

An Exploratory Search for Novel Coronaviruses in Sarawak, Malaysia

by

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Thesis submitted in partial fulfillment of  
the requirements for the degree of  
Master of Science in the Duke Global Health Institute  
in the Graduate School of Duke University

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ABSTRACT

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## **Abstract**

Background: In recent years, emerging zoonotic microbes have gained more attention from the public and policy makers. Explosive outbreaks such as those due to avian influenza viruses, severe acute respiratory syndrome (SARS) virus, swine influenza viruses, Hendra virus, Nipah virus, and Middle East respiratory syndrome (MERS) coronavirus have had tremendous international economic and social impact. In particular, livestock workers have been found to be at increased infection risk and some of the first impacted by a novel pathogen. One of the main obstacles in averting outbreaks of novel microbes is detecting it when it first begins to cross species from animals to man and may not cause severe disease. Often routine diagnostics will fail to detect a new pathogen. The purpose of this research was to evaluate diagnostics for emerging coronaviruses that would be missed with routine diagnostics.

Methods: In 2016, I learned how to run new diagnostics adapted at Duke University to detect novel coronaviruses. I took this molecular technology to Sarawak, Malaysia, where I applied the assays against a panel of human clinical specimens from patients seen at three hospitals for respiratory illnesses. Our collaborators in Sarawak had previously examined these specimens with other assays against human coronaviruses but did not tell me of their results.

Results: In my hands, the new pan-species coronavirus assay detected only one coronavirus among 88 clinical specimens. After I finished my assay work, I learned from our collaborators that 27 of the 88 specimens had been positive for at least one previously recognized human coronavirus. Hence, the sensitivity of the new assay in

my hands was 3.70% (95% confidence interval 0% - 11.91%). However, the assay accurately showed negative results with a specificity of 100%

Conclusion: While this low sensitivity may have been real, it may also been influenced by a number of confounding factors such as specimen nucleic acid degradation with numerous freeze-thaw cycles, imprecise adaptation of an assay to new equipment in a new laboratory, or my or our collaborators' operator error. It is difficult to precisely identify the cause of the discordance. Nevertheless, I learned a great deal about global health in conducting this research in Sarawak and have chronicled some of these lessons in this report.

## **Dedication**

I would like to dedicate this thesis to my parents

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# 1. Introduction

Emerging infectious diseases are one of the most pressing global health challenges of our time (Jones 2008). An emerging infectious disease is one that is present in a population for the first time, one that is drastically increasing in incidence, or one that is affecting new geographic regions (Morse 2001). Approximately 25% of deaths globally occur because of emerging infectious diseases and these disproportionately burden low and middle income countries (WHO 2004). Zoonotic diseases, those that spread from animals (particularly wildlife) to humans, are considered the most prevalent of the emerging infectious diseases (Jones 2008).

## 1.1 *Emerging Infectious Disease*

The emergence of a particular infectious disease is influenced by biological, social, cultural, economic, and environmental factors that can make one region or population more likely to experience an outbreak or an epidemic (Jones 2008). A virus may newly emerge or re-emerge is when it undergoes genetic recombination or re-assortment. Environmental changes over time can influence pathogen emergence. Agricultural development can also provide conditions conducive to the pathogen emergence by increasing contact between humans and animals (Morse 2001). The example of pandemic influenza in China in 2009 illustrates how the two factors can combine to cause pathogen emergence. It became known that waterfowl were acting as reservoirs for influenza, interactions between ducks and pigs caused several pigs in swine farms to become infected. Once the virus entered the pig population, reassortment

occurred which allowed the virus to infect the farm workers interacting with the pigs eventually resulting in a pandemic event (Webster 1992).

Human actions such as migration and urbanization can also promote the spread of an emerging pathogen. Due to rapid migration in recent times, it is estimated that 65% percent of the world's population will live in an urban setting (UN 1991). Cities often contain several features that propagate disease transmission such as standing water and crowded conditions (Morse 2001).

Any combination of these factors can occur to provide to appropriate circumstances for pathogen emergence or re-emergence (Morens 2004). A change in the population of a host species can influence the emergence of an infectious agent. Zoonotic pathogens are particularly known for causing the emergence of a novel pathogen because they often infect a naïve population and change. Once the pathogen has adapted to a human, it can be transmitted person-to-person or form a reservoir in a different host organism (Morse 2001). One example of this which affects human populations in the North America is West Nile Virus (WNV). In this case the pathogen is spread via vector transmission. Though humans are considered "dead end" hosts, many species of infected mosquitos can act as vectors and infect several species of birds which can travel through large spans of land and spread the virus further into human populations (Morens 2004). It has also been found that alligators and chipmunks can act as amplifying reservoirs (Jacobson et al. 2005) (Platt et al. 2007).

## **1.2 Public health in Malaysia**

### **1.2.1 Background and Burden of Disease**

Malaysia is located in Southeast Asia and split into two regions- Peninsular Malaysia and Malaysian Borneo (Tee 2009). Its population, as of 2013, is roughly 30 million people (Malaysia Ministry of Health, 2014). There are several distinct ethnic groups in Malaysia consisting of Malay (50.1%), Chinese (22.6%), indigenous peoples (11.8%), Indian (6.7%), and other non-citizens (8.2%).

Since its independence in 1953, Malaysia has experienced rapid development which has impacted health outcomes for its citizens. This change corresponds to an increasingly sedentary lifestyle, a decrease in physical activity, and an increase in the availability high calorie foods and sugar-sweetened beverages (Davey 2013). According to the Global Burden of Disease the largest contributor to death in Malaysia is ischemic heart disease, accounting for 21% of all deaths. Additionally, there has been a 300% increase in obesity since 1990 to 2011 with no difference between urban and rural population (Davey 2013). Overall, the majority of deaths can be attributed to a non-communicable disease (NCD) (GBD 2015).

Southeast Asia has experienced several zoonotic and vector-borne viral diseases (Mackenzie 2001). Infectious diseases also contribute significantly to the burden of disease in Malaysia. The second largest contributor to annual deaths in Malaysia is lower respiratory infections (LRI), accounting for 11% of all deaths.

Currently, Malaysia faces a double burden of disease; where the burden of infectious diseases and non-communicable diseases (NCD) coexist. This creates a difficult public

health challenge which calls for a nuanced and evidence-based approach to the problem (Tee 2009).

### **1.2.2 Public Health Policies and Infrastructure**

Malaysia instituted a national health policy in 2006 (Malaysia Ministry of Health, 2014). The Malaysian Ministry of Health (MoH) is tax payer-funded and serves about 75% of the population (Malaysia Ministry of Health, 2014). The remaining 25% obtain medical care through the private sector (Malaysia Ministry of Health, 2014). The MoH in Malaysia receives about 4.3% of the total GDP for its budget. There are 141 public hospitals, 1039 health clinics, and 1821 community clinics. Meanwhile, the private sector has 214 hospitals and 6801 medical clinics (Malaysia Ministry of Health, 2014). There are 1.2 physicians for every 1000 people (CIA).

