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Original Article

Development of rapid tools for detection of *Clostridium Botulinum* neurotoxoin

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Abstract

Two rapid immunoassays, BoNT/A colloidal gold immunochromatographic assay and BoNT/A magnetic bead based enzyme immunoassay, were developed to detect botulinum neurotoxins type A (BoNT/A). Both assays were based on the sandwich format using monoclonal antibody for detection of BoNT/A. For BoNT/A colloidal gold immunochromatographic assay, monoclonal antibody (150-3) which had the best capacity to capture BoNT/A was immobilized to a defined detection zone on a porous nitrocellulose membrane while another monoclonal antibody (44.1A) was conjugated to colloidal gold particles which served as detection reagent. The BoNT/A-containing sample was added to the membrane and allowed to react with 44.1A-coated particles. The mixture was then passed along the porous membrane by capillary action past the 150-3 in the detection zone, which would bind the particles that had BoNT/A bound to the surface, giving a red color within this detection zone. The sensitivity of immunochromatographic method reached to 50 ng/ml of BoNT/A, and detection time was less than 10 min. The developed assay showed no cross reaction to type B neurotoxin (BoNT/B) and type E neurotoxin (BoNT/E). As for BoNT/A magnetic bead based enzyme immunoassay, the same pair of monoclonal antibodies was utilized to develop a magnetic bead based enzyme immunoassay for detection of BoNT/A. With this method, 500 pg/ml of BoNT/A was detected in 30 min. In simulated sample test, most of the 1:10 diluted samples do not interfere the sensitivity of these assays.

Keyword：immunochromatographic assays, botulinum toxin A (BoNT/A), colloidal gold particle, monoclonal antibody, magnetic bead based enzyme immunoassay

INSIDE

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Introduction

Clostridium botulinum is a gram-positive rod-shaped bacterium that produces spores and grows in an anaerobic environment. It has 4-9 flagella and no capsule. Botulinum toxin is a exotoxin produced after *Clostridium botulinum* lysed. The toxin is neurotoxic and it is classified into types A-G based on the serologic typing. Apart from deliberately poisoning, the most common botulinum intoxication is due to inappropriate food preservation. In most food-borne cases of botulism, botulinum toxin is absorbed by the gastrointestinal mucosa of people who ingest toxin-contaminated food. Botulism also happens when people ingest spore-contaminated food, especially in infants and the elderly who lack the normal intestinal flora to compete with spores. It is currently known that the botulinum toxin enters the body not only by absorption from intestinal tract but also by contamination of a wound [1]. Botulinum toxin is the most lethal agent known to man. It is hundred million times more lethal than cyanide. The lethal dose 50(LD50) is less than 0.3 ng/ kg [2]. A very small amount of botulinum toxin can result in human mortality. It acts in neuromuscular junction and leads to muscular paralysis. In severe cases of botulism, it leads to paralysis of the diaphragm muscles and causes death. Because of its high lethality, botulinum toxin has the potential to be utilized as a biological weapon. Animal model and human lung epithelial cell model prove that botulism can happen by inhalation of toxin [3], which increases the risk to people in botulinum intoxication. Therefore, it is important to develop a rapid tool for detection of botulinum toxin.

Mechanism of Botulinum toxin

Botulinum toxin is composed of several proteins, known as botulinum neurotoxin complex. The complex consists of one botulinum neurotoxin (BoNT), one nontoxic non-hemagglutinin (NTNH) binding protein and many hemagglutinin (HA) with different molecular weights by non-covalent bond. The binding of nontoxic component (HA and NTNH) and toxic component (BoNT) protects BoNT against the acidity, alkali and protease of the gastrointestinal system [4]. BoNT plays a key role on neurotoxic effect. According to the gene sequences, BoNT/A is a single polypeptide chain with a molecular weight around 150 kDa. After being nicked, it produces two polypeptide chains, a heavy chain with a molecular weight of 100 kDa and a light chain with a molecular weight of 50 kDa. The chains remain connected by a disulfide bound [5]. BoNT has three

domains which possess different functions respectively as shown in Figure 1.

Current researches prove that the 100 kDa heavy chain is responsible for binding with receptors. The C-terminal fragment of the heavy chain has the same binding affinity as the whole heavy chain, which was proved by protein deletion experiments. The antibodies from animals immunized with the C-terminal fragment of the heavy chain can neutralize the toxicity of BoNT. [6]. The N-terminal fragment of the heavy chain of BoNT could form cation channels for translocation of the light chain of BoNT to the cytoplasm [7]. The channels also play a role as chaperon [8], restoring activity of light chain partially destroyed in acidic environment of endosome. According to the amino acid sequences analysis, light chain of BoNT is a metalloprotease with the HEXXH motif. For example, the light chain of BoNT/A is able to cut SNAP-25 [9] which is a component of SNARE protein. The cutting of SNAP-25 by light chain of BoNT/A blocks the fusion of vesicle membrane and presynaptic membrane of muscular junction, and further inhibit acetylcholine release leading to paralysis of muscle as shown in Figure 2.

