疫情報導 Taiwan EB

Taiwan Epidemiology Bulletin

Biweekly August 24 , 2010 / Vol.26 / No.17

Risks of West Nile Virus Infection in Taiwan

Yu-Chi Lin, Jhy-Wen Wu, Ding-Ping Liu

Second Division, Centers for Disease Control, Taiwan

Abstract

West Nile virus (WNV), an arbovirus belonging to the Flaviviridae family, is transmitted by mosquitoes. WNV can cause infection in humans, birds, horses, and other mammals, and lead to vector-borne diseases including West Nile fever and encephalitis in humans. Birds are the primary reservoir and amplifying hosts, and the virus can be maintained in a bird-mosquito-bird cycle. Mosquitoes of the Culex species are the major vectors. Although the virus is widely distributed throughout Africa, Europe, Asia, and Australia, resulting in endemic diseases, it had not been detected in the United States before 1999. The first incursion of WNV into America had caused a severe outbreak of human encephalitis in New York City which made the public health to pay attention to the disease. To date, patients with West Nile fever have never been found in Taiwan, but 5 species of mosquitoes and 46 different birds are potential transmission vectors or reservoirs, according to the studies done by the Centers for Disease Control and Prevention of the United States. Researches on the susceptibility to WNV among mosquitoes and birds in Taiwan are not available at present, but we should keep what had happened in America in mind and be vigilant about the potential of disease outbreak. In addition to routine vector control and elimination of the mosquito larval habitats, establishment of surveillance systems to detect the avian seroprevalence of WNV and the local virus activity are of great importance.

Keywords: West Nile virus, West Nile encephalitis, West Nile fever, Culex, vector-borne diseases

Introduction

Mosquitoes are the major transmission vectors of West Nile virus while birds are the primary reservoir and amplifying hosts. The virus can be maintained in a bird-mosquito-bird cycle. West Nile virus infection used to be endemic in Africa, Asia, and Europe and has caused only febrile illness and sporadic encephalitis until 1999, when it first appeared in New York City with subsequent spread to west coast of the United

The Taiwan Epidemiology Bulletin series of publications is published by Centers for Disease Control, Department of Health, Taiwan (R.O.C.) since Dec 15, 1984.
Publisher : Feng-Yee Chang
Editor-in-Chief : Min-Ho Lai
Executive Editor : Li-Gin Wu, Hsiu-Lan Liu
Telephone No : (02) 2395-9825
Address : No.6, Linshen S. Road, Taipei,Taiwan 100 (R.O.C.)
Website : http://teb.cdc.gov.tw/
Suggested Citation : [Author].[Article title].Taiwan Epidemiol Bull 2010;26:[inclusive page numbers].

States, Central America, the Caribbean, and Canada. Severe outbreaks occurred; more than 16,000 cases of WNV infection were reported in the United States and at least 7,000 patients had neuroinvasive diseases between 1999 and 2004 [1]. Because of the increasing importance of WNV, West Nile fever was announced as category 2 communicable diseases by Taiwan Centers for Diseases Control (Taiwan CDC) on February 10, 2006. Control measures, standard procedures on reporting and laboratory examinations were set up accordingly. During 2006-2009, 12 cases were reported in Taiwan and none of them was positive for WNV. To evaluate the risks of West Nile virus infection in Taiwan, this study addresses the current status of WNV epidemics throughout the world, analyzes what mosquitoes could be vectors, what kind of birds can be amplifying hosts, potential port of entry, and the cross-protection obtained from Japanese encephalitis vaccines.

Characteristics of WNV

West Nile virus, belonging to the genus Flavivirus within the family Flaviviridae, is a member of Japanese encephalitis virus serogroup. The genetic material of WNV is a positive-sense, single- stranded RNA. In addition to Japanese encephalitis virus endemic in Asia, St. Louis encephalitis virus in America, Murray Valley encephalitis virus and Kunjiin virus in Australia all belong to this serogroup [2].

Transmission Routes

WNV is mainly transmitted by mosquitoes. Among the 64 different types of vector mosquitoes identified in the United States since 1999, *Culex pipiens* L., *Cx. quinquefasciatus* Say, and *Cx. restuans* Theobald are the most important. *Cx. tarsalis* Coquillett, *Cx. nigripalpus* Theobald, *Cx. salinarius* Coquillett, *Aedes albopictus* Skuse, *Ae. triseriatus* Say, and *Ae. vexans* Meigen are also capable to transmit WNV [3]. Being the primary reservoir and amplifying hosts, birds infected by WNV may develop viremia. Mosquitoes get infection by biting birds in viremia stage and can transmit the virus to other birds once sufficient viral level is achieved in their salivary glands after approximately 10-days of multiplying. The virus is therefore maintained in nature in a mosquito-bird-mosquito cycle, resulting epizootics or enzootics (Figure) [4]. Infected mosquitoes can bite humans, horses, and mammals and may cause diseases in these incidental hosts or dead-end hosts. Among humans contracting WNV, 80% have asymptomatic infections and 20% develop self-limited febrile illness termed West Nile fever. In addition, viruses may enter the central nerve system and cause West Nile encephalitis. Because WNV cannot multiply

