billions of US dollars [222].

Emerging or re-emerging zoonoses causing outbreaks have occurred more frequently in recent years than ever previously, while control measures are complex and the overall impact can be catastrophic [223,224]. Ongoing COVID-19 (coronavirus disease 2019) pandemic [30] or most recent globally spreading pandemics are caused by zoonotic respiratory pathogens [225] such as SARS in 2003 [226], influenza A/H5N1 virus in 2003 [35], influenza A/H1N1-pdm virus in 2009 [227] and MERS-CoV in 2012 [34], besides pandemics of non-respiratory zoonotic infections like Ebola [228], Zika [229] and HIV/AIDS [230].

4.8.2. Zoonotic emergences and cross-species transmissions

The emergence of zoonotic pathogens in human populations is complex. The zoonotic emergence and spread of zoonotic pathogens in human populations is a result of human contact with infected animals and/or contact with a contaminated environment [231–233]. However, prediction of the zoonotic emergence remains difficult and the underlying mechanisms driving the zoonotic emergence are not well understood [232]. Many factors, including changes in ecology and environment (e.g. changes in climate, landscape characteristics, animal husbandry, food chains, communities of zoonotic hosts and vectors) [234,235], microbial evolution, changes in human demographics and practices, travel, trade, urbanization, human behavior and culture are documented to affect the emergence of zoonotic infections [219,236–239].

The successful emergence of any zoonotic pathogen requires not only human contact with the infected source, but also the ability of cross-species transmission from the microbes. The emergence can fail at beginning of cross-species infection after exposures, or fail to spread human-to-human, or fail to achieve sustained human-to-human transmissions, or simply fail due to lack of exposure by sparse population or limited social connectivity [240] (Figure 3).

The emergence of zoonotic pathogens can be more likely result in a pandemic when it has characteristics of sustained human-to-human transmission, genetic adaptation to the human host and lack of population immunity against the emerging agent [240]. Therefore, the emerging infection causing a pandemic later can be introduced much before the official detection of the infected cases causing the pandemic. Additionally, the viruses with high host plasticity (i.e. high divergence of host species, especially in both wild and domestic of animals) are more likely to have high ability of cross-species transmissions and pandemic potential [241].

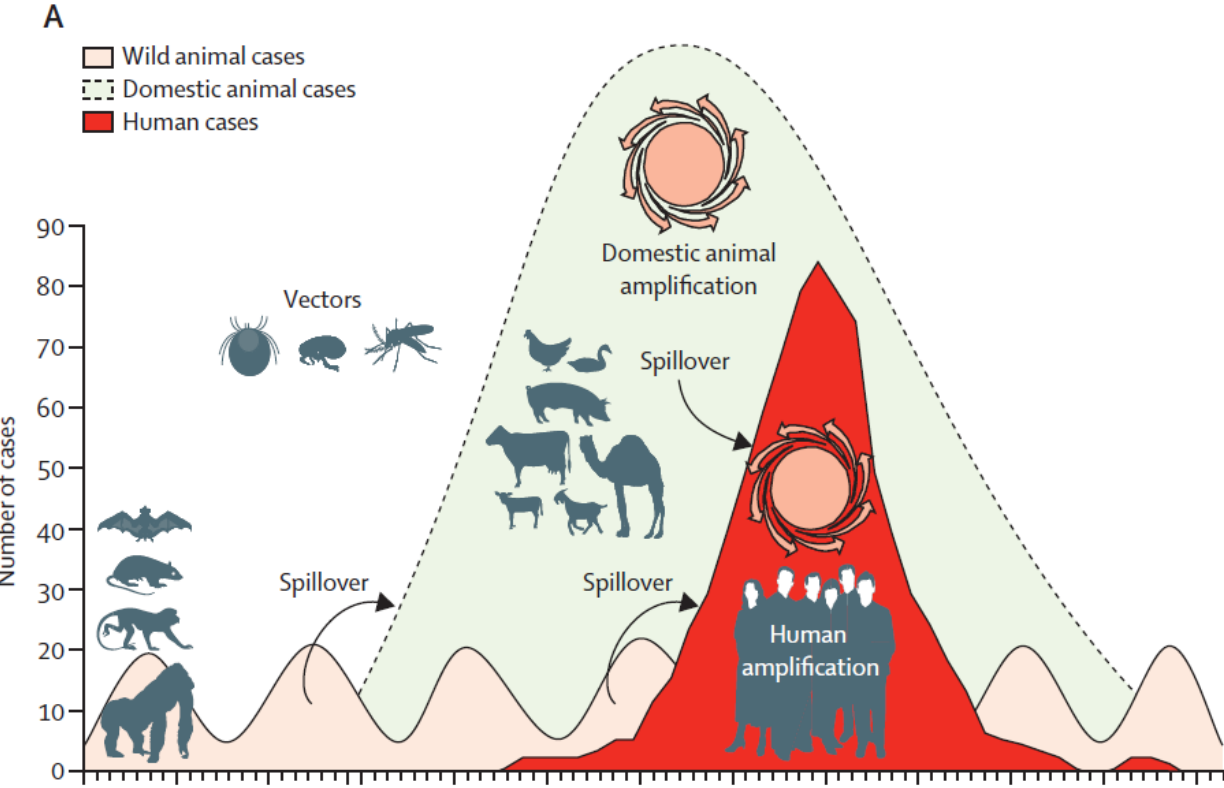


Figure 3: Overview of emergence of zoonotic disease in human happening after a pathogen in wild animals (pink) moving into livestock to result in an outbreak (light green) leading to amplifying in the capacity for transmission of the pathogen to human population (red). It therefore provides the incentive and orientation for early intervention. Copy from Karesh WB, Dobson A, Lloyd-Smith JO, et al. Ecology of zoonoses: Natural and unnatural histories. *The Lancet*. 2012;380(9857):1936-1945. doi:10.1016/S0140-6736(12)61678-X [236]. Reprinted with permission from Elsevier.

4.8.3. Control of zoonotic diseases

Despite the high prevalence of viral zoonoses and high potential to cause pandemics, the early detection of viral pathogens with animal origin exhibiting potential for transmission into human population remains an obstacle [232]. Therefore, just one sector cannot well control the issue.

One Health approach is multisectoral and multi-disciplinary collaboration, including ministries, agencies, stakeholders, sectors, and disciplines. These multiple sectors communicate and work together to obtain better public health outcomes [242]. Therefore, implementation of One Health method with a collaboration of the human health, animal health and environmental health in conducting programs, policies and research in a common goal is considered key to achieve better

understanding, prediction, preparedness, managing and early intervention of zoonotic disease emergence [243,244].

Studies of zoonotic exposure, getting insight of risk of human activities and hazardous behaviour, including the co-sampling of animals, humans and evaluation of zoonotic possibility of the microbes are considered as important factors to understand the emergence [231,232]. Moreover, ongoing pandemic of SARS-CoV-2 highlights the crucial role of active sentinel surveillance in individuals working with animals, or febrile patients and animal species to early detect cross-species transmission the emerging viruses from wildlife and inform the policy makers to prevent it from spreading globally to become pandemic.

It is well admitted that increased contacts between animals and humans provide more opportunity for exposure to zoonotic pathogens and elevate the probability of zoonotic disease emergence [219]. The human population at highest risks of zoonotic infections are therefore those most frequently interacting with animals. For this reason, slaughter-house workers, animal-health workers, livestock-rearing farmers, and those that trade in wildlife are likely at greater risk of zoonotic infection because of sustained occupational exposure [233]. However, the specific features and exposure to potential sources of emerging zoonoses are not well understood. Additionally, the etiologies of acute respiratory infections of people working with animals remain unknown and comprehensive investigations on these high-risk individuals remains a substantial challenge.

4.9. High-risk sentinel cohort study

Southeast Asia is considered as a hotspot for emerging zoonotic diseases [226,231]. Multiple features, including demography, behaviour, attitudes, culture, dense human and animal populations, a high diversity of wild mammalian species, transformation of the agro-ecological landscape associated with economic development and the coexistence of a wide range of diseases in human and animals are thought to be distinctive features of this region that may cause zoonotic disease emergence [231,245]. However, the more precise understanding of the features leading to zoonotic disease transmission is limited.

The high-risk sentinel cohort study is a community-based component of the VIZIONS (Vietnam Initiative on Zoonotic Infections) project. The study focused on individuals at risks of zoonotic infections, consisting of individuals working with animals, such as livestock-rearing farmers, slaughter-house workers, animal health workers and rat traders. The high-risk sentinel cohort study has been conducted in two provinces in Vietnam (Dong Thap and Dak Lak) representing the different geographic regions in Vietnam (the Mekong Delta and the Central Highland,

respectively) [82]. The target of the study is catch zoonotic transmission events in real-time and to provide insight into emergence of the zoonotic diseases in human population with a focus on behavior and demographic factors of high-risk individuals in Vietnam [95] for better prediction and control strategies.

5. AIMS OF THE STUDY

- Goal I: To characterize the nature of exposures to potential sources of zoonotic transmission in individuals with sustained occupational contact with animals in Vietnam.
- Goal II: To determine the spectrum of PCR-detected viral pathogens in respiratory tract of individuals at risk of zoonotic infections.
- Goal III: To gain insights into the virome, including exploring and charactering novel viruses, of acute respiratory infections by mNGS analysis in the individuals.
- Goal IV: To assess zoonotic potential of the novel viruses detected in the respiratory tracts of in the individuals.
- Goal V: To evaluate viral detection of metagenomics and compare viral detection of metagenomics versus (multiplex RT-) PCR in respiratory clinical specimens.

6. MATERIAL AND METHODS

6.1. The high-risk sentinel cohort study

Recruitment (all Publications)

The data and clinical samples analyzed in this study were derived from a high-risk sentinel cohort study [82,95]. The study participants included livestock-rearing farmers, slaughter-house workers and animal-health workers from two study sites in Dong Thap and Dak Lak provinces, representing two different geographical areas: the Mekong Delta and Central Highlands respectively. The individuals selected as representative of the population with sustained occupational exposure to animals in rural Vietnam. The majority of the study participants were livestock-rearing farmers since small-scale animal farming is a common rural livelihood and two thirds of the population

working with animals in these selected areas are farmers [246]. Additionally, rat-traders were also recruited in Dong Thap since this occupation was common in this study site.

Initially, based on the animal farm census, potential participants were invited to an introductory meeting. Those showing interest in the high risk sentinel cohort study were invited to join the study. Signed written consent was then obtained from each study participant. For each farm household, up to four members who work most with animals were recruited. Slaughter-house workers at the central abattoir of each district in the study sites were recruited. Animal-health workers and rat traders were randomly chosen by convenience. The participants were followed for three years, starting from March 2013 in Dong Thap province and March 2014 in Dak Lak province.

Baseline data and sampling (all Publications)

Annually at the beginning of each study year, to establish the baseline data (i.e. when there was no respiratory symptoms reported), the study participants were interviewed to obtain information about demographics, socioeconomic status, animal exposure, attitudes toward risks of zoonotic infections, usage of protective equipment and medical history. Additionally, clinical specimens, including rectal, pooled nasal-throat swabs and blood were collected from each interviewee and their animals. These baseline data were collected from all study participants, except for the farmers, when only one person with most exposure to animals in the household was interviewed and sampled (Figure 4). For this thesis, we focused on only on the nasal-throat swab analysis.

Disease-episode samples (Publication ii, iii, iv)

Participants were asked to report to local study teams if they suffered any symptoms of respiratory tract infection and fever ($\geq 38^0$C). Upon receiving such a report, the site study doctors made a visit to the participant's house within 48 hours. At this visit, information about animal exposures, associated symptoms and participants' medications were obtained. Clinical specimens were also collected from the symptomatic participants and their animals (Figure 4) consisting of rectal, nasal-throat swabs and blood [71,247]. All the specimens were stored at -80^0C. For this thesis, we describe results from the nasal-throat swabs only.

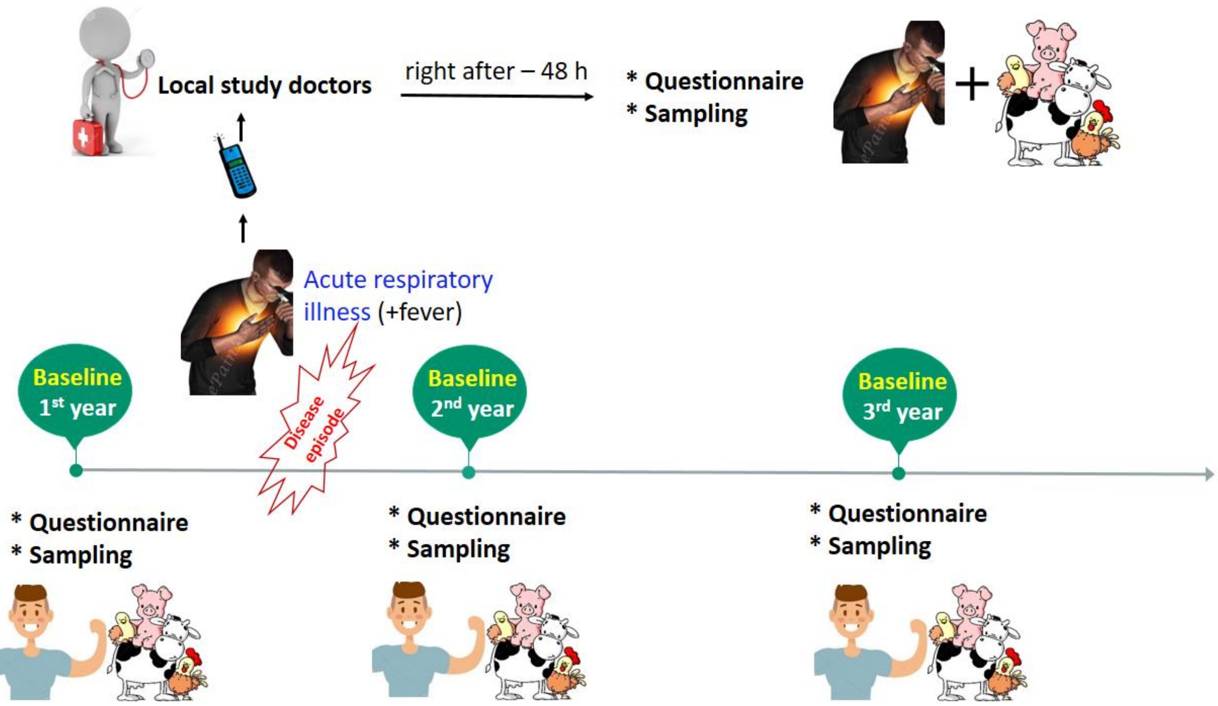


Figure 4: Summary of general information of the high-risk sentinel cohort study. The baseline sampling was at beginning of each study year and when the human participants had no respiratory symptoms. Another sampling was whenever the human participants reported an episode of acute respiratory infections (any symptom of respiratory tract + fever) during the following periods of the 3-year cohort study. The samples were simultaneously collected from both human and their animals. The picture of animals, healthy person, ill person, medical doctor and cell phone were from open-access sources, including Dreamstime [248], Depositphotos[249], ePainAssist [250], Shutterstock [251] and Gifs-animados.es [252], respectively.

6.2. Ethical approvals

The high-risk sentinel cohort study obtained the approval from the Oxford Tropical Research Ethics Committee (OxTREC) (No. 157-12) of the University of Oxford, United Kingdom. The study additionally achieved the approval from the Ethic Boards of the sub-Departments of Animal Health and Dong Thap General Hospital in Dong Thap (No. 850A/QĐ-BVĐT-TCCB) and Dak Lak provinces (No. 5407/UBND-TH), the Hospital of Tropical Diseases in Ho Chi Minh City in Vietnam (No. 137/BVBNĐ-KH).

6.3. Analysis of baseline data on exposure to animals (Goal I; Publication i)

The interview data for baseline from the first, second and third years were combined and analyzed as cross-sectional data, resulting in exposure outcomes in at least one of the three interviews.

6.4. Determining common respiratory viruses by RT-PCRs (Goal II; Publication ii)

Total nucleic acid was isolated from nasal-throat swabs using MagNApure 96 platform (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. The nucleic acid output was recovered in 50 µl buffer and immediately screened for respiratory viruses using multiplex RT-PCR by LightCycler® 480 Instrument II (96 wells) (Roche Molecular Systems, Inc). The assay protocols were derived from previous publications capturing 15 common respiratory viruses and a wide range of their subtypes [253,254,185], including RSV A, RSV B; influenza A virus (including subtypes H3N2, H1N1pdm09, H1N1, and avian/H5); influenza B virus, ADV; EVs; MPV; RV; PIV 1, PIV 2, PIV 3, PIV 4; CoV; BoV and PEV. A cycle threshold (Ct) value ≤40 was considered as positive.

6.5. Explore virome of acute respiratory diseases by mNGS analysis (Goal III, V; Publication iii)

Initially, 200 µ of nasal-throat swabs were treated with 50U of RNase I (Ambion, Life Technology, Carlsbad, CA, USA) and 20U of turbo DNase (Ambion) at 37ºC for 30 min [198]. Then, the nucleic acid was extracted using QIAamp 96 Virus QIAcube HT Kit (QIAgen GmbH, Hilden, Germany) following the manufacturer's instructions. The nucleic acid output was then eluted in 50µl buffer (provided in the QIAamp kit). dsDNA synthesis, random amplification and library preparation were carried out as previously described [72]. The samples in each run were differentiated by the double indexes of Nextera XT Index Kit (Illumina). The prepared library was then sequenced by an Illumina MiSeq platform (Illumina) using MiSeq reagent kit V3.

An in-house viral metagenomic analysis pipeline presented in GitHub: https://github.com/xutaodeng/virushunter/ was used to analyse the MiSeq-generated sequences. Initially, the adaptors, low quality reads and duplicates were removed. The human and bacterial genome-related reads were then subtracted by mapping them to bowtie2 (version 2.2.4). The bowtie2 combined sequences of human reference genomes, mRNA (hg38) and bacterial nucleotide extracted from NCBI nt fasta file (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/, February 2019) from NCBI taxonomy (ftp://ftp.ncbi.nih.gov/pub/taxonomy, February 2019) [255]. The remaining reads were then assembled by de novo alignments in ENSEMBLE software which is a novel

partitioned subassembly method integrating the application of various de Bruijn graph (DBG) and overlap-layout-consensus assemblers (OLC) [256].

Basic Local Alignment Search Tool (BLASTx) with the E score threshold of <0.01 was used to aligned the output contigs (plus single reads) against the viral proteome from NCBI's RefSeq and non-redundant database to screen for viral sequences. To remove tentative viral hits showing better alignments to non-viral sequences, DIAMOND algorithm version 0.9.6 was used to align these tentative matches to viral proteins in the GenBank's entire non-redundant proteome database [257]. Based on NCBI taxonomy of the best hits (lowest E score, threshold of $<10^{-10}$) in the non-redundant proteome database, sequences at output were then classified as viral reads or removed as non-viral reads.

6.6. Genotyping

The Enterovirus Genotyping Tool Version 1.0 [258] was used to serotype of *Enterovirus* genus-related sequences. For subtyping of influenza virus A, CoV and RSV-A related sequences [259–261], their specific sequences for subtyping were firstly located and extracted using the Map-to-reference tool of Geneious 8.1.5 software (Biomatters, San Francisco, CA, USA). The extracted sequences were then subtyped by using BLASTx with the E score threshold of $\leq 10^{-5}$.

6.7. PCR confirmation

mNGS-detected viruses with known human tropism or unknown tropism were confirmed by specific (RT-)PCR with primers (and probes) derived from previous publications [74,119,262] or new designs based on mNGS-obtained contigs. The PCR confirmation was done on nucleic acid newly extracted from original patient samples by MagNApure 96 platform (Roche Diagnostics, Mannheim, Germany) [71].

6.8. Sequencing for complete genome and genome analysis (Goal III; Publication iii)

Inverse and walking primers were used in PCR assays to obtain complete circular virus genomes. De novo and Map-to-reference tool of Geneious 8.1.5 software were used for alignments of the overlapped sequences to obtain the complete genomes and to assess the level of genome coverage and identity.

6.9. PCR screening by new primers designed based on mNGS contigs (Goal III, IV, V; Publication iii, iv)

The primers were new designs based on the mNGS-obtained contigs of the targeted viruses. The PCR screening (confirmed by Sanger sequencing) was conducted on mNGS samples and nasal-throat swabs from their animals, as well as nasal-throat swabs at baseline of the same participants with disease-episode samples in mNGS analysis. The nucleic acid for the screening was newly extracted by MagNApure 96 platform (Roche Diagnostics, Mannheim, Germany) from original patient samples.

6.10. Phylogenetic analysis (Goal III, IV; Publication iii, iv)

MUSCLE algorithm in MEGA software version X [263] was used for sequence alignments. Maximum Likelihood algorithm with bootstrap of 1000 replicates in the MEGA software was applied to build phylogenetic trees.

6.11. Nucleotide sequence accession numbers

The Genbank accession number of raw reads from mNGS analysis is PRJNA639353. The complete genomes and gene sequences were uploaded on GenBank with the accession numbers of MT649483, MT649484, MT649485, MT649486, MT759843, MT823476–MT823478, MW216334–MW216337.

6.12. Statistics

The associations or differences between variables were calculated by the pairwise comparisons of Pearson's Chi-squared test or Fisher exact test or t-test. All these analyses were performed by STATA software (version 12.0). Benjamini and Hochberg method [264] with false discovery rate (FDR) calculator [265] was applied to adjust p values for multiple comparisons. A significant p value was defined as ≤ 0.05. The calculations of 95% confidence intervals of proportions were conducted by Wilson method in EpiTools [266].

7. RESULTS AND DISCUSSION

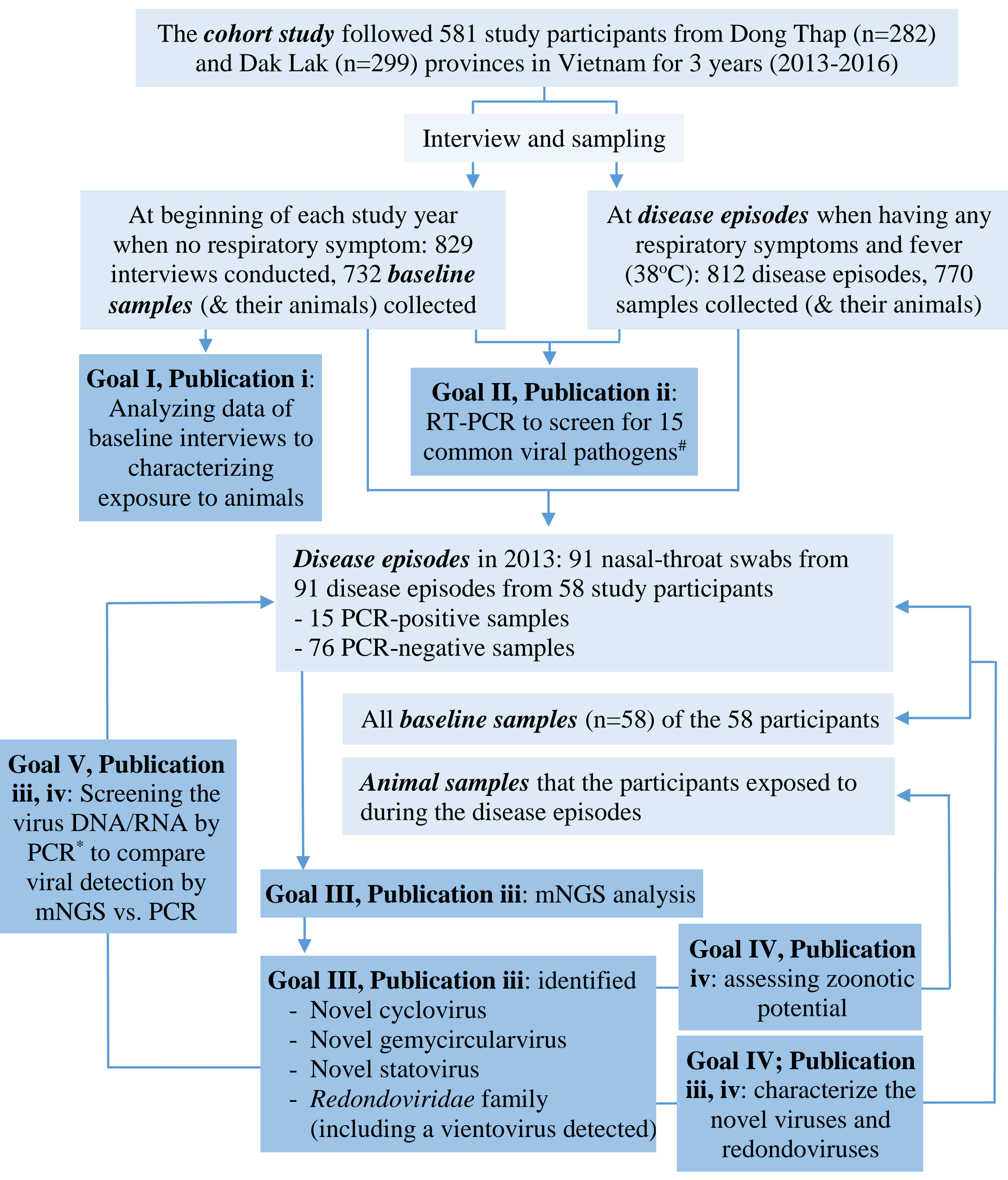


Figure 5: Summary of main methods and results. # the 15 viruses include human coronaviruses subtype OC43 or/and NL63, parainfluenza viruses 1–4, respiratory syncytial virus A and B, adenoviruses, human metapneumovirus, enterovirus, human rhinovirus, human bocavirus, parechoviruses, adenovirus, influenza A virus and influenza B virus. * PCR screening (confirmed by Sanger sequencing) by primers designed based on mNGS contigs.

7.1. General data of study participants, interviews and samples (Publications i, ii, iii, iv)

In total, 581 individuals including 415 (71.4%) livestock-rearing famers, 100 (17.2%) slaughter-house workers, 61 (10.5%) animal health workers and 5 (1.8%) rat-traders were recruited in the high-risk sentinel cohort study. Most of the study participants were ≥16 years old (89.8%; 522/581), median age: 38, range: 2–89 (Table 4).

In over 3 baseline interviews during the 3-year study period (2013–2016), over a half (51.1%; 297) of all 581 study participants were interviewed at least once at the beginning of each study year, corresponding to a total of 829 interviews (Table 4). Consequently, 732 nasal-throat swabs were collected at these annual interviews and considered as baseline samples.

Over the three-year follow-up period, 66.4% (386/581) of the participants reported episodes of acute respiratory infections, corresponding to a total of 812 respiratory episodes (Table 4), with an average of 2.1 episodes per reporting individual, or an average of 1.4 (812/581) episodes per individual over the study period among all participants. Finally, 770 nasal-throat swabs were collected from 94.8% (770/812) of respiratory episodes and considered as disease-episode samples (Table 4).

Table 4: General data of the study participants, interview and samples. Modified with permission from the Journal of Medical Virology [71] (Publications ii).

	All (N=581)	**Dak Lak** (N=299)	**Dong Thap** (N=282)	p value#
Occupation	**N=581**	**N=299**	**N=282**	0.012
Livestock-rearing farmers, n (%)	415 (71)	201 (67)	214 (76)	0.021
Animal-health workers, n (%)	61 (10)	31 (10)	30 (11)	0.915
Slaughter-house workers, n (%)	100 (17)	67 (22)	33 (12)	0.001
Rat traders, n (%)	5 (1)	0	5 (2)	
Median age (range) (in years)	**38 (2-89)**	**39 (2-89)**	**38 (4-76)**	0.995^
Age groups				
≤15, n (%)	59 (10)	24 (8)	35 (12)	0.080
≥16, n (%)	522 (90)	275 (92)	247 (88)	
Sex ratio (male/female)	**1.2 (322/259)**	**1.1 (157/142)**	**1.4 (165/117)**	0.146
No. of participants interviewed annually for baseline*	**N=297^**	**N=162**	**N=135**	p value#
1st year, n (%)	291 (98)	162 (100)	129 (96)	0.042
2nd year, n (%)	273 (92)	150 (93)	123 (91)	0.114
3rd year, n (%)	265 (89)	147 (91)	118 (87)	0.077
No. of study participants reporting respiratory illness	**N=386**	**N=219**	**N=167**	p value#
1st year, n (%)	227 (59)	154 (70)	73 (44)	<0.001
2nd year, n (%)	193 (50)	109 (50)	84 (50)	0.088

3rd year, n (%)	151 (39)	67 (31)	84 (50)	0.043
No. of reported respiratory episodes	**N=812**	**N=394**	**N=418**	p value#
1st year, n (%)	317 (39)	183 (46)	134 (32)	0.017
2nd year, n (%)	317 (39)	129 (33)	188 (45)	0.001
3rd year, n (%)	178 (22)	82 (21)	96 (23)	0.758
No. of collected samples from the episodes	**N=770**	**N=391**	**N=379**	p value#
1st year, n (%)	314 (41)	184 (47)	130 (34)	**0.028**
2nd year, n (%)	281 (37)	127 (33)	154 (41)	**0.016**
3rd year, n (%)	175 (23)	80 (21)	95 (25)	0.808

 # p value (Pearson's Chi-squared or Fisher exact test) of the difference between Dak Lak and Dong Thap.

** a respiratory sample was also collected from each individual interviewed for baseline.*

^ interviewed at least once among three baseline interviews, n=297. The participants interviewed on all three occasions, n=252.

7.2. Exposure to potential zoonotic sources of individuals with sustained occupational contact with animals (Goal I; Publication i)

Exposure by backyard animal farming

The majority (79.8%; 237/297) of the participants interviewed informed raising livestock in their backyard (Figure 6) or on adjoining farmland. Importantly, this backyard animal farming was reported in two third (63.9%, 106/166) of non-occupational farmers, indicating that the small-scale farming is particularly popular in rural Vietnam.

Notably, the animals were kept as a group of several animal species in a narrow area surrounding the household, as is typical in rural Vietnam [267], increasing the contact probability between different types of animals. Viruses with higher animal-host orders have been found to have a greater ability of transmission to humans, as well as initiate human-to-human transmission [241]. Livestock rearing in this way may therefore lead to higher inter-species pathogen transmission events, including zoonoses, and to higher probability of exposure to zoonotic viruses within the study participants. The emergence of avian influenza H5N1 virus in Vietnam in 2008, where the first human case was reported in Dong Thap is an illustration of this [268,269].

Figure 6. Several livestock species in a backyard, typical of rural settings in Vietnam. The photo was from the storage of the high-risk sentinel cohort study, VIZIONS project [95]. Printed with permission from the VIZIONS project.

Exposure by slaughtering, cooking, or consuming exotic animals

Overall, 70% (208/297) of the interviewees reported slaughtering, cooking, or consuming exotic animals (wild pigs, deer, and porcupines as most commonly) within the previous year. Of this group, almost all (99.5%; 207/208) reported consuming such exotic animals. For a proportion of Vietnamese people, the consumption of exotic animals has been thought to provide health benefits such as disease prevention or cure. For example, consumption of porcupine bile is believed to act as an analgesic or porcupine stomach as treatment for stomach pain. Additionally, another reason is probably the desire for the flavor of exotic animal meat and a belief that it is of higher quality.

Exposure by consumption of raw animal blood and meat

Raw blood is been served in Vietnam as a dish named "tiết canh". Almost a quarter (24.6%, 73/297) of the interviewed study subjects reported consumption of raw blood, and over a third

(37%; 110/297) of the subjects consumed raw meat of mammal and bird origin within the year prior to interview. Collectively, over a half of the participants reported of consumption of raw animal blood or meat. The majority of the raw meat consumers thought of it as good for health.