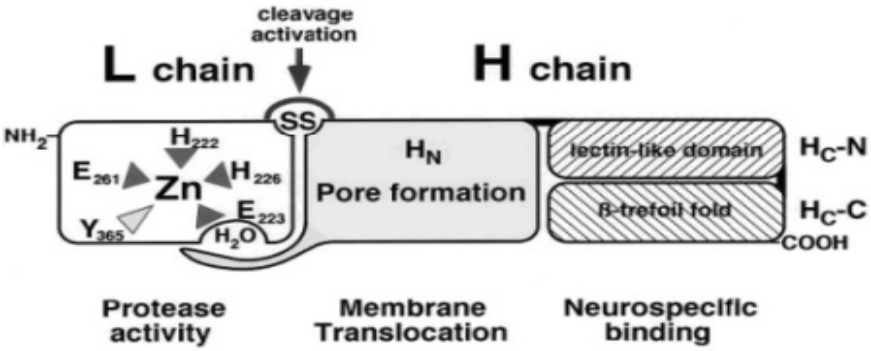

Figure 1. The molecular of botulinum neurotoxin (Fig from Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. Physiological Reviews 2000, 80: 717-767)

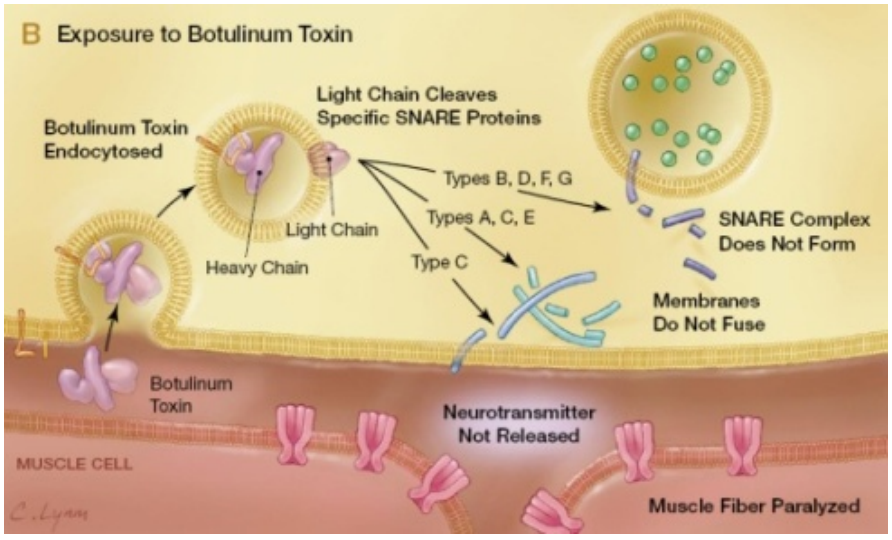

Figure 2. The mechanism of inhibition of acetylcholine release by botulinum neurotoxin (Fig from Arnon SS, Schecchter R, Inglesby TV, et al. Botulinum toxin as a biological weapon: Medical and public health management. JAMA 2001, 285: 1059-1070)

Introduction of current methods for detection of botulinum toxin

Although rare cases of botulism happen each year, but the toxicity of botulinum neurotoxin is very high. In severe cases of botulism, people die of paralysis of diaphragm muscle. Botulinum neurotoxin had been included in the items of biological warfare and food safety because of its high mortality. The botulinum intoxication of *Platalea minor* in Taiwan recently attracted more attention about the environment contamination with botulinum neurotoxin. Nowadays, the golden standard for detection of botulinum toxin is still the mouse bioassay. The sensitivity of mouse bioassay is as high as 5-10 pg/ml toxin [10]. However, mouse bioassay has the following disadvantages. First, it is an *in vivo* assay and needs to sacrifice a large number of mice for BoNT detection, which brings about animal ethic problems. Secondly, it is time consuming to observe the survival of mice in mouse bioassay. Finally, antibodies against different BoNT serotypes are needed in order to differentiate the serotypes of BoNT in the specimens, and more mice might be sacrificed in the neutralization test. As a result of the above drawbacks of mouse bioassay, developing another reliable, sensitive, and rapid tool for detection of BoNT is required. The advantages and disadvantages of current methods in detection of BoNT/A and BoNT/B are listed below. The detection limit of immuno-PCR assay is about 1-20 pg/ml [11], which is more sensitive than mouse bioassay and takes over 10 hours for detection. However, like PCR reaction, this method is likely to get false positive results because of the contamination in the operation procedure. Hence, it gives rise to difficulty in this method. The detection limit of enzyme-linked coagulation assay [12] is also lower than mouse bioassay and it is possible to measure BoNT less than 10 pg/ml. However, the enzyme-linked coagulation assay takes 18 hours to detect the toxin. Besides, coagulation activating enzyme isolated from the venom of Russell's viper limits the application of this method. Time-resolved fluorescence assay [13] uses lanthanide (Europium, Eu3+)-labeled antibody as tracer and its sensitivity reaches 200 pg/ml. Similarly, electrochemiluminescence [14] is utilized as tracer to detect BoNT and its sensitivity reaches 0.78-1.56 ng/ml. Both methods take about 2-2.5 hours to detect the toxin. The traditional ELISA assay has a detection limit of 5 ng/ml [14] and takes roughly 4 hours to complete procedures of ELISA assay. Enzyme assay is capable of detecting BoNT at a concentration of 3.5-5 pg/ml [15] in approximately 2-5 hours. This assay is based on the endopeptidase activity of the neurotoxin's light chain for detection of BoNT. Therefore, it can monitor the endopeptidase activity of BoNT but could be interfered by other protease in specimens. Ganglioside liposome immunoassay has been developed for BoNT detection by interaction between BoNT heavy chain and ganglioside receptors. The results can be read by naked eyes and the detection limit of this assay is about 0.1 ng/ml [16]. Furthermore, it can be measured by densitometry, which the sensitivity could be enhanced to 15 pg/ml and the assay could be completed in less than 20 minutes. The assay possesses rapid,

easily-operated, and sensitive characteristics, but this method could only be applied to toxins with ganglioside as receptors, such as cholera toxin, tetanus toxin and botulinum neurotoxin and thus limits the application.