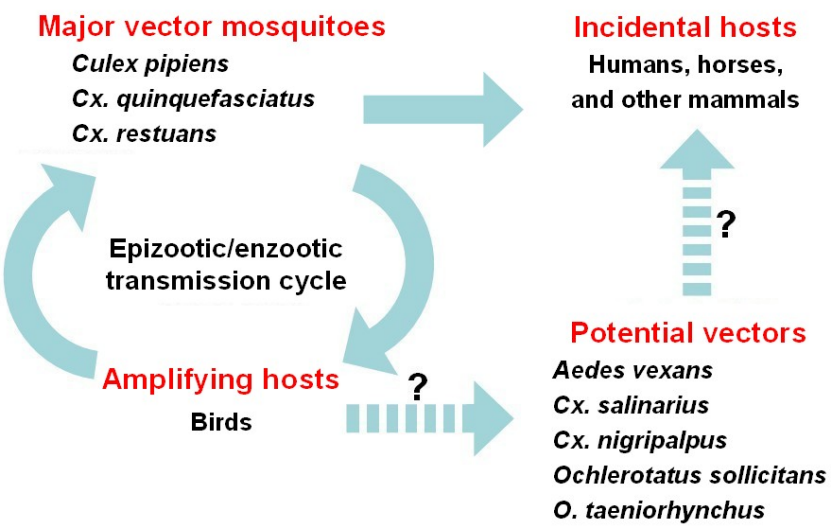

Figure. Transmission routes of West Nile virus [4]

vigorously nor produce viremia in mammals, it cannot be transmitted from human to human through mosquito bites. Contact transmissions between humans, mammals, and birds are not possible, either. However, few cases have been found to get infection through blood transfusion, organ transplantation, breast-feeding, and vertical transmission [3].

Epidemiology

WNV was named after the first isolation from a feverish 37-year-old woman in West Nile District of Uganda in 1937. It was found to be widespread in Africa, Europe, North America, the Middle East areas, southwest Asia, and Australia. WNV caused a severe outbreak in Romania in 1996. Being the largest arbovirus outbreak in Europe in recent years, more than 500 cases were found and the mortality rate was about 10 % [5]. The first appearance of WNV in the western hemisphere was in 1999 with 59 cases occurred in New York City; 37 patients had encephalitis and 7 patients died [6]. Because the virus strain found in the United States was very closely related to the lineage found in Israel in 1998, the outbreak might have originated from the Middle East areas. The transmission route remained unclear [7]. Since the first North American case found in 1999, the virus has been reported throughout the United States. In 2001, 66 cases of WNV infection were reported from 10 states; in 2005, 187 cases for 22 states were reported. The total number of patients increased to 1,356 in 2008, with 44 mortalities; cases were reported from all 50 states except Alaska, Maine, New Hampshire, and Vermont [8].

Risks of West Nile virus infection in Taiwan

To evaluate the risks of West Nile virus infection in Taiwan, we analyze what mosquitoes could be vectors, what kind of birds can be amplifying hosts, potential port of entry, and the cross-protection obtained from Japanese encephalitis vaccines.

A. Mosquitoes vectors in Taiwan

There were 132 species of mosquitoes in Taiwan. Compared with the 64 species that can transmit WNV identified by US CDC

since 1999, 5 of them could be found in Taiwan, including *Aedes albopictus*, *Aedes aegypti* L, *Aedes vexans*, *Culex pipiens molestus*, and *Culex quinquefasciatus* [9]. Different habitual behaviors of different mosquitoes could affect the ability in virus transmission. Because *Aedes albopictus, Aedes aegypti*, and *Aedes vexans* prefer mammals rather than birds, they are minor vectors in transmitting WNV and seldom cause spread of infections in birds. On the contrary, because *Culex pipiens* prefers to bite birds, virus transmission between birds is common. *Culex pipiens* is therefore the major vector of WNV in northeast America, north-central America, and Europe. Infected mosquitoes lodged in public transport can cause worldwide spread of viruses [10]. There is no *Culex pipiens* in Taiwan, but a similar species, *Culex pipiens molestus*, does exist. *Culex pipiens molestus* likes to bite mammals, especially humans [11]. Hybridization between *Culex pipiens* and *Culex pipiens molestus* has been found frequently in the United States, which makes the descendants have equal preferences in birds and humans. The risk of virus transmission from birds to humans may increase [10]. Biting both mammals and birds is the nature of *Culex quinquefasciatus, b*ut the proportion of *Culex quinquefasciatus* infected with WNV was higher in those collected from Bakersfield than those captured from Coachella Valley and Orange County. The efficacy in virus transmission could be different in the same mosquito species, owing to different habitats and distinct geographic distributions [12].

Taiwan CDC had conducted a mosquito surveillance study in 2005, indicating that among the 933 collected mosquitoes, 65 were *Culex quinquefasciatus*, one was *Aedes vexans,* and 249 were *Aedes albopictus*. By using real-time reverse transcription- polymerase chain reaction (RT-PCR) to test WNV, the results were all negative. This could partially explain why community case of WNV infection has never been found. However, because *Culex tritaeniorhynchus* Giles, a major vector of Japanese encephalitis virus which does not exist in America but is common in Taiwan, can not only contract with WNV but also bite both birds and mammals [13, 14], it becomes a potential vector of WNV in Taiwan.

B. Birds that can be amplifying hosts of WNV

According to US CDC, WNV could be isolated or identified in dead bodies of 326 kinds of birds since 1999 [15]. Compared with the Taiwan Wildlife Database established by Council of Agriculture [16] and the Digital Museum of Zoology established by National Taiwan University [17], 46 of the 600 birds ever appeared in Taiwan have been infected with WNV in the US (Table). Four migratory birds, including northern pintail, Eurasian wigeon, mallard, and ruddy turnstone, and 10 resident birds, including cattle egret, rock pigeon, common moorhen, Eurasian jay, barn swallow, nutmeg manikin, black-crowned night heron, osprey, ring-necked pheasant, and winter wren, were common species. Seven were alien species, including Muscovy duck, red lory, budgerigar, cockatiel, crimson rosella, common canary, and rainbow lorikeet. These were all potential hosts of WNV and risks of human infection

Table. Birds found in Taiwan that have been infected with WNV in the US

Colloquial names	Scientific names	Migratory or Resident	Common or rarespecies
Northern goshawk	*Accipiter gentilis*	Vagrant	—
Wood duck	*Aix sponsa*	—	—
Northern pintail	*Anas acuta*	Winter	Common
Eurasian wigeon	*Anas penelope*	Winter	Common
Mallard	*Anas platyrhynchos*	Winter	Common
Greater white-fronted goose	*Anser albifrons*	Vagrant	—
Ruddy turnstone	*Arenaria interpres*	Winter	Common
Short-eared owl	*Asio flammeus*	Winter	Rare
Long-eared owl	*Asio otus*	Winter	Rare
Greater scaup	*Aythya marila*	Winter	Rare
Canvasback	*Aythya valisineria*	Vagrant	—
Canada goose	*Branta canadensis*	—	—
Cattle egret	*Bubulcus ibis*	Resident	Common
Common goldeneye	*Bucephala clangula*	Vagrant	—
Rough-legged hawk	*Buteo lagopus*	Vagrant	—
Muscovy duck	*Cairina moschata*	—	Alien
Rock pigeon	*Columba livia*	Resident	Common
Tundra swan	*Cygnus columbianus*	Vagrant	—
Mute swan	*Cygnus olor*	Vagrant	—
Red lory	*Eos bornea*	—	Alien
Peregrine falcon	*Falco peregrinus*	Transient visitant	Rare
Gyrfalcon	*Falco rusticolus*	—	—
Common moorhen	*Gallinula chloropus*	Resident	Common
Eurasian jay	*Garrulus glandarius*	Resident	Common
Barn swallow	*Hirundo rustica*	Resident / Summer	Common
Caspian tern	*Hydroprogne caspia*	Winter	—
Herring gull	*Larus argentatus*	Winter	Rare
Glaucous-winged gull	*Larus glaucescens*	—	—
Nutmeg mannikin	*Lonchura punctulata*	Resident	Common
Red crossbill	*Loxia curvirostra*	—	—
Budgerigar	*Melopsittacus undulatus*	—	Alien
Smew	*Mergellus albellus*	Vagrant	—
Common merganser	*Mergus merganser*	Vagrant	—
Black-crowned night heron	*Nycticorax nycticorax*	Resident	Common
Cockatiel	*Nymphicus hollandicus*	—	Alien
Osprey	*Pandion haliaetus*	Resident	Common
Common peafowl	*Pavo cristatus*	—	—
Pelagic cormorant	*Phalacrocorax pelagicus*	Vagrant	—
Ring-necked pheasant	*Phasianus colchicus*	Resident	Common
Crimson rosella	*Platycercus elegans*	—	Alien
Bank swallow	*Riparia riparia*	Transient visitant	Rare
Common canary	*Serinus canaria*	—	Alien
Tawny owl	*Strix aluco*	Resident	Rare
European starling	*Sturnus vulgaris*	Vagrant	—
Rainbow lorikeet	*Trichoglossus haematodus*	—	Alien
Winter wren	*Troglodytes troglodytes*	Resident	Common

Remarks:

1. Vagrant birds: Birds that deviate from their routine migratory routes and pass through Taiwan because of typhoon or other factors.
2. —: No records.
3. Winter birds: Birds that fly south in the fall to wintering grounds in warmer regions and fly north in the next spring to breed in the temperate or Arctic summer.
4. Resident birds: Birds that can be observed in the region in the whole year without migration.
5. Transient visitants: Migratory birds that pass through and transient stay in Taiwan in their flyways.
6. Summer birds: Birds that fly north in the spring to breed and fly south in the next fall.

should not be overlooked. In 2006, Animal Health Research Institute, Council of Agriculture used realtime RT-PCR to detect WNV in 4,626 specimens obtained from wild birds in Taiwan. Because the results were all negative, we concluded that the incursion of WNV has not happened by the end of 2006 [18].