The proportion of participants consuming raw blood identified in this study is considered high given that the Vietnamese government banned the sale of the raw blood dishes in 2009 [270]. Furthermore, the majority (61.6%; 45/73) of raw blood consumers considered this activity as not-good for health, perhaps due to widely reported cases of infections by blood-borne zoonotic pathogens such as *Streptococcus suis* [271,272] or *Trichinella spiralis* [273,274]. However, raw blood consumption is a part of traditional culture in several geographic locations, especially in North Vietnam, and raw blood is served as a speciality dish in celebrations or gatherings. This culture originates from distant past as there has be a belief of health benefits, such as boosting the immune system, reducing body temperature, preventing anemia, or treating headaches, coughs, and dysentery [275]. This culturally deep-rooted practice is hard to change based despite health education and rational reasoning.

Exposure by limited use of PPE (personal protective equipment) and bleeding injuries while working with animals

Over two thirds (69/100) of the slaughter-house workers reported never using any piece of PPE (Figure 7) while working with animals at abattoirs and only one of these subjects reported using full PPE, indicating limited PPE use. Over half (58.6%; 174/297) of the study participants interviewed reported recent bleeding injuries whilst working with animals. Importantly, a positive association between no use of PPE and being bitten by animals was found. These data show a pervasive poor understanding by the participants to occupational exposure that increases possibility of zoonotic transmissions. Zoonotic transmission of many pathogens during butchering has been clearly described in Vietnam, for example *Trypanosoma evansi* [276] and rabies [277] in central and northern Vietnam and lack of PPE use was found to be associated with the transmission of *Brucella abortus* from animals to slaughter-house workers [278]. Therefore, the limited use of PPE and bleeding injuries indicate high risks of exposure to zoonotic infections of the participants.

Figure 7. No use of PPE (personal protective equipment) of the slaughter-house workers when slaughtering animals. The photo is from data of the high-risk sentinel cohort study, VIZIONS project [95]. Printed with permission from the VIZIONS project.

High and consistent exposure to a large variety of animals

Besides routine occupational exposure to animals, the study participants had regular contact with a large variety of animals, including 16 types of exotic animals and 16 types of domestic animals. The exotic animals that the participants were most exposed to included wild pigs, porcupines, rice-field rats, deer; and pigs, chickens, dogs, cats, ducks, cattle as domestic animals. All these animals have also been known to be potential reservoirs of zoonotic pathogens [241,279–281].

The data of exposure to zoonotic infections of the participants was consistent over the 3-year study period, indicating high and consistent risks of zoonotic infections for the study participants.

7.3. Common respiratory viruses in respiratory infections in individuals with sustained occupational contact with animals (Goal II; Publication ii)

All of the nasal-throat swabs collected at 3 annual baseline samplings (n=732) and at episodes of acute respiratory infections (n=770) over the 3-year study were screened by RT-PCR assays for 15 most common viruses of respiratory infections (Figure 5).

Frequency and comparisons of respiratory viruses detected at baseline and disease episodes

By RT-PCR, evidence of at least one respiratory virus was detected in 7.9% (58/732) of baseline samples and 17.7% (136/770) disease-episode samples (p<0.001) (Figure 8). The results are in agreement with other studies on adult subjects in other settings [272,282–285]. This may be explained as substantial immunity from previous infections accumulated over their life, resulting in rapid clearance of the viruses in the respiratory tract at the current infections, thereby lowering the virus titers and shortening the time of virus shedding as compared to children [286]. The mixed infections were found in 2 (0.3%) baseline samples and 7 (0.9%) samples at disease episodes (Figure 8).

The median RT-PCR Ct values of samples at baseline and disease episodes were 37.9 (range: 26.4 – 40) and 38.4 (range: 26 – 40), respectively. There was no significant differences of Ct values between samples at baseline and disease episodes (p=0.45), suggesting that Ct values are probably not a good marker for distinguishing between acute and asymptomatic respiratory infections of the viruses.

EVs and RV were the most common viruses detected in both baseline and disease-episode samples, followed by influenza A virus, ADV and CoV. The predominance of EVs and HRV detections we have identified expands our knowledge about the clinical burden induced by the viruses of *Enterovirus* genus in Vietnam beside hand foot and mouth disease [287,288] and central nervous system infections [289,290].

RSV A, RSV B, MPV, influenza B virus, PIV4, BoV were rarely detected, while PIV 1, 2, 3 and PEV were not detected at all over the 3-year study. The low or no detection of RSV, PEV and PIV may be explained by the age structure of the study participants. RSV, PEV and PIV have been well described as pathogens in children [38–42,291,292] while over 92% of the disease episodes reported herein were from study participants all over 16 years old. Additionally, the low detection rates of BoV, MPV and influenza B were in agreement with the other studies [69,293].

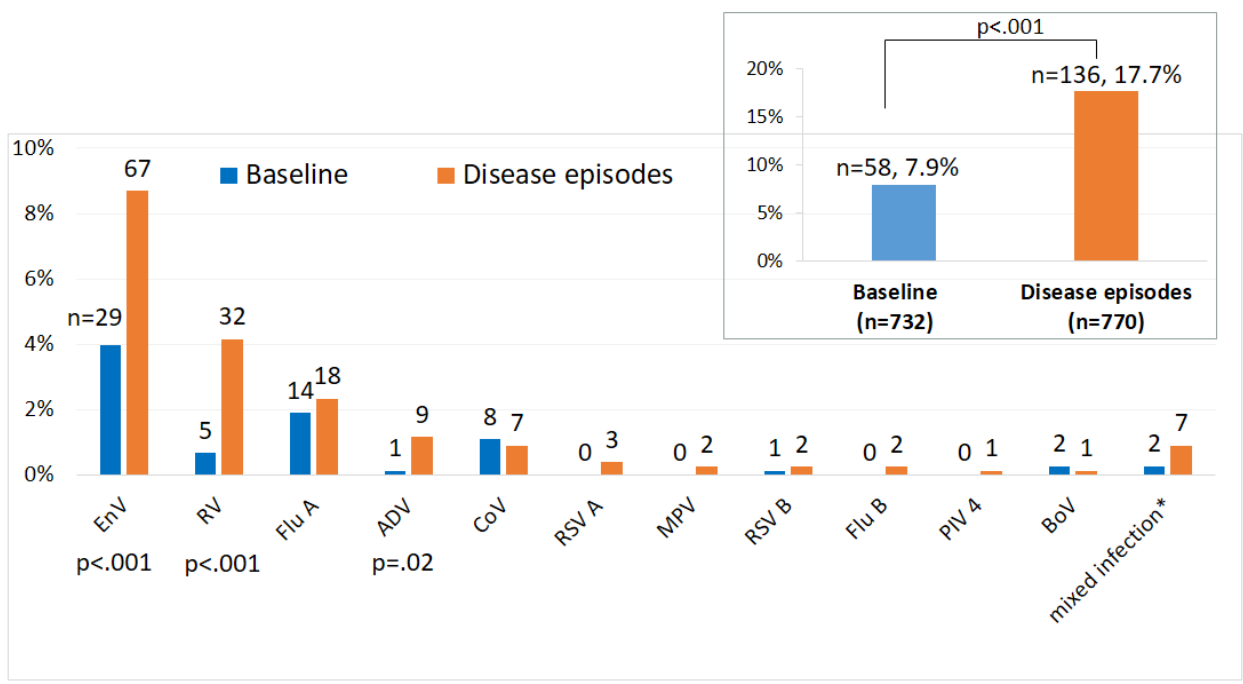


Figure 8. Number (percentage) of participants with detected viruses from nasal-throat swabs. Baseline samples (n=732) were collected annually when the participants had no respiratory symptoms. Samples at disease episodes (n=770) were collected whenever the participants reported acute respiratory infections. PIV 1, 2, 3 and PEV were not detected in all samples. * Mixed infections included one EVs-BoV and one influenza A virus-CoV of baseline samples, and one ADV- influenza B virus, one BoV- influenza A virus, two EVs- influenza A virus, one EVs-RSV B, one EVs-RV and one EVs-ADV-CoV of disease-episode samples.

Seasonal differences in the frequency of detection of respiratory viruses

A seasonal distribution was observed in disease episodes of EVs, influenza A virus and AdV, while there was no difference in detection of HRV and CoV between rainy season (May–October) and dry season (November–April). These are in agreement with previous studies [294–298]. EVs and influenza A virus were detected at significantly higher rates during the rainy season than in the dry period (12.2% (43/353) vs 5.8% (24/417) (p=0.002) for EVs, and 3.7% (13/353) vs 1.2% (5/417), p=0.023 for influenza A virus, respectively), while the AdV detection rates were significantly higher in the dry season than in the rainy period (1.9%, 8/417 vs. 0.3%, 1/353, p=0.044) (Figure 9). The seasonal distribution patterns of the viruses may be informative for more effective prevention and control strategies.

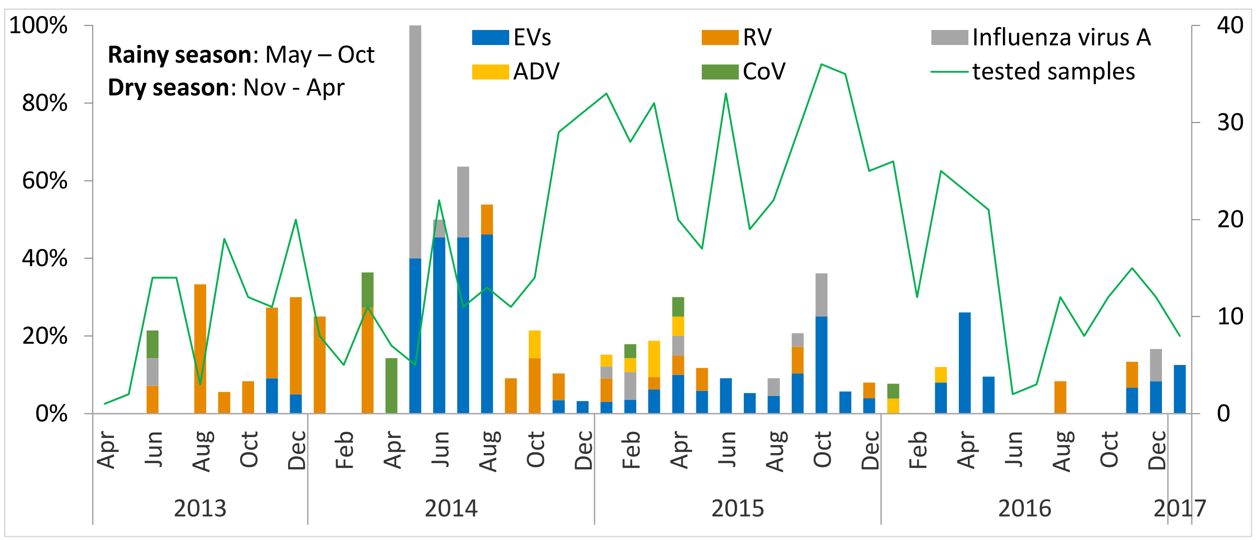


Figure 9. The seasonal distribution of symptomatic infected cases of EVs (enterovirus), RV (human rhinovirus), influenza A virus, ADV (adenovirus) and CoV (coronavirus subtype OC43 and NL63) detected by RT-PCR. Reproduced with permission from the Journal of Medical Virology [71].

7.4. Virome of acute respiratory infections in individuals with high risk of zoonotic infections (Goal III; Publication iii)

The mNGS analysis were conducted on 91 nasal-throat swabs collected from 91 episodes of acute respiratory infections in 58 participants in Dong Thap in the study year 2013. These 91 samples included 76 samples negative for a wide panel of respiratory viruses and 15 samples positive with 6 respiratory viruses detected by RT-PCR (reported in section 7.3) as positive controls to assess the assay performance (Figure 5). One sample (n=1) containing viral transport medium was also included in the analysis as a negative control.

7.4.1. Overview of sequences detected by metagenomics

A total of 31,783,202 raw reads were collected, with a median of 342,524 reads, range: 43,930–718,762 reads/sample. The reads mostly belonged to bacterial, viral and human sequences. Here we focus on viral reads (especially related to eukaryotic viruses) which occupied 2.3% (range: 0.5–12.7%) of the total reads.

Of viral reads, evidence of sequences related to 52 viral species of 31 families was detected. This included 19 virus species of 13 families that have previously been detected in human samples.

These were found in 27 of 91 (29.7%, 95% CI: 21.3–39.7%) samples. After PCR confirmation, 12 virus species of 9 families were found in 22 of 91 samples (24.2%, 95%CI: 16.5–33.9%) (details below).

Additionally, sequences related to fungal viruses (Family *Chrysoviridae, Totiviridae*), bacterial viruses (phages) (Family *Siphoviridae, Inoviridae, Myoviridae, Podoviridae, Leviviridae*) were detected. suggesting a relationship with diet, sequences related to the invertebrate viruses (Family *Iridoviridae, Dicistroviridae*), insect viruses (Family *Baculoviridae, Dicistroviridae, Iflaviridae, Polydnaviridae*), plant viruses (Family *Betaflexiviridae, Bromoviridae, Potyviridae, Partitiviridae*), and algae viruses (Family *Phycodnaviridae*) were also abundantly detected.

7.4.2. Viral detection in controls

Sequences related to 5 virues were detected by mNGS in 13/15 (86.7%, 95%CI: 62.1–96.3%) samples, including 8 RV, 1 EVs, 1 mixed detection of RV and EVs, 1 influenza A virus, 1 CoV and 1 RSV A. Only sequences of RSV and MPV in 2 samples were missed (i.e. not detected by mNGS), but the viruses were detected by RT-PCR with a Ct value of 36.3 and 40, respectively (Table 5). In addition, RSV and MPV sequences were found in 2 mNGS samples which were negative in RT-PCR.

By the mNGS sequences, all viruses detected in 13/15 RT-PCR-positive samples were successfully genotyped/serotyped. The 9 RV belonged to RV B (n=7), RV C (n=1) and RV A (n=1). Additionally, (almost) complete genomes (≥75% coverage) of RV (n=6) and EVs (n=2) were generated. Based on mNGS sequences, cross-detection between EVs and RV by RT-PCRs in two samples was detected and corrected to RV B and EVs-D68, respectively (Table 5).

mNGS detection of most respiratory viruses in (RT-)PCR-positive samples indicates that mNGS is a highly sensitive pan-virus assay of respiratory viruses in clinical samples, in agreement with previous studies [74,198,299]. Only sequences of RSV A and MPV were not detected from mNGS output in the 2 targeted samples (RSV A- and MPV-PCR-positive samples), but identified in only 2 other samples, suggesting that index-hopping was probably happened, although contamination or pipetting mistakes were not excluded. Additionally, detecting and correcting cross-reactivity between EVs and RV by RT-PCR, and genotyping other strains highlight the advantages of mNGS in etiological and epidemiological studies compared to (RT-)PCRs.

RV cluster A and C have been found to cause fatal lower respiratory infections on children [300–302], while our knowledge of RV in adults and RV B remains limited. The identification of RV serotypes in our study therefore highlights predominance of RV B in acute respiratory infections in adults.

Notably, few studies have reported the genetic diversity of HRV circulating in Vietnam, therefore this study expands understanding of the diversity of RV in this locality of the world.

Table 5: Detection of respiratory viruses of mNGS in 15 (RT-)PCR-positive samples. Reproduced with permission from the Journal of Viruses (Publication iii) [303].

No.	Sample ID	Multiplex RT-PCR**		mNGS analysis				
		Virus detected	**Ct value**	**Virus genotype**	**Reads (%)#**	**Total length (bp)**	**Genome coverage (%)**	**Other virus detected##**
1	72	EVs	32.4	Coxsackievirus A21	52,989 (12)	7,440	100.0	
		RV	37.1	RV C56	2,506 (0.6)	7,099	98.1	
2	75	EVs	38.6	RV B	4 (0.0)	598	8.3	
3	5	RV	38.4	RV B3	678 (0.7)	5,512	75.0	Human betaherpesvirus 7
4	33	RV	40	EVs-D68	3,174 (0.7)	5,629	76.2	
5	54	RV	40	RV B	6 (0.0)	723	10.0	
6	73	RV	40	RV B86	6,644 (1.5)	7,212	99.2	Vientovirus
7	83	RV	38.7	RV B79	6,157 (1.8)	5,639	78.2	Novel gemycircularvirus
8	86	RV	38.2	RV B79	19,606 (5.6)	7,224	99.7	
9	91	RV	40	RV A57	2,538 (1.1)	3,450	47.8	
10	92	RV	36.5	RV B35	12,481 (3.1)	7,298	100.0	Bat badicivirus, bat posalivirus
11	4	Influenza A virus	29.3	Influenza A/N2 virus	2 (0.0)	115	0.8	
12	6	CoV*	36	CoV OC43	8 (0.0)	733	2.4	
13	52	RSV-A	30.8	RSV-A genotype ON1	236 (0.1)	5,398	35.4	
14	39	RSV-A	36.3	Not detected	0	0	0	
15	65	MPV	39.5	Not detected	0	0	0	

** OC43 or/and NL63*
*** reported previously*
 # Total reads of the targeted virus (percentage: the total reads of the virus per total raw reads of the sample)
detail of the viruses in Table 6

7.4.3. Viral detection in RT-PCR negative swabs

Amongst 76 (RT-)PCR-negative samples, the sequences related to 12 viral species of 9 families, that have previously been detected in human samples, were found in 16/76 (21.1%) samples. They included both known human viruses (rotavirus, MPV, RSV, torque teno virus, human papillomavirus) and other viruses whose tropism is still unknown (novel cyclovirus, novel gemycircularvirus, novel statovirus, vientovirus, viruses of *Circoviridae* family, gemycircularvirus and statovirus) (Table 6).

In the 15 (RT-)PCR-positive samples, human betaherpesvirus 7, vientovirus, gemycircularvirus, bat badicivirus-like virus and bat posalivirus-like virus were found in 4/15 (26.7%) samples (Table 5 and 6).

Collectively, using specific (RT-)PCR, the presence of rotavirus (n=1), novel cyclovirus (n=1), novel gemycircularvirus (n=3), novel statovirus (n=3), gemycircularvirus (n=3), statovirus (n=2) and vientovirus (n=1) (Table 6) was confirmed in 10 samples (8 of 76 (10.5%) (RT-)PCR-negative and 2 of 15 (13.3%) (RT-)PCR-positive samples) (detection of novel viruses is reported in section 7.5).

This viral survey expands our understanding and provides knowledge about baseline virome in nasal-throat swabs of individuals with high frequency of animal exposure in Vietnam and with acute respiratory infections. Detection of a variety of viruses including several novel or recently identified viral genomes indicates that mNGS is suitable as a highly sensitive pan-virus assay for sequence-independent detection of a variety of viruses including novel ones.

Table 6: mNGS detection of viruses, that have previously been detected in human samples, in nasal-throat swabs negative for human viruses by RT-PCR. Reproduced with permission from the Journal of Viruses (Publication iii) [303].

No.	Sample ID	Detected viruses previously reported in human samples	Confirmed by PCR	No. of reads	Total contig length (bp)	Amino acid identity to Genbank strain (%)	Genome coverage (%)
1	89	Rotavirus	Yes	17	360	98	1.9
2	73	Vientovirus* (vientovirus VZ)	Yes	2	146	53	4.8
3	23	Novel cyclovirus (cyclovirus VIZIONS-2013)	Yes	5	448	61.8	25.9
4	32	Novel gemycircularvirus virus (gemycircularvirus VIZIONS-2013)	Yes	1852	1995	39	91
5	83	Gemycircularvirus VIZIONS-2013*	Yes	120	2000	45	92
6	89	Gemycircularvirus VIZIONS-2013	Yes	1	148	46.9	6.8
7	24	Novel statovirus (statovirus VIZIONS-2013)	Yes	91	1018	42.5	24.6
8	32	Statovirus VIZIONS-2013	Yes	5	231	35	5.6
9	82	Statovirus VIZIONS-2013	Yes	27	2000	49	48.4
10	87	Gemycircularvirus	Yes	39	858	83	39
11	71	Gemycircularvirus	Yes	117	1400	97	63.7
12	88	Gemycircularvirus	Yes	2	300	73	13.6
13	11	Statovirus	Yes	4	351	91	8.5
14	71	Statovirus	Yes	7	812	90	19.6
15	5	Human betaherpesvirus 7*	Not done	2	295	100	0.2
16	15	Human papillomavirus	Not done	73	1280	99.3	17.5
17	17	Human papillomavirus	Not done	6	437	97.9	6
18	2	Torque teno virus	Not done	4	554	88.4	14.6
19	68	Torque teno virus	Not done	2	217	70.6	5.7
20	24	MPV	No	6	417	100	3.1

21	47	RSV A	No	6	468	100	3.1
22	92	Bat badicivirus-like virus*	No	2	204	49	2.3
23	92	Bat posalivirus-like virus*	No	3	182	56	2
24	83	Circular virus of *Circoviridae* family	No	10	167	64	7

* *co-detected with other viruses in 15 (RT-)PCR-positive samples as reported in Table 5*

7.5. Exploring novel viruses in upper respiratory tract of individual with sustained occupational contact with animals (Goal III; Publications iii, iv)

From mNGS analysis, contigs related to vientovirus, cyclovirus, gemycircularvirus and statovirus were detected, sharing low sequence identity with previously known virus species. Further investigations were therefore conducted to identify and explore them (Figure 5).

7.5.1. Vientovirus (Publication iv)

A 146 bp contig from vientovirus-related two reads was obtained in the mNGS analysis from a single nasal-throat sample. By inverse PCR and Sanger sequencing with primers obtained from the mNGS contig, a complete circular DNA genome of 3,054 bp was obtained sharing 79% (2404/3054 bp) whole genome identity with human lung-associated vientovirus AL strain (accession number: QCD25302.1) of *Redondoviridae* family, a recently identified virus family.

The genome structure shows a typical genome feature of a virus of *Redondoviridae* family with 3 ORFs encoding a capsid (Cap), a replication (Rep) protein and an unknown protein (ORF3) (530, 350 and 200 amino acid respectively). A Rep stem-loop structure (with motif "TATTATTTAT") forming immediately upstream of the Rep ORF was identified. The capsid and unknown ORFs were arranged in opposite directions with the replication ORF (Figure 10). The capsid protein shared 97.7 % (509/521) amino acid identity with vientovirus (QCD25303.1) suggesting that it is a strain of *Vientovirus* genus. Its replication protein also shared 59% (207/350) amino acid identity with the vientovirus (QCD25302.1). The species demarcation criteria were proposed to be 50% replication protein identity [81,87]. Additionally, the phylogenetic tree of the replication protein showed genetic distinction from other strains on GenBank (Figure 11). Therefore, it is proposed to be a strain of a strain belonging to vientovirus species of *Torbeviruses* genus, *Redondoviridae* family, and tentatively named *Vientovirus VZ* (accession number: MT759843). This represents first report of vientovirus and a virus of *Redondoviridae* family detected in Vietnam and Asia in general.

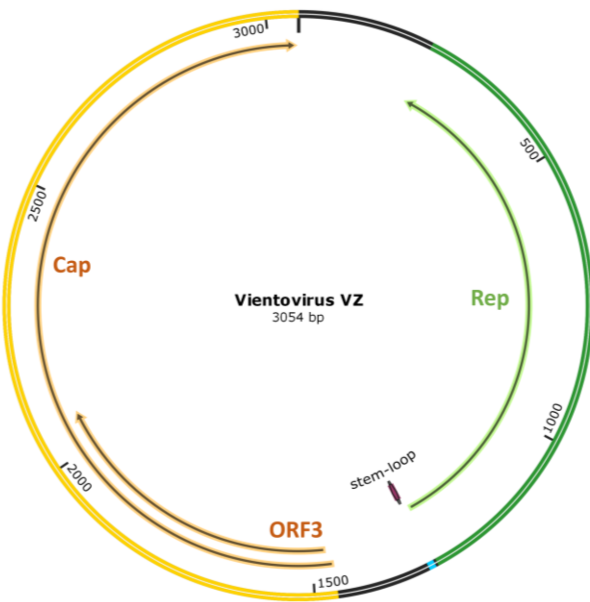


Figure 10: Putative genome organization of vientovirus VZ. Reproduced with permission from the Journal of Viruses (Publication iv).

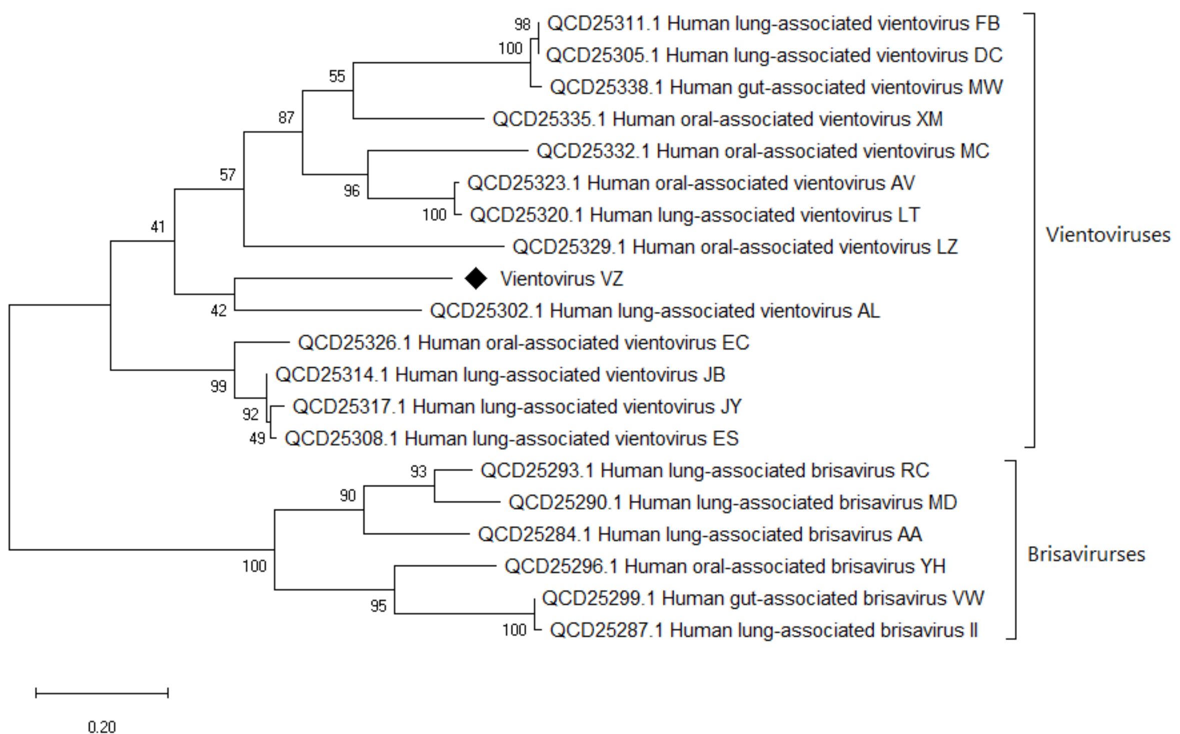
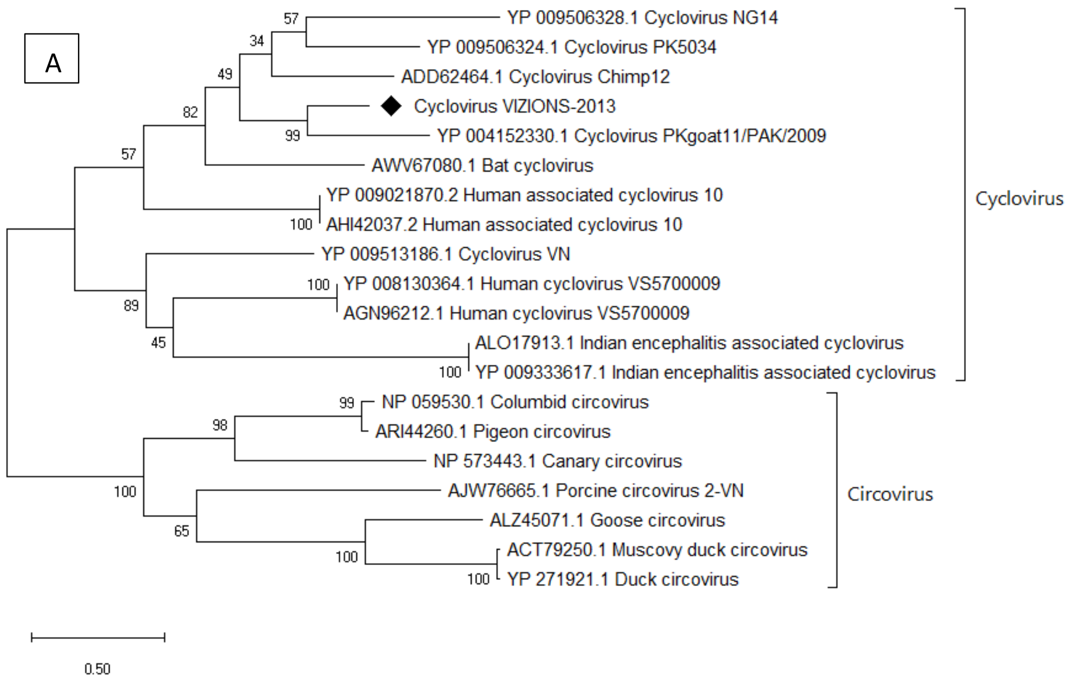


Figure 11: Phylogenetic tree of complete replication protein sequences of vientovirus VZ compared to known redondoviruses on GenBank.

7.5.2. Novel cyclovirus (Publication iii)

Two cyclovirus-related contigs were generated from the mNGS analysis on a single disease-episode sample with (RT-)PCR-unknown etiology. A complete circular DNA genome of 1,740 bp was then obtained by inverse PCR and Sanger sequencing with primers based on the mNGS contigs. The complete genome shared highest nucleotide identity (55%) to cyclovirus NG 14 (accession number: NC_038417) that is lower than species demarcation threshold (80% identity of genome-wide nucleotide sequence) [94]. Phylogenetic analysis of capsid and rep proteins, 216 and 279 amino acids long respectively, confirmed its genetic distinction from other cycloviruses (Figure 12), suggesting that it is a novel cyclovirus species, tentatively named *Cyclovirus VIZIONS-2013* (accession number: MT649485).



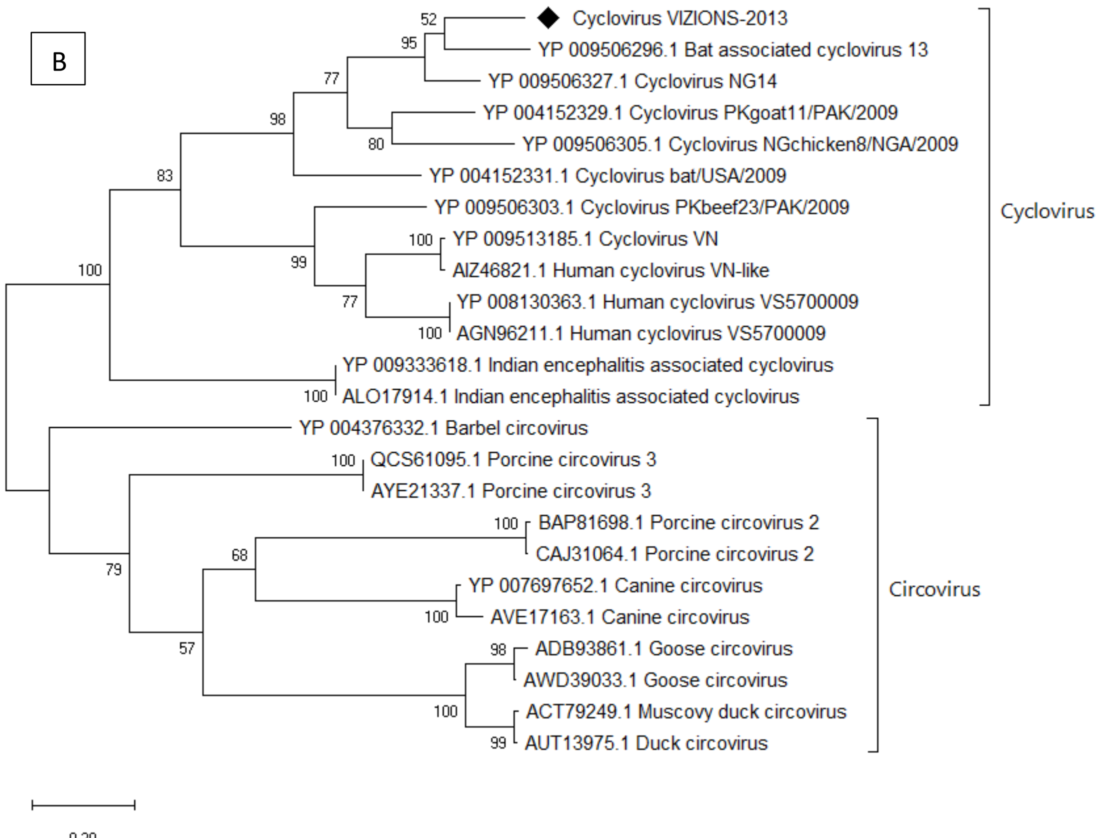


Figure 12. Phylogenetic tree of capsid (A) and replication (B) protein of cyclovirus VIZIONS-2013 compared to known viruses of *Circoviridae* family. Reproduced with permission from the Journal of Viruses (Publication iii) [303].