Introduction of immunochromatographic assay

Report particles commonly used in immunochromatographic assay include colored latex particles, colloidal carbon particles and colloidal gold particles. Colloidal gold particle is produced by reduction of gold ion. Interestingly, because of surface plasma resonance effect, color of gold is shifted from yellow to red when becoming nanoparticle [17]. Generally, gold nanoparticles are produced by reduction of chloroauric acid (HAuCl4) in the laboratory. The gold ions are reduced to neutral gold atoms with the reducing agent. Neutral gold atoms become nuclei and gold gradually starts to aggregate in the form of nano-meter gold particles. The gold particles are surrounded by the residual negative ions in solution, forming a negative charge layer. The electrostatic effect provides the means for the gold particles to keep away one another and to stably stay in suspension, which is named colloidal gold particle [17]. Colors of colloidal gold particles vary from size to size. The smaller particle (2-5 nm) is orange, the medium-sized colloidal gold particle (20-40 nm) is in red wine color, and the color of greater-sized colloidal gold particle (60-80 nm) is blue-purple. The proteins are absorpted to the surface of colloidal gold particle by electrostatic force, hydrophobic force and dative bonding (binding between gold and sulphur, Au-S) [18]. The protein-colloid gold particle conjugate must be stabilized with electrolyte. The concentration of electrolyte cannot be too high since both high salt concentration and surfactant would strip the protein from gold particle. The macromolecule, such as BSA, gelatin, PEG and casein, not only stabilize the conjugation of protein-colloid gold particle, but also reduce nonspecific interactions by blocking the sites on the colloidal surface that are not occupied by the specific protein. Colloidal gold immunochromatogrphic assay is a recently developed diagnostic technique which combines the specific antigen-antibody interaction and chromatographic principle. It is a fast, easy and convenient method. The format of this technique is shown in Figure 3.

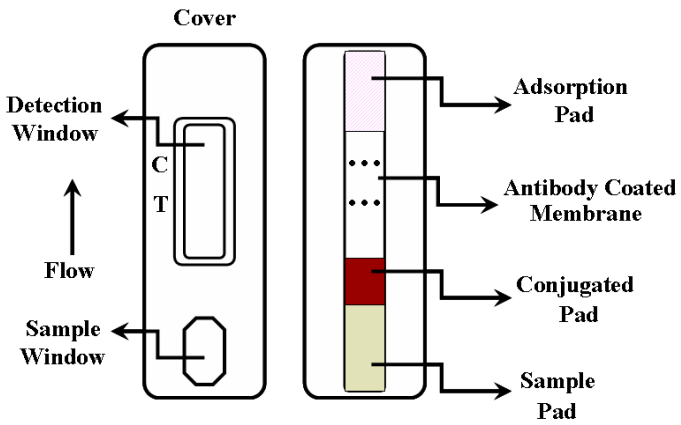

Figure 3. The format of colloidal gold immunochromatogrphic assay.

First, the specific antibody is fixed on one end of the NC membrane, and the antibody labeled with colloidal gold is absorbed on the other end of the membrane (conjugated pad). When the sample is added, the antigen in the sample forms an immune-complex with the antibody labeled with colloidal gold. The complex moves along the NC membrane by the capillary force and then could be captured by the antibody immobilized on the NC membrane. The specific diagnosis is achieved by forming antigen-antibody sandwich format and generate signal by the color of colloid gold particle.

Introduction of magnetic bead based enzyme immunoassay

Magnetic bead is a small and round particle with an iron oxide core. The diameter of magnetic bead ranges from nano-meter to hundreds of micro-meter. The iron oxide of the bead core will cause paramagnetic attraction when the magnetic bead is in magnetic field. The iron oxide core of the bead accounts for 50-60% of the whole bead. The magnetic bead is usually coated with organic polymer, like polyvinyl alcohol (PVA). After modifying the organic polymer layer, magnetic bead will have diverse functional groups. One of the most known commercial products is magnetic bead coated with avidin. The dissociation constant between avidin and biotin is roughly 10^{-14}M and the interaction is not affected by temperature, pH value, organic solvent or even denatured reaction. The magnetic bead has been wildly used for isolation of substance like protein, nucleic acids and cells [19]. In addition, it can be used for immunomagnetic separation-ELISA, such as detection of *Clostridium perfringens* type A enterotoxin [20].

Materials and Methods

Preparation of BoNT/A colloidal gold immunochromatographic assay

The goat anti-mouse antibody and monoclonal antibody 150-3 for BoNT/A was applied linearly on the control (upper) and test (lower) position of a porous nitrocellulose membrane. Antibodies were immobilized to the porous nitrocellulose membrane and dried for one hour. Porous nitrocellulose membrane was soaked in 20 mM Tris pH 7.4 buffer with 1% (v/w) polyvinyl alcohol at room temperature for 30 minutes, and washed once by water and air-dried. Afterward, colloidal gold probe coated with 44.1A anti-BoNT/A IgG was absorbed to the conjugated pad and air-dried. The conjugated pad was bound to the lower end of porous nitrocellulose membrane. In addition, absorption pad was bound to the upper end of the porous nitrocellulose membrane while the conjugated pad was bound to the sample pad end. The prepared colloidal gold immunochromatographic strip was stored at 4℃.

Conclusion

BoNT/A Colloidal gold immuno-chromatographic assay

According to the characteristics of monoclonal antibodies, the 150-3 monoclonal antibody, which has the best capability to capture BoNT/A, is

immobilized on a porous nitrocellulose membrane. While the 44.1A monoclonal antibody, which has the ability to detect BoNT/A, is conjugated to colloidal gold particles. This format is utilized to establish a colloidal gold immunochromatographic assay for detection of BoNT/A. The detection limit of the developed BoNT/A colloidal gold immunochromatographic assay is 50 ng/ml. The BoNT/A colloidal gold immunochromatographic assay showed no cross reaction to type B neurotoxin (BoNT/B) and type E neuroxoin (BoNT/E). Additionally, the sensitivity of BoNT/A colloidal gold-based immunochromatographic assay is not interfered by sample specimen containing 1:1 dilution of serum. As a result, our developed BoNT/A colloidal gold immunochromatographic assay is able to apply to the serum specimen.

BoNT/A magnetic bead based enzyme immunoassay

By using the same pairs of antibodies, magnetic bead M270 is coated with 150-3 monoclonal antibody by covalent bond, and 44.1A monoclonal antibody is conjugated to horseradish peroxidase (HRP). The reaction is developed by adding an HRP substrate to produce a signal, which indicates the quantity of BoNT/A in the sample, and the absorbance is read at 450 nm. The mechanism of BoNT/A magnetic bead based enzyme immunoassay is shown as Figure 4.

The detection limit of magnetic bead based enzyme immunoassay can reach to 500 pg/ml in detection of BoNT/A, which is 100 times lower than colloidal gold immunochromatographic assay. Similarly, the BoNT/A magnetic bead-based immunoassay showed no cross reaction to type B neurotoxin (BoNT/B) and type E neurotoxin (BoNT/E). Furthermore, the sensitivity is not interfered when food samples or clinical specimens are applied. In conclusion, our developed BoNT/A colloidal gold immunochromatographic assay is rapid (taking only 10 minutes), easy to carry, easy to operate and cost saving. The assay could be operated by inexperienced personnel. Besides, only a small amount of specimen (50 ul) is needed for this assay. The results can be read by naked eyes without special

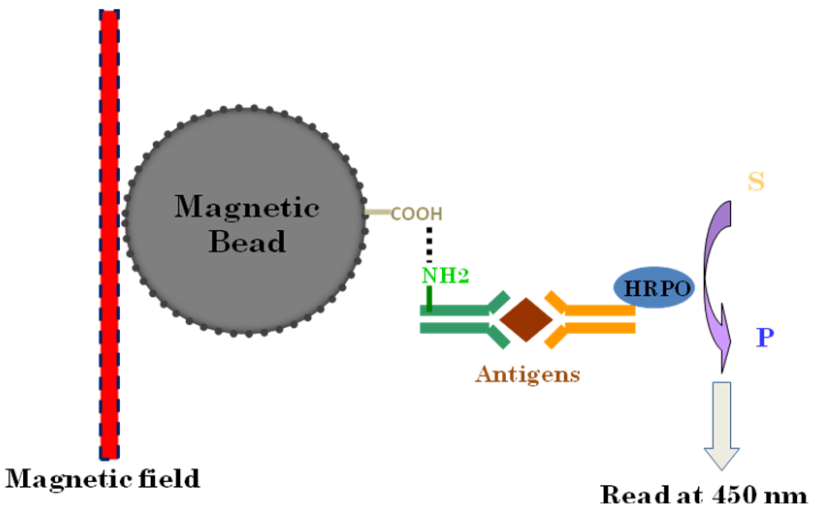

Figure 4. The principle of magnetic bead based enzyme immunoassay

instrument. On the other hand, the BoNT/A magnetic bead based immunoassay also possesses rapid (taking 30 minutes), digitized, and automatic characteristics, but it is more sensitive than BoNT/A colloidal gold immunochromatographic assay. Both useful tools could be provided to first-line responders and laboratory workers for detection of BoNT/A in specimens, which fulfill the detection requirement in fields of biological warfare, anti-terrorism, food industry and environmental monitoring.

Acknowledgements

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Cluster Infection of Novel Influenza A (H1N1) Virus in the Psychiatric Wards of a Hospital in Hualian County of Taiwan, 2011

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Abstract

In late February, 2011, thirty-seven residents with symptoms of upper respiratory infection (URI) in the psychiatric wards of a hospital in Hualian County were reported. Specimens were taken from ten of the thirty-seven cases, and eight of which were confirmed positive for novel influenza A

(H1N1) virus. Since it occurred in a populous institution, the outbreak was classified as a nosocomial cluster caused by novel influenza A (H1N1) virus infection. Thirty-two percent (37/117) of the residents in the psychiatric wards have experienced URI symptoms. The investigation shows that 99.1% of the 117 residents had received 2010-2011 seasonal influenza vaccine (containing strain of A/California/7/2009 (H1N1)-like virus,) but the cluster infection of novel influenza A (H1N1) virus still occurred among them, and was therefore considered an unusual event. This study shows that although residents and workers in populous institutions are the priority objects for seasonal influenza vaccine in Taiwan and have very high vaccination coverage rates, it is still possible for cluster infection of novel influenza A (H1N1) virus to occur under an environment where residents are cared in a concentrated area. Therefore, besides from the fact that residents in a populous institution should maintain a very high vaccination coverage rate, epidemic surveillance and case notification should be improved, infection control measures should be enforced, and the vaccination coverage rate of the workers should be increased to avoid recurrence of the similar cluster infection.

Keywords: novel influenza A (H1N1), seasonal influenza vaccine, cluster infection

Introduction

The novel influenza A (H1N1) epidemic first emerged from Mexico and south-west of United States in April 2009, spreading rapidly throughout the world. On the same year, the World Health Organization (WHO) announced on June 11 that the novel influenza A (H1N1) was a pandemic disease with moderate severity, that is to say the rate of severe cases and fatal cases caused by the novel virus was equivalent to those by influenza virus leading to seasonal epidemics. Therefore, the novel influenza A (H1N1,) in the classification of notifiable diseases in Taiwan, was changed from Category 1 communicable disease down to Category 4 on 19 June 2009. This meant that only those of novel influenza cases meeting the definition for seasonal influenza case with severe complications shall be reported, and the control measures for severe novel influenza cases were same as those for severe seasonal influenza cases. The status of novel influenza A (H1N1) pandemic lasted until 10 August, 2010, when the WHO declared that the disease has entered the post-pandemic period, and stressed that the novel influenza A (H1N1) virus have not yet disappeared, and will probably continue to spread in the same way as the seasonal influenza, but with the potentiality of causing different scaled epidemics in various locations. [1]

