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Establishing a Yellow Fever Serological Testing Method

Abstract

For the rapid diagnosis and early detection of cases, and early control of disease outbreaks, a plan for the establishment of a laboratory testing system for yellow fever was reviewed. Serum specimens were collected from voluntary acceptors of yellow fever vaccine before immunization, and seven, 14 and 48 days after immunization for testing with ELISA (enzyme-linked immunosorbent assay), HI (hemagglutination-inhibition test), PRNT (plaque reduction neutralization test), and virus isolation methods to decide on the most optimum laboratory testing method for yellow fever. The results showed that, by ELISA method, serum collected 14 days after immunization showed ELISA-IgM positive, indicating that both the sensitivity and specificity of the ELISA-IgM system were relatively high. No viruses were detected by virus isolation in sera collected and cultured seven and 14 days after immunization. By using the HI test for testing virus antibodies, it was found that though serum specimens collected before immunization and 14 days after immunization showed cross-reactions of other viruses of the flavivirus genus, antibodies had increased by four-folds. The HI test was thus considered a tool good only for initial screening. When the pairing serum specimens collected before immunization and 14 days after immunization were compared with the PRNT

method, it was found that the neutralizing antibodies of yellow fever had increased by four-folds, and their titers were high, particularly in the case of primary infection or early infection. The type of the flaviviruses that has caused the infection can be detected by using the PRNT to test the titers of the neutralizing antibodies; its sensitivity and specificity are both high, and even higher than those of other serological testing methods are. Results obtained from different experimental methods showed that both the ELISA-IgM and the PRNT were similar in result. They could clearly detect the types of the flaviviruses that had caused the infections. They were higher in sensitivity and specificity than other laboratory testing methods.

Key words: yellow fever, dengue fever, Japanese encephalitis, ELISA, virus isolation, HI, PRNT

Introduction

The rapid development in commerce and industries in the recent years has brought many visitors and tourists from Taiwan to the Southeast Asian countries. Imported communicable diseases have thus become a nightmare to disease control. Strengthened quarantine at ports of entry may not even completely prevent their entry. Yellow fever is classified in Taiwan as a level one notifiable disease. A suspected case must be reported to the local health authorities within 24 hours. Yellow fever, Japanese encephalitis, and dengue fever are all members of the family Flaviviridae.

Yellow fever exists in nature in two transmission cycles, an urban and a sylvan or jungle cycles. The urban yellow fever is primarily transmitted by *Aedes aegypti* mosquitoes[(1)]. It is more common in the humid forests of Africa, and is an endemic in the tropical forests of Africa and Latin America involving mosquitoes and nonhuman primates[(2)]. Since the successful development of 17D vaccines in the 1940's, no cases of *Aedes aegypti*

transmitted yellow fever have been reported in the Americas. The yellow fever outbreaks have also been successfully controlled in the French western African countries by the immunization of the French neurotropic vaccine. In some western African countries, however, for no universal immunization, outbreaks of yellow fever have occurred still.

Clinically, yellow fever is an acute infectious viral disease of short duration and varying severity. The mildest cases are clinically indeterminate. Typical attacks are characterized by symptoms similar to those of dengue fever such as sudden onset, chills, fever, headache, backache, generalized muscle pain, prostration, nausea and vomiting. The pulse may be slow and weak out of proportion to the elevated temperature. Jaundice is moderate early in the disease and is intensified later. Albuminuria and anuria may occur. The overall case-fatality rate among indigenous population in endemic regions is <5%. Fatality can be as high as 50% in foreign population[3,4]. Incubation period is from three to six days. Blood of patients is infective for mosquitoes shortly after onset of fever and for the first 3-6 days of illness. Viruses can even be isolated on the 17th day of onset.

Yellow fever though is more prevalent in the tropical regions of the Americas and Africa, in the tropical and subtropical regions in Asia where dengue fever is endemic, such as Taiwan with a high population density and wide distribution of vector mosquitoes, yellow fever can be easily misdiagnosed as dengue hemorrhagic fever (DHF) for the reason that clinical symptoms of yellow fever and dengue fever or dengue hemorrhagic fever are similar. