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# The Molecular Typing of Legionella Infection from a Spa

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**Abstract**

*Legionella* spp. can be found naturally in environmental waters. The transmission mode is breathing in a mist or vapor that has been contaminated with the bacteria. Public recreational water is a possible source of infection. In Taiwan, people getting *Legionella* infections in whirlpool spas or after shower in public swimming pools were reported lately. In April 2008, a case with positive urinary antigen assay was reported and the isolate from sputum specimen was identified as serogroup 1. Investigation found this patient once stayed in a hotel and went to a whirlpool spa in this hotel. The environmental specimens from water tank were collected. Cultures from the bath yielded at least three different serogroups of *Legionella pneumophila*. Molecular typing by pulsed field gel electrophoresis identified four isolates from environmental specimens and the clinical isolate from sputum specimen sharing similar DNA fingerprinting. The results showed that the infection of this patient was closely related to the contamination of spa with *Legionella* spp., and indicated that the transmission of *Legionella* spp. from recreational water shall be the subject of public health control.

**Keyword**：Legionellosis, *Legionella pneumophila*, pulsed field gel electrophoresis ( PFGE ), travel-associated Legionnaires' disease, whirlpool spa

## Introduction

Since the outbreak occurred from the hotel in Philadelphia in 1976, *Legionella* spp. was considered as an important pathogen of community-acquired and nosocomial pneuomonia [1, 2]. At present, the *Legionellaceae* family has more than 48 species constituting 70 serogroups, including *Legionella pneumophila* and other *Legionella* species, and nearly half of them can cause human respiratory tract infection [2, 3]. *Legionella pneumophila* is the major pathogen of Legionnaires' disease, with serogroup 1 being the primary cause of the disease [4]. The

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transmission mode is breathing in a mist contaminated with the bacteria. Cooling towers, taps, showerheads, and whirlpool spas might be the vector of transmission. Person-to-person transmission has never been documented [5, 6].

Travel-associated Legionnaires' disease has been taken seriously in the past few years. In USA, 8,000-18,000 cases of Legionnaires' disease required hospitalization every year. Among them, twenty percent of the cases were associated with travel. In USA, 40-51% of the travel-associated Legionnaires' diseases were related to hotels between 2005 and 2006 [7]. In UK, about half of the Legionnaires' diseases were travel-associated [8, 9]. A European surveillance study in 2005 which covered 35 countries, presented 755 cases of travel-associated Legionnaires' disease, including 93 cluster outbreaks [10]. Legionnaires' diseases caused by contaminated whirlpool spas were not uncommon in the world [6, 11 - 13]. The study on an outbreak of *Legionella* in whirlpool spas discovered that, if the whirlpool spa is contaminated with bacteria, a longer use of the spa has a higher risk [13].

To identify the source of infection, laboratories usually use molecular typing to compare the DNA phylogenetic relationship between isolates from patients and environmental specimens [6, 13]. Pulsed field gel electrophoresis (PFGE) is highly reproducible and discriminatory between different subtypes, and often applies on molecular typing in bacterial infections [14]. There were two *Legionella* infections caused by recreational waters which proved by PFGE in Taiwan. The first event was a case of *Legionella pneumophila* serogroup 2 due to contaminated spa water in Taipei city, 2005 [6]. The other was a *Legionella pneumophila* serogroup 1 infection event in a public swimming pool in Nantou County, 2007 [15].

In this study, our laboratory utilized PFGE to identify the probable source of *Legionella* infection and confirmed this patient was the second case of *Legionella* infection correlated with whirlpool spas in this country. The study also indicated the traveling history of case will help to trace the infection sources and implement of control measures. It also emphasized the importance of sanitation in public recreational waters.

## Materials and Methods

### A. Case description

The male patient is 60 year-old, he had persistent fever (up to 40 ºC ), nonproductive cough and diarrhea in early April, 2008. Due to respiratory distress and sign of pneumonia, the attending hospital reported him as a suspected case of Legionnaires' disease. The urine antigen test was positive and *Legionella*

*pneumonphila* serogroup 1 was isolated from sputum specimen. Investigation found he had been traveling for 10 days before onset of symptoms. He stayed in a hotel and used the whirlpool spa. The environmental specimens in the hotel were collected. Cultures from the spa water yielded several different serogroups of *Legionella pneumophila*. To facilitate the search of the infection source, these isolates underwent serotyping and molecular typing.

**B. Clinical specimens**

The clinical specimens of the patient consisted of sputum, urine and paired sera. There were eight environmental specimens from the household water, including water tank, tap, shower heads, and drinking water. Four environmental specimens were from the hotel, such as shower heads, tap, drinking water, and water from whirlpool spas. All the specimens were preserved at 4ºC and transported to the laboratory. Tests were processed after receiving the specimens.