In order to protect citizens from suffering severe complications or deaths due to influenza infections, the Centres for Disease Control (Taiwan CDC) of the Department of Health of the Republic of China provided vaccinations against seasonal influenza free of charge for high risk populations, such as elders, infants, young children, health providers, and residents and workers in populous institutions. [2]

In late February, 2011, thirty-seven residents with symptoms of upper respiratory

infection (URI) in the psychiatric wards of a hospital in Hualian County were reported. Specimens were taken from ten of the thirty-seven cases, and eight of which were confirmed positive for novel influenza A (H1N1) virus. Since it occurred in a populous institution, the outbreak was classified as a nosocomial cluster caused by novel influenza A (H1N1) virus infection. The cluster infection attracted much attention from health authorities, because investigation shows that 99.1% of the 117 residents had received 2010-2011 seasonal influenza vaccine. This article describes the process and scale of the occurrence of the cluster infection caused by novel influenza A (H1N1) virus, and explores the possible factors for causing the outbreak.

Case definition

A resident in the psychiatric wards of the hospital in Hualian County developed symptoms of fever (a body temperature above 37.5℃), general body aches, and one of the URI symptoms (including cough, sputum, nasal congestion, runny nose, sore throat, and hoarseness) during the investigation period (17-25 February, 2011) was defined as suspect case. A suspected case that is tested positive for swine H1 (swH1) by real-time PCR test is defined as a confirmed case.

Descriptions of the outbreak

During 17-18 February, 2011, three residents at building B of psychiatric wards of the hospital in Haulian County continually presented symptoms of fever, cough, and sore throat. On 19 February, residents in building A also had symptoms of cough and runny nose, while other residents in building B continually showed signs of URI symptoms. Although most of them were mild cases without dangerous syndrome, with only symptoms of fever, cough, and runny nose, the hospital took relevant measures to monitor and control, to avoid further spread of the outbreak on 20 February, including provision of surgical masks for residents with fever and other URI symptoms, and isolation of these cases in three separate wards. Since these cases were diagnosed as URI, the cases or residents were not provided with antiviral drugs for the treatment or chemoprophylaxis. Up to 25 February, a total of 37 residents with symptoms of fever, cough, and sore throat were identified, therefore the hospital reported the cases to health authorities as a cluster of URI on the same date（Figure）.

Results of investigation and analysis

Cases of the outbreak mainly occurred in wards for patients with mental illness. In total, 117 beds were equipped in these wards, and 117 male patients resided in them. These wards were located in two buildings, A and B. However, the spaces for daily activities and meals of the residents in both of the two buildings were not separated. They shared the same restaurant in building B, and the bathrooms and toilets in buildings A and B.

Through the investigation, authorities found that four workers (three of them had already received seasonal influenza vaccine) of the wards have developed URI symptoms during 11-15 February, 2011, but did not have fever symptoms. Starting from 17 February, the residents continually became ill, and a total of 37 cases were identified up to 25 February. The hospital, therefore, reported to

health authorities as a cluster of URI on 25 February, and took throat swab specimens from ten of the cases on the same date, which were sent to laboratory of the Taiwan CDC for swine H1 real-time PCR test. The result showed that eight of the ten specimens were swine H1 positive. Therefore, the outbreak of the URI occurring among the wards was confirmed to be resulted from novel influenza A (H1N1) virus.

In addition, the hospital reported two more cases with symptoms of URI on 3 March, one physician and one nurse, and the dates of onset were respectively on 1 and 2 March. Based on treatment guidelines, these cases were administered with therapeutic antiviral drugs (oseltamivir 75mg/1 cap Bid for 5 days).

The investigation showed that 117 patients were residing in wards located in both building A and B, and 116 of them have received seasonal influenza vaccines made by same foreign company on 19 October, 2010. Nineteen persons were working for the wards, including eight caregivers, ten nurses, and one physician, twelve of whom had received seasonal influenza vaccines (data on vaccination of residents and workers are shown in Table)

During the investigation period, a total of 37 cases was identified, 17 in the wards of building A, and 20 in building B. The age of the cases ranged from 24-70 years-old (with a median age of 49 years-old.) Cases younger than 40 years of age accounted for 5.4%, in age groups of 40-49 and 50-59 years-old, 60

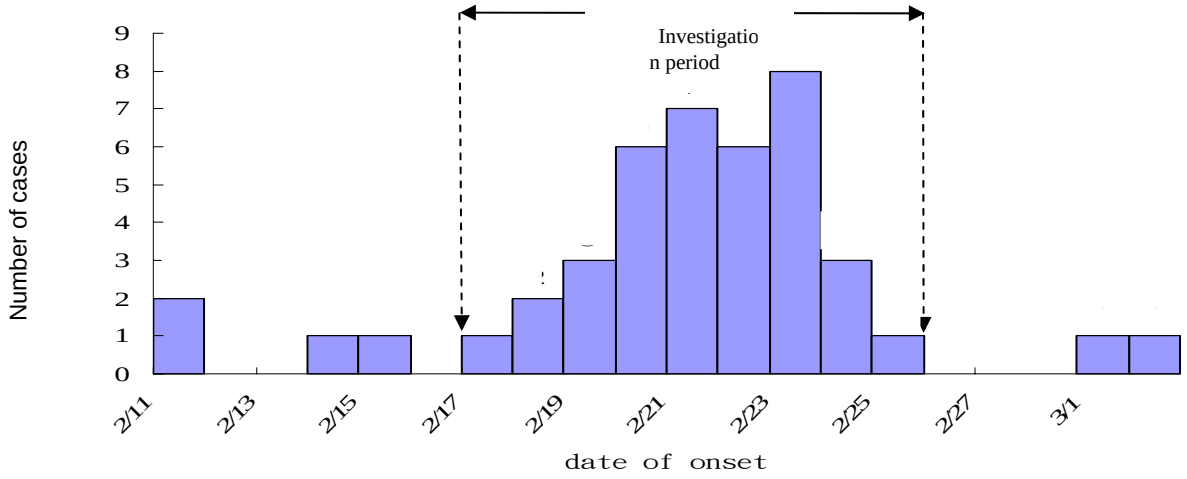

Figure Number of novel influenza A (H1N1) cases in the psychiatric wards of the hospital in Hualian County, by date of onset