### **1.2.3 Infectious Disease Surveillance**

As discussed above, emerging infectious diseases are a significant public health burden. However, a well-developed and effective infectious disease surveillance system can be utilized to potentially avert and control epidemics and pandemics. Early detection is vital for controlling an outbreak. Successful early detection of an outbreak depends on well-developed surveillance infrastructure and rapid notification systems. Diagnostic laboratories are crucial for this success. Identifying the pathogen is essential in determining how to control the outbreak. Additionally, knowing the mode of transmission for the pathogen is critical for informed infection control measures. Crucial to the success of an infectious disease surveillance system is the presence of a national reference laboratory. A national reference laboratory provides essential training to staff

and ensures that other laboratories are maintaining quality standards. An infectious disease surveillance network must be well-integrated to properly achieve its goals. In the past, it has been difficult to conjoin efforts of diseases-specific laboratories, ministries of health, and research institutions. Synergy between these branches leads to more frequent early detection and better informed disease interventions. Additionally, integrated surveillance networks are more cost-effective than fragmented systems. Governments must invest in building capacity for infectious disease surveillance to protect their citizens from disease outbreaks (Chua 2013).

In 2005, the Malaysia MOH released a document outlining guidelines for lab-based infectious disease surveillance. The standardized procedure applies to all public and private hospitals, universities and labs that have culturing capabilities. The head of microbiology will report the pathogen identification to the Surveillance Section of Disease Control Division MOH using a standardized notification form. In conjunction with this reporting, the lab will also send the isolate(s) to a reference laboratory for further typing. The reference lab will type the isolate(s) and report the results to the Surveillance Section of Disease Control Division MOH. The Surveillance Section of Disease Control Division MOH is responsible for producing a weekly report that summarizes these results, particularly information regarding epidemiological trends. This report will be made available to relevant institutions to inform future interventions. Additionally, the Surveillance Section Division is also responsible for investigating potential outbreaks. The process of disease reporting is facilitated by an electronic disease notification system called LabSurv used by the MOH.

### **1.2.4 Previous epidemics in Malaysia**

The South East Asian region has faced several emerging threats in the form of epidemics such as highly pathogenic avian influenza (HPAI) H5N1 virus and severe acute respiratory syndrome (SARS) coronavirus (Tee 2009). Their prevalence in the region makes it essential for pathogen discovery initiatives to exist so that they can be used to inform future preventative measures. A combination of social, ecological, and environmental factors makes Malaysia vulnerable to the threat of emerging infectious diseases (Tee 2009). To avoid and better prepare for future epidemics, it is essential to understand the prevalence and burden of infectious agents such as respiratory viruses. A consistent problem facing Malaysian residents is dengue, a mosquito-borne illness, which is endemic to the country and responsible for a number of outbreaks (Mackenzie 2001). Rapid urbanization has proved to be conducive to the spread of dengue. Standing water, which is a breeding ground for mosquitoes, is more common in urban areas. *Aedes aegypti* is responsible for spreading dengue in urban areas while *Aedes albopictus* spreads dengue in peri-urban areas (Chew 2012). Chikungunya is also responsible for outbreaks and, in fact, is responsible for the first recorded outbreak in Malaysia. The outbreak occurred in the densely populated city of Kuala Lumpur (Mackenzie 2001).

Nipah virus emerged in northern peninsular Malaysia in 1998 causing disastrous results for human and animal health along with negative economic outcomes. Over the course of the next year, the disease spread to the rest of peninsular Malaysia. The outbreak only ended in Malaysia when 1 million pigs were culled but not before 105

people died from the disease. A novel nipah virus was found to be responsible for the outbreak. There are 13 different species of fruit bats found in Malaysia and they are also the host organism for this virus (Mackenzie 2001). Deforestation has resulted in a loss of their natural habitat causing them to interact increasingly with humans in urban populations. This change in behavior is partly responsible for the outbreak (Kaw Bing 2002). Later studies found that the most potent risk factor for nipah virus infection was direct contact with infected pigs (Chew 2000). One lesson to be learned from this outbreak is that animals should be included in disease surveillance as potential reservoirs for pathogens (Kaw Bing 2002). The nipah virus outbreak provides a useful case study for why pathogen discovery initiatives are vital to public health security. Particularly zoonotic viruses, which can lay unnoticed in animal population but cause significant damages to health once they enter a human population. Incorrect diagnosis of the pathogen's identity wasted time, resources, and cost human lives. Timely identification would have averted these unnecessary costs. Lastly, the 1998 Nipah virus outbreak in Malaysia highlights the anthropogenic nature of emerging infectious diseases and how human actions can cause pathogen emergence.

### ***1.3 Coronavirus epidemiology globally and in Malaysia***

Coronaviruses are a single-stranded, positive sense, RNA virus. They are divided into four subgroups; alphacoronavirus, betacoronavirus, deltacoronavirus, and gammacoronavirus. Coronaviruses are known for the glycoproteins ("spikes") that occupy the outer surface of the virus. Structural proteins include spike (S), envelope (E), membrane (M) and nucleocapsid (N). Receptors of glycoprotein are highly diverse

among genera and species. The coronavirus genome is one of the largest known RNA viruses (27 to 31.5 kb) and is polycistronic. This large genome is able to be maintained with relatively few reading errors by the presence of the exoribonuclease function which provides proofreading for the genome.

Coronaviruses typically infect the upper respiratory tract and digestive tract. They usually cause mild respiratory infections and gastroenteritis but can also cause neurological diseases (Zhang et al. 2015). They are widely present in human and animal populations (de Wit 2016). Malaysia is known to have naturally occurring feline coronavirus (FCoV) type I and II in domestic cats (Amer et al. 2012).

### **1.3.1 Human Coronaviruses (HCoV)**

Human coronaviruses circulate globally and are responsible for about 10% of all respiratory tract infections (Dijkman et al 2012). There are six known human coronaviruses (HCoV); OC43, HKU1, SARS-CoV, MERS-CoV, NL63, and 229E. Human coronaviruses fall into two categories of coronavirus- alpha- and betacoronavirus. Alphacoronaviruses include NL63 and 229E, while betacoronaviruses include OC43, HKU1, SARS-CoV, and MERS-CoV.

HCoV OC43 is the most common coronavirus and known to cause lower respiratory tract infections in children and adults. Typically OC43 is responsible for respiratory illnesses but one case showed evidence of the HCoV OC43 being present in the brain tissue of a child with fatal encephalitis (Morfopoulou et al. 2016). This particular strain also has the potential to cause outbreaks as it was responsible for one in France (Vabret et al 2003). OC43 has four known genotypes; A, B, C, and D. These are based on the S, N,

and RNA dependent RNA polymerase genes. There is some evidence to suggest that a new genotype, genotype E, is arising via recombination. Both OC43 and NL63 occur more frequently in children and are thought to potentially create an immunity that protects against subsequent 229E and HKU infection (Dijkman et al 2012). Despite being a human coronavirus, HCoV 229E is able to easily exchange genetic material with viruses that infect bats and alpacas. This information suggests that there is a similar evolutionary history between 229E and MERS-CoV (Corman et al. 2015).

### ***1.3.2 Severe Acute Respiratory Syndrome (SARS)***

In 2003, the discovery of severe acute respiratory syndrome (SARS) occurred in the midst of a pandemic in Asia. The first was found to be in Fushan, China in 2002 (de Wit 2016). SARS coronavirus (SARS-CoV) was identified as the pathogen responsible for the pathogen in July 2003 and during this time there were over 8000 reported cases leading to over 700 deaths in 27 countries (de Wit 2016). The first ever detection of the virus was in masked palm civets and raccoon dogs in a live animal market in China. However, these hosts were not responsible for much further transmission (de Wit 2016). The virus was thought to jump from the palm civets and raccoon dogs to humans through zoonotic transmission (de Wit 2016). Bats are potential reservoirs for this virus but it was largely human-to-human transmission via nosocomial transmission that was responsible for the pandemic (de Wit 2016). In 2003, the spread of SARS could be partially attributed to nosocomial transmission where 33% of all infected cases were healthcare workers (de Wit 2016). Transmission is high in healthcare settings because virus shedding occurs at the onset of symptoms- when people start to seek medical

attention. The virus can also remain viable on surfaces in medical care settings even after patients have received treatment. Additionally, families of patients are also responsible for about 22%-39% of SARS-CoV cases (de Wit 2016).

Symptoms of SARS include fever, body aches, and flu-like symptoms such as coughing and sneezing. Treatment of SARS sometimes involved a combination of the antiviral drug Ribavirin and corticosteroids. However, the efficacy of this treatment is unclear as there was no clinical trial done to assess it.

### ***1.3.3 Middle East respiratory syndrome coronavirus (MERS-CoV)***

Approximately 10 years after the SARS-CoV pandemic, Middle East respiratory syndrome coronavirus (MERS-CoV) was found to be responsible for a death in Saudi Arabia (de Wit 2016). Upon this discovery, several cases which occurred earlier that year in Jordan were retroactively diagnosed as MERS-CoV (de Wit 2016). Knowing that, similar to SARS-CoV, bats can act as reservoirs for MERS-CoV the focus of researchers was to understand the relationship between bats and the spread of MERS-CoV (de Wit 2016). However, several serological studies in Qatar, Oman, and Saudi Arabia showed that dromedary camels had either antibodies against MERS-CoV, RNA material of MERS-CoV, or infectious virus (de Wit 2016). In the Middle East, interactions between humans and camels are far greater due to commercial farms than interaction between humans and bats (de Wit 2016). Thus it was found that dromedary camels are a much more likely reservoir for MERS-CoV (de Wit 2016). Later evidence suggests that an ancestral strain of MERS-CoV crossed over from bats to dromedary camel populations

about 30 years ago. After this, the virus was widely circulated in camel populations giving it easy access to human populations.

For reasons similar to SARS-CoV, much of human-to-human transmission of MERS-CoV can be attributed to nosocomial transmission (de Wit 2016). However, this mostly occurred from patient to patient rather than patient to healthcare worker where 62%-79% of all cases was patient to patient transmission (de Wit 2016). Additionally, 13%-21% of transmission cases occurred between patients and family members (de Wit 2016).

Lessons learned from the SARS pandemic in Asia were clearly still useful at the onset of the Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak. The pathogen was identified before it could into a pandemic of the same proportion as SARS (de Wit 2016). This is largely because the full genome of the virus was known allowing diagnostic assays to be quickly developed and distributed (de Wit 2016). Several steps were taken which allowed timely intervention to limit the proliferation of the virus. These steps included understanding appropriate animal models, treatment efficacy studies, and reservoir identification (de Wit 2016). There are some technologies that show promise for prevention of zoonotic transmission to humans. One such technology is a vaccine that expresses the MERS-CoV spike protein, it was found to potentially limit the viral shedding, thus potentially reducing transmission within animal populations and between human populations (de Wit 2016).

The occurrence of the SARS and MERS outbreaks highlighted the deficiencies of public health preparedness measures. It is clear to see that the process for clinical trials

and development of diagnostic and therapeutic tools needs to be sped up. Additionally, it is important for infectious disease surveillance data to be widely spread and made available to promote collaborative efforts in stopping the spread of disease. Lastly, it is essential for the global health community to increase epidemiological understanding of infectious diseases such as modes of transmission and host and reservoir identification. This understanding can be achieved by supporting pathogen discovery initiatives, they provide crucial, foundational knowledge which can be used to be better equipped for future outbreaks.

#### **1.3.4 Animal Coronavirus**

The ubiquitous nature of coronaviruses means that they can be found in a large variety of organisms acting as hosts or reservoirs. Along with being a significant veterinary health issue, coronaviruses also have an impact on agricultural industries around the world. Notable examples of animals infected by coronavirus are bats and pigs.

Bats lend themselves to be competent reservoirs for several viruses including Nipah virus, Hendra virus, and coronavirus. There are several physiological factors that make bats an appropriate host. First, their long evolutionary history means that they have co-evolved with several viruses in tandem. During periods of hibernation, their metabolic rate slows down and their immune system responses are suppressed. This suppression allows the virus to successfully proliferate within the bat. Additionally, bats also do not have B-cell mediated immune responses so they show no symptoms of infection while

carrying the virus. A lack of symptoms, their ability to fly, and their close proximity to other bats in large colonies make them excellent vehicles for virus transmission (Omrani et al 2015). As discussed above, bats act as reservoirs for MERS-CoV and SARS-CoV, but there are other coronaviruses that can infect bats. New evidence suggests that a new coronavirus- NeoCoV. This virus is a betacoronavirus that is different from MERS-CoV by only one amino acid (Omrani et al 2015).

Pigs are another prominent animal host and reservoir of coronavirus. Their agricultural and economic significance makes swine coronavirus burden a top priority for the global health community and the agricultural industry alike. One such virus is porcine epidemic diarrhea virus (PEDV)- a betacoronavirus. It causes vomiting, watery diarrhea, weight loss and severe enteritis in pigs of all ages. This virus has a high mortality rate, especially among piglets, causing drastically negative economic outcomes. In 2010, there was an outbreak of PEDV and several provinces in China were affected by this virus.