**C. Tests for urine and serum specimens**

*Legionella* Urinary Antigen ELISA kit (BINAX, Maine, USA) was used for detection. Patient urine is added into the microtiter wells coated with rabbit anti-*L. pneumophila* serogroup 1 antibody. If antigen is present, *L. pneumophila* serogroup 1 urinary antigen is captured by antibody. Anti-*Legionella* HRP conjugate is added. After two hours incubation, the wells are decanted and washed to remove the residual urine and unbound conjugate. A color developer is then added. The results are read by spectrometer.

Indirect immunofluorescence antibody assay was used for detecting antibody titer. The reagent was *Legionella* Indirect Antibody Test System (Zeus Scientific, NJ, USA). After serial dilution of serum, the specimen was added in slide with *L. pneumophila* and incubated at 37ºC. Immunofluorescent antibody was added after washing off non-adsorbed serum and incubated at 37ºC. The result was observed with fluorescence microscope after washing off unbound immunofluorescent antibodies. A typical fluorescent reaction indicated that there was *L. pneumophila* antibody in the serum. The antibody titer was determined by end point dilution. A four-fold or greater increase in titer to ≥128 in paired sera obtained during the acute and convalescent phase of disease provided serological evidence of *Legionella* infection.

**D. Isolation and identification of *Legionella* from clinical specimens**

Sputum was treated with 0.2M KCl-HCl (pH 2.0) and neutralized. Plate 0.1 ml of the treated sputum onto PNV selective medium, containing buffered charcoal yeast extract agar (BCYE, from REMEL, Thermo Fisher Scientific, Kansas, USA), L-cysteine (Mast Group Ltd., Merseyside, U.K.), and PNV (polymyxin B, natamycin and vancomycin, from Mast Group Ltd., Merseyside, U.K.). The plate was incubated for 7-14 days at 35 ºC, 5% $CO_2$, 60-90% humidity. Any suspected bacterial colony was subcultured, and tested by Gram's stain, L-cysteine growth requirement, *Legionella* Latex Agglutination test (Oxoid Limited, England), and Direct Fluorescent Assay (DFA) [16].

**E. Processing and culture of environmental specimens**

The water samples (500 ml each) were concentrated by membrane filtration (0.2 μm), and filtered residues were resuspended in 3 ml

sterile water. Of this suspension, 1-ml samples were treated with acid and cultured. The procedure of acid treatment was the same as the sputum specimen. The selective culture medium used was BCYE medium supplemented with L-cysteine and Modified Wadowsky and Yee additive (Mast Group Ltd., Merseyside, U.K.).

**F. Serotyping of *Legionella* isolates**

The method of identification was direct immunofluorescent antibody assay, and the reagent was Direct Fluorescent Antibody Test (Zeus Scientific, NJ, USA). We also used antibody from m-TECH (Monoclonal Technologies, Inc, Alpharetta, GA, USA) to increase the detectable serogroups. *Legionella* isolates which had been cultured for 48 hours were added in 1% neutral formalin and prepared to McFarland No. 1 suspension (about $3 \times 10^8$ cfu / ml). Some suspension was smeared on the slide, air dried and fixed for immunofluorescent staining. Antibody conjugates of different serogroups were added and reacted at room temperature. After 20 minutes, the slide was washed by PBS and distilled water. The slide was then examined by fluorescent microscope after air dried and mounted. If the bacteria appeared as bright yellowish-green, it was interpreted as positive.

**G. Pulsed field gel electrophoresis**

After incubation for 48 hours, adequate amount of *Legionella* isolates were added to 2 ml buffer (100 mM EDTA, 100 mM Tris, pH 8.0) to adjust turbidity. Make a 1% agarose gel with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The bacteria suspension was mixed with equal volume of agarose and filled into a mold. The gel was put into the proteinase K solution (20 mg/ ml proteinase K, 50 mM Tris, 50 mM EDTA, pH 8.0, 1 % sarcosine) and placed in water bath at 56ºC. After 2 hours, the gel was washed by sterile water and TE buffer several times. Every wash was kept in water bath at 56ºC for 15 minutes.

The gel was digested with *Sfi* I restriction enzyme (New England Biolabs, MA, USA) for four hours at 50ºC. Electrophoresis was done with Bio-Rad CHEF MAPPER (Bio-Rad Laboratories, California, USA). The condition of electrophoresis was: voltage gradient 6 V / cm, field pulse angle $120^0$, initial switch time 2 s; final switch time 40 s; and run time 20 h. The product was stained with ethidium bromide and photographed. BioNumerics (Applied Maths, Kortrijk, Belgium) software was used for analysis.

## Results

**A. Clinical Specimens**

The urine was positive for *Legionella pneumophila* serogroup 1. In accordance with the immunofluorescent assay, the antibody titer in the acute stage was less than 1:128, and the titer in the convalescent stage was 1:1,024. Because of the greater than four-fold in titer and ≥128 in paired sera obtained during the acute and convalescent phase, this patient had serological confirmation of *Legionella* infection. The isolation of the sputum specimens also yielded *Legionella pneumophila* serogroup 1, and which subsequently underwent pulsed field gel electrophoresis with environmental isolates.

**B. The isolation and identification of environmental isolates.**

In the culture medium from whirlpool spa specimens, there were quite a few

suspected colonies. These colonies suspected of being *Legionella* were subcultured and stained as Gram negative bacilli, L-cysteine growth requirement test positive, and positive latex agglutination test for *Legionella*. The results of DFA test was shown in figure 1. A total of 33 colonies were defined as *Legionella* spp., such as *Legionella pneumophila* serogroup 1 (19 colonies), serogroup 5 (12 colonies), and serogroup 10 (2 colonies). Among the 12 environmental specimens, *Legionella* was only isolated from water specimens of whirlpool spa. The other environmental specimens, including 8 household specimens, did not isolate any *Legionella* spp. The findings indicated that the whirlpool spa had been contaminated with at least three serogroups of *Legionella*

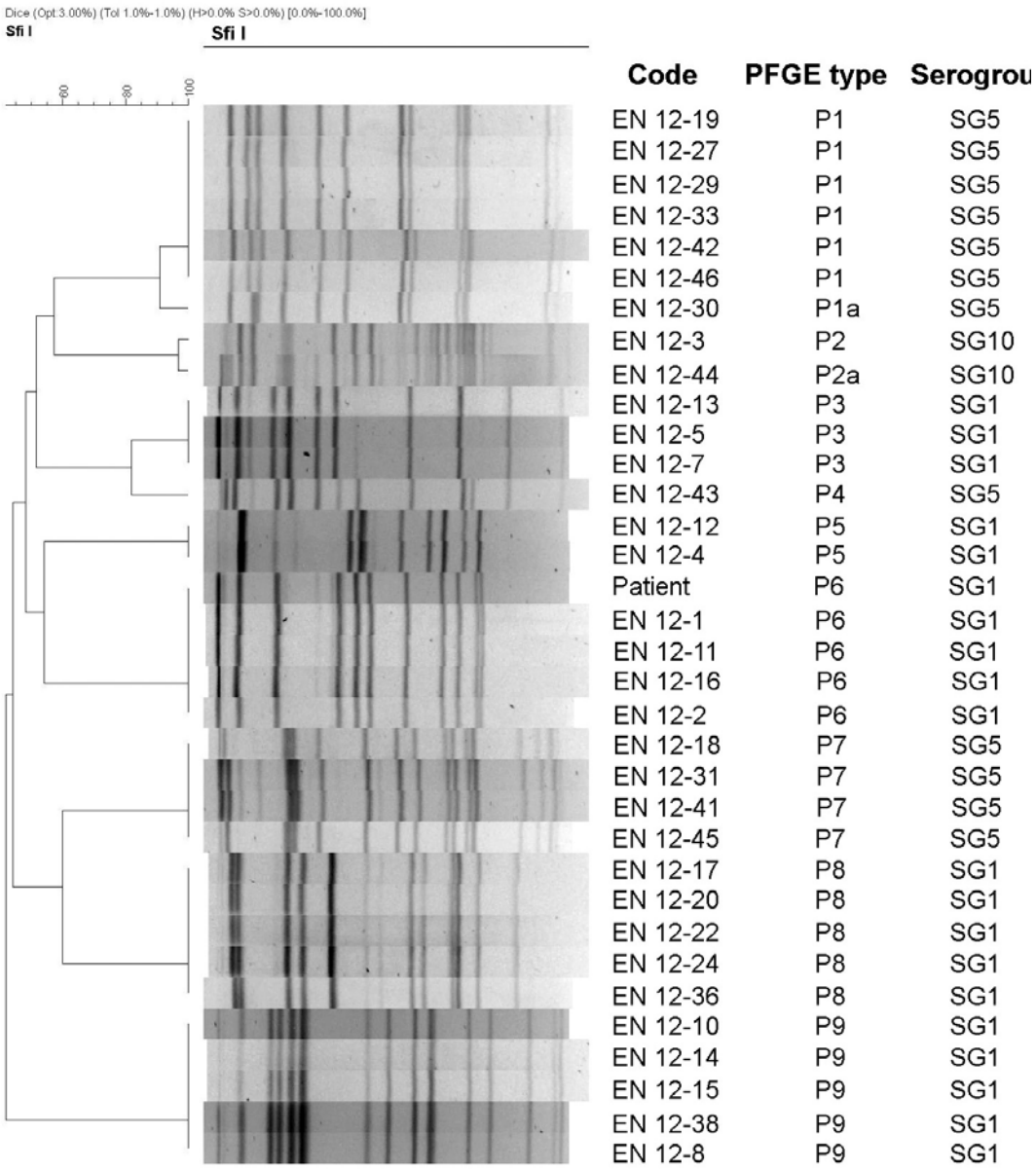


**Figure. The serogroups and PFGE typing of *Legionella pneumophila***

A total of 33 environmental isolates and 1 clinical isolate are divided to 3 serogroups and 11 PFGE types. Four environmental isolates (EN12-1、EN12-2、EN12-11、and EN12-16 ) and clinical isolate have identical DNA fingerprint, indicating the infection of this patient was closely related to the spa water.

*pneumophila*, with serogroup 1 had the highest frequency (57.6%), then serogroup 5 (36.4%), and serogroup 10 (6.1%).

**C. Pulsed field gel electrophoresis patterns**

As shown in Figure 1, the clinical isolate along with 33 environmental isolates could be divided to 11 distinct PFGE types: five for serogroup 1 isolates (P3, P5, P6, P8, and P9), four for serogroup 5 isolates (P1, P1a, P4, and P7) and two for serogroup 10 isolates (P2 and P2a). The similarity between P1 and P1a, as well as P2 and P2a was higher than 90%. Four of the whirlpool isolates (EN12-1、EN12-2、 \EN12-11、EN12-16) and clinical isolates belonged to P6, shared identical DNA fingerprint. In addition, the PFGE types of the 33 isolates in whirlpool spa were distributed evenly and none of the PFGE types had exceeding 20%. Only 12.1% (4/33) of the isolates were subtype P6, which caused *Legionella* infection of the patient.

## Discussion

This article reported the second case of spa-associated *Legionella* infection in Taiwan. *Legionella* spp. is heat-stable, can grow in whirlpool spa and causes long term contamination. Therefore, regular disinfection of the spa and quality control of water play important roles. Disinfection by chlorination is common to maintain spa sanitation. The consumption of chlorine is faster in public pools, as a result, the public pools are easier to be contaminated with bacteria [17]. With the development of laboratory technology and molecular typing, the source of infection can be proved by molecular typing. Recent researches stressed the seriousness of contamination by *Legionella* spp. in recreational water, and it also signified the importance to monitor the *Legionella* spp. in recreational water. The whirlpool spa in this article had been disinfected after isolating *Legionella* spp. In May 2008, the local health staff collected environmental specimens again and no *Legionella* spp. was isolated. Regular monitoring of water and decontamination, installation of new filter, and changing water pipes will aid to prevent further *Legionella* infection.

In this article, the patient is a travel-associated case. Travel-associated Legionnaires' disease is not easily found. Since the visitors usually have symptoms after returning home, it is difficult to trace the source of infection. Furthermore, travel-associated cluster is not easily detected. Visitors are from different residences, having different timing of symptoms onset, making the local health officers hard to identify if all the cases were originated from the same source. For that reason, an integrated surveillance system to collect travel histories of reported cases is needed. This system will help us to find out and prove the common source and implement disinfection and decontamination [13].

The surveillance network of Legionnaires' disease in Europe (EWGLINET) is well-developed. For example, in 2005, 93 outbreaks or clusters were reported by 35 countries, of which 36.6% was detected by the surveillance system [10]. Between 2005 and 2006, US CDC provided a supplementary reporting system for Legionnaires' disease. This system was reported by 32 states and the hospitals through e mail. The purpose of the system is to find out the infection source of

travel-associated Legionnaires' disease in the early stage. The supplementary system recorded ten clusters of travel-associated Legionnaires' diseases during a 2-year period. Of these 10 clusters, seven were associated with hotels, and three were associated with cruise ships. In Taiwan, Legionnaires's disease was classified as a notifiable disease in 1999. In mid-2004, environmental specimens were collected for confirmed cases. The detailed travel history and collection of environmental specimens will help us to trace the infection source of *Legionella* spp.

It is quite common to isolate more than two serogroups of *Legionella pneumophila* in spa water [6,18,19]. In our study, the isolates from environmental specimens were *Legionella pneumophila*, with serogroup 1 the highest frequency (57.6%), then serogroup 5 (36.4%), and serogroup 10 (6.1%). It meant that more than 40% of the isolates from contaminated spa water belonged to non-serogroup 1 *Legionella pneumophila*. Non-serogroup 1 *Legionella* infections in spa were reported in Taiwan and other countries [6, 13]. However, the urinary antigen test can only detect serogroup 1 antigen. Infections caused by other serogroups might be missed by urinary antigen test [20]. Therefore, investigating the event of infection in spa should be considered the possibilities of the underestimated cases caused by non-serogroup 1 infections.

The serogroup 1 isolates from environments could be divided to five distinct PFGE types, such as type P3, P5, P6, P8 and P9. After analyzing by BioNumerics software, the similarity between the different PFGE types was lower than 60%. Does each PFGE type have pathogenic difference? Does P6 isolate have higher pathogenicity? To answer these questions need further study on the molecular pathogenesis.

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# Review of Human Emerging Coronaviruses and Animal Coronaviruses

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**Abstract**

Coronaviruses used to be considered as causing only limited and easily recovered infections. So far, three subgroups and at least 16 species of viruses have been found in its family, including the severe acute respiratory syndrome virus (SARS-CoV). Coronaviruses have the largest non-segment positive stranded RNA among all RNA viruses. It is observed as corona-like particles

in diameter of about 100nm under an electron microscope. Four major structure proteins; spike, envelope, membrane and nucleocapsid proteins, are synthesized using virus replicase and proteinase. Some coronaviruses have haemagglutinin-esterase protein. Virus particles spread mainly through oral-faeces and respiratory routes. Most of coronaviruses replicate as soon as patients get infected. However, SARS-CoV only replicates after the onset of fever and this presents some difficulties in developing commercial diagnostic kits for SARS-CoV. Setting up suitable criteria for SARS infection is still a concern. Real-time polymerase chain reaction can detect the virus particle within 4 days after onset of fever and immuno-chromatography test kits can detect virus particles and associated antibodies within 10 days after onset of fever. To date, five human coronaviruses have been discovered. The major symptom of HCoV-OC42 infections is diarrhea. HCoV-229E, HCoV-HKU1 and HCoV-NL63 are associated with respiratory infections. The latest one, identified as HCo-NH, is still in argument with the relation of Kawasaki disease.

**Keywords**: coronavirus, severe acute respiratory syndrome (SARS), pathogenesis

## Introduction

Coronavirus used to be considered as a group of infective pathogens causing self-limited, localized and easily-recovered infections in mammals. However, the statement is not correct anymore since the discovery of the severe acute respiratory syndrome virus (SARS-CoV), one of five human coronaviruses discovered so far. In this article we will review the species, replication, pathogenesis, clinical signs and infection routes of the genus of *Coronaviruses*. Other emerging human coronaviruses are also discussed, as well as the difficulties in the development of SARS-CoV vaccines and diagnostic kits.

## The discovery of coronaviruses and its family

Coronavirusses were first isolated from chickens in 1937. Because of the difficulty of propagation in cell culture, it's not until 1965 that Tyrrell and Bynoe successfully inoculated the human coronavirus into human ciliated embryonal trachea cells and identified it using electron microscope, in which the virus envelope appears a structure of a crown [1]. The meaning of "crown" in Latin is "corona", thus till 1975, the virus was named as coronavirus [1-3].

The family of *Coronaviridaes* belongs to the order of *Nidovirales*. It includes two genera: *Coronaviruses* and *Toroviruses*. The genus of *Coronaviruses* contains three main subgroups and at least 16 species [4]. The first and second subgroups mainly infect mammals while the hosts of the third subgroup are avian animals. The important viruses in the first subgroup are Human CoV-229E, transmissible gastroenteritis virus (TGE) and porcine respiratory coronavirus (PRCV) in swine, feline coronavirus and its specific type feline infectious peritonitis virus (FIPV), and canine coronavirus. The second subgroup has human CoV-OC43, bovine

coronavirus, murine hepatitis virus (MHV) and rat sialodacryoadenitis virus (RAT-SDAV). Chicken infectious bronchitis virus (IBV) belongs to the third subgroup [5]. Almost all coronaviruses belong to at least one the three subgroups. However, because the genomic sequence of SARS-CoV Toronto strain is similar to HCoV-OC43, the serum reaction is similar to the first subgroup. , Although the position of SARS-CoV is still in argument, it is considered that it either belongs to the subgroup II or forms a new subgroup (the fourth subgroup) because its characters differ from the three main subgroups [3, 6].

**The structure of coronaviruses and their replicative methods**

Coronaviruses are enveloped, single stranded, positive-sense RNA viruses. Its genome (27-31kb) is non-segment and its molecular weight is the highest among all RNA viruses. The spike proteins (S protein) outstand the envelopes and then assemble a corona-like shape. The diameter of a particle ranges from 75 to 160 nm under an electron microscope. The average diameter is 100 nm.

Eleven to seventeen open reading frames (ORF) can be found in coronaviruses but only 7 to 9 mRNAs are translated. The virus transcribes a negative-sense single stranded RNA using the host enzyme system after it entries. The negative single stranded RNA is used as a template to transcribe proteinase and replicase, both of which are located in the first open reading frame. The replicase is responsible for the following transcription and translation [2, 3]. The S protein, haemaglutinnin-esterase, (HE protein), membrane protein (M protein), envelope protein (E protein) and nucleocapsid protein (N protein) are translated according to the order. However, only the viruses in subgroup II have HE protein [4, 5]. Many non-structural proteins are translated during the process as well. Although the function of these proteins are mostly unknown, some act as virulence factors, such as those in SARS-CoV [5]. SARSV-CoV contains 14 ORFs, which encode 4 functional, 8 accessory and 16 nonstructural proteins [3].

**Transmission methods of coronaviruses**

Patients get coronavirus infections from droplets via aerosol route or from feces via oral-fecal route. The droplets are unable to fly further than 1 meter. Thus, the equipments contaminated by infected patients or animals also play a role in spreading the viruses [7]. Other transmission routes are still available for coronaviruses. A queen cat infected by FIP can transmit the virus to her kitten vertically [5]. The survival ability of the virus against its environment is also considerable. Severe acute respiratory syndrome virus can survive in its environment longer than other genera of *Coronaviruses*. It can survive up to 48 hours on the surface of a dry plastic bag or feces, up to 24 hours in urine and about 4 days in a diarrhea stool of a SARS-CoV patient. This is mainly because the structure of its envelope can resist acid and gastrointestinal enzymes [5]. The virus can be inactived by ultraviolet light (UV) at 254 nm, in temperatures higher than 65C or in a pH higher than 14 or lower than 3. Formalin and

glutaraldehyde treatments are also effective in controlling the spread of the virus. Because of its highly contagious nature, the study of SARS-CoV should be restricted in laboratories at biosafety level 3 (BSL3) [8].

**The structural proteins of coronaviruses**

Spike protein is composed of S1 and S2 subunits. When viruses attach target cells, the protein will bind receptors on cell membrane. Consequently, a hole in the membrane will be formed allowing the genome of the virus to enter the cell [8]. The S protein is a type I transmembrane glycoprotein. It is responsible for virus binding, fusion and entry. It is also a major inducer of neutralizing antibodies. The receptors are different and are in different subgroups. The receptor for the subgroup I is aminopeptidase N (APN) [9]. For viruses in subgroup II, such as mouse hepatitis virus, the receptor is carcinoembryonic antigen (CEA). The receptor for SARS-CoV is angiotension-converting enzyme 2 [10, 11]. The S1 subunit attaches this receptor and the S2 subunit is in charge of fusing with the cell. The purpose of S protein is to allow the genome of SARS-CoV to enter the host cell. The attaching efficacy is better in an acidic environment [11].

The glycosylation of E protein and M protein are done in endoplasmic reticulum and then the two proteins are transported to the membrane of the host cells to wait for the assembly of coronaviruses. The N protein will compose with nucleic acid to form a stable helix structure and then the components will move toward to the membranes of host cells. Finally, the first two proteins package the helix structure to form particles before 'budding' them [5]. The nucleo-capsid protein works with S protein to transport the nucleic acid into the host cell while the membrane of the host cells is open. [3].

Some of the subgroup II viruses, such as mouse hepatitis virus, can translate HE protein. It is believed that the gene is originally from the influenza C virus. The main function of this protein is to use acetylesterase to destroy proteins containing sialic acid. Thus the membrane of host cells, such as erythrocytes, will be damaged. It can help S protein to make it easier for viruses to enter the cell. The HE protein is considered as a virulence factor as well [5].

**The outcomes of animal coronaviruses**

The animals infected usually can recover if sufficient nutrition is supplied because the symptoms tend to be self-limited and can easily recover. The prevalence of feline coronavirus in urban cats is higher than 30% and causes un-clinical to mild symptoms. However, when mutation occurs, the virus can penetrate the membrane of guts to abdominal capacity. Then antibody-antigen complexes accumulate in the cavity. Finally, the cat infected dies from cachexia [3]. Mouse hepatitis virus (MHV) is a pathogen with a high mortality and morbidity. The prevalence of it in the UK is 86% in house mice [12]. The virus can be used as a good model to study the transmission of coronaviruses [5].

**The mutant ability of coronaviruses**

The error rate of RNA polymerase while transcription is $1X10^{-4}$. This shows that coronaviruses tends to modify their genomic

sequence. It is observed in the study of feline infectious peritonitis viruses that each FIP virus does not have the same sequence [5]. The result gives us a hint that coronaviruses are able across the barrier of animal species to infect others. The hypothesis is observed in the similarities between the genomic sequences of Human CoV-OC43 and bovine coronavirus [5, 13]. Different open reading frames have different mutant rates. For example, the RNA polymerase is relatively stable. So the part of genes can be used to detect coronavirus using PCR [5].