Table Number of residents and workers receiving seasonal influenza vaccine and having URI symptoms in the psychiatric wards of the hospital in Hualian County

		Workers			
	Residents *	Physician	Nurses	Caregivers	Total
Reception of seasonal influenza vaccine	116（36）**	1（0）	6（0）	5（0）	128（36）
Non-reception of seasonal influenza vaccine	1（1）	0（0）	4（0）	3（0）	8（1）
Total	117（37）	1（0）	10（0）	8（0）	136（37）

Note: * The residents received seasonal influenza vaccine made by same foreign company on 19 October, 2010.
* * Number within the parentheses represents the number of residents with URI symptoms during the investigation period (17-25 February, 2011)

years-old, and older respectively accounted for 45.9%, 35.1%, and 13.5%. All of the cases were male. The attack rate for the cluster was 32% (37/117). Symptoms commonly seen among the cases included fever in 100% of cases (37/37, 17 of them with a body temperature over 38℃), cough in 43% (16/37), runny nose in 24% (9/37), and sore throat, headache, and diarrhoea each in 3% (1/37).

Discussions and Recommendations

The novel influenza A (H1N1) virus spreads between person and person mainly through the route of droplets and contacts. Cases become communicable from the first day before the date of onset of symptoms to the seventh day after the date of onset. However, the communicable period in cases of young children, elders, and immuno-compromised people is probably longer. Although four workers in the psychiatric wards have continually developed URI symptoms during 11-15 February, they were neither taken specimens nor reported to health authorities because they were then considered to not meet the symptoms for flu-like illness. Therefore, we are unable to determine whether they were infected with novel influenza A (H1N1) virus. However, in view of the date of onset of the cases in workers and residents, and in consideration of the working conditions that created a space for close contact with each other, we are unable to exclude the possibilities that the cluster of URI in residents was resulted from a secondary infection of the cases in workers. Although the hospital did not report the cases of URI in the residents until the ending stage of the infection wave, they had undertaken controlled measures, such as case isolation and wearing of surgical masks, on 20 February when they noticed the abnormal occurrences of URI. These measures should have been helpful in preventing the secondary infection from occurring.

The fact that the hospital did not immediately report the cluster infection and take specimens for the cases when three residents developed URI symptoms on 17th and 18th of February, until the number of cases continually increased up to more than 30 cases, indicates that there is room for improvement in the response and management of the cluster. Although the hospital took relevant control measures on 20 February, some more active control actions should have been implemented. In addition, despite the fact that 99.1% of the 117 residents have received 2010-2011 seasonal influenza vaccines, only 63.2% of the workers have been vaccinated. Some workers might be vulnerable to contracting the infection of novel influenza A (H1N1) virus because of insufficient immunity, and were very likely to spread the virus to residents via daily care activities. We, therefore, suggest that the hospital should elevate the coverage of seasonal influenza vaccination among the workers.

In response to the change of the circulating strains of seasonal influenza virus, the WHO, on the basis of the global surveillance data, makes predictions in February or March every year about the dominant strains that may circulate in the coming influenza season, and make recommendation on the virus strains that

should be included in the vaccines for winter season in the northern hemisphere. When the 2009 novel influenza A (H1N1) pandemic came to an end, the WHO recommended that the novel influenza A (H1N1) virus should be included in the 2010-2011 seasonal influenza vaccines for the northern hemisphere. The influenza vaccines used for prevention of 2010-2011 seasonal influenza in Taiwan was produced based on the recommendations made by the WHO on vaccine composition for the northern hemisphere. The influenza vaccines were composed of three inactive strains of the influenza virus, including two influenza A virus (H1N1 and H3N2,) and one influenza B virus (A/California/7/2009 (H1N1)-like virus, A/Perth/16/2009 (H3N2)-like virus, and B/Brisbane/60/ 2008-like virus). [2]

The place where the cluster infection occurred was one of the more populous institutions where the residents, workers, and medical providers in the institution were all the priority objects for 2010-2011 seasonal influenza vaccines, provided free of charge by the Taiwan CDC. The vaccination rates for people in these institutions were generally over 90%. Based on the investigation, twelve of the nineteen workers in the psychiatric wards had received 2010-2011 seasonal influenza vaccines, and 116 of the 117 residents were also vaccinated on 19 October, 2010, with seasonal influenza vaccines of the same batch number produced by the same company. The high overall vaccination coverage rates, 94%, for people in the hospital should be able to provide effective herd immunity. The influenza virus antigen test also showed that the novel influenza virus strain isolated from cases of the cluster is similar to that of included in the vaccines. The result indicated that the virus causing the cluster infection was not a variant strain, which means that the seasonal influenza vaccines given to the residents were still effective for prevention of influenza. Since influenza viruses are RNA viruses that lack a good error-detecting ability in the process of gene duplication, a gene mutation is very likely to occur in the gene duplication. If the mutation has caused the change of amino acid on the antigenic sites of hemagglutinin (HA) and neuraminidase (NA) , the genetic mutation is called antigen drift. However, antigen drift will not usually result in a new subtype of the virus. Therefore, whether the cluster infection was resulted from a minor change in the antigenic sites of the virus still needed to be further explored. Some literatures have indicated that the influenza vaccines were less efficient in elderly people. However, only three (13.5%) of the novel influenza cases identified in the psychiatric wards were older than 60 years of age, which do not completely match the findings of the literatures. Furthermore, the seasonal influenza vaccines had been given to the residents nearly four months before the occurrence of the cluster infection, with a sufficient time for an antibody production. In spite of these conditions, cluster infection of novel influenza A (H1N1) still occurred among the residents. Whether the occurrence of the cluster was associated with factors of vaccine manufacturing, cold chain, or a decreased vaccine protection, it is worthy of further exploration.

Usually, it takes more than two weeks for a host to develop protective antibody after the injection of seasonal influenza vaccine, and the protective antibody can maintain up to six months. However, the vaccinated host will still have the risk of acquiring influenza before the development of a protective antibody. Based on previous studies, the efficacy of the influenza vaccine on average was 30-80%. However, it will changed, depending on the host's age or health conditions. For healthy adults, the efficacy can reach 70-90%. For elderly people, influenza vaccinations will make a reduction of hospitalization due to severe or complicated influenza up to 50-60%, and decrease mortality rate as high as 80%. [3]

The study on clinical symptoms of novel influenza A (H1N1) cases conducted in the USA, Canada, and Japan in 2009 showed that 87-94% of the cases showed symptoms of fever, 59-92% have coughs, and 39-60% experience sore throats. [4-5] Analysis on clinical symptoms of novel influenza A (H1N1) cases in Taiwan indicated that 78.7-89.6% of the cases have symptoms of fever, 75.7-82% have coughs, 20-50% also presented sore throats, runny noses, headaches, and sore muscles, with less than 10% of cases experiencing vomiting and diarrhoea. [6-8]

Although novel influenza A (H1N1) virus was inferred to be the agent causing the cluster infection based on laboratory data, 13 of the 37 cases presented only the symptom of fever and no other symptoms. Moreover, the percentages of cases with symptoms of fever (≧38℃, 46%), cough (43%), and runny nose (24%) were obviously lower than those found among general novel influenza A (H1N1) cases without receiving influenza vaccines. The cases occurred in the cluster infection also had milder symptoms, and no severe cases were found among them, as compared with the general unvaccinated novel influenza A (H1N1) cases. Whether these findings were associated with the fact that the majority of cases of the cluster infection had received influenza vaccines, a more complete data was necessary for further exploration. Based on Taiwan CDC surveillance data, an accumulated 1,751 influenza cases have been admitted to hospital for treatment up to 8 April, 2011, with 47 of them having received seasonal influenza vaccines; and, of the 130 fatal cases, only three have received seasonal influenza vaccines, with only two of the 94 fatal cases resulted from the infection of novel influenza A (H1N1) virus having received seasonal influenza vaccines. These data revealed that unvaccinated people have an obviously higher risk of becoming severe cases, while acquiring infection of influenza virus and the influenza vaccination can definitely prevent occurrences of serious complications, and decrease the number of hospitalizations. Most of the data in previous studies show that vaccination is still the most effective and safest measure to take in prevention of the infection of influenza up to now. Therefore, to ensure health for yourself and your family, people at a high risk of infection should complete the seasonal influenza vaccinations within the scheduled time period.

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Biosafety and Biosecurity

Health Management Recommendations for Laboratorian working on *Brucella*

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Brucella species are Gram-negative, rod-shaped bacteria. They can cause zoonotic brucellosis, mainly infecting cattle, goats, pigs, camels and dogs. Humans are accidental hosts. People are infected usually due to contacting with infected animal tissues or consume contaminated dairy products. *Brucella* belongs to Risk group 3 (RG3) microorganisms with approximately 31% attack rate to humans [1]. Laboratory personnel can get infection via inhaling or contact with the agent or its aerosols. *Brucella* is the most common pathogen that causes laboratory-acquired infections [2].

Taiwan's first human case of brucellosis was reported in 1978 [3] when a veterinary graduate student was accidentally infected at the laboratory of a university. During 1979 to 1981, cows in 14 Taiwan’s pastures were infected by *Brucella*, while 16 people were

infected via direct contact with cattle or specimens, including nine laboratory staffs.

The Centers for Disease Control, Taiwan (Taiwan CDC) received report of three imported brucellosis human cases from three hospitals during May to July, 2011. Since no human brucellosis infection has been seen in Taiwan for 30 years, most laboratories do not have the experience of testing *Brucella*. Taiwan CDC used questionnaires and telephone interviews to investigate the experimenter's protective measures during the operation process in the hospitals where the reported cases were involved in. The results showed that only one hospital directly transferred patient's blood samples to Taiwan CDC for testing, while the other two hospitals had cultured the patients' samples, identified the bacterial strain and done nucleic acid sequencing either in a Biosafety Level 2 (BSL-2) laboratory or at a negative pressure BSL-2 laboratory, respectively.

According to the operation guidelines for *Brucella* cultures established by U.S. Centers for Disease Control and Prevention, it is recommended to perform *Brucella* at BSL-3 laboratories [4], and list the personnel who conducts testing and research of *Brucella* as the high-risk group of brucella infection, for example, those who directly contacts with, inhales or inoculates *Brucella*, who operates *Brucella* on the bench with less than five feet distance, and who is in the laboratory during the process of potential infectious aerosols generating are personnel at high-risk. Particularly it should be reported to the relevant authorities when possible exposure to the environment of infection source is found. And it is suggested that taking post-exposure prophylaxis (PEP), such as taking a valid dose of Doxycycline and Rifampin for successive 21 days, and regularly taking serological examination, body temperature and monitoring symptoms for six months after exposed [5].