Adult and young pigs were affected alike and 100% of suckling piglets were found to be ill. A similar outbreak occurred in the United States in 2014, affecting 23 states with over 2500 reported cases. This outbreak, which disproportionately affected piglets, was responsible for significant economic damages to the swine farming industry (Wang 2014). It was later found that PorCoV HKU15, a deltacoronavirus, was also found to be complicit in the outbreak which occurred in pig farms in Ohio in 2014 (Wang et al. 2014). This was the first time this particular virus was associated with any clinical symptoms in pigs (Wang et al. 2014). Research suggests that that this virus is present among all pig populations in major pig-producing states in the U.S., therefore, more research needs to

be done to fully understand the human and animal health implications of this virus (Wang et al. 2014).

#### **1.4 Molecular Procedures**

The procedure used in this study is a reverse transcriptase polymerase-chain-reaction (RT-PCR). This is a molecular assay which is able to convert extracted RNA into complimentary DNA (cDNA) for the purpose of detecting gene expression of a particular pathogen. The Saif protocol employs a conventional RT-PCR method while the Perera assay uses a real-time RT-PCR method. A real-time RT-PCR allows the user monitor amplification of the DNA as the procedure is happening rather than waiting for the procedure to finish to get the results. Typically, a real-time RT-PCR is thought to be a more sensitive procedure as it requires a smaller amount of nucleotides for detection.

#### **1.5 Study Aims**

The purpose of this study was to evaluate a pan-species conventional RT-PCR molecular assay whose purpose was to detect the presence of any coronavirus in a sample. The assay was adapted from a paper published by Dr. Linda Saif in 2011 (Vlasova et al. 2011). This assay, henceforth referred to as the “Saif assay”, would be able to detect both human and animal coronavirus. The results of the assay were validated against the molecular results from a real-time assay adapted by Dr. Perera at UNIMAS, henceforth referred to as the “Perera assay”. From August 2015 to May 2016, the researcher was trained in One Health laboratory techniques including how to use the Saif assay. Additionally, from my year-long research assistantship at Duke One Health

Research Laboratory, the assay used in Malaysia was a validated protocol using standard operating procedure in the Duke One Health lab. Working in Sarawak, Malaysia required ordering necessary reagents from a Singaporean chemical company which took several weeks to arrive. Positive controls were received from the Duke One Health Laboratory in Singapore at Duke-NUS. Upon their arrival, the assay was adapted to the equipment available to me in the lab as well as to the general layout of the lab. Every effort was made to ensure that the procedures could be followed as expected. The assay was effective in the lab in Sarawak because the positive controls showed up as bright bands on the gel image taken after the assay was completed. The study took place in Universiti Malaysia Sarawak (UNIMAS) in Kota Samarahan, Malaysia under the supervision of Dr. David Perera. Dr. Perera is a Doctor of Philosophy in Medical Biotechnology and the principle investigator on several projects at UNIMAS. He was consulted many times during this study for his technical expertise.

## **2. Methods**

For a 3-year period (from November 2012 to October 2015) samples were collected from 3 hospitals in Sarawak, Malaysia- Sarawak General Hospital, Bintulu General Hospital, and Sibul General Hospital. Samples were later processed at Universiti Malaysia Sarawak (UNIMAS) in the Institute for Health and Community Medicine. Patients under the age of 5 exhibiting acute respiratory tract infection (ARTI) symptoms were recruited. Genetic material (RNA) of any virus present was extracted from the primary samples. All subsequent fieldwork assays were completed on the extracted RNA samples.

The samples size consisted of 88 nasopharyngeal aspirates (NPA), nasopharyngeal swabs (NS) and/or endotracheal tube secretions (ETT) samples that were putatively positive for the presence of coronavirus using a real-time RT-PCR assay designed to detect human coronaviruses. Of these 88 samples, 27 were putatively positive for coronavirus, the rest were positive for other respiratory viruses. These 88 samples were chosen because their initial results were not conclusive until sequencing was done to confirm the identity of the pathogen. In running the Saif assay against the panel of 88 human specimens this study sought to see how this conventional RT-PCR assay compared with the Perera real-time assay and to see if we could detect novel or animal-reservoir coronaviruses in the human specimens.

### **2.1 Procedures**

The RT-PCR procedure used by Dr. Perera and his team was based on a paper by Buecher et al. using a Qiagen OneStep RT-PCR kit. The original primer used by Dr.

Perera and team at UNIMAS was developed from a highly conserved region in coronavirus- the *pol* genes found in the GenBank database (Buecher et al. 2005). The PCR cycling conditions were as follows: denaturation occurred at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds. Annealing occurred at 50°C for 30 seconds and extension occurred at 60°C for 2 minutes.

For my research, the RT-PCR was conducted using a Superscript® III One Step RT-PCR System with Platinum® Taq DNA Polymerase and a BIORAD instrument.

Conventional RT-PCR procedure was followed according to the Saif assay. This pan-species assay targets a 390 bp fragment of the nucleoprotein gene present in all coronaviruses (Vlasova et al. 2011) (Vijgen et al. 2008). The extracted RNA products were incubated at 42°C for 30 minutes. The products were preheated for 5 minutes at 94°C. 35 cycles were done at 94°C for 1 minute, then 50°C for 1 minute, and 72°C for 1 minute. Extension of PCR products was done at 72°C for 7 minutes. This was done 48 samples at a time in PCR tubes with a total volume of 25 µL. When running the samples through the RT-PCR assay a human and porcine positive control was included along with a negative control.

After the RT-PCR procedure was done a gel electrophoresis as conducted to see if any of the samples contained human or porcine coronavirus. PCR products were visualized on a 2% agarose gel.

A positive result was considered to be any gel image showing a bright band of a similar molecular weight and breadth as the positive control. Positive results led to the sample being extracted and purified from the gel. Once the sample was extracted, the

nucleic acid was sequenced. The resulting sequence was inputted into the BLAST search engine to further confirm the identity of the virus present in the sample.

## **2.2 Differences in Methodology**

One notable difference between the two assays was that the Perera team assay used a real-time RT-PCR assay whereas the Saif assay was a conventional RT-PCR assay. Real-time assays are generally thought to be able to detect fewer nucleic acid target molecules compared to conventional RT-PCR assays. Also, different conditions were used for denaturing, annealing, and extension. Additionally, the Perera team's assay used 40 cycles for denaturation whereas the assay used for this project used 35 cycles. The primer used by Perera and team targeted 440-bp fragment of the RNA-dependent RNA polymerase (*pol*) genes whereas the primer used in this project targeted the polymerase region and 390 bp fragment of the nucleoprotein (N) gene (Vlasova et al. 2011). Lastly, it is important to note that both assays were conducted successfully in their respective labs.