7.5.3. Novel gemycircularvirus (Publication iii)

From output of the mNGS analysis of a single disease-episode sample with unknown etiology by (RT-)PCR, multiple gemycircularvirus-related contigs were detected. A complete circular DNA genome of 2,171 bp was then generated from PCR and Sanger sequencing with inverse primers. The complete genome showed highest nucleotide identity (48.3%) to a murine feces-associated gemycircularvirus 2 (accession number: MF416388.1). The current species demarcation of gemycircularviruses is 78% genome-wide pairwise identity [118], while all of the proposed species (n=43; 73 strains) of the genus *Gemycircularvirus* share at least 56% whole genome identity with each other [118]. Additionally, the phylogenetic analyses of the capsid (298 amino acid) and replication (333 amino acid) proteins show a highly genetic distinction from other viruses of the *Genomoviridae* family (Figure 13). Collectively, these suggest that it is a novel gemycircularvirus, tentatively named *Gemycircularvirus VIZIONS-2013* (accession number: MT649486).

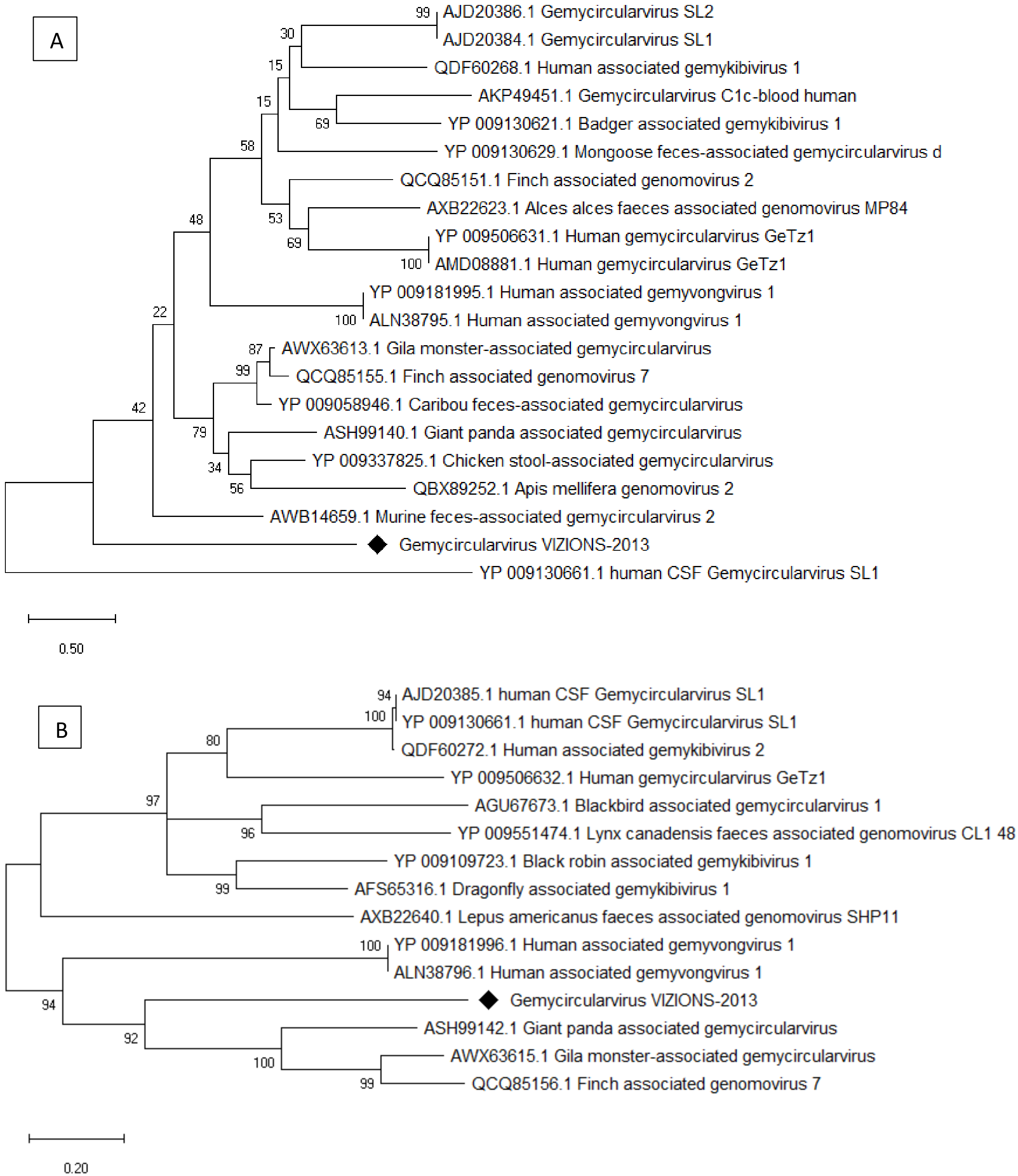


Figure 13. Phylogenetic tree of capsid (A) and replication (B) proteins of the gemycircularvirus VIZIONS-2013 compared to the viruses of *Genomoviridae* family. Reproduced with permission from the Journal of Viruses (Publication iii) [303].

7.5.4. Novel statovirus (Publication iii)

From the mNGS analysis of 3 (RT-)PCR-negative samples, seven statovirus-related contigs were collected. Using De novo alignment, partial proposed RdRp and coat protein sequences were obtained with the length of 249 and 260 amino acid, respectively, sharing 40.4 and 45% amino acid identity in comparison with other statoviruses on GenBank. There is currently no available species or genus demarcation for statoviruses [119]. Together with the low identity of RdRp protein sequence of this strain with other statoviruses and the distinction in phylogenetic analysis (Figure 14), it is suggested as novel statovirus species, tentatively named Statovirus VIZIONS-2013 (accession number: MT649483, MT649484).

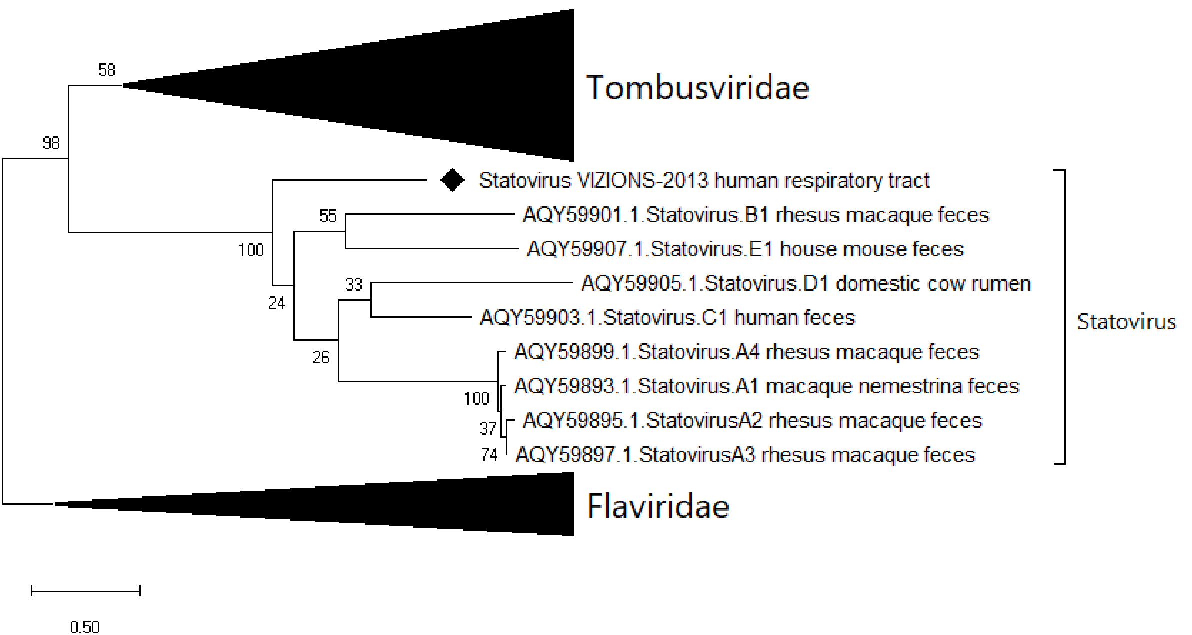


Figure 14. Phylogenetic tree of 249 aa RdRp protein sequences of statovirus VIZIONS-2013 compared to the statoviruses on Genbank and viruses of *Tombusviridae* and *Flaviviridae* family. Reproduced with permission from the Journal of Viruses (Publication iii) [303].

The detection of these novel viruses and a virus of the recently discovered virus family, *Redondoviridae*, expands our understanding of the human respiratory virome. Additionally, since the majority of the acute respiratory infections remains the unknown etiology despite of intensive laboratory diagnostics, it is therefore necessary for further characterization.

7.6. Comparison of detection of novel viruses by mNGS versus PCRs (Goal V; Publications iii, iv)

By mNGS analysis and PCR (with Sanger sequencing), vientovirus VZ, cyclovirus VIZIONS-2013, gemycircularvirus VIZIONS-2013 and statovirus VIZIONS-2013 were identified as novel viruses or a virus of recently discovered *Redondoviridae* family (section 7.5). PCRs (confirmed by Sanger sequencing) were used to screen these (novel) viruses in the same mNGS-analyzed samples to assess detection ability of mNGS (Figure 5).

For redondoviruses, the PCRs firstly target the region of capsid gene for detection of redondoviruses. Redondovirus-positive samples were then applied in PCRs targeting whole replication gene for species identification. Sanger sequencing was then used to confirm the PCR results and to achieve the amplified replication-gene sequences for species identification. The primers were newly designed based on the complete genome achieved above and all available redondovirus sequences on Genbank [81].

Redondoviridae **family (including vientovirus VZ)** (Publication iv): Redondovirus DNA were only detected in 1 of 91 samples in mNGS analysis (section 7.5). However, by PCR screening, redondovirus DNA was additionally detected in 29 of 90 (32.2%) mNGS-negative samples.

Cyclovirus VIZIONS-2013 (Publication iii): DNA of Cyclovirus VIZIONS-2013 was only detected in 1 of 91 samples in mNGS analysis (section 7.5). However, PCR (confirmed by Sanger sequencing) additionally detected the virus DNA in 5 of 90 (5.6%) mNGS-negative samples.

Gemycircularvirus VIZIONS-2013 (Publication iii): Besides detection of Gemycircularvirus VIZIONS-2013 DNA in 1 of 91 samples analyzed by mNGS (section 7.5), DNA of this virus was detected in mNGS output of two other samples. By PCR, DNA of gemycircularvirus VIZIONS-2013 was additionally found in 12 of 88 (13.6%) mNGS-negative samples.

Statovirus VIZIONS-2013 (Publication iii): Besides detected in 3 of 91 samples analyzed by mNGS (section 7.5), DNA of statovirus VIZIONS-2013 was not detected by PCR assays in any of mNGS-negative samples.

Collectively, more frequent detection of the (novel) viruses by PCR than by mNGS in the same samples suggests that PCR currently remains the most sensitive test for the viruses with genomes already known. The main advantages of mNGS are therefore the ability to detect and sequence all viral genomes simultaneously rather than performing an extensive set of different (RT-)PCRs and to detect novel viruses as no virus specific primers are required.

7.7. Characterization of the detected novel viruses (Goal III; Publications iii, iv)

Due to high prevalence of the novel viruses (vientovirus VZ, cyclovirus VIZIONS-2013, gemycircularvirus VIZIONS-2013 and statovirus VIZIONS-2013) in disease-episode samples collected in 2013 (section 7.6), to better understand the characterization of these novel viruses, the PCR assays (confirmed by Sanger sequencing) (same as in section 7.6) were utilized to screen these viruses in baseline samples (collected when no acute respiratory symptoms) in 2013 from the same participants taken at the disease episodes.

***Redondoviridae* family** (Publication iv)

**** Prevalence of redondoviruses***

Collectively from section 7.6, redondovirus DNA was detected in 30 of 91 (32.7%) disease-episode samples. Of 27 (86.7%; 27/30) replication-gene PCR amplicons, 9 (33.3%; 9/27) belong brisavirus and 18 (66.7%; 18/27) are vientovirus.

Of baseline samples (n=58) of the same participants (n=58) taken at disease episodes, redondovirus DNA was detected in 29 samples (50%). Of 26 (89.7%; 26/29) replication-gene PCR amplicons achieved, 6 (23.1%) belong to brisavirus, and 20 (76.9%) are vientovirus.

Collectively, combining the detection results from baseline and disease-episode samples, redondoviruses were detected in the majority of participants (34/58; 58.6%). Vientovirus were more frequently detected than brisavirus, 72.7% (24/33) vs. 27.3% (9/33), respectively (the efforts of species identification for one sample were unsuccessful). The prevalence of viruses of *Redondoviridae* family herein is higher compared with the previous detections of around 15% in healthy American [81,87], 11% in Italy [88] and 2% in Spain [88].

**** Genetic diversity of redondoviruses***

To explore the genetic relationship in this study, 16 complete replication protein sequences of Vietnamese redondoviruses described above were compared against the sequences available on GenBank (from the US, Spain and China) [81,86–88]. No large-scale geographical clustering was found between Vietnamese and the US, Spanish and Chinese strains (Figure 15), suggesting a wide geographic distribution and high genetic diversity of redondoviruses.

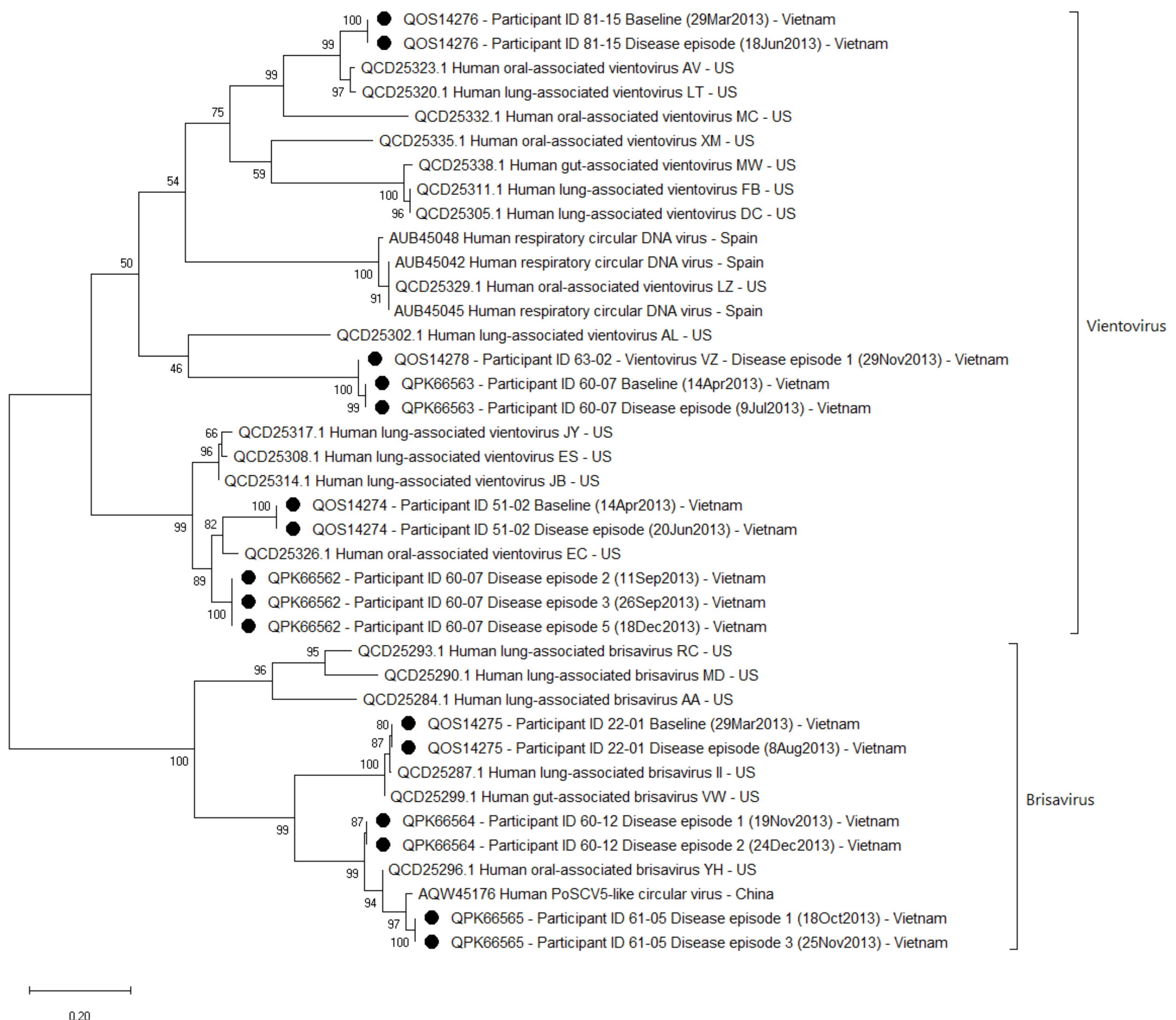


Figure 15: Phylogenetic tree of complete redondovirus replication protein sequences in this study compared to known redondoviruses in the US, Spain and China on Genbank [87]. Black circle: Vietnamese redondovirus strains detected in this study; the strains with same accession number had identical replication gene. Reproduced with permission from the Journal of Viruses (Publication iv).

<u>* Possible persistence of redondoviruses in nasopharynx</u>

Of 23 participants with at least 2 of their longitudinal samples positive with redondoviruses, 10 (43.5%) had the same replication gene as redondovirus strains (610–1306 bp, equal to 58–100% of whole replication gene) detected repeatedly in their longitudinal samples (window of 35–132 days) (Figure 16). Of these 10 redondovirus strains, 4 belong to the brisavirus and 6 belong the

vientovirus (Figure 16). No identical replication gene sequence was detected in samples of different participants.

	Study year 2013						
	Baseline	Disease episode 1	Disease episode 2	Disease episode 3	Disease episode 4	Disease episode 5	Duration of persistence (days)
Participant ID 60-07	VienV VZ 14-Apr	VienV VZ 09-Jul	VienV S39 11-Sep	VienV S39 26-Sep	RedonV 15-NoV	VienV S39 18-Dec	86 and 98, respectively
Participant ID 48-01	VienV S19 14-Apr	VienV S19 10-Jul					87
Participant ID 81-15	VienV S8 29-Mar	VienV S8 18-Jun					81
Participant ID 49-01	VienV S15 14-Apr	VienV S15 20-Jun					67
Participant ID 51-02	VienV S17 14-Apr	VienV S17 20-Jun					67
Participant ID 22-01	BrisaV S32 29-Mar	BrisaV S32 08-Aug					132
Participant ID 81-23	BrisaV S4 07-Apr	BrisaV S4 05-Jun	10-Jul				59
Participant ID 61-05	RedonV 14-Apr	BrisaV S56 18-Oct	RedonV 08-Nov	BrisaV S56 25-Nov			38
Participant ID 60-12	14-Apr	BrisaV S83 19-Nov	BrisaV S83 24-Dec				35

Figure 16. Chart of identical replication-gene sequences of brisavirus and vientovirus detected in samples at baseline and disease episodes. RedonV: Redondoviruses; VienV: Vientovirus; BrisaV: Brisavirus. Vientovirus or brisavirus with same name and in the samples collected from the same participants had identical replication-gene sequences (610–1306 bp, equally to 58–100% of whole replication gene). Boxes with the name of redondoviruses were samples positive for redondoviruses by PCR, but no PCR-replication sequence was achieved for species identification. Boxes with only date were negative for redondoviruses. Reproduced with permission from the Journal of Viruses (Publication iv).

Cyclovirus VIZIONS-2013 (Publication iii)

From results presented in section 7.6, collectively, evidence of cyclovirus VIZIONS-2013 was detected in 6/91 disease-episode samples (6.6%, 95%CI 3.1–13.7%). Matching participants' baseline and disease episodes, the virus DNA was found in 6 of 58 (10.3%) baseline samples.

We analysed all 12 nucleotide sequences of the 206 bp capsid gene PCR amplicon from the virus-positive samples. The amplicons shared 92–98% identity compared with original cyclovirus VIZIONS-2013. Mixed PCR results with other viruses were detected in several samples, however,

no significant difference between the viruses with and without mixed detection was found ($p \geq 0.47$).

Gemycircularvirus VIZIONS-2013 (Publication iii)

Collectively from section 7.6, sequences of gemycircularvirus VIZIONS-2013 sequence were detected in 15/91 (13.6%) disease-episode samples. Matching participants' baseline and disease episodes, DNA of this novel virus was also found in 8/58 (13.8%) baseline samples.

The 423-bp replication gene PCR amplicons of the 23 strains of gemycircularvirus VIZIONS-2013 shared 94.3–98% identity with each other. No significant difference between the viruses with and without mixed detection was found ($p \geq 0.47$) although mixed detections with other viruses were found.

Statovirus VIZIONS-2013 (Publication iii)

From results presented in section 7.6, collectively, sequences of this novel virus were detected in 3/91 (3.3%) disease-episode samples. Baseline samples collected from these three individuals with positive disease-episode samples were also positive with this virus. Thus, the detection proportion of statovirus VIZIONS-2013 at baseline was 3/58 (5.2%). Notably, all the 420bp PCR amplicons of the statovirus-positive samples from the same individuals both at disease episodes and baseline were 100% identical.

Overall, these further characterizations expand our understanding about prevalence of these novel viruses and provide better prediction of their pathogenic potential. Similar prevalence of redondoviruses in samples at baseline and disease episodes of the study participants is in agreement with those previously reported [81].

7.8. Assessment of zoonotic potential of the viruses detected in respiratory tract of the individuals (Goal IV; Publication iv)

Viruses of *Redondoviridae* family (Publication iv)

All nasal-throat swabs (n=53) from animals to which the 6 farmers testing positive for redondovirus had been directly exposed immediately before their disease episodes (presented in

section 7.6), were screened for redondoviruses by PCR (same as screening on human samples at section 7.6 and 7.7). The animals were pigs, chicken, Muscovy ducks, ducks and dogs from 6 farmer households. They included 27 samples from 27 pigs of 5 household, 13 sample pools from 27 chicken of 5 households, 8 sample pools from 17 Muscovy ducks of 2 households, 1 sample of 1 duck, and 4 sample pools from 6 dogs of 4 households. No evidence of redondoviruses was detected in any sample. This is in line with the reported screening [81] from available database of metagenomics analyses of animal samples. It suggests low probability of a zoonotic transmission from these close animal contacts.

Gemycircularvirus VIZIONS-2013 (unpublished data)

By PCR screening (see details in 7.6 and 7.7), the gemycircularvirus was detected in 2/11 pigs that were in close contact with the two study participants who were also positive for the gemycircularvirus (Figure 17). Subsequent analysis of the obtained sequences of the PCR products (370 bp) showed that the gemycircularvirus sequences obtained from animal samples shared 100% identity with those obtained from the human samples.

In contrast, no evidence of the virus was found in 7 nasal-throat swab pools from 16 pigs that 3 virus-negative participants were exposed to during their disease episodes (Figure 17). There is no significant difference (p=0.23) in clinical symptoms and animal-exposure characteristics between the participants with and without gemycircularvirus VIZIONS-2013 detected in their animals.

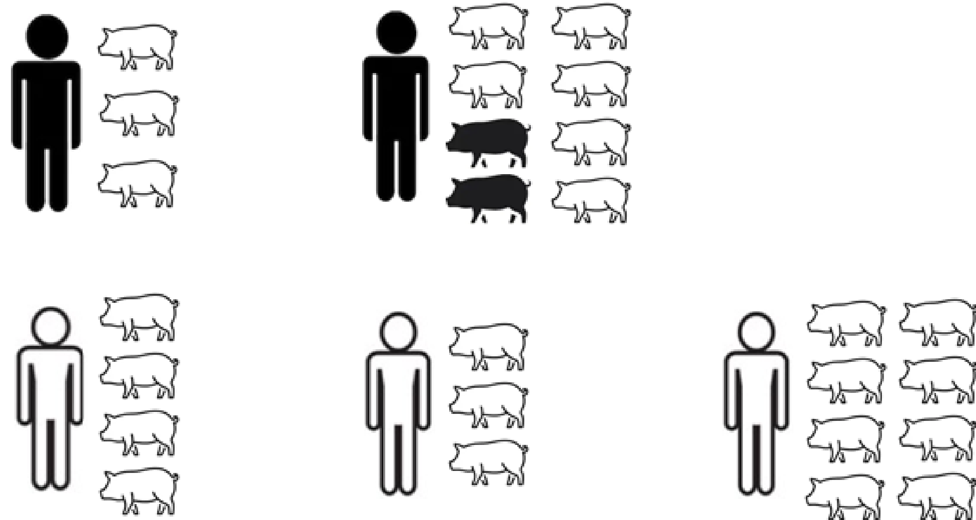


Figure 17. Relationship of gemycircularvirus VIZIONS-2013 in respiratory samples of participants and their close contact pigs. Black human or pig pictures indicate their respiratory samples are positive with gemycircularvirus VIZIONS-2013. White human or pig pictures indicate their respiratory samples testing negative for the virus. A participant and their two pigs had 100% identical PCR amplicons (370 bp) of the virus. The animation pictures of human and animals were copied from open-access sources, Shutterstock [251].

The detection of identical sequences in humans and their animal contacts points to the possibility of cross species transmission, although whole genome sequence based analysis is needed to conclusively define the relatedness between these viral strains. However, it should be further studied whether the virus indeed infects animals and cause a zoonotic transmission in humans. Other possible explanations may be that the virus colonizes the respiratory tract or gemycircularvirus-infected eukaryotic microbes (e.g. fungi, parasites) release viruses in the respiratory track of both human and animals.

Cyclovirus VIZIONS-2013 (unpublished data)

Cyclovirus VIZIONS-2013 were randomly screened in 27 nasal-throat swabs of 27 pigs (from the screening of redondoviruses above) which were close contacts of 5 participants who was negative for the virus at their disease episodes (see section 7.6 and 7.7 for more details about the screening assays). Cyclovirus VIZIONS-2013 was detected in samples from 11 pigs that were in close contacts with 4 study participants (Figure 18). The obtained sequences of PCR products shared 92.5–96.2% identity compared to the original sequence of the virus obtained from the study participants (section 7.5.2). This points to the possibility of zoonotic potential of the cyclovirus VIZIONS-2013, although its ability to infect and pathogenicity remains to be determined and asymptomatic presence or contamination of this novel cyclovirus in both human and animals can not be excluded.

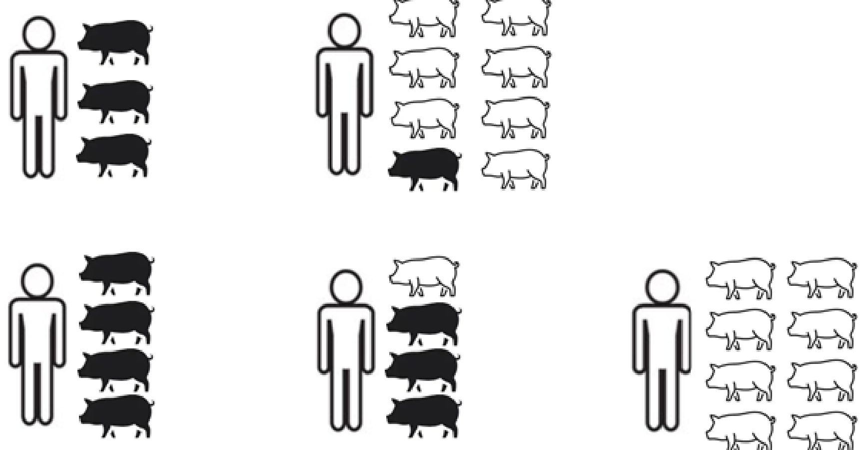


Figure 18. The detections of cyclovirus VIZIONS-2013 in respiratory samples of study participants and their close contact animals (pigs). Black pig icons indicate their nasal-throat swabs are positive with cyclovirus VIZIONS-2013. White human or pig icons indicate their nasal-throat samples are negative with this virus. The virus PCR amplicons (206 bp) detected in the pigs shared 92.5–96.2% identity with each other. The animation icons of human and animals were copied from open-access sources, Shutterstock [251].

Statovirus VIZIONS-2013 (unpublished data)

Sequence of statovirus VIZIONS-2013 was screened in 8 nasal-throat swabs of 8 pigs which a virus-positive participant directly contacted during disease episode. Additionally, the screening was also conducted in 19 nasal-throat swabs of 19 pigs that 4 statovirus VIZIONS-2013-negative participants directly contacted at their disease episodes. No evidence of the virus was found. This suggests a low potential of a zoonotic transmission from pigs through respiratory route of this novel statovirus.

8. CONCLUDING REMARKS

Acute respiratory tract infection is a major health issue worldwide. A majority of all known human diseases and most of diseases associated with recent epidemics or pandemics were of zoonotic origin [219,221]. The ongoing COVID-19 pandemic highlights the importance of gaining insight into exposure to zoonotic sources and active surveillance to characterize viromes, novel viruses and enabling rapid evaluation of zoonotic potentials of emerging viruses to inform policy makers. Additionally, feasible laboratory diagnostic tools, available for rapid detection of emerging viruses are crucial for prompt identification and control of future outbreaks.

This thesis aimed to understand the underlying exposures driving zoonotic emergence, explore virus populations associated with acute respiratory infections in individuals working with animals, as well as to evaluate respiratory virus detection by mNGS and to compare the viral detection of mNGS versus PCR in one of the world's zoonotic emergence hotspots.

The results of animal exposure show that the study participants were frequently exposed to zoonotic sources by occupational activities (as animal raising farmers, animal health workers, slaughter-house workers and rat traders) and non-occupational activities (e.g. farming in backyard, slaughtering/cooking or consuming exotic animals and consumption of raw animal blood and meat). Additionally, no or limited use of PPE was recorded indicating limited knowledge of the risks of zoonotic exposure. The exposure to a large variety of animal species documented may also increase possibilities of zoonotic transmission. This study, therefore, illustrates exposure characteristics to possible zoonotic sources and indicates a high risk of zoonotic infection for the study participants.

The multiplex RT-PCR screening for 15 most common respiratory viruses identified EVs and RV as the predominant viruses throughout the study both at baseline and in disease-episode samples. The findings underline their role in respiratory infections and further expand our knowledge of clinical burden of *Enterovirus* genus beside being the culprit of hand, foot and mouth disease [287,288] and as major causes of central nervous system infections [289,290] resulting in substantial morbidity and mortality worldwide.