In response to the recurrence of similar cases in the future, Taiwan CDC took some international recommendations and guidelines as references to establish "The risk assessment and post-exposure prophylaxis guidelines for laboratory personnel work on *Brucella*" [6]. It is posted on Taiwan CDC's website and hence the public can easily follow, and it is also helpful in strengthening the safety awareness of the laboratory personnel when operating *Brucella* and ensuring them to take appropriate post-exposure preventive measures and health monitoring. In addition, to protect the safety of laboratory personnel, it is suggested to conduct strain identification or nucleic acid sequencing for pathogen cultures with unknown RG level in a BSL-3 laboratory. If BSL-3 laboratory is unavailable, it is recommended to send the specimens to Taiwan CDC or organization equipped with a BSL-3 laboratory for confirmation. Nevertheless, the culture specimens should be delivered intact without colony transfer in order to protect the operators' safety.

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An Introduction to the Laboratory Biosafety Accreditation Systems in China

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As biotechnology develops, the researches involving with biological or animal specimens have increased significantly, and indirectly prompting the staff to pay more attention to laboratory biosafety issues at the same time. After the outbreak of severe acute respiratory syndrome (SARS), the WHO [1] had warned the state members that laboratories might be the risky areas in transmitting SARS, and recommended the state members to be vigilant on the management of laboratory biosafety measures.

Chinese government issued a series of laws and regulations on laboratory biosafety construction and management since SARS occurred in 2003. For example, the State Council of the People's Republic of China issued Decree No. 424 of "Regulation on the biosafety management of pathogenic microbiology laboratory" [2] in 2004 that stipulated the health department in State Council to be in charge of human health-related laboratories and the supervision of laboratory biosafety, while the veterinary department to be responsible for animal-related laboratories and the supervision of laboratory biosafety. The categorization management in pathogenic microorganisms, the laboratory grading management, and biosafety standards were also established. The laboratories and their respective authorities are responsible for routine management of the laboratory activities; establish robust safety management systems; inspect and maintain the laboratory facilities and equipments; and control of laboratory infections. The Article 20 under the regulation also stipulated that Biosafety Level 3 (BSL-3) and BSL-4 laboratories should be validated by national laboratory accreditation system for every 5 years.

In the same year, China National Accreditation Board for Laboratory (CNAL), which is the predecessor China National Accreditation Service for Conformity Assessment (CNAS), organized and contacted with the relevant institutions, associations and industries to establish and announced the China national standards for laboratory biosafety - General laboratory

safety requirements (GB19489-2004) [3]. The CNAL also followed the Article 20 of “Regulation on the biosafety management of pathogenic microbiology laboratories” to draft and establish the national laboratory biosafety accreditation system. Initial planned laboratory accreditation norm (CNAL / AC 30; 2005) [4] consisted of two parts: the first part directly adopted the contents from the General laboratory safety requirements (GB19489-2004); the second part adopted partial of the “Regulation on the biosafety management of pathogenic microbiology laboratories” promulgated by Chinese State Council. The general laboratory safety requirements (GB19489-2004) were framed mainly referenced to WHO Laboratory Biosafety Manual, 3rd Edition [5], and International Organization for Standardization (ISO) 15190: 2003 Medical Laboratory - Requirements for Safety [6], U.S. CDC’s Biosafety in the Microbiological and Biomedical Laboratories [7], and Canada’s Laboratory Biosafety Guideline [8].

The ISO released the ISO 15190:2003 Medical Laboratories - Safety requirements in November 2003 that regulates the laboratory construction, the facilities, the environment, personnel operations, environmental management and utilization, corresponding to the biological, chemical, and radiological safety requirements. The contents were mostly concerning general safety requirements in medical laboratories, applicable to the BSL-1 and BSL-2 laboratories.

Realizing that the concept of biosafety management needs internationalization to meet biosafety and biosecurity management, CNAS amended the General laboratory safety requirements (GB19489-2004) in 2007 to the contents of "risk assessment", "BSL-1 and BSL-2 laboratories”, "The type of BSL-3 laboratories", " BSL-4 laboratories”, "automation requirements for biosafety laboratories”, "laboratory biosafety management system" and "standard revision". After that, CNAS officially launched the newly revised “General laboratory safety requirements” (GB19489-2008) [9] on December 26, 2008, and also revised “Laboratory biosafety accreditation norm” (CNAS-CL05: 2009) [10]. To corresponding to different laboratory activities, the purposes of the contents of the 2008 edition is mainly to add the classification of different laboratories with primary personal protection and basic protective equipments, enhance the requirements of laboratory automation system, increase the requirements of laboratory biosafety management system, deleted the chapters with repeated contents, and classify the hazard degrees.

The concept of laboratory biosafety management was originated from the “Laboratory biorisk management standards” (CWA 15793:2008) [11] established by European Committee for Standardization (CEN) in 2008, which combines the thinking of continuous improvement in organizational operation process along with the laboratory biosafety system. With this concept, through the management system within an agency or

unit, the biosafety operation can be achieved by using self-auditing and maintenance. The analysis of laboratory biosafety accidents by CNAS discovered that more than 90% of incidents were caused from the management problems. Therefore, to establish a systematic management system for laboratory biosafety is indispensable to ensure safe operation in laboratories. The previous General laboratory safety requirements (GB19489-2004) did not provide explanation and requirement from a systematic perspective. This brought a greater challenge to laboratories on the practical application process.

CNAS is the executive agency that implements the laboratory biosafety accreditation system in China. The first BSL-3 laboratory was accredited in June, 2005. By October 2010, 26 BSL-3 laboratories and two BSL-2 laboratories had been accredited.

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