### 3. Results

Of the 88 samples tested, one sample produced a bright band (sample R535). The sample was extracted from the gel (Figure 1a and 1b) and the purity was tested using a Thermo Scientific NanoDrop 2000. The purity was found to be 1.6uL. The sample was subsequently sequenced and shown below:

```
TGGGATTACCCTAAGTTTTTGTCTGCTATGCCAAACATACTACGTATTGTTAGTA
GTTTGGTATTAGCCCGAAAACATGAGACATGTTGTTTCGCAAAGCGATAGGTTTTA
TCGACTTGCGAATGAATGCGCACAAGTTTTGAGTGAAATTGTTATGTGTGGtGGC
TGTTATTATGTTAAGCCTGGtGGCACTAGTAGtGGTGAtGCAACTACTGCTTTTGCT
AATTCAGTCTTTAACATATGTCAAGCTGTTTCAGCCAAtGTATGTGCCTTAaTGTCa
TGCAATGGCAATAAGATTGAARATCTTARTATACGTGCTCTTCAGAAGCGCTTAT
ACTCACATGKGTATAGAATGATAARGTTGATTCAACCTTTGTCACAGAATATTAT
GAATTTTTAAATAAGCATTTTAGTATGATGATTTTGAGTGACGACACCGTTGTCT
GCTAA
```

By employing BLAST, the identity of the virus was confirmed **to be human coronavirus OC43**. The BLAST (figure 2) search results can be found below. Table 1 below shows all 88 samples tested, including the 27 supposedly positive results.

The sensitivity of the assay used in this project was found to be 3.70% (0%,11.91%). Of the 27 positive samples tested, one true positive was found. The specificity of the assay was 100%

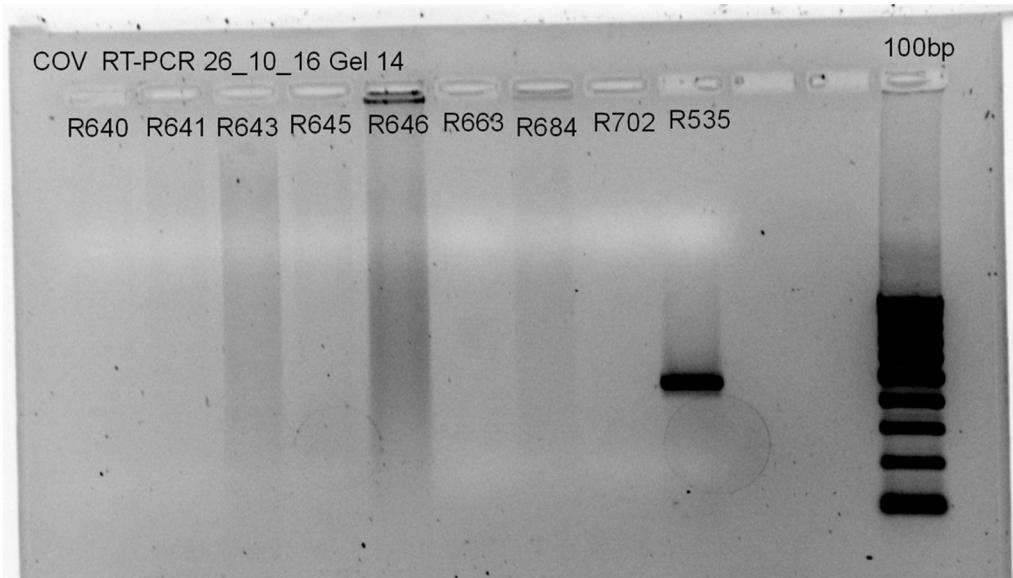


Figure 1a: Positive Result Gel Image

This figure shows the positive result for sample R535 as a bright band on the gel image.

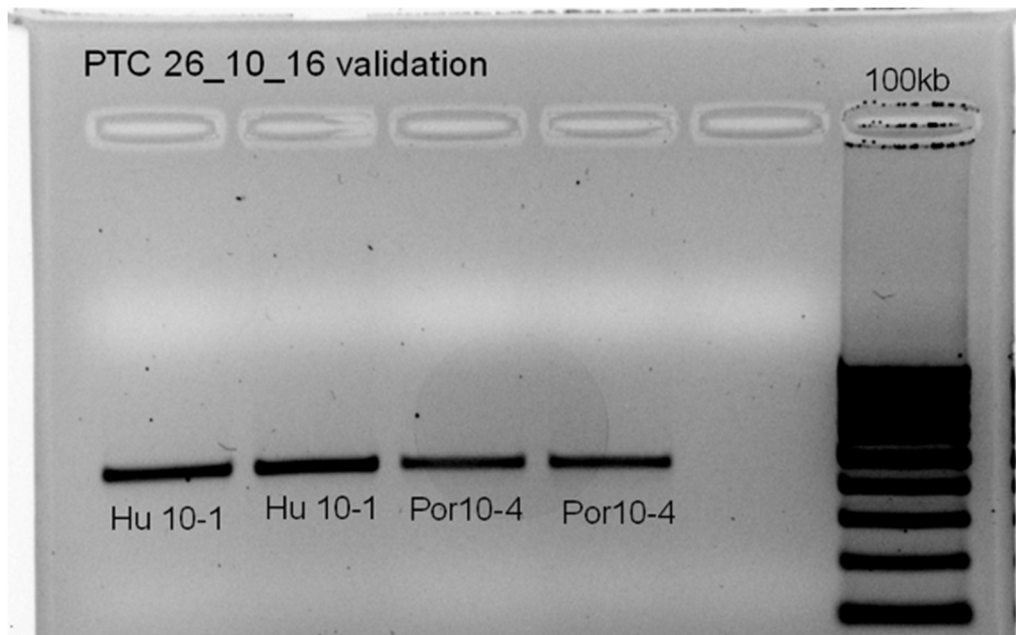


Figure 1b: Positive Control Gel Image

This figure shows that the four positive controls, made from the same mastermix, had successfully appeared on the gel image. This result further validates the positive result for sample R535. The positive controls are DNA positive oligo based on the primer alignment and the reference strain sequences. Porcine positive control is sourced from NR-43286 Porcine respiratory coronavirus -- ISU-1. The human positive control is sourced from NR-470 Human Coronavirus NL63.