There are normally two main species of human coronaviruses. The first one is HCoV-OC43. It causes gastroenteropathic symptoms, such as diarrhea in infants and children. The virus only infects 1/3 of cilia cells in the gut. Diarrhea occurs because of mal-absorption. Infants younger than 12 months can present milk diarrhea. Human CoV-229E usually infects epithelial cell and causes mild upper respiratory symptoms. It is difficult for human CoV-229E to be differentiated from flu-like infections but a specific symptom is that very few patients infected by HCoV-229E will develop neurologic syndromes [5]. This infection is seasonal and mainly occurs in winter. The virus can spread to other organs through macrophages after which, infections can occur in the liver, kidney, heart and eyes. There is no serum cross protection among serotypes so a patient may be repeatedly infected by many different serotypes [5].

**The symptoms and its pathogenesis of SARS-CoV**

SARS-CoV is notorious for causing severe lower respiratory syndromes. However, it also causes gastrointestinal symptoms. Twenty-seven percent of patients suffer from diarrhea and thirteen percent of patients suffer from abdominal pain. The latent period of SAR-CoV is about 6.4 days. The mortality rate for patients younger than 60 years old is 13.2% but it increases up to 43.3% for people over 60 years old [14]. According to Gu et al., SARS-CoV can infect the lungs, the epithelial cells in kidneys, mucous cells in the guts, neural ganglia cells and immune cells. The death is associated with multi-organ failure. The main target of the virus is the lungs and the pathological changes are associated with cell mediated immune response. The infected cells present apoptosis and have auto-antibodies. The inadequate interferon response, higher concentration of cytokines in the lungs and mass macrophages and T lymphocytes are observed in the lung and are all related to the diffuse alveolar damage. A lot of cells in the lungs are destroyed and then interstitial pneumonia and cell vacuolation are observed. Finally, the lungs fill up with tissue fluid and the patient dies of ventilation failure [15].

**The evolution of SARS-CoV**

When the SARS outbreak was fist reported to the WHO in 2003, scientists had no clue about what pathogen was causing this epidemic. At first, the influenza virus was suspected. Then, the coronavirus particle was observed under electron microscope. Hence, it was recognized that the genomic sequence is 99% similar to the coronavirus of civet cats. Although it is still believed that the virus is originally from the coronavirus of civet cats,

their relationship is still in debate and further research is required. In addition, the transmission model between animals has not been set up [5]. The SARS-CoV can infect domestic cats, macaque monkeys, ferrets and other animals [5], however limited upper and lower respiratory symptoms are observed in well-designed experimental studies [3].

When a virus crosses the barrier of species, it usually results in high mortality and morbidity in the new host. With time, a pathogen will modify its way to adopt into the new host or the transmission will die out. When the balance point is reached, the mutant rate of a virus will reduce to a relatively stable stage. This progress is called 'host adaption'. The evolution of SARS-CoV fits the hypothesis [5]. Zhang et al pointed out that nucleotide substitution per site per year in SARS-CoV is $0.8\text{-}2.38X10^{-3}$ [16]. According to the evolution of S protein, the SARS outbreak can be divided into three stages: 02-04 interspecies epidemic, 03-early-mid epidemic and 03-late epidemic groups. Positive selection was observed in the first and second stage. The mutant rate of this protein is high and the result of translation tends to be different. The purpose of this is to overcome the barrier between species and then try to adopt in the new species. The direction of the mutation in the second stage is also an attempt to try and improve its infective ability towards humans. Finally, the purifying selection was detected in the 03-late epidemic stage. This direction's purpose is to find a way to survive and gain the ability to propagate in the new hosts. The study presents a clear model about the evolution of a virus crossing the interspecies barrier [16]. Groneberg et al. states that the mutant rate of SARS-CoV is relatively stable compared with other coronaviruses because its replicase can function as an exonuclease to re-correct error. The hypothesis can be supported by the fact that only two lineages, which are the Hong Kong and Canadian Toronto groups, are identified. The fact that the mutant rates of proteins translated are slower than in the first stage can also support the hypothesis [5]. This information indicates that vaccines against SARS-CoV could be developed in the future.

**The current development of SARS-CoV vaccine**

Although there is still a tough road ahead in terms of developing a robust vaccine against SARS-CoV, scientists still believe that vaccines based on recombinant, DNA, vector platforms, inactive or attenuated coronavirus could be commercialized. Inactive, recombinant, DNA or vector vaccines have been tried. The efficacy of antibodies using passive immune protection by animal models has been studied widely [3].