C. Potential ports of entry of WNV

Taiwan is an important hub for migratory birds in East Asia. Winter birds from Siberia fly south in the fall, pass through China, Korea, Japan, and lodge in Taiwan; summer birds from India fly north in the spring, cross China and stay in Taiwan. Because WNV infection have been reported in Siberia and India, migratory birds infected with WNV can transmit viruses to humans or birds in Taiwan through mosquito bites. Imported ornamental birds from endemic areas can also contract WNV. Smuggled ornamental birds that escape from quarantine regulations, such as parrots or canaries, also are possible source of infections and can spread the virus through mosquito bites. Mosquitoes infected with WNV lodged in public transportation such as airplanes and ships can bring the virus into Taiwan as well. Therefore, quarantine of imported birds and elimination of mosquitoes in airports and harbors are of great importance.

D. Cross-protection obtained from Japanese encephalitis vaccines

Japanese encephalitis vaccines manufactured using inactivated viral strains have been approved in many countries. Because WNV is a member of Japanese encephalitis virus serogroup, some studies have tried to analyze the cross-protection effects and positive results could be found in hamster models. Hamsters receiving inactivated Japanese encephalitis vaccines have lower viral levels during viremia stage, fewer cases of encephalitis, and lower mortality after challenged with WNV compared with control groups [19]. Others indicated that although humans receiving Japanese encephalitis vaccines could not produce neutralizing antibodies against WNV, the disease severity in those who did get infection was lower than those who did not receive vaccinations [20]. The public health departments in Taiwan have put Japanese encephalitis vaccine into the regular vaccination schedule since 1968. The seropositive rate for anti-Japanese encephalitis antibody in citizens aged between 15 to 90 years was 71% in 2004 [21]. Four doses of Japanese encephalitis vaccines are required for each child. In 2008, the vaccine coverage rates for the second, third, and fourth dose was 94.9%, 91. 8%, and 99.1% respectively [22]. It is possible that partial immunity derived from Japanese encephalitis vaccines can protect citizens from risks of WNV infection. This is also a reasonable explanation for the lower incidence of WNV infection in Asian countries which the vaccination coverage rates for Japanese encephalitis are very high.

Discussion

Because WNV must be transmitted through mosquito bites, vectors are pivotal in spreading the virus. Warm and humid weather between June and November in the United States fits mosquitoes perfectly to breed and grow, so West Nile fever occurs more

frequently in these seasons. Of all the vector mosquitoes that carried WNV between 2001 and 2004, 80% was Culex. The proportion of *Culex quinquefasciatus* increased drastically from 2.1% in 2001 to 51.1% in 2004 [1]. Because *Culex quinquefasciatus* and *Culex pipiens molestus* are quite common in Taiwan, *Culex pipiens molestus* may hybridize with alien *Culex pipiens*, and mosquitoes' susceptibility to WNV may increase, risks of West Nile fever should not be overlooked, although currently we do not know how susceptible the mosquitoes are.

Birds are the major hosts of WNV. Four common winter birds flying south to Taiwan in the fall have the potential to get infection, including northern pintail, Eurasian wigeon, mallard, and ruddy turnstone. The number of mosquitoes in falls and winters is usually lower than that in summer, but mosquitoes are not uncommon in southern Taiwan in winters because of the tropical / subtropical location. Migratory birds may bring WNV into Taiwan, and three common resident birds here are potential hosts, including rock pigeons, barn swallow, and tree sparrows. In addition, rock pigeons' nests built at vents of air conditioners or water towers of skyscrapers and high buildings make the pigeons live more close to humans. Seropositive rate for anti-WNV antibodies in 499 rock pigeons in Atlanta, USA was 25.7% during 2002-2003; WNV could be detected in 11 of the 269 serum specimens obtained during epidemic seasons (4.1%). This study confirmed the possibility that WNV can infect rock pigeons [23]. Barn swallows are also common in Taiwan. Some of them are residents; the others are summer birds coming in the spring. Because the season migratory barn swallows come to Taiwan is simultaneous with the time mosquitoes breed and multiply, the risk of vector transmission of WNV may further increase. A nest built at a house by bran swallows is a symbol of good fortune in Taiwan, so instead of driving them away, people sometimes assist them to support the nests with wood boards or wire meshes. Fortunately, 1000 barn swallows in France were all negative for anti-WNV antibodies in 2009 [24]. Infectivity of WNV among barn swallows could be lower than our expectation. House sparrow (*P. domesticus*) in America is another host for WNV according to the study done by US CDC. Although there is no house sparrow in Taiwan, tree sparrows are quite common, especially on cables around houses. Tree sparrows in Poland have been tested for anti-WNV antibodies, 12.1% of the 33 birds were positive [25], so we should also consider tree sparrows as potential viral hosts. Rock pigeons, barn swallows, and tree sparrows are common in Taiwan. Because their lives and habitual behaviors have well adapted to the urbanized environments, they could pose a higher risk in transmitting WNV.

Case of West Nile fever has never been confirmed from Taiwan and the neighboring Asian countries, including Japan, Korea, and mainland China. A nationwide WNV surveillance conducted in Japan between April 2004 and March 2007, detecting virus using RT-PCR, did not found any positive results in 742 dead birds and 32,145 mosquitoes [26]. This study indicated that there is no incursion of WNV into Japan so far.

In conclusion, considering the birds and vectors in Taiwan already known to be WNV hosts in US, and the possibility of viral

spreading through the other birds and mosquitoes without records of infection, we should never underestimate the risk of WNV outbreak. Breeding sites of mosquitoes should be eliminated regularly, seroprevalence for anti-WNV antibodies and viral surveillance system among birds should be established.

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Molecular Epidemiologic Investigation of Hospital-Acquired Legionellosis with Multiple Serogroups of Legionella in Chiayi County

Lei-Ron Tseng, Jui-Hsin Chang, Jei-Kai Tan, Ying-Yan Chen, Jung-Jung Mu, Chuen-Sheue Chiang, Ho-Sheng Wu

Research and Diagnostic Center, Centers for Disease Control, Taiwan

Abstract

Hospital-acquired Legionellosis has received significant attention in western countries. The major source of hospital-acquired Legionellosis is the potable water supply system and micro-aspiration of contaminated water is the mode of transmission. The fatality rate of hospital-acquired Legionellosis is twice as much as that of community-acquired infection. Patients with chronic illnesses and organ transplant recipients are at greater risk of infection. Few cases of hospital-acquired legionellosis have been reported in Taiwan and the actual incidence might be underestimated. Water quality monitoring and the routine cultures from environmental water samples have emerged as an effective strategy for prevention of hospital-acquired legionellosis. Taiwan Centers for Disease

Control (Taiwan CDC) received a case report of *Legionella pneumophila* from a hospital in Chiayi County in August, 2007, later confirmed as serogroup 1 infection. Isolates of *Legionella pneumophila* serogroup 1 and serogroup 6 had also been cultured from tap water of that hospital. When comparing the genetic fingerprints of bacterial isolates from environments with those from the patient using pulsed field gel electrophoresis (PFGE), the results were quite similar. Another case had been reported to Taiwan CDC in January 2008, and *Legionella pneumophila* serogroup 6 had been isolated from the patient's sputum. The second patient was treated in the same hospital as the first case for 22 days prior to the onset of illness, and the genetic fingerprint of the second patient's bacterial isolate was almost identical with the isolate from environmental culture of the hospital. Molecular subtyping of the clinical and environmental isolates revealed high possibility of hospital-acquired infection of these two patients. This study is the first published case report of hospital-acquired infection with multiple serogroups of Legionella in Taiwan. Based on this study, appropriate surveillance of water supply systems in hospitals has proved to play an important role in controlling hospital acquired infection, and molecular subtyping has also made it easier to identify the source of infection.

Keywords：hospital-acquired legionellosis, *Legionella pneumophila*, pulsed-field gel electrophoresis (PFGE), molecular typing, serogroup 1, serogroup 6

Introduction

Legionella is a common pathogen of community-acquired and hospital-acquired pneumonia, with at least 48 species and 70 serogroups identified. Among them, *Legionella pneumophila* serogroup 1 is most important [1]. From 1980 to 1998, 25% to 45% patients with legionellosis were hospital-acquired and the fatality rate was 28%, which was twice as much as that of community-acquired legionellosis in USA [2]. Inhalation or aspiration of contaminated water was the mode of transmission and the major sources in the hospital were the potable water supply system and the cooling towers [3]. However, experts have raised doubts about the link between cooling towers and hospital acquired legionellosis because potable water supply system has been found to be the actual source of infection in many outbreaks since 1985 [4,5]. For example, 19 of the 20 hospital acquired outbreaks that occurred in New England and Wales of the United Kingdom from 1982 to 1990 virtually have been linked to potable water [6].

Risk factors for hospital acquired legionellosis included hospital size, organ transplant recipients, and colonization rate of distal sites of water supply systems [7]. Dr. Best *et al*. have pointed out that risks of hospital acquired infection significantly increase once colonization rate of distal sites exceeds 30% [8]. To the contrary, hospital acquired infections have never happened in hospitals with zero colonization rate [9, 10]. Therefore, monitoring colonization rate of distal sites of water supply systems for legionella in hospitals is the most effective strategy for prevention of hospital acquired

legionellosis. In 2007, Dr. Stout *et al.* have also demonstrated a significant association between colonization rate of distal sites of water supply systems in the hospital and the incidence of hospital acquired legionellosis [11].