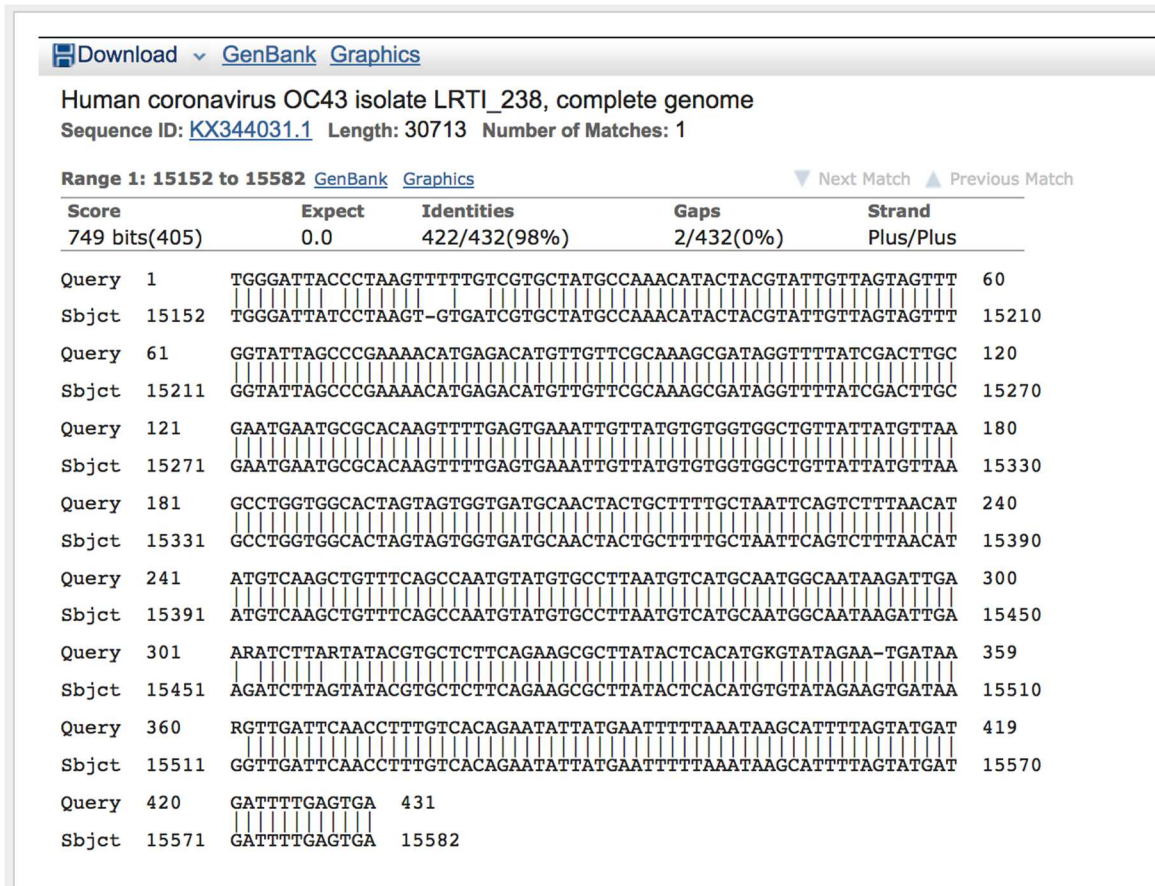


Figure 2: Blast Search Result Image

Specimen R535's sequence had a 98% match for the identity score with human coronavirus OC43.

Table 1: List of Samples Tested

Results of previous molecular assay work performed by Dr. Perera and team. The following 88 samples were tested in a blinded fashion using the pan-species RT-PCR assay against all human and animal coronavirus to potentially find evidence of new coronavirus and to further validate the positive coronavirus results.

Sample No.	Hospital	Sex	Age	Date taken	Sample type	Clinical Diagnosis	Multiplex PCR (1st Def)
R31/1	Bintulu	M	4 mth 3 weeks	21-Nov-12	NS	Pneumonia	RSV
R34/1	SGH	M	1 yr 2 mths	28-Nov-12	NS	Acute Bronchiolitis	HCoV-NL63
R38/1	Sibu	F	5 mths	17-Nov-12	NS	Acute Bronchiolitis	Neg
R42/1	Sibu	F	1 yr 6 mths	26-Nov-12	NS	Pneumonia	HBoV
R74/1	Bintulu	F	2 mths 20 days	22-Dec-12	NS	Pneumonia	AdV, RSV, HCoV-NL63
R86/1	Bintulu	M	8 mths	Dec-12	NS	Acute Bronchiolitis	AdV, RSV
R114/1	Sibu	M	10 mths	14-Dec-12	NS	Pneumonia	HRV, HCoV-NL63
R124/1	Sibu	F	2 yrs	30-Dec-12	NS	Pneumonia	AdV
R127/1	SGH	M	5 mths	17-Jan-13	NS	Pneumonia	RSV, PIV 4
R128/1	SGH	F	4 yrs old	21-Jan-13	NS	Pneumonia	Neg
R131/1	Bintulu	F	5 yrs	17-Jan-13	NS	Pneumonia	Neg
R163/1	Sibu	F	10 mths	15-Jan-13	NS	Pneumonia	AdV, RSV
R170/1	Bintulu	F	11 mths	Jan-13	NS	acute exacerbation of bronchial asthma secondary to RTI	Neg
R171/1	Bintulu	F	4 yr 5 mths	23-Jan-13	NS	Pneumonia	RSV
R172/1	Bintulu	F	3 mths	23-Jan-13	NS	Pneumonia	RSV

R173/1	Bintulu	M	1 mth 18 days	Jan-13	NS	Acute Bronchiolitis	RSV
R182/1	SGH	M	2 yrs 5 mths	04-Feb-13	NS	Acute exacerbation of bronchial asthma sec to RTI	HRV
R223/1	Bintulu	F	2 yr 1 mth	06-Mar-13	NS	Acute exacerbation of bronchial asthma sec to RTI	AdV
R227/1	Bintulu	M	4 mths	10-Mar-13	NS	pneumonia	AdV, HRV
R241/1	SGH	F	10 mths	01-Apr-13	NS	Pneumonia	HMPV
R250/1	SGH	M	10 mths	08-Apr-13	NS	Pneumonia	HCoV_229E
R299/1	Sibu	F	2 mths	12-Mar-13	NS	Pneumonia	RSV, HCoV-229E
R304/1	Sibu	F	2 yr	19-Mar-13	NS	Pneumonia	HCoV-OC43
R323/1	Bintulu	M	1 yr 5 mths	03-May-13	NS	Pneumonia	Neg
R324/1	Bintulu	F	1 mth 4 d	05-May-13	NS	Pneumonia	HCoV_229E
R329/1	SGH	F	1 yr 9 mths	13-May-13	NS	Viral Wheeze	AdV
R330/1	SGH	M	1 yr 7 mths	13-May-13	NS	Acute exacerbation of Bronchial Asthma with concurrent RTI	AdV
R331/1	SGH	M	3 yr 3 mths	May-13	NS	Pneumonia	PIV 3
R332/1	SGH	F	2 yrs 5 mths	16-May-13	NS	Pneumonia	AdV
R336/1-R	SGH	M	1 yr 6 mths	21-May-13	NS	Pneumonia	AdV
R337/1-R	SGH	M	3 yrs 7 mths	20-May-13	NS	Pneumonia	AdV
R339/1	SGH	F	1 yr 3 mths	28-May-13	NS	Pneumonia	AdV

R340/1	SGH	M	1 yr 4 mths	30-May-13	NS	Pneumonia	Neg
R341/1	SGH	M	2 yrs 7 mths	05-Jun-13	NS	Pneumonia	AdV
R342/1	SGH	M	1 yr 7 mths	06-Jun-13	NS	Pneumonia	AdV
R343/1	Bintulu	M	1 yr 2 mths	26-May-13	NS	Pneumonia	RSV
R344/1	Bintulu	F	1 yr 9 mth	Jun-13	NS	Pneumonia	AdV
R350/1	Sibu	M	10 mth	25-Apr-13	NS	Acute Bronchiolitis	RSV
R351/1	Sibu	F	5 mth	21-Apr-13	NS	Pneumonia	RSV
R352/1	Sibu	F	5 yr	19-Apr-13	NS	Pneumonia	AdV
R353/1	Sibu	F	4 yr	19-Apr-13	NS	Pneumonia	RSV
R358/1	Sibu	F	1mth	16-Apr-13	NS	Pneumonia	RSV
R366/1	Sibu	M	1 yr	25-Mar-13	NS	Pneumonia	RSV
R367/1	Sibu	F	1 mth	24-Mar-13	NS	Pneumonia	RSV
R368/1	Sibu	F	1 yr 11mths	20-Mar-13	NS	Pneumonia	RSV
R369/1	Sibu	M	6 mth	20-Mar-13	NS	Acute Bronchiolitis	Neg
R370/1	Sibu	F	10 mth	20-Mar-13	NS	Acute Bronchiolitis	AdV, PIV 3
R371/1	Sibu	M	4 yr 11 mth	20-Mar-13	NS	Acute exacerbation of bronchial asthma sec to RTI	PIV 3
R372/1	Sibu	F	1 yr 9 mth	19-Mar-13	NS	Acute Bronchiolitis	AdV
R373/1	Sibu	M	1 yr 2 mths	08-Mar-13	NS	Newly diagnosed bronchial asthma with concurrent RTI	RSV
R374/1	Sibu	M	7 mth	17-Feb-13	NS	Pneumonia	Neg
R375/1	Sibu	F	7 mth	15-Feb-13	NS	Pneumonia	RSV

R376/1	Sibu	M	1 yr 7 mths	13-Feb-13	NS	Pneumonia	RSV
R377/1	Sibu	M	1 yr	Jun-13	ETT	Pneumonia	AdV
R380/1	Sibu	M	6 mth	Jun-13	NS	Pneumonia	Neg
R381/1	Sibu	F	1 yr 6 mths	07-Jun-13	NS	Pneumonia	Neg
R384/1	Bintulu	F	1 yr 5 mths	10-Jun-13	NS	Pneumonia	AdV
R385/1	Bintulu	M	9 mth 22 days	10-Jun-13	NS	Acute exacerbation of bronchial asthma sec to RTI	AdV
R387/1	Sibu	M	7 mths	06-May-13	NS	Pneumonia	HRV
R391/1	Sibu	F	4 yr 1 mth	17-May-13	NS	Pneumonia	Neg
R396/1	Sibu	F	1 yr 9 mths	12-May-13	NS	Pneumonia	AdV, HRV, HCoV-229E
R426/3	Bintulu	M	7 mth	22-Jul-13	NS	Pneumonia	HCoV-OC43
R440/1	Sibu	F	2 yr 8 mths	11-Jun-13	NS	Pneumonia	HCoV-229E
R512/1	Sibu	F	9 mth	1-Jul-13	NS	Pneumonia	AdV
R513/1	Sibu	M	1 yr 6 mths	1-Jul-13	NS	Pneumonia	AdV, HRV, RSV
R514/1	Sibu	M	1 yr 1 mth	9-Jul-13	NS	Pneumonia	AdV, PIV 3, IAV
R515/1	Sibu	F	2 yr 3 mth	11-Jul-13	NS	Pneumonia	AdV, HMPV
R516/1	Sibu	F	3 yr 1 mth	13-Jul-13	NS	Pneumonia	AdV
R517/1	Sibu	M	1 yr 3 mth	14-Jul-13	NS	Pneumonia	HRV
R521/1	Sibu	M	8 mths	19-Jul-13	NS	Pneumonia	AdV, HCoV-OC43, HRV
R535/1	Bintulu	M	2 yr 2 mth	3-Oct-13	NS	Pneumonia	HCoV-OC43

R624/1	Bintulu	M	9 mth	29-Jan-14	NS	Newly diagnosed bronchial asthma with concurrent RTI	AdV, RSV
R625/1	Bintulu	M	10 mth	30-Jan-14	NS	Pneumonia	RSV
R626/1	Bintulu	F	9 mth	30-Jan-14	NS	Pneumonia	AdV, RSV
R627/1	Bintulu	M	1 yr 1 mth	3-Feb-14	NS	Pneumonia	AdV, RSV
R628/1	Bintulu	M	6 mth	3-Feb-14	NS	Pneumonia	RSV
R629/1	Bintulu	M	1 yr 2 mths	3-Feb-14	NS	Acute Exacerbation of bronchial asthma secondary to RTI	RSV
R630/1	Bintulu	M	2 yr 5 mth	4-Feb-14	NS	Pneumonia	Neg
R631/1	SGH	F	1 yr 10 mths	6-Feb-14	NS	Pneumonia	RSV
R632/1	SGH	M	1 yr 3 mths	10-Feb-14	NS	Acute exacerbation of bronchial asthma sec to RTI	IBV
R636/1	Bintulu	F	1 yr 8 mths	6-Feb-14	NS	Pneumonia	RSV
R640/1	Bintulu	M	1yr 1 mth	6-Feb-14	NS	Pneumonia	RSV
R641/1	Bintulu	M	10 mth	13-Feb-14	NS	Pneumonia	RSV
R643/1	Bintulu	M	2 mth	14-Feb-14	NS	Pneumonia	RSV
R645/1	Bintulu	M	7 mth	17-Feb-14	NS	Acute Bronchiolitis	HMPV
R646/1	Bintulu	M	10 mth	18-Feb-14	NS	Pneumonia	IBV
R663/1	Sibu	F	4mth	4-Feb-14	NS	Pneumonia	HCoV-OC43
R684/1	Sibu	F	4 yr	4-Mar-14	NS	Pneumonia	RSV
R702/1	Sibu	M	1yr 1 mth	28-Nov-13	NS	Pneumonia	neg
R703/1	Sibu	M	5 mth	3-Dec-13	NS	Acute Bronchiolitis	HMPV, PIV 1
R704/1	Sibu	F	4yr	8-Dec-13	NS	Pneumonia	AdV, RSV
R705/1	Sibu	F	1yr 2 mths	11-Dec-13	NS	Pneumonia	Neg