Despite a wide panel of respiratory viruses screened by multiplex RT-PCR, the majority of the tested specimens did not provide a viral etiology to the disease episodes. This was in agreement with previous studies [69,70,282–285]. This may be explained by most of the study participants being adults and already acquired substantial immunity to the respiratory viruses during their life, leading to the rapid clearance of the viruses from their respiratory tract, thereby lowering the virus titter and shortening the viral shedding [286,294]. Alternatively, non-viral pathogens may be responsible as bacterial pathogens were not tested.

Besides the common viruses detected by RT-PCR, a variety of viruses including uncommon or novel emerging viruses were additionally detected by mNGS analysis. This thesis therefore expands our understanding of virus populations and provide knowledge about the virome associated with acute respiratory infections in individuals at risk of zoonotic infections. To the best of my knowledge, my research represents one of the first (if not the first) to explore the viral contents in respiratory samples of people in close contact with animals in Vietnam, one of the world's hotspots of zoonotic emerging infections.

The detection of the targeted viruses in 13 of 15 (RT-)PCR-positive samples and a variety of uncommon and novel viruses indicate that the mNGS protocol/pipeline applied here is a highly sensitive pan-virus assay for detection of a variety of viruses including novel ones. The results therefore emphasize the importance of a sequence-independent diagnostic test to identify uncommon or novel emerging viruses. Additionally, by genetic sequences simultaneously obtained in mNGS output, EVs and RV in RT-PCRs were cross-detected and species genotyping was done, highlighting the advantages of mNGS in etiological and epidemiological studies as compared to (RT-)PCR which is sequence-dependent detection. However, more frequent detection of the novel viruses (novel cyclovirus, novel gemycircularvirus and viruses of *Redondoviridae* family) by PCR than by mNGS in the same samples shows that PCR remains the most sensitive diagnostic test for viruses with genomes already known. The main advantages of mNGS is its ability to detect and sequence simultaneously a variety of viral genomes rather than perform an extensive set of different (RT-)PCRs and without requiring to know the virus sequences.

The identification, in this study, of the novel viruses (such as cyclovirus VIZIONS-2013, gemycircularvirus VIZIONS-2013 and statovirus VIZIONS-2013) or recently discovered virus and vientovirus VZ) contributes to a better understanding of the respiratory virome in individuals with high risk of zoonotic infections in the world. Similar virus prevalence between samples collected during disease episodes and at baseline implies that these viruses are probably not highly pathogenic. However, the higher copy numbers of redondoviruses in critically ill patients compared with in healthy individuals has been recently reported [81]. Therefore, copy numbers of cyclovirus VIZIONS-2013, gemycircularvirus VIZIONS-2013, statovirus VIZIONS-2013 and redondoviruses at baseline and disease-episode samples remained to be elucidated and compared. Moreover, sequences related to viruses of the phylum *Cressdnaviricota* are common contaminants of metagenomic reagents [304,305]. Thus, whether these viruses infect human cells, other non-human cells in the lungs, or reflect passive contamination of the respiratory tract will require further studies.

Frequent detections of identical replication-gene sequences of redondoviruses for up to over 3 months in longitudinal samples of the same but not in different participants suggest possible chronic shedding of the viruses in human respiratory tract. An airborne environmental contamination seems unlikely since the closely contacting animals tested PCR negative. Additionally, the viruses were detected in respiratory tract of a majority of the human participants, while no evidence of the virus was neither detected in any animal samples in this study, nor in environmental and animal samples widely analyzed previously [81]. This study therefore provides additional evidence supporting the suggestion[81] that human respiratory tract is the exclusive site of redondoviruses, or its host if not human cells (e.g. redondovirus infected eukaryotic parasites).

No evidence of redondoviruses was found in nasopharynx of the domestic animals, to which the redondovirus-positive participants were directly exposed to. It provides an important evidence, suggesting that these animals are less likely to be the hosts of redondoviruses and possibility evidence of host restriction to humans exclusively of the virus family, although rectal swabs or meat produced from these animals remain to be determined for presence of redondoviruses. However, DNA of all others of CRESS DNA viruses have been widely detected in animal samples. Moreover, a range of viruses, like the case of deltaviruses that human was assumed to be strictly their host before identified in birds, snakes, fish, amphibians, and invertebrates [90,91]. Therefore, it is possible that redondoviruses will be found in animals in the future. Virus surveillance is recommended in various species of animals.

The possibility of redondovirus replication in human cells also remains likely since *Circoviridae* (a virus family of CRESS-DNA viruses) is well-known to infect mammals and redondoviruses were previously suggested to relate to several human disorders. Moreover, phylogenetic analysis shows no geographical clustering between Vietnam and GenBank strains (from the US, Spain and China), indicating wide geographical spread and diversity of this virus family. Collectively, this study provides new insight into this novel virus family, demonstrating high prevalence, wide geographic distribution, the possibility of chronic shedding in the human respiratory tract and no evidence of zoonotic transmission from close animal contacts. However, the tropism and pathogenicity of this virus family remain to be determined.

In contrast with redondoviruses, the DNA of cyclovirus VIZIONS-2013 and gemycircularvirus VIZIONS-2013 was frequently detected in pigs. Notably, the identical 370bp sequence of gemycircularvirus VIZIONS-2013 was found in respiratory samples of human participants and their close porcine contacts, suggesting that a zoonotic transmission was probably detected. However, more evidence is needed to elucidate the possibility of passive contamination of the respiratory tract of both humans and pigs, since the host of cycloviruses and gemycircularviruses have not been determined yet [85,118].

Since we did not recruit human participants without animal exposure as control group, we cannot determine whether the disease episodes, viromes identified and zoonotic potential detected here are unique to individuals working with animals or can be generalized to a wider population. Additionally, raising exotic (or non-domestic local) animals is common and permitted in Vietnam, so we did not distinguish between exotic animals by farming or hunting activities. Moreover, we did not check if PPE was even available at the slaughters for the use, therefore, the data of limited PPE use of the slaughter-house workers can be a bias.

Collectively, these studies illustrate sustained exposure of the participants to zoonotic sources that possibly drive zoonotic emergence. The study participants, slaughter-house workers, animal health workers, livestock-rearing farmers, and rat-traders, are therefore suitable to be used as sentinels for research and surveillance of zoonotic diseases. Additionally, this study expands understanding of virome in acute respiratory infections and zoonotic potential of the viruses in individuals working with animals. Moreover, this study helped to verify that the mNGS protocol/pipeline applied here is sensitive to detect a variety of respiratory viruses, including novel viruses and therefore feasibly support for prompt detection of emerging viruses in future outbreaks. Further research is therefore critical to explore the tropism, pathogenicity and natural hosts of these novel viruses.

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Original Contribution

Occupational Animal Contact in Southern and Central Vietnam

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Abstract: Despite the global zoonotic disease burden, the underlying exposures that drive zoonotic disease emergence are not understood. Here, we aimed to assess exposures to potential sources of zoonotic disease and investigate the demographics, attitudes, and behavior of individuals with sustained occupational animal contact in Vietnam. We recruited 581 animal workers (animal-raising farmers, slaughterers, animal health workers, and rat traders) and their families in southern and central Vietnam into a cohort. Cohort members were followed for 3 years and interviewed annually regarding (1) demography and attitudes regarding zoonotic disease, (2) medical history, (3) specific exposures to potential zoonotic infection sources, and (4) socioeconomic status. Interview information over the 3 years was combined and analyzed as cross-sectional data. Of the 297 cohort members interviewed, the majority (79.8%; 237/297) reported raising livestock; almost all (99.6%; 236/237) reported being routinely exposed to domestic animals, and more than a quarter (28.7%; 68/237) were exposed to exotic animals. Overall, 70% (208/297) reported slaughtering exotic animals; almost all (99.5%; 207/208) reported consuming such animals. The consumption of raw blood and meat was common (24.6%; 73/297 and 37%; 110/297, respectively). Over half (58.6%; 174/297) reported recent occupational animal-induced injuries that caused bleeding; the use of personal protective equipment (PPE) was limited. Our work demonstrates that individuals working with animals in Vietnam are exposed to a wide range of species, and there are limited procedures for reducing potential zoonotic disease exposures. We advocate better education, improved animal security, and enforced legislation of PPE for those with occupational animal exposure in Vietnam.

Keywords: Cohort, Emerging infections, Zoonosis, Exposure risk, Vietnam, Slaughterers, Animal health workers

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Introduction

Zoonoses are infections that can be transmitted from vertebrate animals to humans and vice versa (WHO 2018). Globally, zoonotic infections are responsible for a high disease burden; approximately 60% of all known human diseases and 75% of diseases associated with recent epidemics or pandemics were zoonoses (Woolhouse and Gowtage-Sequeria 2005; Taylor et al. 2009; WHO 2017). Despite the high prevalence of zoonoses, the emergence of zoonotic disease remains difficult to predict and the underlying mechanisms that drive these processes are not well-understood. Studies of zoonotic exposure and hazardous behavior, including the co-sampling of animals, humans, and food products with animal origins, are one approach for better predicting and ultimately intervening in zoonotic disease outbreaks. Contact with infected animals, and/or exposure to contaminated environments, contributes to the emergence and spread of zoonotic diseases in human populations. It is additionally known that increased contact between animals and humans provides more opportunity for exposure to zoonotic pathogens (WHO 2017). Accordingly, the human populations at the highest risk of zoonotic infections are those that have the most frequent interactions with animals. For this reason, slaughterers, animal health workers, animal-raising farmers, and those that trade in wildlife are likely at greater risk of zoonotic infection than those outside of the agricultural industry.

Southeast Asia is considered to be a major hotspot for emerging zoonotic diseases (Morse et al. 2012; Horby et al. 2013). Demography, behavior, attitudes, culture, large animal populations, a high diversity of wild mammalian species, and the coexistence of a broad spectrum of diseases in human and animals are distinctive features of this region, which may lead to the more frequent emergence of zoonotic disease (Morse et al. 2012; Horby et al. 2013; Morand et al. 2014). However, we have a poor understanding of the specific features that lead to zoonotic disease transmission, such as the behavior of those that have sustained contact with animals. Here, we aimed to assess human exposure to animal sources which may be potential reservoirs of zoonotic disease. Additionally, we aimed to investigate the demographics, attitudes, and behavior of assumed high-risk individuals (those with a sustained occupational exposure to animals) living in Vietnam, a country located within the Southeast Asian epicenter of zoonotic diseases. Therefore, we accessed data from a high-risk sentinel cohort (HRSC) study, which was a component of the VIZIONS (Vietnam Initiative on Zoonotic InfectIONS) program (Carrique-Mas et al. 2015; Rabaa et al. 2015) to assess how cohort members interacted with animals and identify potential disease exposure risks.

Methods

Ethics

The ethics boards of Dong Thap Hospital, Dak Lak Hospital, the Sub-Departments of Animal Health in Dong Thap and Dak Lak, and the Hospital of Tropical Diseases in Ho Chi Minh City provided ethical approval for this study. The protocol associated with HRSC study was additionally approved by the Oxford Tropical Research Ethics Committee (OxTREC) (No. 157-12) in the UK.

Study Design

The details of design and implementation of the HRSC study have been described previously (Carrique-Mas et al. 2015). Animal-raising farmers, slaughterers, animal health workers, and rat traders residing in Dong Thap and Dak Lak provinces in the southern and central region of Vietnam, respectively (representing two different geographical and ecological areas), were recruited into the HRSC study (Carrique-Mas et al. 2015; Rabaa et al. 2015). These individuals were broadly representative of people working with animals in rural Vietnamese provinces and were considered to have common occupations associated with continued exposure to animals. Small-scale animal farming is a substantial form of livelihood in these rural provinces and farmers comprise more than two-thirds of the population in the selected areas (GSO—General Statistics Office of Vietnam 2013). Therefore, it was determined that animal-raising farmers should account for approximately two-thirds of the cohort members.

Individuals working in four identified sectors (animal-raising farmers, slaughterers, animal health workers, and rat traders) in selected districts were randomly selected and invited to attend meetings introducing the study. Those with an interest in the study were formally invited to participate; family members of animal farmers were also invited to participate in the study. Animal slaughterers were selected from central slaughter points within each district of the province. Rat traders and animal health workers were

selected by convenience. The HRSC study followed the cohort members for three years, starting in June 2013 in Dong Thap province and February 2014 in Dak Lak province. In total, the HRSC was comprised of 581 individuals, including 131 animal-raising farmers, 284 family members of animal-raising farmers, 100 slaughterers, 61 animal health workers, and 5 rat traders. Only adult cohort members working with animals were interviewed ($n = 297$). All cohort members were interviewed on enrollment (first year) and were approached for additional interviews on subsequent years (second and third years); farming households were an exception, only those responsible for raising animals were interviewed.

Data Collection

The baseline questionnaire used for all participants was comprised of four sections: (1) demography and general information and attitudes regarding animal exposures (2) existing and previous medical history, (3) specific exposures to potential sources of zoonotic infection through primary and secondary occupations, the use of personal protective equipment (PPE) while working with animals, perceived high-risk food-consuming habits, occupational injuries, attitudes to potential exposure risks, and (4) socioeconomic status. The interview data from the first, second, and third year were combined and analyzed as cross-sectional data, resulting in exposure outcomes in at least one of the three interviews.

Data Preparation and Analysis

Data were prepared by Microsoft Excel (version 2013) and analyzed using STATA statistical software version 12.0. Pearson's Chi-squared test or Fisher's exact test was used for pairwise comparisons of categorical variables; the latter when there was a small sample size (< 5) in any of the cells in the contingency table. The Bonferroni method was used for error correction of multiple comparisons (Armstrong 2014). McNemar's test was used to evaluate the consistency of exposures to animals for the cohort members over the study period. 95% confidence intervals for the percentages/proportions were calculated by the Wilson method (Brown et al. 2001); $P \leq 0.05$ was considered significant. Members of the cohort that were rat traders ($n = 5$) were excluded from analyses of association, difference, or consistency due to an insufficient sample size.

Results

Demographic Characteristics

Over the three-year study period, approximately half (51.1%; 297/581) of the cohort members in the two provinces were interviewed on at least one occasion (Table 1). Responses were recorded from 31.6% (131/415) of the animal-raising farmers (one representative on each farm was interviewed) and from all cohort members with other occupations (slaughterers; $n = 100$, animal health workers; $n = 61$, and rat traders; $n = 5$). The median age of those interviewed was 43 years, with an age range of 16–73 years. The majority (53.6%; 156/291; no data from six individuals) of participants had a medium level of education (defined as middle/high-school level), 24.4% (71/291) had a low education level (none/primary level), and 22% (64/291) had a high education level (defined as post-high-school level). 15% (43/291) of participants suffered from at least one underlying chronic disease, including heart disease, diabetes, kidney disease, liver disease, malignancy, lung disease, alcoholism, chronic stomach pain, gastrointestinal disease, and sinusitis.

Exposure to Live Animals

Of the 297 cohort members interviewed on at least one occasion, the majority (79.8%; 237/297) reported raising livestock either in their backyard, in the area surrounding their household, or on adjoining farmland (Table 2). 63.9% (95% CI 56.3–70.8%; 106/166) of the interviewed non-professional farmers also reported raising animals (i.e., small-scale backyard farming) (Table 2). Almost all (99.6%; 236/237) of the cohort members reported being routinely exposed to domestic animals, and over a quarter (28.7%; 68/237) were exposed to exotic (non-domestic) animals. The most common exotic animal exposures were wild pigs (61.8%; 42/68), wild birds (30.9%; 21/68), deer (20.6; 14/68), and porcupines (16.2%; 11/68). Dogs (85.2%; 201/236), chickens (79.2%; 187/236), pigs (53.8%; 127/236), and cats (53.8%; 127/236) were the most common domestic animals that cohort members were exposed to.

Table 1. General characteristics of animal exposures in the cohort members.

		Province			Occupation				Sex			Age group				
	All	Dong Thap	Dak Lak	*P* value	Farmer	Animal health worker	Slaughterer	*P* value	Female	Male	*P* value	≤ 15	16–44	45–59	≥ 60	*P* value
Study population	581	282	299		415	61	100		259	322		59	308	168	46	
Number interviewed[a]	297	135	162	0.128	131	61	100	< 0.001	93	204	< 0.001	0	163	114	20	0.003
Raising live animals at households or on farms	237 (80)	127 (94)	110 (68)	< 0.001	131 (100)	39 (64)	63 (63)	< 0.001	71 (76)	166 (81)	0.317	0	123 (75)	96 (84)	18 (90)	0.342
Exotic animals	68 (23)	7 (5)	61 (38)	< 0.001	62 (47)	2 (3)	4 (4)	< 0.001	23 (25)	45 (22)	0.611	0	23 (14)	33 (29)	10 (50)	< 0.001
Domestic animals	236 (79)	110 (81)	126 (78)	0.431	130 (99)	39 (64)	63 (63)	< 0.001	71 (76)	165 (81)	0.369	0	123 (75)	95 (83)	18 (90)	0.447
Bleeding injuries while working with animals	174 (59)	70 (52)	104 (64)	0.031	50 (38)	35 (57)	85 (85)	< 0.001	51 (55)	123 (60)	0.376	0	106 (65)	62 (54)	6 (30)	0.018
Slaughtering/cooking and consuming exotic animals	208 (70)	58 (43)	150 (93)	< 0.001	90 (69)	42 (69)	71 (71)	1	66 (71)	142 (70)	0.812	0	117 (72)	79 (69)	12 (60)	1
Slaughtering/cooking	65 (22)	22 (16)	43 (27)	0.033	40 (31)	10 (16)	11 (11)	0.003	16 (17)	49 (24)	0.188	0	36 (22)	23 (20)	6 (30)	1
Consuming	207 (70)	57 (42)	150 (93)	< 0.001	90 (69)	42 (69)	70 (70)	1	67 (72)	140 (69)	0.553	0	115 (71)	79 (69)	12 (60)	1
Consumed raw blood or meat	139 (47)	56 (41)	83 (51)	0.093	64 (49)	31 (51)	39 (39)	0.678	22 (24)	117 (57)	< 0.001	0	87 (53)	41 (36)	11 (55)	0.039
Raw blood	73 (25)	7 (5)	66 (41)	< 0.001	32 (24)	16 (26)	25 (25)	1	8 (9)	65 (32)	< 0.001	0	44 (27)	24 (21)	5 (25)	1
Raw meat	110 (37)	53 (39)	57 (35)	0.469	51 (39)	23 (38)	31 (31)	1	16 (17)	94 (46)	< 0.001	0	69 (42)	31 (27)	10 (50)	0.051

The values are shown in format of number (percentage). The denominator for the percentages is the value on the top row.

[a]Cohort members interviewed at least once among three baseline interviews (n = 297), including at enrollment (first year, 2013–2014, n = 291), second (2014–2015, n = 273) and third year (2015–2016, n = 265) in the high-risk sentinel cohort (HRSC) study (n = 581), cohort members interviewed on all three occasion (n = 252).

Table 2. Exposure to live animals in the cohort.

	Occupational exposures (n = 131)	Non-occupational exposures (n = 166)			Total	95% CI
	Farmers	Animal health workers	Slaughterers	Rat traders		
Interviewed cohort members[a] N	131	61	100	5	297	
Raising reported[d]	131 (100.0)	39 (63.9)	63 (63.0)	4 (80.0)	237 (79.8)	74.9–84.0
Raising of exotic animals	62 (47.3)	2 (5.1)	4 (6.3)		68 (28.7)	23.3–34.8
Wild pig	37 (28.2)	2 (5.1)	3 (4.8)		42 (61.8)	49.9–72.4
Other wild birds[b]	17 (13.0)	2 (5.1)	2 (3.2)		21 (30.9)	21.2–42.6
Deer	14 (10.7)				14 (20.6)	12.7–31.6
Porcupine	11 (8.4)				11 (16.2)	9.3–26.7
Jungle fowl	5 (3.8)				5 (7.4)	3.2–16.1
Monkey	2 (1.5)				2 (2.9)	0.8–10.1
Civet	2 (1.5)				2 (2.9)	0.8–10.1
Bamboo rat[c]	1 (0.8)	1 (2.6)			2 (2.9)	0.8–10.1
Bat	1 (0.8)				1 (1.5)	0.3–7.9
Pheasant	1 (0.8)				1 (1.5)	0.3–7.9
Raising of domestic animals[e]	130 (99.2)	39 (100.0)	63 (100.0)	4 (100.0)	236 (99.6)	97.7–99.9
Dog	118 (90.8)	35 (89.7)	44 (69.8)	4 (100.0)	201 (85.2)	80.1–89.1
Chicken	128 (98.5)	26 (66.7)	31 (49.2)	2 (50.0)	187 (79.2)	73.6–83.9
Pig	87 (66.9)	18 (46.2)	22 (34.9)		127 (53.8)	47.4–60.1
Cat	83 (63.8)	18 (46.2)	23 (36.5)	3 (75.0)	127 (53.8)	47.4–60.1
Duck	51 (39.2)	4 (10.3)	14 (22.2)	2 (50.0)	71 (30.1)	24.6–36.2
Muscovy duck	51 (39.2)	3 (7.7)			54 (22.9)	18.0–28.7
Pigeon	25 (19.2)	5 (12.8)	3 (4.8)		33 (14.0)	10.1–19.0
Cattle	15 (11.5)	5 (12.8)	10 (15.9)		30 (12.7)	9.1–17.6
Goose	21 (16.2)	1 (2.6)	3 (4.8)		25 (10.6)	7.3–15.2
Goat	12 (9.2)	2 (5.1)	2 (3.2)		16 (6.8)	4.2–10.7
Rabbit	15 (11.5)				15 (6.4)	3.9–10.2
Buffalo	1 (0.8)		5 (7.9)		6 (2.5)	1.2–5.4
Quail	2 (1.5)				2 (0.8)	0.2–3.0
Turkey	2 (1.5)				2 (0.8)	0.2–3.0

[a]The cohort members interviewed at least once among three baseline interviews, including at enrollment (first year), second and third years. The values are shown in format of number (percentage: 95% CI). The empty cells equal to "0".
[b]Other wild birds than pheasants.
[c]*Rhizomys sumatrensis.*
[d]Denominators of analyses of raising of any exotic animals groups.
[e]Denominators of corresponding subgroups analyses.

There was a significant difference in exposure to exotic animals by occupation, age group, level of education, and area of residence. Notably, farmers (45.8%; 60/131) were significantly more commonly exposed to exotic animals than slaughterers (4%; 4/100) and animal health workers (3.3%; 2/61) ($P < 0.001$) (Table 1). Similarly, cohort members in the 60 + (50%; 10/20) and 45–59 year age groups (29%; 33/114) were more regularly exposed to exotic animals than those in the 16–44 year age group (14.1%; 23/163) ($P < 0.001$). Cohort members in Dak Lak were significantly more exposed to exotic animals than cohort members in Dong Thap; 37.7% (61/162) and 5.2% (7/135) ($P < 0.001$), respectively.

Animal Exposure in Animal Health Workers and Slaughterers

Animal slaughterers (n = 100) and animal health workers (n = 61) were exposed to 17 different animal species; the most common were chickens, ducks, Muscovy ducks, pigs, and cattle (Table 3). We observed a significant difference in animal exposure by area of residence. Animal health workers residing in Dak Lak were more frequently exposed to beef cattle than those in residing in Dong Thap (P = 0.024). Alternatively, animal health workers in Dong Thap were more commonly exposed to Muscovy ducks than animal health workers in Dak Lak (P = 0.013). Furthermore, slaughterers in Dak Lak were more commonly exposed to beef cattle, buffaloes, and geese than those residing in Dong Thap (P = 0.001, 0.014, and 0.001, respectively) (Table 3).

Slaughtering, Cooking and Consuming Exotic Animals

Of all the interviewed cohort members, 70% (208/297) reported slaughtering, cooking, or consuming exotic animals within the year prior to interview (Table 1). The majority (99.5%; 207/208) reported consuming such animals, and 31.3% (65/208) reported slaughtering or cooking these animals. The most common exotic animals that the cohort members were exposed to through slaughtering, cooking, or consuming were wild pigs, deer, and porcupines (Table 4). These animals were generally raised in their backyards, the area surrounding their own household, or on specific wildlife farms (Table 2). Porcupines, civets, deer, jungle fowl, squirrels, and pangolins were slaughtered/cooked in Dak Lak only. Correspondingly, only participants in Dak Lak reported consuming civets, squirrels, jungle fowl, pangolins, and wild rabbits (Table 4). Cohort members in Dak Lak were significantly more likely to slaughter, cook, and consume exotic animals than those in Dong Thap (26.5% (43/162) vs. 16.3% (22/135); and 92.6% (151/163) vs. 41.5% (56/135), respectively; P = 0.033 and $P < 0.001$, respectively) (Table 1).

Consuming of Raw Animal Blood or Raw Meat

Raw animal blood is commonly consumed in Vietnam as a dish named "tiet canh." Almost a quarter (24.6%; 73/297) of interviewed cohort members reported the consumption of raw blood, and over a third (37%; 110/297) had consumed raw mammal or bird meat within the year prior to interview (Table 5). Cohort members typically reported consuming raw blood 1–3 times per year (72.6%; 53/73), and over a half (53.6%; 59/110) reported eating raw meat $\geq$ 4 times per year. The most commonly consumed animal blood was (sequentially) from pigs (40.1%; 61/152), ducks (38.2%; 58/152), rabbits (7.2%; 11/152), Muscovy ducks (5.9%; 9/152), goats (3.9%; 6/152), deer (1.3%; 2/152), and beef cattle (1.3%; 1/152). The most commonly consumed raw meat was from beef cattle (91%; 142/156); the consumption of raw meat from pigs (5.1%; 8/156), goats, rabbits, chickens, and quails were less common (< 2% each, data not shown). Cohort members in Dak Lak reported consuming raw blood more commonly than those in Dong Thap (40.7% (66/162) vs. 5.2% (7/135), respectively, $P < 0.001$) (Table 1). Men were more likely to consume raw blood (31.9% (65/204) or raw meat (46.1%; 94/204) than women (8.6% (8/93), $P < 0.001$ and 17.2% (16/93), $P < 0.001$, respectively). The majority (61.6%; 45/73) of raw-blood consumers considered this activity to be not good for their health, the remainder thought it was healthy, were not sure, or had no opinion (38.3%; 28/73) (Table 5). Regarding the consumption of raw animal meat, 33.6% (37/110) of consumers acknowledged that it was probably not good for their health, while the majority (51.8%; 57/110) thought that eating raw meat was good for their health.

Bleeding and Biting Injuries

Over half (58.6%; 174/297) of the 297 interviewed cohort members reported recent occupational injuries that induced bleeding while working with the animals (Table 6). The majority of these cohort members (70.1%; 122/174) reported being injured 1–3 times per year; more than a quarter (29.9%; 52/174) reported being injured $\geq$ 4 times a year. Cohort members were most frequently bitten by pigs, chicken, ducks, beef cattle, buffalo, wild pigs, dogs, and rats. Other bleeding injuries induced by working with the animals were associated with knives, needles, and skin abrasions. Overall, cohort members in Dak Lak (64.2%; 104/162) were injured more frequently than those in Dong Thap (51.9%; 70/135) (P = 0.031). Slaughterers (85%; 85/100) experienced significantly more bleeding injuries than animal health workers (57.4%; 35/61) and farmers (38.2%; 50/131) ($P < 0.001$). Members in the 60 + year age group (30%; 6/20) were less regularly injured than those in the 45–59 (54.4%; 62/114) and 16–44 year age groups (65%; 106/163) (P = 0.018) (Table 1).

Table 3. Animals exposures in slaughterers and animal health workers.

	Slaughterers			Animal health workers		
	Dong Thap	Dak Lak	Total	Dong Thap	Dak Lak	Total
Interviewed cohort members	33	67	100	30	31	61
Reported exposure, *N*	33	67	100	30	31	61
Chicken	18 (54.5)	35 (52.2)	53 (53.0: 43.3–62.5)	29 (96.7)	31 (100.0)	60 (98.4: 91.3–99.7)
Duck	18 (54.5)	35 (52.2)	53 (53.0: 43.3–62.5)	29 (96.7)	26 (83.9)	55 (90.2: 80.2–95.4) ($P = 0.013$)
Muscovy duck	18 (54.5)	30 (44.8)	48 (48.0: 38.5–57.7)	22 (73.3)	13 (41.9)	35 (57.4: 44.9–69.0)
Pig	15 (45.5)	31 (46.3)	46 (46.0: 36.6–55.7)	29 (96.7)	31 (100.0)	60 (98.4: 91.3–99.7)
Cattle		16 (23.9)	16 (16.0: 10.1–24.4) ($P = 0.001$)	25 (83.3)	31 (100.0)	56 (91.8: 82.2–96.5) ($P = 0.024$)
Geese		16 (23.9)	16 (16.0: 10.1–24.4) ($P = 0.001$)	8 (26.7)	5 (16.1)	13 (21.3: 12.9–33.1)
Buffalo		11 (16.4)	11 (11.0: 6.3–18.6) ($P = 0.014$)	6 (20.0)	11 (35.5)	17 (27.9: 18.2–40.2)
Rabbit		8 (11.9)	8 (8.0: 4.1–15.0)			
Pigeon		2 (3.0)	2 (2.0: 0.6–7.0)			
Cat		1 (1.5)	1 (1.0: 0.2–5.5)	8 (26.7)	1 (3.2)	9 (14.8: 8.0–25.7)
Rice field rat	1 (3.0)		1 (1.0: 0.2–5.5)			
Dog		1 (1.5)	1 (1.0: 0.2–5.5)	21 (70.0)	10 (32.3)	31 (50.8: 38.6–62.9)
Goat					8 (25.8)	8 (13.1: 6.8–23.8)
Porcupine				1 (3.3)	2 (6.5)	3 (4.9: 1.7–13.5)
Wild pig				1 (3.3)	1 (3.2)	2 (3.3: 0.9–11.2)
Monkey				1 (3.3)		1 (1.6: 0.3–8.7)
Other wild bird				1 (3.3)		1 (1.6: 0.3–8.7)

The values are shown in format of number (percentage: 95% CI). Empty cells equal to "0." Statistically significant differences between variables at 5% level are shown.

The Use of Personal Protective Equipment

Over two-thirds (69/100) of slaughterers at abattoirs reported never using PPE, and only one worker reported using full PPE. When used, gloves were the most common piece of PPE, followed by boots, face masks, and aprons. In contrast, < 5% of slaughters reported using a mob caps/hats or goggles (Table 7). We found that animal slaughterers in Dong Thap reported not using PPE (93.9%; 31/33) more commonly than animal slaughterers in Dak Lak (56.7%; 38/67) ($P < 0.001$) (Table 7). There was a significant association between those reported being bitten by animals and those using PPE; slaughters not using any PPE were bitten to the point of bleeding (13.04%; 9/69) more commonly than those reporting the use any piece of PPE (0%; 0/69) ($P = 0.054$).

Despite most cohort members reporting direct contact with animals on a daily basis, one-fifth (20.3%; 59/291) reported doing nothing, did not answer, or did not know what to do when bitten. Almost a quarter (22%; 64/291) reported using no gloves, facemasks, or protective clothing when routinely touching animals. Additionally, over two-thirds of members (68.7%; 200/291) thought, or did not know, that they could not get an infection from having contact with apparently healthy animals. Over a quarter (28.2%; 82/291) of the cohort members thought they could not contract an infection through direct contact with diseased animals.

Exposure Consistency Over the Study Period

Over a half (51.1%; 297/581) of the cohort members were interviewed at least once, and 84.8% (252/297) of the cohort members were interviewed on all three occasions; 89.2% (265/297) members were interviewed at year three. The reporting of direct animal exposures reported was

Table 4. Exposure to exotic animals by slaughtering, cooking, or consuming.