In Taiwan, few sporadic case reports of hospital acquired legionellosis have been published [12, 13]. The first reported large scale outbreak occurred in one hospital in southern Taiwan in 2000, with 81 suspected cases detected, and the potable water supply system had been proven to be the source of infection [14]. In 2008, the first article about 16 hospitals environmental surveillance was published. In the 16 hospitals undergoing investigation, the colonization rate of distal sites of water supply system has been found to be 63% (10/16) and the colonization rate was more then 30% in 3 of them [15]. Based on this survey, colonization rate of legionella in hospitals of Taiwan was high and the actual incidence might be underestimated.

The laboratory of Research and Diagnostic Center of Taiwan CDC identified two nosocomial events by molecular subtyping in 2007; both of them were caused by *Legionella pneumophila* serogroup 1 and the potable water supply systems were the sources of infection. In this article, we reported two unusual cases of hospital acquired legionellosis caused by different serogroups of *Legionella pneumophila*, serogroup 1 in the first case and serogroup 6 in the other case. The hospital environmental cultures were also colonized with legionella of the same two subtypes as were found in the patients. Therefore we use molecular subtyping to exam the clinical and environmental isolates to delineate the hospital colonizing legionella as the source of infection in these two cases.

Material and Methods

A. Case description

Patient A, a 55-year-old female with heart disease and undergoing regular hemodialysis, was hospitalized in a hospital in Chiayi County for 27 days, from July 10 to August 6, 2007, with onset of pneumonia on July 27. The patient was reported to Taiwan CDC as a suspected case of legionellosis and clinical specimen was sampled on August 3.

Patient B, a 73-year-old male, was hospitalized in the same hospital in Chiayi County because of cardiac events for 22 days, from December 5 to December 26, 2007, with onset of pneumonia on December 26. He was transferred to another hospital on December 26 where he was then reported as a suspected case of legionellosis on January 10, 2008. Clinical specimen was also sampled on the date of report. Definite diagnosis of legionellosis in both patients was established through standard methods routinely used [16]. Since onset of pneumonia occurred after 18 and 22 days of hospitalization, respectively, both patients were qualified as hospital acquired infections [17].

B. Clinical specimens

Clinical specimens included sputum, urine, initial serum samples, and convalescent serum samples. Three environmental water specimens, from water dispenser of the nursing station (EN1), faucet of the bathroom of Patient A's ward room (EN2), and shower nozzle of Patient A's ward room (EN3), were also sampled on August 13, 2007. All specimens were preserved at 4ºC and

transported to the Bacteriology Laboratory of Taiwan CDC for further examinations.

C. Examinations of urine and serum samples

Detection of legionella antigen in urine was performed by Legionella Urine Antigen ELISA kit (BINAX, Scarborough, ME, USA) according to the user manual. By using Legionella Indirect Antibody Test System (Zeus Scientific, NJ, USA), the indirect immunofluorescence assay was used to detect the antibody titers against legionella in serum. For the latter, 15μL of two-fold serial dilution of the serum samples with phosphate buffer solution (PBS) was used for immunofluorescence assay, followed by examination with fluorescence microscopes. Diagnosis of legionellosis was established if seroconversion, defined as an increase in antibody titers of greater than or equal to fourfold and the highest antibody titer was greater than or equal to 128, was achieved [16].

D. Identification and differentiation of bacterial isolates from sputum specimens [16]

After pretreatment with acid, 0.1mL sputum was inoculated onto selective culture plates, including BCYE (Buffered charcoal yeast extract agar, REMEL, Thermo Fisher Scientific, Lenexa, KS, USA), L-cycteine, a necessary supplement for growth (Mast Group Ltd., Mereyside, UK), and PNV, an antimicrobial additive (polymyxin B, natamycin, and vancomycin, Mast Group Ltd.). The culture plates were incubated at 35ºC with 2.5-5.0% CO_2, and a relative humidity between 60-90%. The culture plates were checked daily and suspected bacterial isolates were sub-cultured and further examined with Gram's stain, L-cysteine requirement assay, a latex agglutination test, and direct immunofluorescence assay (DFA).

E. Culture and management of environmental water specimens

Water sample of 500mL was filtered with 0.2μm membrane, and resuspended in 3mL sterile water by vortexing. One mL of the suspension was pretreated with acid and culture. The pretreatment procedures of the environmental specimens were same as that of sputum specimens. Selective culture media were used, containing BCYE, L-cysteinie, and antimicrobial agents MWY (Modified Wadowsky and Yee, Mast Group Ltd.). The subsequent culture methods, identification and differentiation of bacterial isolates were similar to those of sputum specimens. Since the purpose of environmental culture was to find out all the possible sources of infection, the more suspicious isolates examined was the better. Direct immunofluorescence antibody assay could be used to delineate the species and subtypes of those isolates.

F. Identification of serogroups

The reagents of direct immuno-fluorescence antibody assay used included Direct Fluorescent Antibody Test (Zeus Scientific, NJ, USA), and m-TECH antibody (Monoclonal Technologies, Inc., Alpharetta, GA, USA). Bacteria were resuspended in 1% formalin and fixed on slides after 48 hours of growth. Antibodies for various bacterial serogroups were used. Twenty minutes after reaction, the slides were washed with distilled water and PBS, air-dried, and examined with fluorescence microscope.

G.. Pulsed-field gel electrophoresis to discriminate subtypes

After 48 hours of growth, bacteria were

resuspended in 2mL of buffer solution (100mM EDTA, 100mM Tris, pH 8.0) with suitable turbidity. An equal volume of 1% agarose solution was dissolved in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) and bacterial solution was injected into the mould to form a gel block. The gel block was treated with Proteinase K solution (20mg/mL Proteinase K, 50mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosine) for 2 hours at 56℃, then washed twice with sterile water and four times with TE Buffer , for 15 minutes each time with shaking at 56℃. Ten units of *Sfi* I restriction enzyme (New England Biolabs, MA, USA) in 200μL reaction buffer was added to the gel block to react for 4 hours at 50℃. The gel block was molded into 1% agarose gel after completion of all reactions. By using Bio-Rad CHEF MAPPER (Bio-Rad Laboratories, Hercules, CA, USA), the electrophoresis was performed at 6V/cm, electric field angle at 120°, pitch change from 2 to 40 seconds, with the total duration being 20 hours. The gel was stained with ethidium bromide after electrophoresis, photographed and analyzed by using BioNumerics (Applied Maths, Kortrijk, Belgium).

Results

A. Results of clinical specimens

The serologic test result of Patient A was positive. Her antibody titers to legionella, both IgM and IgG, were less then 32 at initial phase but were 128 at convalescent phase. Urinary specimens were not available. Because culture of respiratory specimen revealed growth of *Legionella pneumophila* serogroup 1, definite diagnosis of this patient was confirmed.

The serologic test result of Patient B was negative. His antibody titers, both IgM and IgG, were less then 128 at initial and convalescent phases. Urinary antigen was not detected in his specimen. Because culture of his sputum revealed growth of *Legionella pneumophila* serogroup 6, definite diagnosis of this patient was confirmed.

B. Results of environmental specimens

Specimens sampled from hospital environments were sent for culture. Legionella was isolated from tap water (EN2) and shower nozzle (EN3) obtained in Patient A's ward. These bacterial isolates were sent for subculture and identification of serogroups and the results were listed in Table. Thirteen isolates were confirmed to be legionella, among which 8 were *Legionella pneumophila* serogroup 1, 4 were *Legionella pneumophila* serogroup 6, and 1 was *Legionella erythra*. The potable water supply system was contaminated by at least 3 types of legionella. Among the bacterial isolates, 61.5% (8/13) was *Legionella pneumophila* serogroup 1, which was the most common type, and 38.5% (5/13) was *Legionella spp.* other than serogroup 1.

Table. Culture results of hospital environmental water specimens and the identification of serogroups

Sample code	Location (type)	Culture test	Serogroup (no. of strains)
EN1	Nursing station: tap	−	None
EN2	Patient A's ward: tap	+	*L. pneumophila* serogroup 1 (1); *L. pneumophila* serogroup 6 (4); *L. erythra* (1)
EN3	Patient A's ward: shower	+	*L. pneumophila* serogroup 1 (7)

C. Pulsed field gel electrophoresis (PFGE) patterns

As shown in Figure 1, the PFGE pattern of clinical specimen of Patient A (pattern A) was only one band different from that of environmental specimen EN3-2 (pattern B). The two specimens bore a similarity up to 90.9%. Compared the same clinical specimen with the other 7 environmental specimens (pattern C), there were a 2-band difference and the similarity was 86.3%. As shown in Figure 2, the PFGE pattern of clinical specimen of Patient B (pattern D) was two bands different from that of hospital environmental specimens (pattern E). They bore a similarity of 90.0%.

According to the article published in 1995 by Tenover *et al.*, two bacterial isolates were considered to be closely related if the difference in the number of bands between them was 1-3 [18]. The PFGE patterns in both Patient A and Patient B were closely related to the hospital environmental specimens, therefore legionella infection of these two patients were probably associated with the hospital environment contaminated by legionella.