Recombinant vector-based vaccines based on the study of S protein are considerable and many of them are in the preclinical trial stage. A DNA vaccine encoding the full length of S protein could induce neutralizing antibodies and protect mice against SARS-CoV challenge [17]. Intranasal or intramuscular inoculations of attenuated modified vaccinia virus Ankara vaccines integrated with S protein also produce neutralizing antibodies [18]. Mucosal immunization of African green

monkeys with an S protein integrated attenuated parainfluenza virus can protect animals against SARS-CoV challenge [19]. Although it has not yet moved into the clinical trial stage, all of the results have proven that the vaccine developed should be useful in protecting humans against SARS-CoV [3].

**The other emerging human coronaviruses**

There have been at least three emerged human coronaviruses discovered since SARS-CoV was first reported [4]. Dominguez et al, discovered the fourth human coronavirus in Netherland and named it as HCoV-NL63. The virus was identified in children's respiratory systems in other countries [20]. The HCoV-NL63 is very similar to HCoV-229E according to their genomic sequences and then it is sorted into the subgroup I. The disease is most often reported in winter and the most susceptible are children younger than 5 years old. The main symptoms are upper respiratory infection and bronchitis. Few cases developed pneumonia [13]. The pathogen may be related to the cause of stridor in children [3]. The virus grows very slowly in cell culture and then the main identification method is the PCR technique [12]. The virus was also identified as HCo-NH by Esper et al in New Haven. It was considered as the cause of Kawasaki disease in children [3, 21, 22]. The fifth human coronavirus is called HCoV-HKU1 and it was discovered in 2004 in Hong Kong in a 71 year old elderly person suffering from pneumonia. The virus is grouped into the second subgroup [3] and proved that it is related to upper respiratory and gastroenteropathic problems [14].

**The development of SARS-CoV diagnostic kits**

Generally, a virus will try to replicate as soon as possible after it enters a host. Clinical signs would be observed during this period. The SARS-CoV replicates slowly and very few virus particles can be detected in the latent stage. After the onset of fever, the virus begins to replicate faster [23, 24]. This phenomenon is the obstacle of setting up an ideal diagnostic kit. In addition, no cases have been reported recently. Hence, a diagnostic method developed is not able to be tested. In the end, clinical signs are the only criteria with which to define a suspected 'SARS' case [25]. Since SARS is classified as category 1 notifiable diseases in Taiwan, it is still important to develop a suitable and sensitive detection method in order to prevent any more outbreaks.

Although a fast and sensitive method is yet to be developed, there are still some other methods. Using an electron microscope is reliable and fast with the only downside being its low sensitivity. The turn over time of this method is about 2 hours. Using negative stain, virus particles can be seen when there are more than $10^7$ particles in a microlitre. However, it is difficult to use the results as firm evidence. The advantage is it can be used as a tentative diagnostic method to support a direction when all other methods are unavailable. The discovery of SARS-CoV is based on the electronic microscopic method [6].

Cell culture is considered as the gold standard but it is not a fast diagnostic kit. The

time and labor required for this method is a major issue. Its sensitivity is low, and in particular, the cytopathic effect may be hard to see. Moreover, some viruses, such as SARS-CoV, are highly contagious pathogens so this is not the most suitable way when the public health issue is the concern.

The polymerase chain reaction technique can amplify the target nucleotide sequence within 4 hours. The samples can be collected from the nasal cavity, oral cavity or from feces. This method is the standard diagnostic method certified by the WHO to detect SARS-CoV [26]. It is the only method that can guarantee detection of the virus within 10 days of the onset of symptoms. The first advantage of this method is its turn over time of 2 to 4 hours. Secondly, it can supply a solid result when the patient infected is within the first few days the onset of symptoms. The disadvantage of the PCR technique is that it requires special equipment and can only be performed in special laboratories certified by the government or the WHO. The need for well trained handlers is also an issue.

The strength of a good serological detect method is that it can screen mass samples in a short time without loss of its accuracy. It is useful for discovering an infection and allows it to be treated as early as possible. The fluorescent antibody test is the earliest method developed to detect SARS-CoV. It can show a positive result after 10 days of the onset of fever [26]. The Enzyme link immunosorbent assay (ELISA) and Immuno-chromatography test (ICT) have been developed well but are unable to meet the criteria set by the WHO. According to the characters of SARS-CoV and the suggestions of the WHO, the best diagnostic method is to combine clinical signs and epidemiological information to screen first and then combine a laboratory tool, such as a reverse transcriptase polymerase chain reaction, to do the tentative diagnosis. If there is a SARS suspected case, the person in charge must send the case to reference laboratories to confirm the result [25]。

## Conclusion

Because it is difficult to propagate coronaviruses *in vitro* and its faster mutation rate, it is possible that more emerging virus will be discovered and that there will be more possibilities of a virus jumping into a new host. To understand the pathogenesis, the developments of vaccines and diagnostic kits will be of benefit to people in the field of public health and they should utilize their previous experiences to overcome and prevent future outbreaks.

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