		Farmers	Animal health workers	Slaughterers	Rat traders	Total
Interviewed cohort members, N		131	61	100	5	297
Reported exposure[a]		90 (68.7)	42 (68.9)	71 (71.0)	5 (100.0)	208 (70.0: 64.6–75)
Slaughtering and cooking	All exposed[b]	39 (43.3)	10 (23.8)	11 (15.5)	5 (100.0)	65 (31.3: 25.3–37.8)
	Wild pig	25 (64.1)	6 (60.0)	3 (27.3)		34 (52.3: 40.4–64.0)
	Rice field rat	4 (10.3)	5 (50.0)	7 (63.6)	5 (100.0)	21 (32.3: 22.2–44.4)
	Porcupine	8 (20.5)	1 (10.0)			9 (13.8: 7.5–24.3)
	Civet	2 (5.1)	2 (20.0)			4 (6.2: 2.4–14.8)
	Bamboo rat	1 (2.6)	1 (10.0)	1 (9.1)	1 (20.0)	4 (6.2: 2.4–14.8)
	Deer	2 (5.1)	2 (20.0)			4 (6.2: 2.4–14.8)
	Jungle fowl	3 (7.7)				3 (4.6: 1.6–12.7)
	Squirrel	2 (5.1)				2 (3.1: 0.9–10.5)
	Pangolin	1 (2.6)				1 (1.5: 0.3–8.2)
	Other wild bird	1 (2.6)				1 (1.5: 0.3–8.2)
Consuming	All exposed[c]	90 (100.0)	42 (100.0)	70 (98.6)	5 (100.0)	207 (99.5: 97.3–99.9)
	Wild pig	65 (72.2)	31 (73.8)	56 (80.0)		174 (73.4: 78.5–88.4)
	Deer	38 (42.2)	16 (38.1)	18 (25.7)		72 (34.8: 28.6–41.5)
	Porcupine	31 (34.4)	17 (40.5)	16 (22.9)		64 (30.9: 25.0–37.5)
	Rice field rat	25 (27.8)	15 (35.7)	12 (17.1)	4 (80.0)	56 (27.1: 21.5–33.5)
	Civet	9 (10.0)	7 (16.7)	4 (5.7)		20 (9.7:6.3–14.5)
	Bamboo rat	4 (4.4)	4 (9.5)	2 (2.9)	1 (20.0)	11 (5.3: 3.0–9.3)
	Jungle fowl	6 (6.7)	1 (2.4)	1 (1.4)		8 (3.9: 2.0–7.4)
	Squirrel	2 92.2)	2 (4.8)			4 (1.9: 0.8–4.9)
	Other wild bird	3 (3.3)	1 (2.4)			4 (1.9: 0.8–4.9)
	Pangolin	1 (1.1)		1 (1.4)		2 (1.0: 0.3–3.5)
	Bat	2 (2.2)				2 (1.0: 0.3–3.5)
	Monkey	2 (2.2)				2 (1.0: 0.3–3.5)
	Wild rabbit		1 (2.4)			1 (0.5: 0.1–2.7)

At least once among three baseline interviews, including at enrollment (first year), second and third years. The values are shown in format of number (percentage: 95% CI). The empty cells equal to "0".

[a]Denominators of analyses of "All exposed" groups.

[b]Denominators of analyses of "Slaughtering and cooking" groups.

[c]Denominators of analyses of "Consuming" groups.

consistent over the three-year study period ($P > 0.05$). However, the consumption of raw animal blood declined significantly between year one and year two versus year three (17.9% (45/251) vs. 8.1% (20/231), $P = 0.0005$), and (13.8% (35/254) vs. 7.9% (20/254), $P = 0.01$, respectively). The same trend between year one and year three was observed for raw-meat consumption (22.3% (58/260) vs. 16.2% (42/260), $P = 0.048$). Additionally, slaughters reported using PPE more commonly in the first year than the third year (57.3% (51/89) vs. 32.6% (29/89), $P = 0.0001$). Overall, cohort members reported getting bitten or other animal-induced injuries significantly less in year two and three than year one (bitten: 3.3% (9/269) vs. 17.1% (46/269), $P < 0.0001$ and 3.1% (8/261) vs. 17.2% (45/261), $P < 0.0001$, respectively) (other injuries: 20.9% (56/268) vs. 38.1% (102/268), $P < 0.0001$ and 18.8 (49/260) vs. 38.1% (99/260), $P < 0.0001$, respectively).

Discussion

Our results indicate that, besides their own occupational exposures, cohort members in the selected locations were regularly exposed to a large variety of differing animals. Farmers were the most commonly interviewed group;

Table 5. Raw-blood and raw-meat consumption.

	Farmer	Animal health worker	Slaughterer	Rat trader	Total
Interviewed cohort members, *N*	131	61	100	5	297
Reported consumption	64 (48.9)	31 (50.8)	62 (62.0)	5 (100.0)	162 (54.5: 48.9–60.1)
Raw-blood consumption					
None	99 (75.6)	45 (73.8)	75 (75.0)	5 (100.0)	224 (75.4: 70.2–80.0)
Yes[a]	32 (24.4)	16 (26.2)	25 (25.0)		73 (24.6: 20.0–29.8)
1–3 times	21 (65.6)	15 (93.8)	17 (68.0)		53 (72.6: 61.4–81.5)
$\geq$ 4 times	11 (34.4)	1 (6.3)	8 (32.0)		20 (27.4: 18.5–38.6)
Opinion about raw-blood consumption					
Good	4 (12.5)	1 (6.3)	8 (32.0)		13 (17.8: 10.7–28.1)
Not good	23 (71.9)	12 (75.0)	10 (40.0)		45 (61.6: 50.2–72.0)
No opinion or not sure	5 (15.6)	3 (18.8)	7 (28.0)		15 (20.5: 12.9–31.2)
Raw-meat consumption					
None	80 (61.1)	38 (62.3)	69 (69.0)		187 (63.0: 57.3–68.3)
Yes[b]	51 (38.9)	23 (37.7)	31 (31.0)	5 (100.0)	110 (37.0: 31.7–42.7)
1–3 times	25 (49.0)	9 (39.1)	13 (41.9)	4 (80.0)	51 (46.4: 37.3–55.7)
$\geq$ 4 times	26 (51.0)	14 (60.9)	18 (58.1)	1 (20.0)	59 (53.6: 44.4–62.7)
Opinion about raw-meat consumption					
Good	28 (54.9)	10 (43.5)	18 (58.1)	1 (20.0)	57 (51.8: 42.6–60.9)
Not good	18 (35.3)	10 (53.5)	9 (29.0)		37 (33.6: 25.5–42.9)
No opinion or not sure	5 (9.8)	3 (13.0)	4 (12.9)	4 (80.0)	16 (14.5: 9.2–22.3)

At least once among three baseline interviews, including at enrollment (first year), second and third years, and their opinions about the consumption. The values are shown in format of number (percentage: 95% CI). The empty cells equal to "0".

[a]Denominators of analyses of "raw-blood consumption" frequency (1–3 times and $\geq$ 4 times) and "opinions about raw-blood consumption" opinions (Good, Not good or No opinion or not sure).

[b]Denominators of analyses of "raw-meat consumption" frequency (1–3 times and $\geq$ 4 times) and "opinions about raw-meat consumption" opinions (good, not good, or no opinion or not sure).

Table 6. Bleeding and biting injuries when working with animals.

		Farmers	Animal health workers	Slaughters	Rat traders	Total
Interviewed cohort members, *N*		131	61	100	5	297
Reported injuries*		50 (38.2)	35 (57.5)	85 (85.0)	4 (80.0)	174 (58.6: 52.9–64.0)
Bleeding injuries	Bitten	22 (44.0)	21 (60.0)	9 (10.6)	3 (75.0)	55 (31.6: 25.2–38.9)
	Other injuries	36 (72.0)	29 (82.9)	85 (100.0)	4 (100.0)	154 (88.5: 82.9–92.4)
	1–3 times	41 (82.0)	30 (85.7)	51 (60.0)		122 (70.1: 62.9–76.4)
	$\geq$ 4 times	9 (18.0)	5 (14.3)	34 (40.0)	4 (100.0)	52 (29.9: 23.6–37.1)

At least once among three baseline interviews, including at enrollment (first year), second and third years. The values are shown in format of number (percentage: 95% CI). The empty cells equal to "0".

[a]Denominators of subsequent analyses of corresponding variables/groups.

therefore, we unsurprisingly found that the principal animal exposures in this population came from raising livestock. More than three quarters of all interviewed participants (animal-raising farmers, slaughterers, animal health workers, and rat traders) and over two-thirds of the interviewed subjects, with the exception of animal-raising farmers, reported raising exotic or domestic animals in their backyard or around the family household. Exposure to exotic animals was greater in Dak Lak province than in Dong Thap province, which largely reflects the distinct

Table 7. The use of personal protective equipment (PPE) at abattoirs.

	Dong Thap	Dak Lak	Total
Interviewed cohort members N	33	67	100
No usage of any piece of PPE	31 (93.9)	38 (56.7)	69.0: 59.4–77.2) ($P < 0.001$)
Full PPE		1 (1.5)	1 (1.0: 0.2–5.5)
Gloves	5 (15.2)	59 (88.1)	64 (64.0: 54.2–73.7)
Boots	5 (15.2)	54 (80.6)	59 (59.0: 49.2–68.1)
Face mask	6 (18.2)	50 (74.6)	56 (56.0: 46.2–65.3)
Apron		22 (31.8)	22 (22.0: 15.0–31.1)
Hat/mob cap		4 (6.0)	4 (4.0: 1.6–9.8)
Goggles		2 (3.0)	2 (2.0: 0.6–7.0)

At least once among three baseline interviews, including at enrollment (first year), second and third years. Each separate piece of PPE indicates that at least this PPE was used. The values are shown in format of number (percentage: 95% CI). The empty cells equal to "0." The statistically significant differences between variables at 5% level are shown.

profiles of the two locations. Dong Thap is closer to Ho Chi Minh City; therefore, there is a greater demand for non-exotic meat. In contrast, Dak Lak is more remote and caters more for the local rural population.

Beside routine occupational exposures to animals, cohort subjects had contact with a large variety of animal types, including fifteen types of exotic and domestic animals. Moreover, the animal species that the cohort members were most exposed to (wild pigs, porcupines, rice field rats, deer, pigs, chickens, dogs, cats, ducks, and cattle) are known to be potential reservoirs for zoonotic pathogens (Hart and Trees 1997; Acha and Szyfres 2003; Meng et al. 2009; Kreuder Johnson et al. 2015). These interactions with a range of animals are related to the fact that more than two-thirds of those in the cohort practiced backyard farming. This proportion was higher than the average within the Vietnamese population; approximately 50% of all households in Vietnam are estimated to farm animals (GSO—General Statistics Office of Vietnam 2013), and small-scale animal production is particularly common in rural Vietnam. This industry is maintained by the higher prices (often more than double) demanded for "home reared" animal meat in comparison to animals raised in industrial facilities. Additionally, rural Vietnamese people like to support their community and purchase local produce. A large range of activates in this industry is highlighted by the fact that the majority of farming families reported raising several animal species in small numbers in or around their households (Phuong et al. 2015). These data suggest a low level of biosecurity with the potential for the mixing of multiple animal species; we speculate that this increases the risk of pathogen transfer between animals and may lead to a greater likelihood for exposure to zoonotic diseases in those farming animals. The presence of a large variety of animal types in small areas, particularly in single households farming a mixture of animal species, may increase opportunity for species-crossing pathogen transmission events, as illustrated by the emergence of avian influenza H5N1 virus in Vietnam in 2003, with the first reported case coming from Dong Thap.

We found that safety procedures for those handling live animals or involved in slaughtering or butchering were inadequate. Notably, a low proportion of cohort members reported not using any PPE when handling animals. These data indicate a pervasive poor understanding of occupational exposure that may result in increased potential for zoonotic disease transmission if animals are infected with zoonotic pathogens. This lack of PPE usage was specifically common among the animal slaughterers, where contact with fresh blood is a sustained occupational hazard. The risk of such activities is highlighted by recent reports of the transmission of *Trypanosoma evansi* and rabies in central and northern Vietnam, which likely occurred during the butchering of raw meat, and processing of the animal brain or via contact with animal saliva, respectively (Wertheim et al. 2009b; Chau et al. 2016). A lack of PPE was also significantly associated with being bitten by animals; while this is not surprising, the proportion of other animal-induced injuries also corresponded with low PPE usage. A study conducted by Rui Carlos and co-workers (Schneider

et al. 2013) found that a lack of PPE usage in Brazil was associated with the transmission of *Brucella abortus* from animals to slaughterers at slaughterhouses. Therefore, this lack of PPE usage may indicate a higher exposure risk for zoonotic infections among cohort subjects. This observation is particularly concerning, and we advocate better education for PPE use in animal worker and slaughters. We suggest that these workers are a key population for exposure to common zoonotic pathogens, such as *Brucella,* which has been recently found endemic in Vietnam and poses a major risk to human health (Campbell et al. 2017). Additional follow-up studies encompassing serological testing are required to test this hypothesis.

In Vietnam, animal-product consumers enjoy many foods that would be considered atypical in the west; exotic animals are a particular delicacy. Almost all of the interviewed cohort members had consumed at least one of thirteen different types of exotic animal over the three-year study period. The thought is that the consumption of exotic animals has preventative health benefits and/or a positive medical treatment effect. For example, the consumption of porcupine stomach is believed to cure stomach pain, and porcupine bile is used as an analgesic. Furthermore, grated deer horn is thought to treat a multitude of chronic diseases and prolong life. A general increase in income across the population and permission from the Vietnamese government for wildlife farming in recent years (Ministry of Agriculture and Rural Development—Vietnam) have played a major role in the rise of exotic animal consumption. The overlap between the types of animals raised, slaughtered, cooked, and consumed by the cohort subjects indicates that increasing consumption requires more intensive farming, slaughtering, cooking, and the hunting exotic animals to supply this increasing demand. As the current economic trajectory is predicted to continue to improve across Vietnam (The World Bank in Vietnam 2017), it is probable that the consumption of exotic animals will increase. This increase in the rearing intensity of exotic animals may have a possible knock-on effect for disease exposures within the general population.

An additional specific practice in Vietnam that may increase the risk of zoonotic exposures is consumption of raw animal blood or meat. More than a half of the cohort members reported the consumption of raw animal blood or meat over the study period. This proportion was high, especially considering that sale of raw blood dishes was banned by the Vietnamese government in 2009 (The Agriculture Ministry of Vietnam). The consumption of raw animal blood is found to have a higher possibility of infections from blood-borne zoonotic pathogens such as *Streptococcus suis* (Wertheim et al. 2009a; Navacharoen et al. 2009; Trung et al. 2011), *Trichinella spiralis* (Taylor et al. 2009; De et al. 2015), and *Brucella* spp. (Njeru et al. 2016). Counter-intuitively, we also found that the majority of the raw-blood consumers considered that eating raw blood was not good for their health. Raw-blood consumption in Vietnam is largely cultural and is a common dish at special gatherings or celebrations in several geographic areas. Raw animal blood is also thought to have potential health benefits, such as boosting the immune system, reducing body temperature, preventing anemia, and as treating headaches, coughs, and dysentery (Thi et al. 2014). These social factors show that culture strongly drives consumer tastes, and restricting the population eating raw animal blood is more of a social issue than a scientific one.

An analysis of demography in the cohort members further demonstrated potential risks of zoonotic disease exposure. Approximately a quarter of the subjects had a low education level, which has been previously associated with a higher incidence of numerous communicable diseases (Zimmerman and Woolf 2014; Bruce et al. 2019). These data, together with a high exposure to potential sources of zoonotic disease, indicate a possible elevated risk of zoonotic infections in the cohort subjects. We also identified significant differences between exposures to potential zoonotic sources by location, sex, age group, education level, and profession; high frequency of contact with animals is associated with a likelihood of increased zoonotic transmission (Howard and Fletcher 2012; WHO 2017). Therefore, it is important to elucidate the demographic/social factors that drive zoonotic infections to induce feasible interventions and to determine whether consistency or variation in exposure over time results elevated or reduced risk. To achieve this, it will be essential to further gather information on the incidence of zoonotic disease in those that work with animals and also measure the exposure to given pathogens.

Our study contains limitations. This was a cohort consisting of individuals with a perceived risk of zoonotic disease, and control subjects without animal exposures were not recruited. Additionally, we did not distinguish between those farming or hunting exotic animals, as raising many types of exotic animals is common and permitted in Vietnam. Furthermore, we did not evaluate disease episodes with a suitable control group (those not working with animals) or measure serological exposure; therefore, we

cannot estimate whether those working with animals do have an increased incidence of infectious disease from an animal origin.

Despite the apparent limitations, our study illustrates that, in addition to occupational exposures, individuals that work with animals in Vietnam are frequently exposed to a range of animal species which is likely to increase their risk of zoonotic disease exposures. Sustained animal exposure and a large variety of animal species demonstrate that slaughterers, animal health workers, animal-raising farmers, and rat traders are sentinel professions for performing zoonotic disease surveillance. Additionally, the attitudes and behavior of the cohort members show that they have limited knowledge of potentially zoonotic disease exposures. The data presented here, in combination with further sero-epidemiological and molecular studies, will aid in elucidating the potential factors associated with a comparatively high incidence of emerging zoonotic disease in Southeast Asia. Ultimately, our findings will be useful for better preparedness, intervention plans, disease prediction models, and the development of future research into zoonotic infections in Southeast Asia.

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RESEARCH ARTICLE

Respiratory viruses in individuals with a high frequency of animal exposure in southern and highland Vietnam

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Abstract

Active surveillance for zoonotic respiratory viruses is essential to inform the development of appropriate interventions and outbreak responses. Here we target individuals with a high frequency of animal exposure in Vietnam. Three-year community-based surveillance was conducted in Vietnam during 2013-2016. We enrolled a total of 581 individuals (animal-raising farmers, slaughterers, animal-health workers, and rat traders), and utilized reverse transcription-polymerase chain reaction to detect 15 common respiratory viruses in pooled nasal-throat swabs collected at baseline or acute respiratory disease episodes. A respiratory virus was detected in 7.9% (58 of 732) of baseline samples, and 17.7% (136 of 770) of disease episode samples ($P < .001$), with enteroviruses (EVs), rhinoviruses and influenza A virus being the predominant viruses detected. There were temporal and spatial fluctuations in the frequencies of the detected viruses over the study period, for example, EVs and influenza A viruses were more often detected during rainy seasons. We reported the detection of common respiratory viruses in individuals with a high frequency of animal exposure in Vietnam, an emerging infectious disease hotspot. The results show the value of baseline/control sampling in delineating the causative relationships and have revealed important insights into the ecological aspects of EVs, rhinoviruses and influenza A and their contributions to the burden posed by respiratory infections in Vietnam.

KEYWORDS
asymptomatic, cohort study, viral etiology, respiratory disease, Vietnam, zoonoses

1 | INTRODUCTION

Annually, acute respiratory tract infections are responsible for more than 3 million deaths worldwide.[1] In Vietnam, a developing country in Southeast Asia, mortality attributed to acute respiratory infections accounted for half of that attributed to the other infectious diseases combined in 2016.[1]

Viruses are regarded as the most common causes of acute respiratory diseases, and some emerging respiratory diseases as the Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS), both related to coronaviruses (CoVs), are listed in the WHO's List of Blueprint priority diseases[2] because of their pandemic potential. While the reported patterns of the etiological agents vary between geographic locations and age groups, generally, respiratory syncytial virus (RSV)-A, RSVB, influenza A virus, influenza B virus, adenovirus (ADV), enterovirus (EVs); human metapneumovirus (MPV), human rhinovirus (HRV), parainfluenza virus

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(PIV)1-4, human CoV (including subtypes OC43 and NL63), human bocavirus (BoV) and parechovirus (PEV) are the most common viruses detected in respiratory samples worldwide.[3-9] Of these, influenza A virus, influenza B virus, and CoV have been reported as the most common viruses detected in people over 5 years old,[10-13] while RSV and PIVs have been regarded as the leading causes of respiratory infections in children under 5 years old in South East Asia.[3,14,15]

Zoonotic infections are of global concern, and approximately 60% of known infectious diseases in humans are of zoonotic origin.[16] In addition, Southeast Asia, including Vietnam, is one of the hotspots of emerging infectious diseases. Indeed, many of the recent respiratory outbreaks are linked with zoonotic viruses as SARS-CoV,[17] avian influenza A virus H5N1,[18] pandemic influenza A virus subtype H1N1,[19] and more recently MERS-CoV,[20] with the majority being first reported in Asia. Collectively, active surveillance for (novel) zoonotic viruses in this vulnerable part of the world is of both medical and public health significance. As such, for the detection of novel zoonotic viruses in humans and animals, during 2012-2015 the Vietnam Initiative on Zoonotic InfectiONS (VIZIONS) project, consisting of the various hospital- and community-based studies, was conducted across Vietnam.[21,22] Herein, we focus our analysis on a community-based study, which was designed to capture the cross-species transmission events of zoonotic viruses among individuals with a high risk of zoonotic infections in southern and highland Vietnam. In this study, our aim was to describe the frequency of common respiratory viruses in clinical samples collected from these individuals, later called cohort members, at baseline and when a respiratory disease episode was reported during the study period.

2 | METHODS

2.1 | Study design and inclusion criteria

This study was a part of the High-Risk Sentinel Cohort (HRSC) study which was a community-based component of the VIZIONS project.[21] The HRSC study was first commenced in June 2013 in Dong Thap and then in February 2014 in Dak Lak. These are provinces located in southern and highland of Vietnam, respectively, representing two different geographic areas in Vietnam.

Animal-raising farmers, animal health workers, and slaughterers were eligible to be enrolled in the study since these are common occupations in rural Vietnam with frequent occupational exposure to animals. Rat traders in Dong Thap were additionally recruited due to the commonality of this occupation in this locality. The animal-raising farmers accounted for about two-third of the population with occupational exposure to animals in these study provinces.

On the basis of the animal farm census, letters were sent out to invite potential participants to attend an introductory meeting. The consent forms were then obtained from those who were willing to join the HRSC study. For each farmer household, up to four members having the highest frequency of working with animals were recruited. The slaughterers were recruited from all local central abattoirs or slaughter points. The animal-health workers and rat traders were selected by convenience. Consequently, a total of 581 individuals (median age in year, 38; range, 2-89), including 415 (71.4%) animal-raising farmers, 100 (11.7%) slaughterers, 61 (10.5%) animal-health workers, and 5 (1.8%) rat-traders, were recruited. Each cohort members were followed up annually for up 3 years since recruitment.

2.2 | Data collection

Annually, to establish the baseline data (ie, no disease episode reported), the cohort members were interviewed, and clinical specimens, including rectal, pooled nasal, and throat swabs and blood were also collected from each interviewee. These baseline data were collected from all cohort members, except for the farmers, for which only one person mostly working with animals per household was interviewed and sampled.

During the study period, whenever getting illness (diarrhea and respiratory infection) defined as any signs/symptoms of respiratory tract infections (eg, sneezing, coughing or sore throat), plus fever (≥38°C), the cohort members informed the local study teams. Within 48 hours, the site study doctors made a visit to the participant houses and collected information about animal exposures, associated symptoms, and medication. In addition, clinical specimens, including blood, and (when relevant) rectal- or pooled nasal and throat swabs were collected. All the specimens were stored at −80°C until analysis. Here, we focused on respiratory episodes. As such, only pooled nasal-throat swabs of each individual were analyzed.

2.3 | Respiratory virus detections by real-time polymerase chain reaction analysis

To detect common respiratory viruses in pooled nasal and throat swabs, we first isolated total nucleic acid (NA) from patient samples using MagNA Pure 96 platform (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. The NA output was then eluted in 50 μL of elution buffer and immediately screened for respiratory viruses using multiplex real-time polymerase chain reaction (RT-PCR) assays.

The RT-PCR assays used in the present study were derived from previous publications,[23-26] which captured 15 common respiratory viruses and a wide range of their subtypes, including RSVA, RSVB; influenza A virus, influenza B virus, ADV; EVs; MPV; HRV; PIV-1, PIV-2, PIV-3, PIV4; CoV subtype OC43 and NL63; BoV and PEV.[23-25] Influenza A virus-positive samples were further tested for (zoonotic) subtypes, including H3, H1N1pdm09, H1, and avian/H5 [25,26] (primer and probe sequences are listed in Table S2). All the RT-PCR reactions

were carried in a LightCycler 480 Instrument II (96-wells) (Roche Molecular Systems, Inc).

2.4 | Data analysis

The data were analyzed by STATA software, version 12.0.[27] The pairwise comparisons of categorical variables were calculated by Pearson's χ^2 test (or Fisher exact test when the sample size was less than five in any of the cells of a contingency table) or two-sample *t* test with equal variances. The errors of multiple comparisons were corrected by the Bonferroni method.[28] $P \leq .05$ was considered the significance. EpiTools[29] were used to calculate 95% confidence intervals for the odds ratio. The rat traders (n = 5) were excluded from these tests because of an insufficient sample size.

2.5 | Ethics

The HRSC study was approved by the Oxford Tropical Research Ethics Committee (OxTREC) in the United Kingdom, and by the Ethics Committees of Dong Thap Hospital, Dak Lak Hospital, the sub-Departments of Animal Health in Dong Thap and Dak Lak, and the Hospital of Tropical Diseases in Ho Chi Minh City in Vietnam. Written informed consent was obtained from each study participant.

3 | RESULTS

3.1 | Collection of respiratory swabs and reports of disease episodes

The detailed characteristics of the cohort members are briefly summarized in Table 1. Approximately half (51.1%; 297 of 581) of the study population was annually interviewed during 2013-2015, corresponding to a total of 829 interviews conducted (291, 273, and 265 interviews in 2013, 2014, and 2015, respectively) (Table 1). Consequently, 732 pooled nasal-throat swabs were collected at these annual interviews for respiratory virus detection. Herein, these samples were considered as baseline samples.

Over the 3-year period, 66.4% (386 of 581) of the cohort members reported having respiratory infections, corresponding to a total of 812 respiratory episodes (Table 1), or an average of 2.1 episodes per reporting individual, and 1.4 (812/581) episodes per individual among all cohort members. The slaughterers (225/100) were more likely to have respiratory diseases than the animal-health workers (92/61) and the farmers (491/415) ($P < .003$). In total, of the 812 reported respiratory episodes, 770 pooled nasal-throat swabs were collected for respiratory virus detection.

TABLE 1 Baseline characteristics

	All	Dak Lak	Dong Thap	*P* value[a]
Occupation	N = 581	N = 299	N = 282	.012
Farmers, n (%)	415 (71)	201 (67)	214 (76)	**.021**
Animal-health workers, n (%)	61 (10)	31 (10)	30 (11)	.915
Slaughterers, n (%)	100 (17)	67 (22)	33 (12)	**.001**
Rat traders, n (%)	5 (1)	0	5 (2)	
Median age (range), y	38 (2-89)	39 (2-89)	38 (4-76)	.995[b]
Age groups				
≤15, n (%)	59 (10)	24 (8)	35 (12)	.080
≥16, n (%)	522 (90)	275 (92)	247 (88)	
Sex ratio (male/female)	1.2 (322/259)	1.1 (157/142)	1.4 (165/117)	.146
No. of cohort members interviewed annually for baseline[c]	N = 297	N = 162	N = 135	
1st year, n (%)	291 (98)	162 (100)	129 (96)	**.042**
2nd year, n (%)	273 (92)	150 (93)	123 (91)	.114
3rd year, n (%)	265 (89)	147 (91)	118 (87)	.077
No. of cohort members reporting respiratory illness	N = 386	N = 219	N = 167	
1st year, n (%)	227 (59)	154 (70)	73 (44)	**<.001**
2nd year, n (%)	193 (50)	109 (50)	84 (50)	.088
3rd year, n (%)	151 (39)	67 (31)	84 (50)	**.043**
No. of reported respiratory episodes[d]	N = 812	N = 394	N = 418	
1st year, n (%)	317 (39)	183 (46)	134 (32)	**.017**
2nd year, n (%)	317 (39)	129 (33)	188 (45)	**.001**
3rd year, n (%)	178 (22)	82 (21)	96 (23)	.758

[a]*P* value (Pearson's χ^2 or Fisher exact test) of the difference between Dak Lak and Dong Thap.
[b]*t Test.*
[c]At these follow-up time points, a respiratory sample was collected from each individual.
[d]A total of 770 samples were collected and included in polymerase chain reaction analysis, with 314, 281, and 175 samples in first, second, and third years, respectively.

TABLE 2 Number (percentage) of cohort members with detected viruses from tested pooled nasal and throat swabs

	Whole study				1st year				2nd year				3rd year			
	Baseline (N = 732)	Disease episode (N = 770)	*P* value	OR (95% CI)	Baseline (N = 290)	Disease episode (N = 314)	*P* value	OR (95% CI)	Baseline (N = 240)	Disease episode (N = 281)	*P* value	OR (95% CI)	Baseline (N = 202)	Disease episode (N = 175)	*P* value	OR (95% CI)
EVs	29 (4.0)	67 (8.7)	<.001	2.3 (1.5-3.6)	2 (0.7)	12 (3.8)	.013	5.7 (1.3-26)	10 (4.2)	40 (14.2)	<.001	3.8 (1.8-7.8)	17 (8.4)	15 (8.6)	.96	1 (0.5-2.1)
HRV	5 (0.7)	32 (4.2)	<.001	6.3 (2.4-16)	2 (0.7)	21 (6.7)	<.001	10.3 (2.4-44)	1 (0.4)	9 (3.2)	.024	7.9 (1-63)	2 (1.0)	2 (1.1)	1	1.2 (0.2-8.3)
Influenza A virus	14 (1.9)	18 (2.3)	.568	1.2 (0.6-2.5)	4 (1.4)	4 (1.3)	1	1 (0.2-3.7)	6 (2.5)	11 (3.9)	.365	1.6 (0.6-4.4)	4 (2.0)	3 (1.7)	1	0.8 (0.2-3.9)
H3	3 (21.4)	12 (66.7)	.016	7.3 (1.5-36)	0	1 (0.3)	1	NA	0	10 (3.6)	.002	NA	3 (1.5)	1 (0.6)	.63	0.4 (0-3.7)
H1-seasonal	0	0	NA	NA	0	0	NA	NA	0	0	NA	NA	0	0	NA	NA
H1-pan09	0	0	NA	NA	0	0	NA	NA	0	0	NA	NA	0	0	NA	NA
H5	0	0	NA	NA	0	0	NA	NA	0	0	NA	NA	0	0	NA	NA
ADV	1 (0.1)	9 (1.2)	.021	8.6 (1.1-68)	1 (0.3)	4 (1.3)	.375	3.7 (0.4-33)	0	2 (0.7)	.502	4.3 (0.2-90)	0	3 (1.7)	.1	8.2 (0.4-160)
CoV[a]	8 (1.1)	7 (0.9)	.72	0.8 (0.3-2.3)	1 (0.3)	4 (1.3)	.375	3.7 (0.4-33)	6 (2.5)	3 (1.1)	.314	3.2 (1.3-8)	1 (0.5)	0	1	NA
RSVA	0	3 (0.4)	.25	NA	0	3 (1.0)	.250	NA	0	0	NA	NA	0	0	NA	NA
MPV	0	2 (0.3)	.5	NA	0	1 (0.3)	1	NA	0	1 (0.4)	1	NA	0	0	NA	NA
RSVB	1 (0.1)	2 (0.3)	1	1.9 (0.2-21)	1 (0.3)	0	1	0.3 (0-7.5)	0	2 (0.7)	.502	NA	0	0	NA	NA
Influenza B virus	0	2 (0.3)	0.5	NA	0	0	NA	NA	0	0	NA	NA	0	2 (1.1)	.22	NA
PIV4	0	1 (0.1)	1	NA	0	0	NA	NA	0	1 (0.4)	1	NA	0	0	NA	NA
BoV	2 (0.3)	1 (0.1)	.615	0.5 (0-5.3)	1 (0.3)	0	.48	NA	0	1 (0.4)	1	NA	1 (0.5)	0	1	NA
(+) ≥1 virus	58 (7.9)	136 (17.7)	<.001	2.5 (1.8-3.5)	12 (4.1)	45 (14.3)	<.001	3.9 (2-7.5)	22 (9.2)	67 (23.8)	<.001	3.1 (1.8-5.2)	24 (12)	24 (13.7)	.64	1.2 (0.6-2.1)
(+) ≥2 viruses[b]	2 (0.3)	7 (0.9)	.18	3.3 (0.7-16)	0	2 (0.6)	.5	4.6 (0.2-97)	1 (0.4)	4 (1.4)	.38	3.4 (0.4-31)	1 (0.5)	1 (0.6)	1	1.2 (0-18.6)

Note: The OR was the disease episodes vs baseline. *P* values were calculated by Pearson's χ^2 or Fisher exact test.