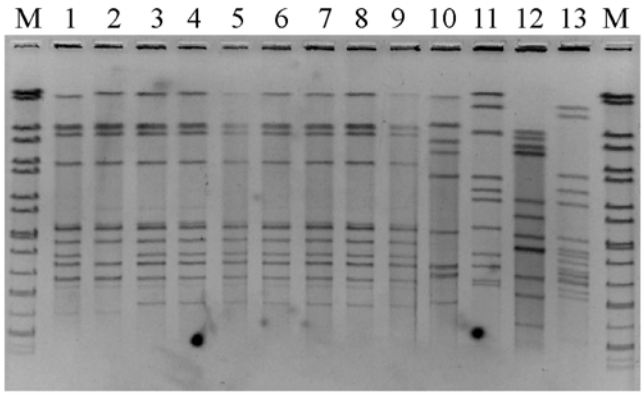

Figure 1. PFGE pattern of *Legionella pneumophila* serogroup 1 of bacterial isolates from both clinical and environmental specimens

Lane M: size marker.
Lane 1: bacterial isolate from clinical specimens of Patient A.
Lane 2-9: bacterial isolates, EN3-2, EN2-10, EN3-3, EN3-5, EN3-6, EN3-7, EN3-8, EN3-9, from the hospital environmental specimens.
Lanes 10, 11: bacterial isolates from clinical specimens, not associated with this infection.
Lanes 12, 13: bacterial isolates from environmental specimens, not associated with this infection.

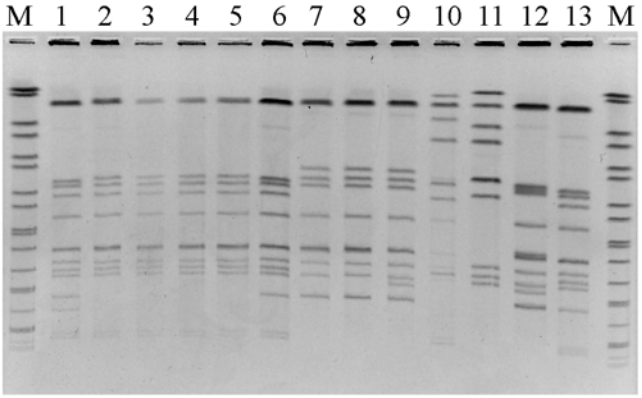

Figure 2. PFGE pattern of *Legionella pneumophila* serogroup 6 of bacterial isolates from both clinical and environmental specimens

Lane M: size marker.
Lane 1: bacterial isolate from clinical specimens of Patient B.
Lane 2-5: bacterial isolates, EN2-2, EN2-3, EN2-4, EN2-5 from the hospital environmental specimens.
Lanes 6-9: bacterial isolates from clinical specimens not associated with this infection.
Lanes 10-13: bacterial isolates from environmental specimens not associated with this infection.

Discussion

This is the first published article in Taiwan addressing hospital acquired legionellosis with multiple serogroups of legionella. Patient A was infected by *Legionella pneumophila* serogroup 1 and Patient B was infected by *Legionella pneumophila* serogroup 6. As for bacterial isolates from environmental specimens, 61.5% was *Legionella pneumophila* serogroup 1 and 38.5% was *Legionella pneumophila* other than serogroup 1. Although the most common subtype of bacterium among hospital acquired legionellosis was *Legionella pneumophila* serogroup 1 in the literature [3], quite a few cases of hospital acquired legionellosis caused by other serogroups have been reported worldwide [19-21]. Therefore, isolates of *legionella spp.* other than serogroup 1 should not be overlooked.

Detection of urinary antigen by a commercial kit produced by Binax (Portland, USA) has become the most widely used test for diagnosis of legionellosis. The major limitation of the test is that it only detects the antigen of *Legionella pneumophila* serogroup 1. For example, Patient B's urinary antigen test was negative, but he turned out to be infected by *Legionella pneumophila* serogroup 6, which was confirmed by culture. Because of the lower sensitivity and more complex processing of culture method, the actual incidence of *Legionella pneumophila*, other than serogroup 1, could be underestimated.

Both cases in this article were reported to Taiwan CDC more then 20 days after admission. The lack of prompt and effective medication could contribute to the persistent pneumonic symptoms during hospitalization. Clinical presentations of legionellosis were not unique [22]. Empiric use of ineffective antibiotics might lead to poorer treatment outcomes [23]. Delayed initiation of effective antimicrobials could result in higher case fatality [24]. Erythromycin is one of the most frequently-used antibiotic, but quinolones are actually more effective [25].

To prevent hospital acquired legionellosis, a disinfection process and a comprehensive surveillance system could be a good start. Lots of effective disinfection methods have been reported in recent years [26, 27]. They could not only decrease colonization rate of legionella in hospital environments but also reduce hospital acquired infections. To monitor the water quality, water sampling from hospital environments and isolation of the colonizing legionella are mandatory. Many developed countries have adopted this approach on a regular basis based on several concerns: first, this survey can uncover the actual status of colonization and prompt the authorities to do disinfection rigorously; second, if legionella colonization can be documented, doctors will be more vigilant and more willing to consider this diagnosis; third, the bacterial isolates can be useful resource in establishing a serogroup database for research and outbreak investigations.

Although the environmental sampling was not based on a regular active surveillance, bacteria isolated from hospital environments and subsequent molecular subtyping facilitated the investigation. The PFGE patterns, which indicated environmental isolates and clinical isolates were closely

related, confirmed the source of infection in this hospital outbreak. This article demonstrates the important role of molecular epidemiology in infectious diseases control.

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