R706/1	Sibu	F	2yr 3 mth	13-Dec-13	NS	Pneumonia	AdV, RSV
R750/1	SGH	F	10 mth	30-Jul-14	NS	Viral Croup	HCoV_229E, PIV 1
R792/1	Sibu	M	1 mth	21-Apr-14	NS	Pneumonia	RSV
R794/1	Sibu	M	1yr 1mth	29-Apr-14	NS	Pneumonia	Neg
R797/1	Sibu	M	3 mths	5-May-14	NS	Pneumonia	RSV
R798/1	Sibu	M	7 mths	5-May-14	NS	Pneumonia	PIV 1
R799/1	Sibu	F	6 mths	1-May-14	NS	Pneumonia	PIV 3, ADV
R813/1	Sibu	M	1 yr 1 mth	16-Jun-14	NS	Pneumonia	Neg
R816/1	Sibu	M	2 yr 6 mths	24-Jun-14	NS	Pneumonia	RSV
R821/1	Sibu	F	1 yr 2 mths	18-Jul-14	NS	Pneumonia	RSV
R826/1	Sibu	F	2 mth	4-Aug-14	NS	Pneumonia	RSV, HCoV-229E
R847/1	Bintulu	M	1 yr 13 day	1-Dec-14	NS	Pneumonia	RSV, HCoV-OC43
R858/1	SGH	M	4 mth 23 days	14-Jan-15	NS	Pneumonia , TRO Pentussis	HCoV-OC43
R920/1	Bintulu	M	1 mth 2 weeks	21-Jan-15	NS	Pneumonia	HCoV-OC43
R996/1	Bintulu	M	9 mth	11-Jun-15	NS	Pneumonia	RSV, PIV 1, AdV
R1003/1	Bintulu	M	4 mth	22-Jun-15	NS	Pneumonia	HCoV-NL63
R1008/1	Bintulu	M	3 mth 25 days	28-Jun-15	NS	Pneumonia	RSV, HCoV-OC43
R1100/1	Bintulu	F	1 yr 1 mth	30-Jul-15	NS	Acute exacerbation of bronchial asthma sec to RTI	RSV, HCoV-229E
R1112/1	SGH	M	1 yr 17 day	26-Jul-15	NS	Pneumonia	HCoV-229E, HboV, AdV, RSV

R1123/1	Bintulu	M	6 weeks	18-Aug-15	NS	Pneumonia	HRV, HcoV-NL63, AdV
R1142/1	SGH	M	1 yr 1 mth	31-Aug-15	NS	Acute Bronchiolitis	RSV, PIV 3, HRV, HCoV-NL63
R1143/1	SGH	F	6 mth	4-Sep-15	NS	Pneumonia	RSV, PIV 3, HcoV-NL63
R1152/1	Bintulu	F	1 yr 11 mths	21-Sep-15	NS	Pneumonia	HCoV-OC43
R1183/1	Sibu	F	2 yr 3 mth	20-Jul-15	NS	Pneumonia	RSV, HCoV-NL63, AdV

Legend: ADV: Adenovirus RSV: Respiratory syncytial virus HCoV: Human Coronavirus Neg: Negative for any virus  
HBoV: Human bocavirus HRV: Rhinovirus PIV: Human parainfluenza viruses HMPV: Human  
metapneumovirus

## 4. Discussion

Studying zoonotic transmission of viruses is particularly important because it accounts for a significant portion of emerging pathogens. Coronaviruses are also known to move from wildlife populations to livestock populations *and sometimes to humans*. It is particularly important to search for novel coronaviruses because they have the potential to cause catastrophic outbreaks. Coronaviruses have been responsible for several outbreaks such as the SARS-CoV outbreak in 2002. These outbreaks can have devastating effects on human and animal health and cause significant economic losses. In my study of 88 human specimens, one sample was found to be positive for human coronavirus OC43. The assay had a low level of sensitivity which could be explained by a number of factors. Future studies could potentially examine patients experiencing acute respiratory tract infection (ARTI) who are known to have contact with animals. Despite these results, it is imperative to continue such exploratory studies due to the public health relevance discussed earlier.

Potential flaws in the way the project was conducted could be responsible for the results described above. First, since the specimens were archived, they were frozen and thawed multiple times. Continuous changes in temperature may have degraded the structural integrity of the RNA in the samples, thus leading to false negative results. Despite using a PCR room to prepare the mastermix with no introduction of inhibitors, it is possible that some trace amounts of inhibitors may have unintentionally contaminated the mastermix. Contamination would have given false negative test results and decreased the specificity of the assay. Positive and negative controls were used consistently throughout the experiment and validated before starting and at several points during the experiment.

One aspect of the study which could have been improved was to target patients exhibiting signs of ARTI who were known to come into contact with animals. This would have potentially shown more zoonotic transmission of respiratory viruses. Another way to strengthen the study would have been to use a real-time RT-PCR which is considered to be the gold standard method for nucleic acid measurement (Schmittgen et al. 2008).

Living and working in Sarawak, Malaysia showed me that public health was one of the biggest priorities of the government and the general public. This prioritization was evidenced by government-sponsored advertisements encouraging citizens to limit their salt and sugar intake when preparing food. Additionally, there were several free events promoting physical activity for people of all ages promoted on the radio. These radio ads emphasized the physical and mental health benefits of physical activities such as yoga and Zumba. It was also interesting to see children with apparent cases of hand, foot, and mouth disease in markets. In Malaysia, hand, foot, and mouth disease is typically caused by enterovirus 71 (Chan et al. 2000) Airports also frequently contained several large displays warning for symptoms of Zika, dengue, and even Ebola. Public outreach such as this shows that the MOH recognizes the burden and potential risks involved with infectious diseases and take action to educate the public on these risks. I also attended a conference on the preparedness of publically funded hospitals to deal with patients suspected of MERS-CoV infection. The conference was well-attended by healthcare professionals of all levels including doctors, nurses, paramedics, and epidemiologists. During this conference, a patient simulation was conducted to evaluate the preparedness of a nearby public hospital to deal with a patient exhibiting symptoms of MERS-

CoV. My experiences over the course of 10 weeks show that Malaysia is indeed a quintessential example of a country facing a double burden of disease.

## 5. Conclusion

Based on the results and analysis of this project, it seems likely that my coronavirus assay had very low sensitivity. There are a number of reasons why this could have occurred and the precise cause could not be ascertained by me during the brief travel experience.

Respiratory viral illnesses contribute significantly to the burden of disease in southeast Asia and studies like this are crucial to better understand and address this burden. Additionally, this information is important to know to better inform infectious disease surveillance methods to be better prepared for the next emerging infectious disease outbreak.

Over the course of this project, it was interesting to note how lab procedures were similar to my previous lab experience at Duke One Health Research Laboratory. Spending time working in an infectious disease lab and briefly shadowing a former state epidemiologist, Dr. Andrew Kiyu, at a conference about influenza and MERS-CoV gave me a good idea of the current challenges concerning infectious disease in Malaysia. It is important to reiterate that public health community should include pathogen discovery as part of their larger global health security agendas to be able to better protect human and animal populations globally.

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