Abbreviations: ADV, adenovirus; BoV, bocavirus; CI, confidence interval; CoV, coronavirus; EV, enterovirus; HRV, human rhinovirus; OR, odds ratio; PEV, parechovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

[a]Subtype OC43 and NL63.

[b]Including one EVs-BoV and one influenza A virus-CoV in the baseline, and one AdV-influenza B virus, one BoV-influenza A virus, two EVs-influenza A virus, one EVs-RSVB, one EVs-HRV and one EVs-AdV-CoV in the disease episodes PIV-1, -2, -3, and PEV were not detected in all samples.

3.2 | Frequency of respiratory viruses detected at baseline and the disease episodes

Evidence of a respiratory virus by RT-PCR analysis was documented in 7.9% (58 of 732) of samples collected at the baseline, and 17.7% (136 of 770) of samples collected when a respiratory disease episode was reported ($P < .001$) (Table 2). In addition, mixed infections were recorded in 2 (0.3%) and 7 (0.9%) samples collected at baseline and disease episodes, respectively (Table 2).

Of the detected viruses, EVs, HRV and influenza A virus were the most common viruses detected in samples collected at both baseline and disease episodes, followed by ADV and CoV (Table 2). There were significant differences in the frequencies of EVs, HRV and ADV detected in the two groups; 29 of 732 (4%) at baseline vs 67 of 770 (8.7%) at disease episodes ($P < .001$) for EVs, 5 of 732 (0.7%) vs 32 of 770 (4.2% ($P < .001$) for HRV, and 1 of 732 (0.1%) vs 9 of 770 (1.2%; ($P = .021$) for ADV (Table 2). In addition, of the influenza A virus RT-PCR positive cases, subtype H3 was detected at a higher frequency at disease episodes than at baseline, 66.7% (12 of 15) vs 21.4% (3 of 14), $P = .016$. Remaining influenza A virus-positive cases were RT-PCR negative for specific RT-PCR for the other tested subtypes (H1N1pdm09, H1N1, and H5) (Table 2).

3.3 | Clinical signs/symptoms of cohort members in acute respiratory diseases with the detected viruses

For the altogether 770 reported respiratory episodes, cough and sneezing were the most common symptoms recorded, present in 76% (585 of 770) and 74.7% (575 of 770) of cases, respectively, followed by sore throat (65.3%; 503 of 770), headache (51.4%; 396 of 770), body aches (41.8%; 322 of 770), and dyspnea (7.4%; 57 of 770) (Table 3). In addition, gastrointestinal symptoms were recorded in 7.3% (56 of 770), but watery diarrhea was more often recorded in cohort members without a virus detected than in those with a positive finding, 52 of 634 (8.2%) vs 4 of 136 (2.9%), $P = .029$ (Table 3).

Of the virus-positive cases, watery diarrhea was only recorded in those positive for EVs and HRV, whilst sore throat was predominantly recorded in those positive for influenza A virus. Otherwise, there were considerable similarities in age and clinical presentations of cohort-member groups who were positive for different viruses (Table 3).

3.4 | The frequency of respiratory viruses detected by provinces

To assess the differences in the frequencies of respiratory viruses under investigation between Dong Thap and Dak Lak, which represent the two distinct geographic localities in Vietnam, we stratified the data for these two individual provinces (Table 4). Subsequently, EVs, HRV and influenza A virus remained the leading viruses detected in the tested samples from these provinces, while the detection rates of EVs and HRV in disease episode samples collected in Dong Thap were significantly higher than that in Dak Lak (11.1% [42 of 379] vs 6.4% [25 of 391]; $P = .021$, and 6.1% [23 of 379] vs 2.3% [9 of 391], $P = .009$, respectively). In Dong Thap, EVs and HRV were significantly more often detected in samples collected at disease episode than at baseline; $P < .001$ for both EVs and HRV. In Dak Lak, no significant differences were found (Table 4).

3.5 | Temporal and seasonal differences in the frequency of detection of respiratory viruses

There were some fluctuations in the detection of the most common viruses (especially EVs and HRV; Table 2) over the study period. Of particular note was the significant increase in the frequency of EVs from baseline to disease episodes in the first 2 years (from 0.7% at baseline to 3.8% at disease episodes in the first year, and from 4.2% to 14.2% in the second year, respectively) (Table 2). In year 3, the detection of EVs remained high but was comparable in samples collected at baseline (8.4%, 17 of 202) and disease episodes (8.6%, 15 of 175) (Table 2). In contrast to EVs, there was a downward trend of HRV detection over time, while the frequency of influenza A virus was relatively stable over the 3-year period (Table 2).

In terms of seasonality, overall, there were some clear trends in the seasonality of the most common viruses (especially EVs and influenza A virus, Figure 1). More specifically, EVs and influenza A virus were significantly more often found in rainy season (May-October) than in dry season (November-April); the detection rates were 12.2% (43 of 353) vs 5.8% (24 of 417) ($P = .002$) for EVs, and 3.7% (13 of 353) vs 1.2% (5 of 417), $P = .023$ for influenza A virus, respectively. In contrast to EVs and influenza A virus, ADV was more often found in the dry season than in the rainy season (1.9%, 8 of 417 vs 0.3%, 1 of 353, $P = .044$) (Figure 1).

3.6 | Animal exposure

Overall, the cohort members were exposed to a wide range of animals, including 11 types of exotic animals and 11 types of domestic animals, within ≤1 month prior to the disease episode (n = 770) (Table S1). There was no difference in the patterns of animal exposure among cohort members who were positive for the predominant viruses (EVs, HRV, and influenza A virus). The numbers of the remaining viruses were insufficient to informatively assess their associations with age, seasonality, clinical presentation, and animal exposure.

TABLE 3 Number (and percentage) for demographics and clinical characteristics of cohort members reporting respiratory episodes

	Tested samples (N = 770)	Positive samples (N = 136)	Negative samples (N = 634)	*P* value	OR (95% CI)	EVs (N = 67)	HRV (N = 32)	Influenza A virus (N = 18)	ADV (N = 9)	CoV[a] (N = 7)
Age ≤ 15	60 (7.8)	15 (11)	45 (7.1)	.121	1.6 (0.9-3)	8 (11.9)	3 (9.4)	2 (11.1)	2 (22.2)	2 (28.6)
Fever	770 (100)	136 (100)	634 (100)	NA	NA	67 (100)	32 (100)	18 (100)	9 (100)	7 (100)
Cough	585 (76.0)	102 (75.0)	483 (76.2)	.770	0.9 (0.6-1.4)	49 (73)	23 (72)	15 (83)	8 (89)	6 (86)
Sneezing	575 (74.7)	105 (77.2)	470 (74.1)	.455	1.2 (0.8-1.8)	50 (75)	25 (78)	15 (83)	6 (67)	7 (100)
Sore throat	503 (65.3)	90 (66.2)	413 (65.1)	.818	1.1 (0.7-1.6)	46 (69)	16 (50)	15 (83)	4 (44)	5 (71)
Dyspnea	57 (7.4)	8 (5.9)	49 (7.7)	.456	0.8 (0.3-1.6)	4 (6)	2 (6)	0	0	0
Headache	396 (51.4)	70 (51.5)	326 (51.4)	.991	1 (0.7-1.5)	30 (45)	20 (63)	11 (61)	3 (33)	3 (43)
Body aches	322 (41.8)	48 (35.2)	274 (43.2)	.089	0.7 (0.5-1.1)	21 (31)	11 (34)	9 (33)	0	2 (29)
Watery diarrhea	56 (7.3)	4 (2.9)	52 (8.2)	.029	0.3 (0.1-1)	2 (3)	2 (6)	0	0	0
Nausea	55 (7.1)	8 (5.9)	47 (7.4)	.529	0.8 (0.4-1.7)	3 (4)	1 (3)	2 (11)	1 (11)	1 (14)
Antibiotic use[b,c]	199 (25.8)	37 (27.2)	162 (25.6)	.689	1.1 (0.7-1.7)	18 (26.9)	5 (15.6)	4 (22.2)	1 (11.1)	2 (28.6)

Note: Four patients have no data on gender and age. *P* values were calculated by Pearson's χ^2 or Fisher exact test. The difference in each viral infection inducing each clinical symptom was not significant ($P > .05$). The OR was the disease "positive samples" vs "negative samples".

Abbreviations: ADV, adenovirus; CI, confidence interval; CoV, coronavirus; EV, enterovirus; HRV, human rhinovirus; OR, odds ratio.

[a]Subtype OC43 and NL63.

[b]The antibiotic use of the patients from the first symptoms to the incidence interview/sampling.

[c]Antibiotic types used Cephalosporin, Amoxicillin, Clarithromycin, Ampicillin, Augmentin, Azithromycin, Chloramphenicol, Ciprofloxacin, Erythromycin, Ofloxacin, Spiramycin.

TABLE 4 Number (percentage) of cohort members with different detected viruses at baseline and disease episodes in Dak Lak and Dong Thap

	Dak Lak				Dong Thap				Baseline (Dak Lak vs Dong Thap)		Disease episodes (Dak Lak vs Dong Thap)	
	Baseline (N = 434)	Disease episode (N = 391)	*P* value	OR (95% CI)[a]	Baseline (N = 298)	Disease episode (N = 379)	*P* value	OR (95% CI)[a]	*P* value	OR (95% CI)[b]	*P* value	OR (95% CI)[b]
EVs	18 (4.1)	25 (6.4)	.147	1.6 (0.9-2.9)	11 (3.7)	42 (11.1)	<.001	3.3 (1.6-6.4)	0.756	1.1 (0.5-2.4)	.021	0.6 (0.3-0.9)
HRV	4 (0.9)	9 (2.3)	.112	2.5 (0.8-8.3)	1 (0.3)	23 (6.1)	<.001	19 (2.6-143)	0.653	2.8 (0.3-25)	.009	0.4 (0.2-0.8)
Influenza A virus	6 (1.4)	9 (2.3)	.324	1.7 (0.6-4.8)	8 (2.7)	9 (2.4)	.798	0.9 (0.3-2.3)	0.206	0.5 (0.2-1.5)	.947	1 (0.4-2.5)
H3	0	4 (44.4)	.103	NA	3 (37.5)	8 (88.9)	.050	13.3 (1.1-166)	0.067	0.1 (0-1.9)	.256	0.5 (0.1-1.6)
H1-seasonal	0	0	NA	NA	0	0	NA	NA	NA	NA	NA	NA
H1-pan09	0	0	NA	NA	0	0	NA	NA	NA	NA	NA	NA
H5	0	0	NA	NA	0	0	NA	NA	NA	NA	NA	NA
ADV	1 (0.2)	5 (1.3)	.107	5.6 (0.7-48)	0	4 (1.1)	.135	NA	1	2.1 (0.1-50)	1	1.2 (0.3-4.6)
CoV[c]	4 (0.9)	3 (0.8)	1	0.8 (0.2-3.7)	4 (1.3)	4 (1.1)	.736	0.8 (0.2-3.2)	0.722	0.7 (0.2-2.8)	.721	0.7 (0.2-3.3)
RSVA	0	0	NA	NA	0	3 (0.8)	.26	NA	NA	NA	.119	0.1 (0-2.7)
MPV	0	0	NA	NA	0	2 (0.5)	.506	NA	NA	NA	.242	0.2 (0-4)
RSVB	1 (0.2)	0	1	NA	0	2 (0.5)	.506	NA	1	2.1 (0.1-50)	.242	0.2 (0-4)
Influenza B virus	0	0	NA	NA	0	2 (0.5)	.506	NA	NA	NA	.242	0.2 (0-4)
PIV4	0	0	NA	NA	0	1 (0.3)	1	NA	NA	NA	.492	0.3 (0-8)
BoV	1 (0.2)	0	1	NA	1 (0.3)	1 (0.3)	1	0.8 (0.1-12)	1	0.7 (0-11)	.492	0.3 (0-8)
(+) ≥1 virus	33 (7.6)	48 (12.3)	.024	1.7 (1.1-2.7)	25 (8.4)	88 (23.2)	<.001	3.3 (2.1-5.3)	0.699	0.9 (0.5-1.5)	<.001	0.5 (0.3-0.7)
(+) ≥2 viruses[d]	2 (0.5)	2 (0.5)	.917	1.1 (0.2-7.9)	0	5 (1.3)	.071	NA	0.517	3.4 (0.2-72)	.28	0.4 (0.1-2)

Note: Other viral pathogens were not showed as they were detected in less than 10 samples. *P* values were calculated by Pearson's χ^2 or Fisher exact test

Abbreviations: ADV, adenovirus; CI, confidence interval; CoV, coronavirus; EV, enterovirus; HRV, human rhinovirus; OR, odds ratio.

[a]The OR was the disease episodes vs. baseline.

[b]The OR was Dak Lak and Dong Thap.

[c]Subtype OC43 and NL63.

[d]Including one EVs-BoV and one influenza A virus-CoV in the baseline, and one AdV-influenza B virus, one BoV-influenza A virus, two EVs-influenza A virus, one EVs-RSVB, one EVs-HRV, and one EVs-AdV-CoV in the disease episodes.

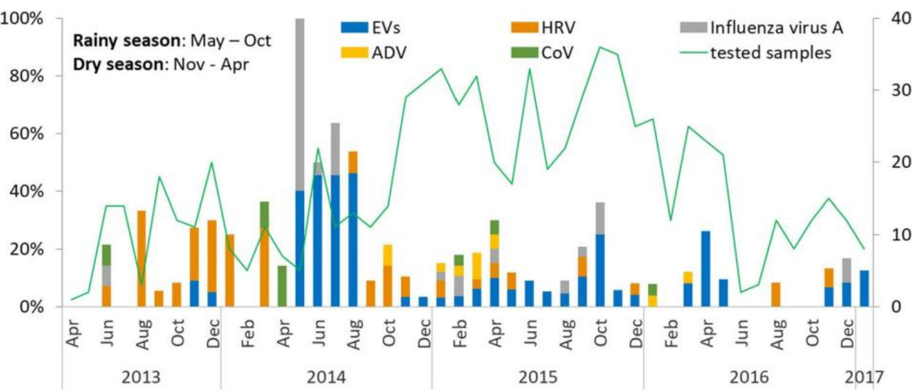

FIGURE 1 The seasonal distribution of symptomatic EVs-, HRV-, influenza A virus-, ADV-, and CoV (subtype OC43 and NL63)-infected cases detected by RT-PCR assay. The bars show the proportion of the viruses detected among total samples tested (the line chart) each month. EVs and influenza A virus were more likely detected in the rainy season than in the dry season ($P = .002$ and $P = .023$, respectively), while the ADV detections were more frequent in the dry season as compared with the rainy one ($P = .044$). There was no significant difference in the detections of HRV and CoV (subtype OC43 and NL63) between dry and rainy seasons ($P = .333$ and .227, respectively). ADV, adenovirus; CoV, coronavirus; EV, enterovirus; HRV, human rhinovirus; RT-PCR, real-time polymerase chain reaction

4 | DISCUSSION

Here we describe the frequency of common human viruses causing acute respiratory infections in people with high exposure to animals in Dong Thap (Southern) and Dak Lak (Highland) provinces. We showed that EVs, HRV and influenza A virus were the predominant viruses detected in respiratory samples of the cohort members in both localities and that their detection rates were significantly higher in respiratory samples collected at respiratory disease episodes than in those collected at baseline. In addition, the results have also revealed important insights into the ecological characteristics of these predominant viruses. More specifically, our analysis shows that EVs and influenza A virus were more often found in the rainy season (from May to October), and there were fluctuations in the detection of EVs and HRV over time, while influenza A virus activity was relatively stable over the study period, suggesting that these viruses may have interacted with the immune landscape of the study population.

Although viral detection in upper respiratory samples like pooled nasal and throat swabs may merely reflect the carriage of such viruses in these body cavities, a higher detection frequency in samples collected at disease episodes than at baseline suggests an association between the detected viruses and the reported respiratory episodes. As such, the high frequency of EVs and HRV detected in samples collected at disease episodes in the present study further expand our knowledge about the clinical burden posed by viruses of the genus *Enterovirus* in Vietnam. Indeed, an outbreak of enterovirus associated diseases like hand foot and mouth disease (HFMD) have been frequently reported in Vietnam and Asia since 1997.[30,31] Likewise, enteroviruses have been reported to be one of the leading causes of central nervous system infections and respiratory illness in Vietnam.[5,32,33] In addition, in line with the observed cyclical epidemic patterns of HFMD in Vietnam and Asia,[30,31] for which the underlying mechanism remains unknown, the fluctuations in the detection EVs and HRV over the study period and between Dong Thap and Dak Lak suggest an interplay between the pathogens and the proportion of susceptible individuals in respective provinces.

The higher detection of influenza A virus subtype H3 in samples collected at the disease episodes than in those collected at baseline points to the association between subtype H3 with respiratory illness in Vietnam. In contrast to the prevalence of influenza A virus subtype H3, the result showing an overall comparable prevalence of influenza A virus in both sample groups suggests that there is a high level of asymptomatic infection of influenza A virus in the general population, in agreement with previous reports.[34,35] The difference in sensitivities between RT-PCR assays used may explain our failure to identify the specific influenza A virus subtypes in the remaining pan-influenza A virus RT-PCR positive samples.

The low prevalence or absence of respiratory viruses like PIVs, PEV, RSVA, and RSVB in the present study may be attributed to the age structure of the present cohort. Indeed, while, these viral species are well-established agents of (respiratory) infections in children, and to some extent in elderly people (eg, in case of PIVs),[3,10,14,15,36-38] over 92% of the respiratory disease episodes reported in this study were among cohort members aging ≥16 years. In terms of seasonal distribution of the predominant viruses as EVs, influenza A virus, HRV and ADV, our report supports previous findings.[39-43]

Our overall RT-PCR yield of 17.7% of viral agents in respiratory samples of the cohort members with the majority age from 16 years or above is in agreement with the diagnostic yields of previous studies.[44-49] The results suggest that it is probably because adults have acquired substantial immunity during their life, leading to the rapid clearance of the infecting viruses from their respiratory tract, thereby shortening the duration of viral shedding.

Our study has some limitations. First, no human subjects without animal exposure were recruited as controls. Therefore, we were unable to assess the effect (if any) of animal exposure on the

frequency of the respiratory disease incidence, as well as the observed viral patterns. Second, despite a holistic effort, nonviral agents as bacterial pathogens were not tested. Third, a slight decrease in sensitivity of the multiplex RT-PCR platforms used in the present study as compared with that of the corresponding monoplex RT-PCRs have previously been reported,[23] which may in part explain the absence of respiratory viruses in some of the tested samples. Collectively, future studies should explore if unbiased pan-pathogen assays, namely metagenomic next-generation sequencing-based approach could improve the etiological detection in patients presenting with respiratory infection; the usefulness of this approach has already been shown for other diseases worldwide, especially in low- and middle-income countries like Vietnam.[50]

5 | CONCLUSION

We reported the detection of common respiratory viruses in individuals with a high frequency of animal exposure in two distinct geographic regions in Vietnam, representing one of the broad-range, prospective and controlled screenings for viral etiologies of respiratory illnesses in people with unique animal contacts in a setting where zoonotic emerging infections are likely to occur. The results show the value of baseline/control sampling in analyzing causative relationships and have revealed important insights into the ecological aspects of EVs, HRV and influenza A and their contributions to the burden posed by respiratory infections in Vietnam.

THE VIZIONS CONSORTIUM MEMBERS

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Publication 3

Article

The Virome of Acute Respiratory Diseases in Individuals at Risk of Zoonotic Infections

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Abstract: The ongoing coronavirus disease 2019 (COVID-19) pandemic emphasizes the need to actively study the virome of unexplained respiratory diseases. We performed viral metagenomic next-generation sequencing (mNGS) analysis of 91 nasal-throat swabs from individuals working with animals and with acute respiratory diseases. Fifteen virus RT-PCR-positive samples were included as controls, while the other 76 samples were RT-PCR negative for a wide panel of respiratory pathogens. Eukaryotic viruses detected by mNGS were then screened by PCR (using primers based on mNGS-derived contigs) in all samples to compare viral detection by mNGS versus PCR and assess the utility of mNGS in routine diagnostics. mNGS identified expected human rhinoviruses, enteroviruses, influenza A virus, coronavirus OC43, and respiratory syncytial virus (RSV) A in 13 of 15 (86.7%) positive control samples. Additionally, rotavirus, torque teno virus, human papillomavirus, human betaherpesvirus 7, cyclovirus, vientovirus, gemycircularvirus, and statovirus were identified through mNGS. Notably, complete genomes of novel cyclovirus, gemycircularvirus, and statovirus were genetically characterized. Using PCR screening, the novel cyclovirus was additionally detected in 5 and the novel gemycircularvirus in 12 of the remaining samples included for mNGS analysis. Our studies therefore provide pioneering data of the virome of acute-respiratory diseases from individuals at risk of zoonotic infections. The mNGS protocol/pipeline applied here is sensitive for the detection of a variety of viruses, including novel ones. More frequent detections of the novel viruses by PCR than by mNGS on the same samples suggests that PCR remains the most sensitive

diagnostic test for viruses whose genomes are known. The detection of novel viruses expands our understanding of the respiratory virome of animal-exposed humans and warrant further studies.

Keywords: virome; acute respiratory disease; NGS; metagenomics; zoonoses; novel cyclovirus; novel statovirus; novel gemycircularvirus

1. Introduction

Acute respiratory infections are the leading cause of morbidity and mortality worldwide [1,2]. Despite intensive laboratory investigations, a substantial proportion of acute respiratory infections are of unknown etiology, resulting in difficulties in clinical management [3–6]. Metagenomics is an unbiased (independent of specific sequences) approach increasingly being applied in virus discovery as well as for molecular diagnostics [6–8].

Viruses are the main causes of acute respiratory infections with the potential to cause pandemics [9–12]. Notably, most emerging viral agents of acute respiratory diseases are of zoonotic origin and pose a major threat to human health [10–13]. The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exemplifies problems resulting from emerging zoonotic pathogens [9]. Individuals with frequent and sustained contact with animals are considered at higher risks of infections with zoonotic pathogens and are therefore suitable targets for emerging virus surveillance programs [13]. The early detection of emerging viral pathogens of animal origin that exhibit potential for human-to-human transmission remains a difficult but essential step to mitigate their propagation.

Here, we characterized the eukaryotic virome of respiratory specimens taken from patients presenting with acute respiratory infections in a cohort with a high level of animal exposure [14] in southern Vietnam. Additionally, we compared viral detection by mNGS versus PCR to assess the utility of mNGS in routine diagnostics.

2. Materials and Methods

2.1. Ethical Approvals

All study subjects gave their informed consent for inclusion before participating in the study. The study was approved by the Oxford Tropical Research Ethics Committee (OxTREC) (No. 157-12), the United Kingdom, and the Ethic Committees of Dong Thap Hospital in Dong Thap provinces, Vietnam.

2.2. The Sentinel Cohort Study and Samples

The clinical samples used in this study were derived from a sentinel cohort study described previously [5,14]. The cohort study is a community-based component of The Vietnamese Initiative on Zoonotic Infections (VIZIONS) project [15,16], which was conducted to detect potential zoonotic transmission. In brief, individuals, including animal-raising farmers, slaughterers, animal health workers, and rat-traders from Dong Thap and Dak Lak provinces in Vietnam, were recruited in the cohort study and followed for 3 years, 2013 to 2016 [14].

Starting each study year, to create baseline data, the cohort members were interviewed and them plus their animals (all without symptoms and sign of respiratory disease) were sampled. During the follow-up period, whenever the cohort member got any signs/symptoms of respiratory tract infections and fever (≥38 °C), specimens from the diseased individual and their animals were collected. The clinical specimens collected from each cohort member and their animals consisted of rectal, pooled nasal and throat swabs,

and blood [5,14]. Here, we focused on nasal-throat swabs sampled at respiratory disease episodes during 2013 (Figure 1).

2.3. Metagenomic Next-Generation Sequencing (MNGs) Assay

Initially, 200 μ of nasal-throat swabs collected at disease episodes and a negative control containing viral transport medium were first treated with 20 U of turbo DNase (Ambion, Life Technology, Carlsbad, CA, USA) and 50 U of RNase I (Ambion) at 37 °C for 30 min [17]. Viral RNA was then isolated from nuclease-treated materials using a QIAamp 96 Virus QIAcube HT Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions for nucleic acid extraction. The nucleic acid output was then recovered in 50 μL of elution buffer (provided with the QIAamp kit).

Double-stranded DNA synthesis, random amplification, and library preparation were carried out as previously described [6]. The prepared library was sequenced using the MiSeq reagent kit V3 in an Illumina MiSeq platform (Illumina, San Diego, CA, USA. The double indexes of Nextera XT Index Kit (Illumina) was used to multiplex and differentiate the samples in each run.

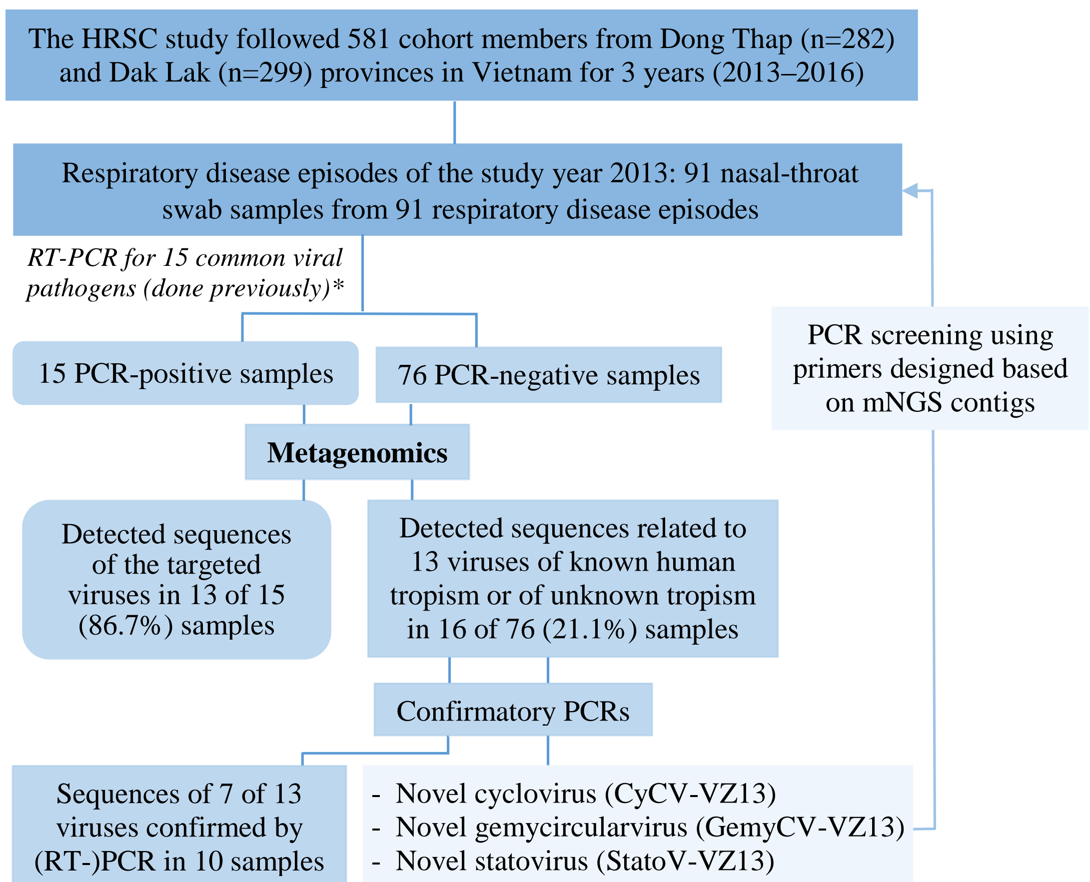


Figure 1. Overview of the methods and main results. HRSC: high risk sentinel cohort.* see [5], the 15 viruses include human rhinovirus (HRV), enterovirus (EVs), coronavirus (CoV) subtype OC43 and NL63, respiratory syncytial virus (RSV) A, RSV B, human metapneumovirus (MPV), influenza A virus, influenza B virus, adenovirus, parainfluenza virus (PIV)1–4, human bocavirus, and parechovirus.

2.4. Analysis of mNGS Sequence Data

An in-house analysis pipeline was used to analyze sequence data that is posted in GitHub: https://github.com/xutaodeng/virushunter/. Briefly, the adaptors, low-quality reads, and duplicate reads

were firstly removed. The reads related to human and bacterial genomes were subtracted by mapping reads using bowtie2 (version 2.2.4) to concatenated human reference genome sequence and mRNA sequences (hg38), and bacterial nucleotide sequences extracted from NCBI nt fasta file (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/, February 2019) based on NCBI taxonomy (ftp://ftp.ncbi.nih.gov/pub/taxonomy, February 2019) [18]. The remaining reads were de novo assembled using ENSEMBLE software [19], which uses a partitioned subassembly approach to integrate the use of various de Bruijn graph (DBG) and overlap-layout-consensus assemblers (OLC) [19]. To allow for sensitive screening of viral sequences, the resulting contigs (plus single reads) were aligned against the viral proteome of the NCBI's RefSeq and the viral proteome of the non-redundant database by the Basic Local Alignment Search Tool (BLASTx). Matches with E score <0.01 were retained. To filter out tentative viral hits that showed better alignments to non-viral sequences, these tentative matches to viral proteins were then aligned to the GenBank's entire non-redundant proteome database using DIAMOND algorithm version 0.9.6 [20]. Sequences were then classified as viral or removed as non-viral according to the NCBI taxonomy of the best hits (lowest E score) in the non-redundant proteome database. Viral reads described here have E scores to viral proteins $<10^{-10}$.

2.5. PCR Confirmatory Testing of Viruses Detected by Metagenomics and Genome Sequencing

mNGS-detected viruses that were previously reported in human samples were confirmed by specific RT-PCR using previously published or newly designed primers/probes, followed by Sanger sequencing of the obtained amplicons (if applicable). The PCR confirmatory experiments were carried out on newly extracted nucleic acid from original patient samples using MagNApure 96 platform (Roche Diagnostics, Mannheim, Germany) [5]. Inverse primers were used to amplify and then sequence complete circular virus genomes.

2.6. PCR Screening by New Primers Designed Based on mNGS Contigs

For eukaryotic viral genomes detected in mNGS output, PCRs (confirmed by Sanger sequencing) were applied to screen for their genetic sequences. The primers were designed based on the mNGS contigs of the viruses of interest and PCRs conducted on mNGS-negative samples (Figure 1). PCR screening was carried out on the nucleic acid re-isolated from the original respiratory sample using a MagNApure 96 platform (Roche Diagnostics) [5].

2.7. Viral Genotyping

Sequences related to the enterovirus genome were classified using the Enterovirus Genotyping Tool Version 1.0 [21]. For influenza virus A, coronavirus (CoV), and respiratory syncytial virus (RSV)-A, the read sequences for subtyping [22–24] were located and extracted from mNGS output using the Map-to-reference tool of Geneious Prime 2020.0.2 software (Biomatters, Auckland, New Zealand). The recovered sequences were then used to compare against the NCBI non-redundant protein sequence database using BLASTx (E value $\leq 10^{-5}$).

2.8. Phylogenetic Analysis

Sequence alignment was conducted by the MUSCLE algorithm of MEGA software version X. Phylogenetic trees were built by the Maximum Likelihood (bootstrap 1000) algorithm of the MEGA

2.9. Nucleotide Sequence Accession Numbers

The raw NGS reads were deposited in the database of Genbank (PRJNA639353). The GenBank accession numbers for the novel viral genomes described here are MT649483 and MT649484 (novel statovirus), MT649485 (novel cyclovirus), and MT649486 (novel gemycircularvirus).

2.10. Statistics

Pearson's Chi-squared test or Fisher exact test or t-test was applied in the calculation of associations or differences between variables by pairwise comparisons. The *p* values were adjusted for multiple comparisons using the Benjamini and Hochberg method [25] with a false discovery rate (FDR) calculator [26]. A $p \leq 0.05$ was considered significant. The Wilson method in EpiTools [27] was used to calculate 95% confidence intervals. Pearson's correlation coefficients were calculated by STATA software 12.0 to measure the correlation of normally distributed variables. The normality was tested with normal Q-Q plots by STATA.

3. Results

3.1. Characteristics of the Cohort Members and Clinical Samples

We first applied mNGS to nasal-throat swab samples collected during disease episodes in 2013. These samples consisted of 94 samples from 94 disease episodes from 60 study participants residing in Dong Thap province. A convenience sample size of 91 samples from 58 individuals was selected from these 94 samples of 60 participants for metagenomics analysis. Of these, 15 were positive for at least one respiratory viral pathogen and were included as positive controls for mNGS analysis alongside the remaining 76 RT-PCR negative swabs. The demographic and baseline characteristics of the participants are presented in Table 1.

- **Table 1.** General characteristics of the 58 cohort members, and comparison between clinical symptoms recorded at respiratory-disease episodes of PCR-positive and -negative individuals.

	mNGS analysis			
	Total	**PCR positive**	**PCR negative**	***p* value[#^]**
No. of cohort members	***N* = 58**	***N* = 14**	***N* = 51**	
Median age (range) (in years)	35.5 (7–76)	31 (13–58)	38 (7–76)	0.465[##]
Sex ratio (male/female)	2.6 (42/16)	1.3 (8/6)	3.3 (39/12)	0.465
Occupations				
Animal health worker	12 (20.7)	2 (14.3)	11 (21.6)	1
Animal-raising farmer	26 (44.8)	6 (42.9)	22 (43.1)	1
Slaughterer	18 (31.0)	5 (35.7)	17 (33.3)	1
Rat-trader	2 (3.4)	1 (7.1)	1 (2.0)	0.829
Having chronic diseases	4 (6.9)	0	4 (7.8)	1
Respiratory disease episodes	***N* = 91**	***N* = 15**	***N* = 76**	
Frequency of clinical signs				
Fever	91 (100)	15 (100)	76 (100)	-
Cough	75 (82.4)	8 (53.3)	67 (88.2)	**0.015**
Sneezing	69 (75.8)	14 (93.3)	55 (72.4)	0.465
Sore throat	49 (53.8)	8 (53.3)	41 (53.9)	1
Dyspnea	9 (9.9)	1 (6.7)	8 (10.5)	1
Headache	57 (62.6)	12 (80)	45 (59.2)	0.465
Body aches	47 (51.6)	7 (46.7)	40 (52.6)	1
Watery diarrhea	11 (12.1)	0 (0)	11 (14.5)	0.5
Nausea	2 (2.2)	0 (0)	2 (2.6)	1

- The value shows in format of number (percentage).
- [#] between PCR-positive vs PCR-negative columns conducted by Pearson's Chi-squared test or Fisher exact test.
- [##] by t-test.

- ^ the p values of multiple comparisons were corrected by the Benjamini and Hochberg method for false discovery rate (FDR) correction.

3.2. Overview of Sequences Detected by Metagenomics.

A total of 31,783,202 raw reads were obtained, with a median read of 342,524 and range of 43,930–718,762 reads/sample. Most of the reads were ~145–150bp length. Reads belonged to viral, bacterial, and human as well as unclassifiable sequences. We focused on viral reads from eukaryotic viruses, which in total accounted for 2.3% (range: 0.5–12.7%) of the total reads obtained from individual samples.

Evidence of the sequences related to 52 viral species from 31 families (including 19 viral species from 13 families that have previously been reported in human samples) was found in 27 of 91 (29.7%, 95% confidence interval (CI): 21.3–39.7%) samples but not in the negative control. After confirmatory PCR for a subset of viruses, the presence of 12 virus species from 9 families could be confirmed in 22 of 91 samples (24.2%, 95% CI: 16.5–33.9%) (Tables 2, 3).

Sequences related to those of viruses of invertebrates, plants, fungi, algae, and bacteria were also detected (Supplementary Table S4).

3.3. Viral Detection in Positive Controls

Sequences related to 5 viral pathogens detected by diagnostic RT-PCRs were detected by mNGS in 13 of 15 (86.7%, 95% CI: 62.1–96.3%) samples, including 8 human rhinovirus (HRV), 1 enterovirus (EVs), 1 mix-detection of HRV and EVs, 1 influenza A virus, 1 coronavirus (CoV), and 1 respiratory syncytial virus (RSV) A. mNGS failed to detect RSV (cycle threshold (Ct): 36.3) and human metapneumovirus (MPV) (Ct: 40) in 2/15 RT-PCR-positive samples (Table 2) but detected in only 2 other samples, which were negative in RT-PCR (Table 3).

- **Table 2.** Detection of respiratory viral pathogens of mNGS in 15 RT-PCR-positive samples where human viral pathogens were previously detected by diagnostic RT-PCR [5]. HRV: human rhinovirus, EVs: enterovirus, CoV: coronavirus subtype OC43 and NL63, RSV: respiratory syncytial virus, MPV: human metapneumovirus.

No.	Sample ID	Multiplex RT-PCR**		NGS analysis				
		Virus detected	Ct value	Virus genotype	Reads (%)[#]	Total length (bp)	Genome coverage (%)	Other virus detected[##]
1	72	EVs	32.4	Coxsackievirus A21	52,989 (12)	7440	100.0	
		HRV	37.1	HRV C56	2506 (0.6)	7099	98.1	
2	75	EVs	38.6	HRV B	4 (0.0)	598	8.3	
3	5	HRV	38.4	HRV B3	678 (0.7)	5512	75.0	Human betaherpesvirus 7
4	33	HRV	40	EVs-D68	3174 (0.7)	5629	76.2	
5	54	HRV	40	HRV B	6 (0.0)	723	10.0	
6	73	HRV	40	HRV B86	6644 (1.5)	7212	99.2	Vientovirus
7	83	HRV	38.7	HRV B79	6157 (1.8)	5639	78.2	Novel gemycircularvirus (GemyCV-VZ13)
8	86	HRV	38.2	HRV B79	19,606 (5.6)	7224	99.7	
9	91	HRV	40	HRV A57	2538 (1.1)	3450	47.8	
10	92	HRV	36.5	HRV B35	12,481 (3.1)	7298	100.0	Bat badicivirus, bat posalivirus
11	4	Influenza A virus	29.3	Influenza A/N2 virus	2 (0.0)	115	0.8	
12	6	CoV*	36	CoV OC43	8 (0.0)	733	2.4	
13	52	RSV-A	30.8	RSV-A genotype ON1	236 (0.1)	5398	35.4	
14	39	RSV-A	36.3	Not detected	0	0	0	
15	65	MPV	39.5	Not detected	0	0	0	

- * OC43 or/and NL63.
- ** reported previously [5].
- [#] Total reads of the targeted virus (percentage: the total reads of the virus per total raw reads of the sample).
- [##] detail of the viruses in Table 3.

Using the mNGS sequences, the viruses detected in 13/15 patients by diagnostic RT-PCRs were successfully genotyped (Table 2). Based on mNGS sequences, cross-detection between EVs and HRV by RT-PCRs in two samples was detected and corrected to HRV-B and EV-D68, respectively (Table 2), and generated (almost) complete genomes (≥75% coverage) of HRV (n = 6) and EVs (n = 2) (Table 2). Phylogenetic analysis of the six HRV sequences revealed that they belong to species B (n = 4), species A (n = 1) and species C (n = 1) (Supplementary Material Figure 1).

3.4. Viral Detection in RT-PCR-Negative Swabs and Results of Confirmatory PCR

Of the 76 RT-PCR-negative samples, sequences related to 12 viral species of 9 families that have previously been reported in both sterile and non-sterile human samples were found in 16 of 76 (21.1%) samples. They included both known human viruses (rotavirus, MPV, RSV, torque teno virus, human papillomavirus) and other viruses whose tropism is still unknown (novel cyclovirus, novel gemycircularvirus, novel statovirus, viruses of the *Circoviridae* family, gemycircularvirus, and statovirus) (Table 3).

- **Table 3.** Metagenomic detection of viruses that have previously been detected in human samples in nasal-throat swab samples negative for human viral pathogens by diagnostic RT-PCR [5].

No.	Sample ID	Detected viruses previously reported in human samples	Confirmed by PCR	No. of reads	Total contig length (bp)	Amino acid identity to GenBank strain (%)	Genome coverage (%)
1	89	Rotavirus	Yes	17	360	98	1.9
2	73	Vientovirus*#	Yes	2	146	53	4.8
3	23	Novel cyclovirus (CyCV-VZ13)	Yes	5	448	61.8	25.9
4	32	Novel gemycircularvirus virus (GemyCV-VZ13)	Yes	1852	1995	39	91
5	83	GemyCV-VZ13	Yes	120	2000	45	92
6	89	GemyCV-VZ13	Yes	1	148	46.9	6.8
7	24	Novel statovirus (StatoV-VZ13)	Yes	91	1018	42.5	24.6
8	32	StatoV-VZ13	Yes	5	231	35	5.6
9	82	StatoV-VZ13	Yes	27	2000	49	48.4
10	87	Gemycircularvirus	Yes	39	858	83	39
11	71	Gemycircularvirus	Yes	117	1400	97	63.7
12	88	Gemycircularvirus	Yes	2	300	73	13.6
13	11	Statovirus	Yes	4	351	91	8.5
14	71	Statovirus	Yes	7	812	90	19.6
15	5	Human betaherpesvirus 7*	Not done	2	295	100	0.2
16	15	Human papillomavirus	Not done	73	1280	99.3	17.5
17	17	Human papillomavirus	Not done	6	437	97.9	6
18	2	Torque teno virus	Not done	4	554	88.4	14.6
19	68	Torque teno virus	Not done	2	217	70.6	5.7
20	24	MPV	No	6	417	100	3.1
21	47	RSV A	No	6	468	100	3.1
22	92	Bat badicivirus-like virus*	No	2	204	49	2.3
23	92	Bat posalivirus-like virus*	No	3	182	56	2
24	83	Viruses of *Circoviridae* family	No	10	167	64	7

- * co-detected with other viral pathogens in 15 positive-control samples as reported in Table 2.
- # will be described in a separate paper.

In the 15 control samples where human viral pathogens were previously detected by diagnostic RT-PCR, we also detected by mNGS the following viruses: human betaherpesvirus 7, vientovirus, gemycircularvirus, bat badicivirus-like virus [28], and bat posalivirus-like virus [28] in 4 of 15 (26.7%) samples (Tables 2 and 3).

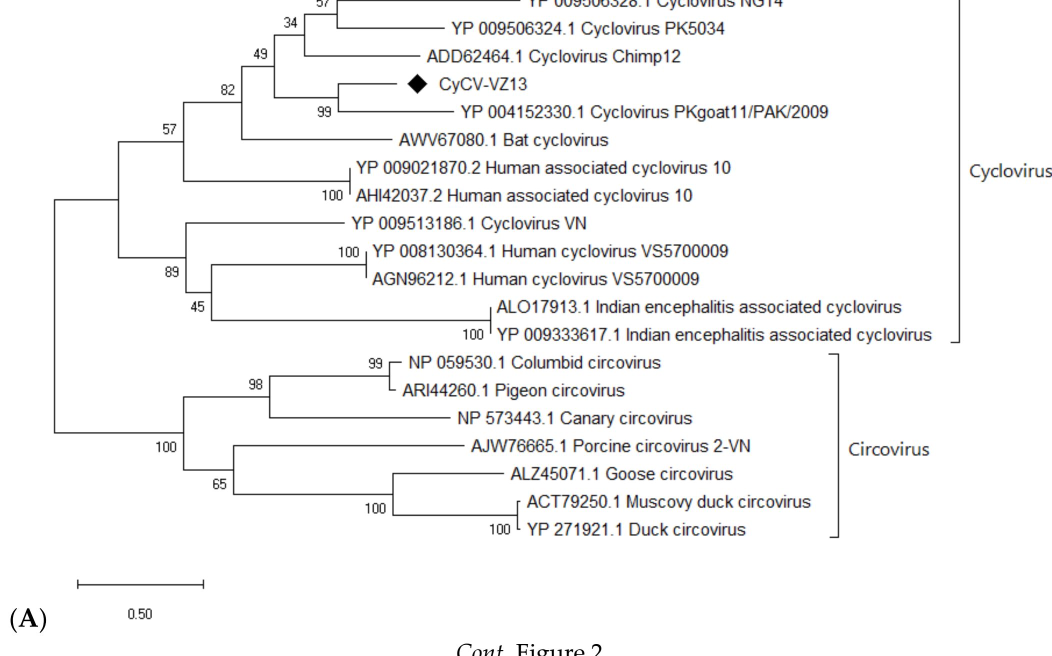
Using specific PCR, we were able to confirm the presence of rotavirus (n = 1), novel cyclovirus (n = 1), novel gemycircularvirus (n = 3), novel statovirus n = 3), gemycircularvirus (n = 3), statovirus (n = 2), and

vientovirus (n = 1) (Table 3) in 10 samples (8 of 76 (10.5%) RT-PCR-negative and 2 of 15 (13.3%) RT-PCR-positive samples).

3.5. Detection and Genomic Characterization of Novel Viruses

3.5.1. A Novel Cyclovirus

Two cyclovirus-related contigs were generated from the NGS output of a single sample of acute respiratory disease with an unknown (diagnostic RT-PCR negative) etiology. A complete circular DNA genome of 1740 bp was then obtained by inverse PCR and Sanger sequencing (Supplementary Table S1). The complete genome shared the highest nucleotide identity (55%) to cyclovirus NG 14 (accession number: NC_038417), which is lower than the species demarcation threshold (80% identity of the genome-wide nucleotide sequence) [29]. Phylogenetic analysis of capsid and rep proteins, 216 and 279 amino acids long, respectively, confirmed its genetic distinction from other cycloviruses (Figure 2), suggesting that it is a novel cyclovirus species, tentatively named Cyclovirus VIZIONS-2013 (CyCV-VZ13).



(A)

Cont. Figure 2.

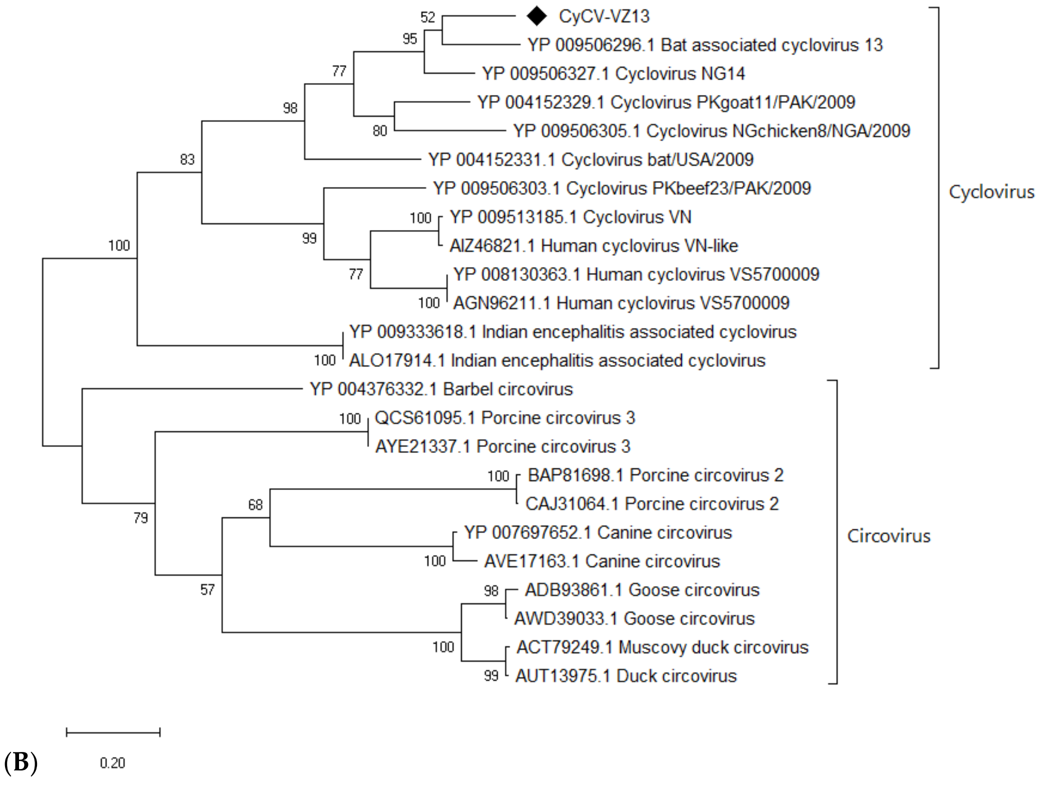


Figure 2. Phylogenetic tree of capsid (**A**) and replication (**B**) protein of Cyclovirus VIZIONS-2013 (CyCV-VZ13) compared to known viruses of the *Circoviridae* family.

Besides being detected in 1 of 91 samples by mNGS analysis, subsequent PCR screening with primers based on mNGS contigs detected the CyCV-VZ13 genome in 5 of the 90 mNGS-negative samples (5.6%). Mix detections with other viruses were found in several samples (Supplementary Tables S2 and S3).

3.5.2. A Novel Gemycircularvirus

Multiple gemycircularvirus-related contigs were detected in the mNGS output of a single sample of acute respiratory disease with a RT-PCR-unknown etiology. Based on PCR with inverse primers (Supplementary Table S1), and Sanger sequencing, a complete circular DNA genome of 2171 bp was generated. The complete genome shared the highest nucleotide identity (48.3%) to a murine feces-associated gemycircularvirus 2 (GenBank: MF416388.1). The species demarcation of gemycircularvirus is a 78% genome-wide pairwise identity [30]. All of the proposed species (n = 43; 73 strains) within the genus *Gemycircularvirus* share 56–77% whole-genome similarity with each other [30]. These suggest that a member of a novel gemycircularvirus species was discovered, which we tentatively named gemycircularvirus VIZIONS-2013 (GemyCV-VZ13). The phylogenetic analyses of the capsid (298 amino acid) and replication proteins (333 amino acid) were in agreement with this suggestion (Figure 3).

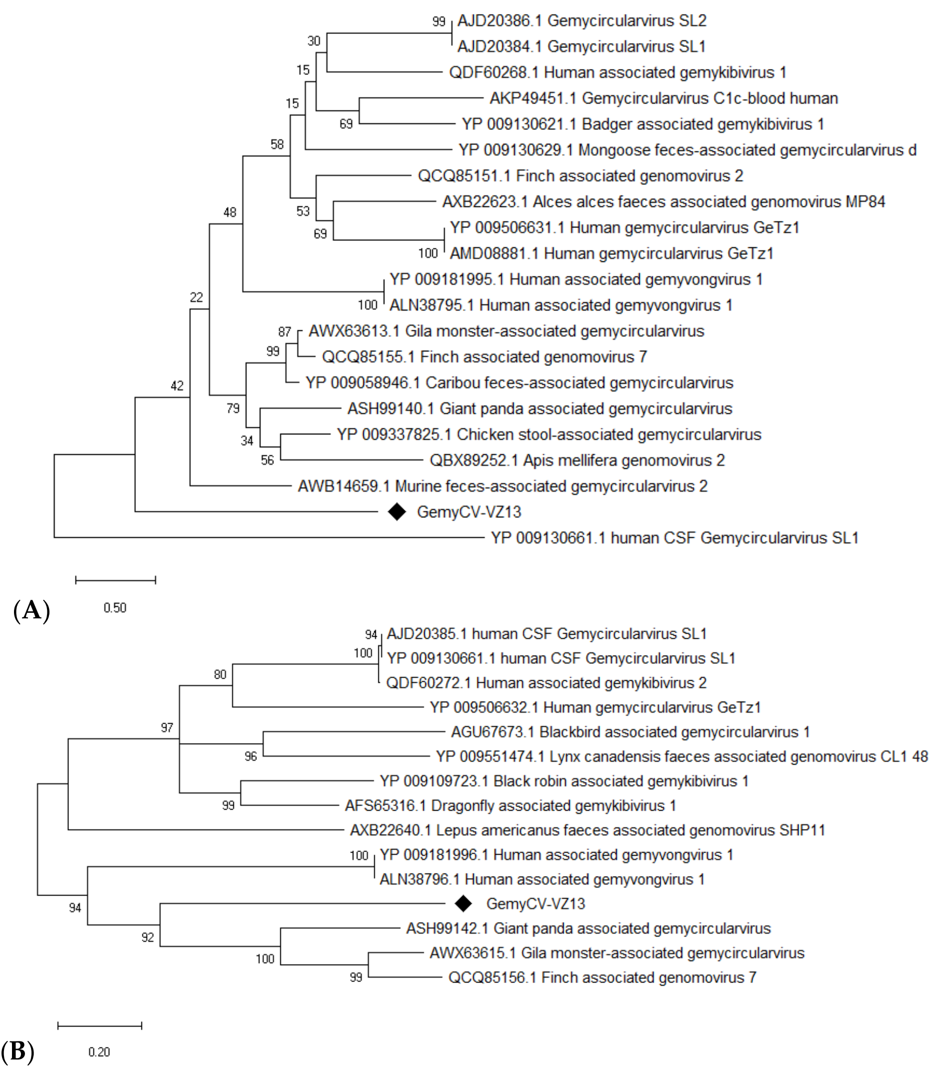


Figure 3. Phylogenetic tree of capsid (**A**) and replication (**B**) proteins of the gemycircularvirus VIZIONS-2013 (GemyCV-VZ13) compared to the viruses of the *Genomoviridae* family.

The sequences of GemyCV-VZ13 were found in the mNGS output and confirmed by PCR of two more samples. Besides being detected in 3 of 91 samples in the mNGS analysis, subsequent PCR screening yielded evidence of GemyCV-VZ13 in 12 of 88 (13.6%) mNGS-negative samples. Mix detections with other viruses were also found (Supplementary Tables S2 and S3).

3.5.3. A Novel Statovirus

Seven statovirus-related contigs were detected in mNGS output from three nasal-throat swab samples with negative diagnostic RT-PCR. Subsequently, partial RNA-dependent RNA polymerase (RdRp) and coat protein sequences were generated with 249 and 260 amino acids in length, respectively, sharing 40.4% and 45% amino acid identity with available statoviruses sequences. Currently, no species/genus demarcation of statoviruses is available [31]; however, based on the low identity of the RdRp protein sequence with other statoviruses and the distinction in the phylogenetic tree (Figure 4), we proposed this as a novel statovirus species, tentatively named statovirus VIZIONS-2013 (StatoV-VZ13).

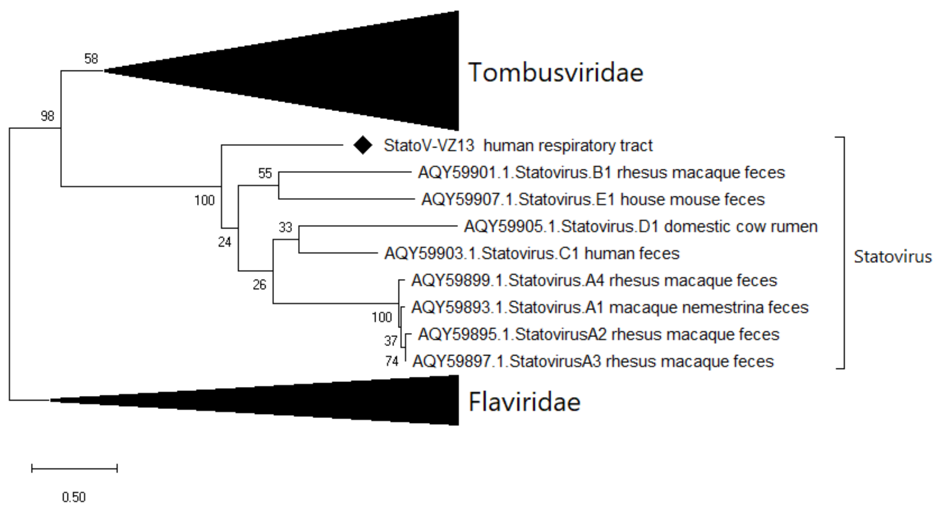


Figure 4. Phylogenetic tree of 249 amino acid partial RNA-dependent RNA polymerase (RdRp) protein sequences of statovirus VIZIONS-2013 (StatoV-VZ13) compared to the statoviruses on GenBank and viruses of the Tombusviridae and *Flaviridae* family.

Besides being detected in 3 of 91 nasal-throat swab samples by mNGS, additional PCR screening with primers based on mNGS contigs did not detect StatoV-VZ13 in any of the mNGS-negative samples. Thus, StatoV-VZ13 was detected in 3/91 (3.3%) samples collected at disease episodes.

4. Discussion

We describe here viral nucleic acids in nasal-throat swab samples from cases of acute respiratory diseases of unknown etiology from people at risk of zoonotic infections from Dong Thap province of Vietnam in 2013. We identified 12 species from 9 families of viruses that have previously been reported in various human samples. Sequences related to bacterial viruses, invertebrate viruses, fungal viruses, insect viruses, plant viruses, and algae viruses were also detected in the samples. Therefore, this viral survey expands our understanding of virus populations in acute respiratory diseases, particularly in people at risk of zoonotic infections, in Vietnam.

Metagenomic detection of most respiratory viral pathogens detected by RT-PCR indicated that the mNGS pipeline/protocol applied here is a sensitive pan-pathogen assay of respiratory viral pathogens in clinical samples, in agreement with previous studies [6,17,32]. Only sequences of RSV A and MPV were not detected from metagenomic output in the two diagnostic RT-PCR positive samples (RSV A- and MPV-PCR-positive samples) but were instead identified in only two other samples, suggesting that index-hopping probably happened, although contamination or pipette mistakes were not excluded. Additionally, detecting and correcting cross-reactivity between EVs and HRV in RT-PCR results, and genotyping other strains highlights one of the advantages of metagenomics in etiological and epidemiological studies compared to RT-PCR. Indeed, the analysis of HRV based on the obtained sequences suggested the predominance of HRV B in acute respiratory diseases in adults and imported HRV into Vietnam from several independent events. Notably, few studies reported the genetic diversity of HRV circulating in Vietnam. As such, our data has also shed light on the diversity of HRV in this locality.

The detection of several novel or recently identified viral genomes, including CyCV-VZ13, GemyCV-VZ13, StatoV-VZ13, and vientovirus, show that metagenomics is suitable as a sensitive pan-pathogen assay for sequence-independent detections of a variety of viruses, including novel ones. However, more frequent detections of the novel viruses by PCR than by metagenomics on the same samples suggests that PCR currently remains the most sensitive test for the diagnosis of those viruses whose genomes are already

known. The main advantages of metagenomics are therefore the ability to detect and sequence all viral genomes simultaneously rather than perform an extensive set of different RT-PCRs.

Currently, there are no robust criteria that can reliably define a true positive metagenomic result without the requirement of conducting confirmatory experiments [8,33]. As an exploratory study, we pragmatically took into account short viral reads presenting in the tested samples at any frequency for subsequent confirmatory PCR testing. This led to the discovery of several new viruses (CyCV-VZ13, StatoV-VZ13, and GemyCV-VZ13) in the present study, and the correct detection of influenza A virus in an RT-PCR-positive nasal-throat swab. Of note, a novel cyclovirus has previously been discovered and characterized based on a single initial read [34]. Collectively, the data thus suggest that even a single or a few viral reads generated by metagenomics can be a reliable marker for pathogen detection and discovery provided that the sequence similarity is high enough or used as an initial step towards generating a longer contig.

Cycloviruses belong to the *Circoviridae* family The closely related circoviruses are well known as pathogens in swine and birds and several other animals [35,36]. The natural hosts and pathogenic potentials of members of the *Cyclovirus* genus have not been definitely determined [36]. However, cyclovirus sequences have been detected in blood [37], cerebrospinal fluid [34,38], human respiratory tract [34,39], and persistent detection of identical sequences in the serum of immunodeficient patients [40]. Similarly, whether gemycircularviruses can infect humans is unknown. The germycircularvirus genome was identified in a wide range of host, in the feces of different animals, plants, insects, sewage, in the human respiratory tract [41], in blood from a patient with multiple sclerosis, and in the cerebrospinal fluid of encephalitis patients [41–45]. Statoviruses belong to a novel taxon of RNA viruses and have been detected in stool samples of diverse mammals, including human, macaque, mouse, and cow, but not in public sequencing datasets from bacteria, fungi, plants, unicellular eukaryotic organisms, or environmental samples and in the human respiratory tract [31]. The identification of novel viruses, including CyCV-VZ13, GemyCV-VZ13, and StatoV-VZ13, contributes to a better understanding of the respiratory virome in this part of the world. However, sequences related to viruses of the phylum Cressdnaviricota are ubiquitous contaminants of commonly used metagenomic reagents [46,47]. Thus, whether these genomes infect human cells, other non-human cells in the lungs, or reflect passive contamination of the respiratory tract will require further studies.

5. Conclusions

Our study demonstrates the presence of known and novel viruses in patients with acute respiratory diseases at risk of zoonotic infections. mNGS is a sensitive pan-pathogen assay for sequence-independent detection of respiratory viral pathogens in clinical samples. The detection of several novel viruses further contributes to our understanding of the human respiratory virome, and warrants further research to ascribe the clinical significant potential of these novel viruses.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Phylogenetic tree of partial VP1-VP3 protein sequences (90% coverage) from HRV sequences obtained from mNGS herein against corresponding sequences from The US on GenBank; Table S1: Title: Primer (and probe) sequences of the RT-PCRs used; Table S2: Detail characteristics and virus detections of the samples at respiratory-disease episodes; Table S3: Mix-detection of viruses detected by NGS with PCR confirmation; Table S4: Detection of viruses of invertebrate, insect, plant, fungi, bacteria and algae.

Author Contributions: Conceptualization, methodology and visualization, N.T.K.T., A.-M.K.V., O.V., S.B., L.V.T.; Formal analysis, investigation and resources, N.T.K.T., N.T.T.H., N.T.H.N., T.M.P., P.T.T.T., H.R.vD., H.D.T.N., D.T.H., D.A.H., L.T.T.H., X.D., L.V.T.; Data curation and validation, N.T.K.T., E.D., A.-M.K.V., O.V., L.V.T.; Writing—original draft preparation, N.T.K.T.; Writing—review and editing, N.T.K.T., G.T., E.D., A.-M.K.V., O.V., S.B., L.V.T.; Supervision, A.-M.K.V., O.V., L.V.T.; Project administration, S.B., L.V.T.; Funding Acquisition, S.B., L.V.T.; Final approval of the submitted manuscript, all authors.

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Article

Redondoviridae: High Prevalence and Possibly Chronic Shedding in Human Respiratory Tract, But No Zoonotic Transmission

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Abstract: *Redondoviridae* is a recently discovered DNA virus family consisting of two species, *vientovirus* and *brisavirus*. Here we used PCR amplification and sequencing to characterize redondoviruses in nasal/throat swabs collected longitudinally from a cohort of 58 individuals working with animals in Vietnam. We additionally analyzed samples from animals to which redondovirus DNA-positive participants were exposed. Redondoviruses were detected in approximately 60% of study participants, including 33% (30/91) of samples collected during episodes of acute respiratory disease and in 50% (29/58) of baseline samples (with no respiratory symptoms). Vientovirus (73%; 24/33) was detected more frequently in samples than brisaviruses (27%; 9/33). In the 23 participants with at least 2 redondovirus-positive samples among their longitudinal samples, 10 (43.5%) had identical redondovirus replication-gene sequences detected (sampling duration: 35–132 days). We found no identical redondovirus replication genes in samples from different participants, and no redondoviruses were detected in 53 pooled nasal/throat swabs collected from domestic animals. Phylogenetic analysis described no large-scale geographical clustering between viruses from Vietnam, the US, Spain, and China, indicating that redondoviruses are highly genetically diverse and have a wide geographical distribution. Collectively, our study provides novel insights into the *Redondoviridae* family in humans, describing a high prevalence, potentially associated with chronic shedding in the respiratory tract with lack of evidence of zoonotic transmission from close animal contacts. The tropism and potential pathogenicity of this viral family remain to be determined.

Keywords: redondoviruses; vientovirus; brisavirus; persistence; respiratory; animals; zoonosis

1. Introduction

Acute viral respiratory infections are associated with a significant global disease burden and are associated with the majority of epidemics and pandemics [1,2], including the ongoing SARS-CoV-2 pandemic [3]. Often, the etiological agent in the majority of the patients presenting with acute respiratory infections remains undetermined [4–7]. Therefore, it is critical to assess the potential clinical significance of newly discovered viruses, particularly to inform clinical management and health policymakers.

Redondoviridae is a novel virus family within the circular Rep-encoding single-stranded (CRESS) group of DNA viruses [8]. This family consists of only one genus, *Torbevirus*, which is divided into two species, vientovirus and brisavirus. The accepted species demarcation is ≤50% sequence similarity of the replication protein [8,9].

Redondoviruses have been exclusively detected in samples from humans, especially those collected from the respiratory tract [8,10–12]. Redondovirus DNA was detected in 15% (9/60), 11% (22/209), and 2% (2/100) of oropharyngeal samples taken from healthy adults in the US [8], Italy [10], and Spain [11]. Higher loads of redondovirus DNA were detected in respiratory samples from critically ill patients than in those from healthy individuals [8]. Redondoviruses may also be associated with periodontal disease because their abundance was noted to decrease with standard periodontal treatment [8]. Moreover, persistent detection of redondoviruses in serial endotracheal aspirates from critically ill subjects over 2–3 weeks has been documented [8].

Existing data suggest that redondoviruses are unlikely to be bacteriophage because they carry no prokaryotic ribosome binding site [8]. There is currently no evidence regarding the targeted detection of redondoviruses in animals, fresh water, marine, air, or soil samples [8]. Screening is generally performed via metagenomic sequence analysis, but PCR amplification remains the gold standard for the targeted detection of microbes. Additionally, data regarding the host range, prevalence, and key characteristics of this recently discovered virus family remain scarce.

Collectively, given the pathogenic potential of redondoviruses, as well as existing knowledge gaps regarding their epidemiology and evolution, we aimed to investigate their genetic diversity, epidemiological features, and potential for zoonotic transfer. These data might aid the prioritization of appropriate intervention strategies in the future.

2. Materials and Methods

2.1. The High-Risk Sentinel Cohort Study

Samples from this investigation were derived from a previously described cohort study conducted in Vietnam [6,13]. In brief, the cohort comprised healthy individuals working with animals in Dong Thap Province (n = 282) and Dak Lak Province (n = 299) in Mekong Delta and central highlands of Vietnam, respectively. Recruitment was initiated in March 2013 in Dong Thap Province and from February 2014 in Dak Lak Province. The study participants were followed for 3 years (4/2013–4/2016 for the Dong Thap site and 2/2014–2/2017 for the Dak Lak site).

We collected respiratory samples (nasal and throat swabs) from the participants and their animals at the beginning of each year when no respiratory symptoms were present. These samples were defined as baseline samples. Over the 3-year follow-up period, we collected disease-episode samples from the diseased participants and their animals whenever the participants reported they had an acute respiratory infection. Acute respiratory infection was defined as any signs/symptoms of respiratory tract infections with fever (≥38 °C).

Here we focused on nasal/throat swabs collected during all respiratory disease episodes reported in 2013 (n = 91). These samples were collected from 58 study participants residing in Dong Thap Province. Additionally, all baseline samples (n = 58) of these participants were analyzed. To assess the zoonotic potential of detected redondovirus, we tested nasal/throat swabs collected from

animals to which the redondovirus-positive participants (farmers) were exposed during each specific disease episode.

2.2. Whole-Genome Amplification by Inverse PCR

The complete viral genome was amplified by inverse PCR using specific primers (Table 1) designed from metagenomic contigs. The PCR was conducted in a final 25 μL volume reaction mixture, containing 18 μL of Platinum™ PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA, USA), 1 μL of each reverse and forward primer at a concentration of 10 μM each, and 5 μL of extracted nucleic acid. PCR reactions were performed using a Mastercycler (Eppendorf, Hamburg, Germany) (Table 1).

Additionally, we employed a primer-walking strategy to close gaps within the genomes (Table 1). PCR amplicons were detected using 1% agarose gels and sequenced using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) on an ABI377 automatic sequencer (Applied Biosystems), following the manufacturer's instructions.

To minimize the likelihood that vientovirus sequences were derived from nucleic acid extraction kits, which has been previously reported [14,15], we used 2 nucleic acid extractions. One source was newly extracted from the original sample using a MagNApure 96 platform (Roche Diagnostics, Mannheim, Germany) [13]. The other comprised residual nucleic acid materials after mNGS sequencing extracted by the QIAamp 96 Virus QIAcube HT Kit (QIAGEN GmbH, Hilden, Germany) [16].

Table 1. Newly designed primer sequences for the PCRs.

Primer Name	For Purpose	Sequence	PCR Products (bp)	Target (Regions)	Thermal Cycles
Vientovirus VZ-inverse_F	Whole genome	TATTTGTGGCCTTACTCCTTGT	3000	Replication gene (2628–2649′)	95 °C for 2 m; 45 cycles of 95 °C for 15 s, 52 °C for 30 s, 72 °C for 2 m 45 s; 72 °C for 5 m
Vientovirus VZ-inverse_R	Whole genome	GGACATATAGCAGAAAAAGGTGATG		Replication gene (2577–2552′)	
Vientovirus VZ-walking_F	Whole genome	AGACTTGCTTCTATGGTTTGTAGT	1400	Capsid gene (268–291′)	95 °C for 2 m; 45 cycles of 95 °C for 15 s, 48 °C for 30 s, 72 °C for 2 m; 72 °C for 5 m
Vientovirus VZ-walking_R	Whole genome	TGATACACAATTCTTTTACCGTTGT		Capsid gene (1777–1752′)	
Vientovirus VZ-close gap_F	Whole genome	GGGGCCCTTGAACCACATTA	750	Replication gene (2352–2372′)	95 °C for 2 m; 45 cycles of 95 °C for 15 s, 52 °C for 30 s, 72 °C for 1 m 15 s; 72 °C for 5 m
Vientovirus VZ-close gap_R	Whole genome	GCAGCCCTCTTAAGCCTGTA		Replication gene (132–112′)	
Redondovirus-capsid gene_F	PCR screening	GGCTTAAGAGGGCTGCTAGG	460	Capsid gene (116–136′)	95 °C for 5 m; 45 cycles of 95 °C for 20 s, 52 °C for 30 s, 72 °C for 1 m; 72 °C for 5 m
Redondovirus-capsid gene_R		TCCTTGGATGCCATGAAACT		Capsid gene (575–555′)	
Redondovirus-replication gene_F	Genetic characterization	GTTGTCACTTGTGAAACGATGA	1400	Replication gene (1711–1733′)	95 °C for 5 m; 45 cycles of 95 °C for 20 s, 50 °C for 30 s, 72 °C for 2 m; 72 °C for 5 m
Redondovirus-replication gene_R		TCGACGATAAACTCTCTTTCTTGA		Replication gene (43–19′)	

2.3. PCR Screening and Genetic Characterization of Redondoviruses in Respiratory Samples and Animal Contacts

We used residual nucleic acid extractions from human disease-episode samples [13] for the PCR screening of redondoviruses. We extracted nucleic acid using the MagNApure 96 platform. For samples collected at baseline or from animals, nucleic acid was freshly isolated from the original materials using the QIAamp viral RNA kit (QIAgen GmbH, Hilden, Germany), following the manufacturer's instructions.

To investigate the prevalence of redondoviruses in human and animal samples, we employed a generic single-round PCR assay targeting a conserved region of the capsid protein-coding gene. The primer sequences are described in Table 1.

To genetically characterize the amplified redondovirus nucleic acid, we applied a generic PCR to amplify the entire replication protein-coding gene in samples positive by the capsid-gene PCR (Table 1). The PCR primers were newly designed from the complete genome generated as part of the initial experiment described above and available redondovirus sequences deposited in the GenBank [8].

We used Sanger sequencing to sequence the generated PCR amplicons. The PCR and sequencing procedures used were comparable to those used for confirmatory PCR and sequencing above, with some modifications to the thermal cycling conditions (Table 1). Negative controls were included in each PCR detection experiment. The PCR-associated experiments were conducted in unidirectional molecular diagnostic facilities consisting of three physically separated laboratories for reagent preparation, nucleic acid extraction, and amplification to minimize the risk of contamination.

2.4. Phylogenetic Analysis

Sequence alignments were conducted in MUSCLE available in MEGA version X. Phylogenetic trees were constructed using the generated nucleotide for genetic characterization using the Maximum Likelihood method available in the MEGA software with a bootstrap value of 1000 replicates.

2.5. Nucleotide Sequence Accession Numbers

The redondovirus genomes and replication coding sequences described here were submitted to GenBank under the Accession Numbers MT759843, MT823476–MT823478, and MW216334–MW216337.

2.6. Statistics

Statistical associations and differences between variables were calculated using Pearson's Chi-squared test or Fisher's exact test for categorical data and t-test for continuous data, respectively, by pairwise comparisons in STATA software (version 12.0). *P*-values were adjusted for multiple comparisons by the Benjamini and Hochberg method [17] with a false discovery rate (FDR) calculator [18]. A value of $p \leq 0.05$ was considered significant.

2.7. Ethics

The high-risk sentinel cohort study received approvals from the Ethics Committees at the University of Oxford, United Kingdom, and at the sub-Departments of Animal Health and General Hospital in Dong Thap Province and Dak Lak Province and in the Hospital of Tropical Diseases in Ho Chi Minh City in Vietnam, as reported previously [16,19]. Written consent was obtained from each study participant.

3. Results

3.1. Detection and Genetic Characterization of a Vientovirus

We previously detected a contig derived from two reads related to the human lung-associated vientovirus AL strain (Accession Number: MK059760.1) in one sample using metagenomic sequencing [16]. Using inverse PCR, we recovered a full circular genome of this virus, which was 3054-bp. A sequence comparison found that the generated sequence was closely related to the reported genomes of vientovirus of the family *Redondoviridae* (sharing a 79% sequence identity (2404/3054 bp)). The obtained sequence possessed a typical genomic structure of this viral family, containing three open reading frames (ORF1-3) encoding for capsid, replication, and a protein of unknown function (530, 350, and 200 AA, respectively). The coding region of the capsid protein and the protein of unknown function was arranged in an opposite orientation to the replication protein (Figure 1). Additionally, a typical stem-loop structure ("TATTATTTAT") was identified upstream of the 5′ end of the replication protein-coding region (Figure 1).

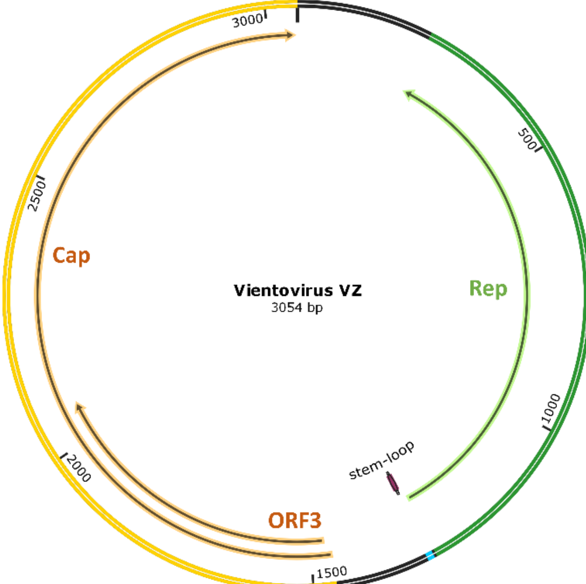

Figure 1. Putative genome organization of vientovirus VZ. Vientovirus VZ has typical genome features of a virus of the *Redondoviridae* family. Cap: capsid protein; Rep: replication protein; ORF3: open reading frame 3 encoding an unknown protein.

A pairwise comparison demonstrated that the capsid and replication protein sequences share the highest similarity (97.7% and 59.1%) with respective protein sequences (Accession Numbers: QCD25327.1 and QCD25302.1, respectively), corresponding with 98.1% and 66.6% of similarities at the nucleotide level of the vientovirus (Accession Numbers: MK059768 and MK059760). Phylogenetic analysis of replication-gene nucleic acid showed a close relatedness with previously reported vientovirus sequences (Figure 2). The detected virus was confirmed as vientovirus, which we named vientovirus VZ (Accession Number: MT759843).

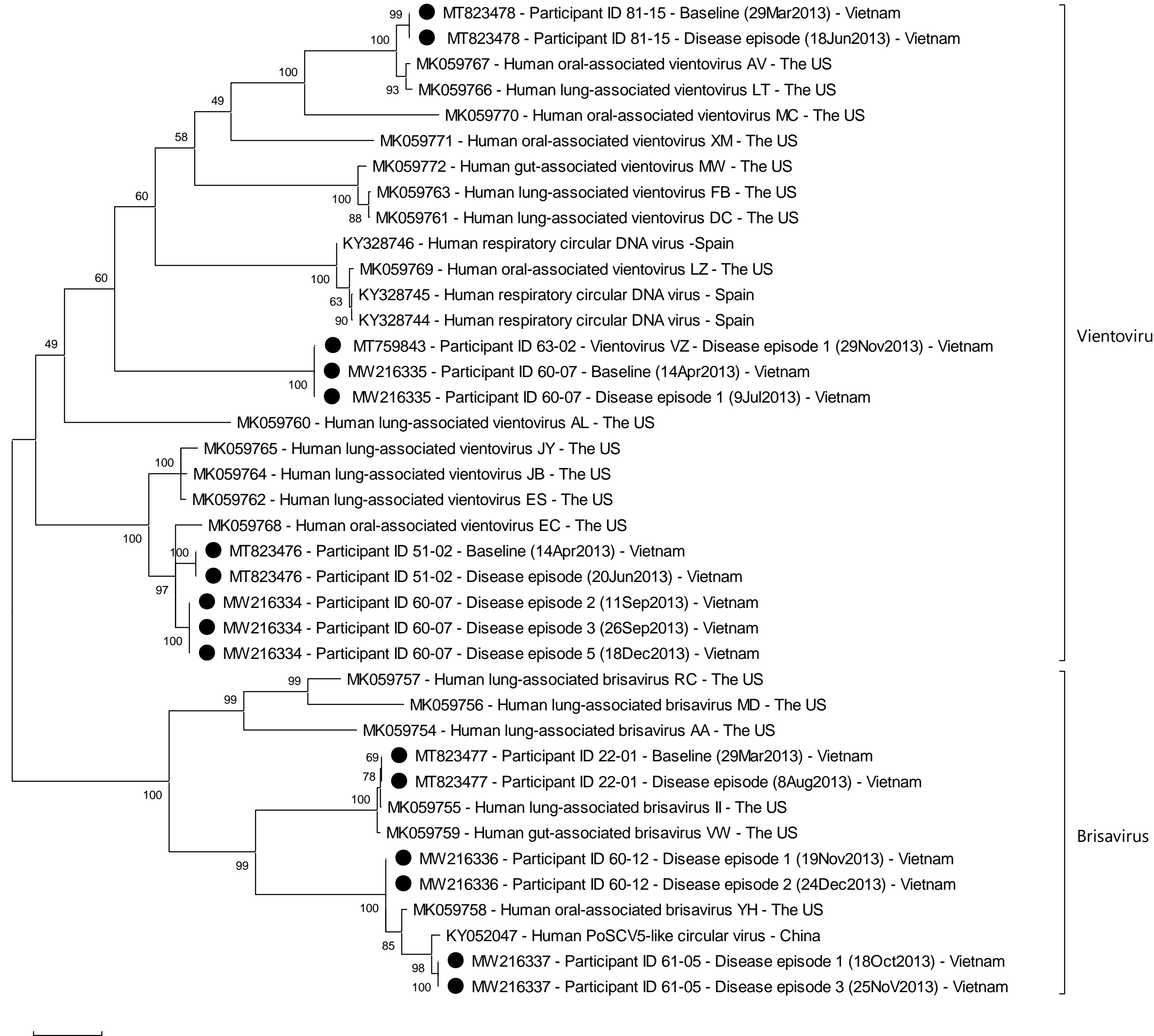

Figure 2. Phylogenetic tree of complete nucleic acid sequences of the replication protein-coding gene of redondoviruses. The sequence Accession Numbers are included on the tips of the tree. Black circles denote redondovirus strains detected in the present study.

3.2. Detection of Redondoviruses in Respiratory Samples

We performed subsequent PCR screening and detected redondovirus DNA in 29 of 58 (50%) baseline samples from 58 participants (Table 2). We additionally detected redondovirus DNA in 30/91 (32.7%) disease-episode samples from the same participants (Table 2).

Overall, after combining the data from the baseline and disease-episode samples, we detected redondoviruses in at least one longitudinal sample collected at baseline and disease episodes in over half of the participants (33/58; 56.9%) (Table 2).

Sequencing of the PCR amplicons was successful in 26/29 and 27/30 positive samples at baseline and during disease episodes, respectively. Of the 26 sequences obtained from the baseline samples, 6 (23.1%) belonged to brisavirus, and 20 (76.9%) belonged to vientovirus (Table 2). Of the 27 sequences obtained from the disease-episode samples, 9 sequences (33.3%) belonged to brisavirus, and 18 (66.7%) belonged to vientovirus (Table 2).

Table 2. Detection of redondoviruses from the study participants and each of the baseline and clinical samples.

	Redondoviruses Negative	Redondoviruses Positive				Total
		Brisavirus	Vientovirus	Undefined *	Subtotal	
Study participants ^	25	9	23	1	**33**	58
Baseline samples	29	6	20	3	**29**	58
Disease-episode samples	61	9	18	3	**30**	91

* Redondovirus-screening PCR was positive, but no PCR sequence was obtained for species identification. ^ Number of participants who never got infected (negative) or got infected with redondoviruses at least once (positive) during the entire study are shown.

3.3. The Genetic Diversity of Redondoviruses

We next compared 16 complete replication protein-coding sequences of redondoviruses that we obtained in the present study with those isolated from the US, Spain, and China available in GenBank. A pairwise comparison and phylogenetic analysis revealed that there was no extensive geographical clustering among viruses detected in Vietnam, the US, Spain, and China (Figure 2).

3.4. Evidence of Possible Persistence of Redondoviruses in Nasopharynx

Of the 23 participants with at least two longitudinal samples that were positive for redondoviruses, 10 (43.5%) provided evidence of having an identical replication gene of redondovirus (610–1306 bp, equivalent to 58–100% of complete nucleic acid sequence coding replication protein) detected in their longitudinal samples within a window of 35–132 days (Table 3). In one patient (ID 60-07), we detected vientovirus VZ with the same replication protein-coding gene in nasal/throat swabs collected at baseline and disease episode No. 1. However, in subsequent disease episodes, a genetically related but nonidentical vientovirus was detected (Table 3).

	Study Year 2013						
	Baseline	**Disease Episode 1**	**Disease Episode 2**	**Disease Episode 3**	**Disease Episode 4**	**Disease Episode 5**	**Duration of Persistence (Days)**
Participant ID 60-07	VienV VZ 14-Apr	VienV VZ 09-Jul	VienV S39 11-Sep	VienV S39 26-Sep	RedonV 15-NoV	VienV S39 18-Dec	86 and 98, respectively
Participant ID 48-01	VienV S19 14-Apr	VienV S19 10-Jul					87
Participant ID 81-15	VienV S8 29-Mar	VienV S8 18-Jun					81
Participant ID 49-01	VienV S15 14-Apr	VienV S15 20-Jun					67
Participant ID 51-02	VienV S17 14-Apr	VienV S17 20-Jun					67
Participant ID 22-01	BrisaV S32 29-Mar	BrisaV S32 08-Aug					132
Participant ID 81-23	BrisaV S4 07-Apr	BrisaV S4 05-Jun	10-Jul				59
Participant ID 61-05	RedonV 14-Apr	BrisaV S56 18-Oct	RedonV 08-Nov	BrisaV S56 25-Nov			38

Participant ID 60-12	14-Apr	BrisaV S83 19-Nov	BrisaV S83 24-Dec	35

Table 3. Chart showing identical replication-gene sequences of brisavirus and vientovirus detected in samples at baseline and disease episodes. RedonV: redondoviruses; VienV: vientovirus; BrisaV: brisavirus. Vientovirus or brisavirus written with the same name and in the samples collected from the same participant have identical replication-gene sequences. Boxes with redondoviruses are samples positive with redondoviruses by PCRs, but no PCR-replication sequences were achieved for species identification.

3.5. The Demographics of Participants with and without Redondoviruses Detected in at Least one of Their Longitudinal Samples Taken at Baseline and Disease Episodes

The demographics of the 58 study participants with redondoviruses detected in at least one of their serial samples at both baseline and disease episodes are presented in Table 4. Notably, the redondovirus-positive participants were significantly older than those negative for redondoviruses (43.8 vs. 33.8, $p = 0.02$) (Table 4). The participants were more likely to test positive for redondoviruses if their occupation was a slaughterer (45.5% vs. 15%, $p = 0.02$) (Table 4).

Table 4. The demographics of the study participants.

	Total	Redondoviruses Positive *	Redondoviruses Negative	*p*-Value
Number of participants	58	33	25	NA ^
Having chronic diseases (%)	4 (6.9)	1 (3)	3 (12)	0.3
Occupation (%)				
Animal-raising farmer	26 (44.8)	13 (39.4)	13 (52)	0.3
Animal-health worker	12 (20.7)	5 (15.2)	7 (28)	0.1
Slaughterer	18 (31)	15 (45.5)	3 (12)	0.02
Rat trader	2 (3.4)	0 (0)	2(8)	NA
Females/males (ratio)	16/42 (0.4)	11/22 (0.5)	6/19 (0.3)	1
Median age in year (range)	35.5 (7–76)	43.8 (23–76)	33.8 (7–72)	0.02#

* Number of participants who got infected with redondoviruses at least once during the entire study. ^ NA: not applicable. The value is shown in a number format (percentage). *P*-values were calculated using Pearson's Chi-squared test or Fisher's exact test. The p-values were adjusted for multiple comparisons using the Benjamini and Hochberg procedure; # by *t*-test.

3.6. Clinical Symptoms of Redondovirus-Infected Patients during Disease Episodes

Coughing was the most common clinical symptom recorded in the redondovirus-infected patients, followed by sneezing and a sore throat. Dyspnea and watery diarrhea were recorded in 10% (3/30)) and 13% (4/30) of the participants, respectively. There was no significant difference in respiratory symptoms between individuals with and without a redondovirus detected in respiratory samples ($p = 0.24$; (Table 5)). Likewise, there was no significant difference in clinical symptoms between the brisavirus- and vientovirus-positive participants (Table 5).

Table 5. Clinical symptoms from 58 patients at 91 disease episodes with and without redondoviruses detected.

	No. of Disease Episodes	Redondoviruses Positive				Redondoviruses Negative	*p*-Value#
		Total	Brisavirus*	Vientovirus *	*p*-Value		
	$N = 91$	$N = 30$	$N = 9$	$N = 18$	NA	$N = 61$	NA

Fever	91 (100)	30 (100)	9 (100)	18 (100)	1	61 (100)	1
Cough	75 (82.4)	24 (80)	8 (88.9)	14 (77.8)	1	51 (83.6)	1
Sneezing	69 (75.8)	22 (73.3)	5 (55.6)	15 (83.3)	0.743	47 (77.0)	1
Sore throat	49 (53.8)	19 (63.3)	5 (55.6)	13 (72.2)	1	30 (49.2)	1
Dyspnea	9 (9.9)	3 (10.0)	1 (11.1)	2 (11.1)	1	6 (9.8)	1
Headache	57 (62.6)	24 (80.0)	8 (88.9)	14 (77.8)	1	33 (54.1)	0.243
Body aches	47 (51.6)	19 (63.3)	9 (100)	10 (55.6)	0.261	28 (45.9)	0.666
Watery diarrhea	11 (12.1)	4 (13.3)	2 (22.2)	2 (11.1)	1	7 (11.5)	1
Nausea	2 (2.2)	0 (0)	0 (0)	0 (0)	NA	2 (3.3)	NA

The value is shown in a number format (percentage). NA: not applicable. *P*-values were conducted using Pearson's Chi-squared test or Fisher's exact test and adjusted for multiple comparisons using the Benjamini and Hochberg procedure; * 3 disease episodes with a redondovirus detected, but no PCR sequence was obtained for species identification; # between column "Total" of "Redondoviruses positive" vs. column "Redondoviruses negative".

3.7. Coinfection in Samples Having Redondoviruses Detected with Other Respiratory Viruses

Taking into account the results of our previous PCR screening [13] and mNGS analysis [16], we identified a mixed infection of redondoviruses and other viruses in 28 samples. The codetected viruses included gemycircularvirus VIZIONS-2013, cyclovirus VIZIONS-2013, human rhinovirus, statovirus VIZIONS-2013, RSV A, gemycircularvirus, enterovirus, statovirus, and influenza A virus (Table 6).

Table 6. Codetection of redondoviruses and other viruses in the respiratory samples analyzed in this study.

	Redondoviruses Positive *				**Redondoviruses Negative**	***p*-Value #**
	Total	**Brisavirus**	**Vientovirus**	***p*-Value**		
	33	9	23	NA	25	NA
Gemycircularvirus VIZIONS-2013^	8 (24.2)	2 (22.2)	5 (21.7)	1	7 (28)	0.7
Cyclovirus VIZIONS-2013	4 (12.1)	1 (11.1)	3 (13)	1	5 (20)	0.5
Rhinovirus	4 (12.1)	0 (0)	4 (17.4)	0.3	1 (4)	0.4
Respiratory syncytial virus A	2 (6.1)	0 (0)	2 (8.7)	1	0 (0)	0.5
Statovirus VIZIONS-2013	2 (6.1)	1 (11.1)	1 (4.3)	0.5	0 (0)	0.5
Statovirus	2 (6.1)	0 (0)	2 (8.7)	1	0 (0)	0.5
Enterovirus	1 (3)	0 (0)	1 (4.3)	1	1 (4)	1
Influenza A virus	1 (3)	1 (11.1)	0 (0)	0.3	0 (0)	1
Metapneumovirus	1 (3)	0 (0)	1 (4.3)	1	0 (0)	1
Gemycircularvirus	1 (3)	0 (0)	1 (4.3)	1	0 (0)	1
Coronavirus OC43	0 (0)	0 (0)	0 (0)	NA	1 (4)	0.4

* Number of participants who got infected with redondoviruses at least once during the entire study. NA: not applicable. The value is shown in a number format (percentage). *P*-values were calculated using Pearson's Chi-squared test or Fisher's exact test. The p-values were adjusted for multiple comparisons using the Benjamini and Hochberg procedure; # by *t*-test. ^ 1 sample with a redondovirus detected, but no PCR sequence was obtained for species identification. # between column "Total" vs. column "Redondoviruses negative".

3.8. Detection of Redondoviruses in Respiratory Samples of Animals

We screened 27 samples from 27 pigs from 5 households, 13 pooled samples from 27 chickens from 5 households, 8 pooled samples from 17 Muscovy ducks from 2 households, 1 sample from a duck, and 4 pooled samples from 6 dogs from 4 households for redondovirus by generic PCR. None tested positive.

4. Discussion

Here we report the detection and genetic characterization of several redondovirus species of the recently discovered *Redondoviridae* family [8,12] in longitudinal upper respiratory tract samples of individuals at potential risk of zoonotic disease exposure and their animal contacts [6]. We found that nearly 60% of tested human participants were positive for either brisavirus or vientovirus of the family *Redondoviridae*, while none of the animals tested were positive for these viruses; these data are largely in agreement with a previous report [8]. Notably, we identified the same redondovirus replication protein-coding gene in longitudinal samples of 10 participants for up to 5 months. In a previous study, redondovirus DNA was detectable in serial samples collected from several patients over 2–3 weeks [8]. Collectively, these data suggest the persistence of the redondoviruses in the human respiratory tract, although sequence comparison at the whole-genome level is needed to confirm the relatedness between these redondovirus strains. Collectively, this study provides additional evidence supporting the possibility that redondoviruses, or their host(s) if not human cells, can colonize the human respiratory tract. Therefore, their pathogenic potential for humans warrants further research.

The prevalence (56.9%) of redondoviruses detected in our study participants was higher than the reported prevalence of 15% in the oropharynx of healthy Americans [8], 11% among Italians [10], and 2% among Spanish subjects [11]. However, phylogenetic analysis found no large-scale geographical clustering between viruses detected in Vietnam, the US, Spain, and China, indicating the wide geographic distribution and genetic diversity of redondoviruses.

Additionally, we observed a higher proportion of redondoviruses detected in samples at baseline than during disease episodes of the study participants. However, higher copy numbers of redondovirus DNA were previously reported in oropharyngeal samples of critically ill patients versus those of healthy individuals [8]. Thus, future studies should assess the kinetics of redondoviral loads over the course of the illness as well as between disease episodes and at baseline.

This work represents the first PCR screening study for redondoviruses in domestic animals from one of the recognized global hotspots of emerging infections. The sampled domestic animals were from households of study participants who tested positive for redondovirus. We found no evidence for redondoviruses in the respiratory tracts of these domestic animals. The absence of redondovirus in animal samples is in line with a recent report that used metagenomics [8]. The data also suggest that cross-species transmission was unlikely to occur among our study subjects. However, sequences of CRESS-DNA viruses have been widely found in animals [20,21]. More recently, deltaviruses that were theoretically confined to humans were detected in birds, snakes, fish, amphibians, and invertebrates [22,23]. Notably, we found that redondovirus-positive individuals were more likely to be animal slaughters. Therefore, whether similar or more divergent redondoviruses can be detected in animals merits further research.

Whether redondoviruses replicate in humans, other eukaryotic cellular residents of the respiratory tract, or are passively inhaled and deposited on respiratory surfaces remains unknown. An airborne environmental source seems unlikely given that closely associated animals tested PCR negative. Replication of redondoviruses in human cells also remains a possibility as a related family of CRESS-DNA viruses, the *Circoviridae*, includes members known to infect mammals [24,25].

There were no significant differences in clinical symptoms of acute respiratory illness in patients with and without redondoviruses detected in their samples, indicating, as is true for most respiratory pathogens, that clinical symptoms cannot be used to identify different etiologies. Additionally, we cannot exclude the possibility that the symptoms were caused by non-Redondoviridae viruses. Evidence for any association or causal relationship between this virus family and acute respiratory or other diseases, or lack of such association, still needs more studies; this is true also for some other newly found viruses, such as anelloviruses [8].

We found a significant difference in the detection of redondoviruses along with other respiratory viruses in this study. A previous publication demonstrated that anelloviruses were often codetected with redondoviruses [8]. Therefore, we propose the further screening of samples for redondoviruses

and anelloviruses to provide a better understanding of the interaction between redondoviruses and anelloviruses.

5. Conclusions

Our study adds to the growing body of knowledge regarding the epidemiological features and genetic diversity of the new *Redondoviridae* family. Importantly, we found no evidence of cross-species transmission between humans and their animal contacts. Whether redondoviruses are associated with respiratory or other infections in humans requires further research.

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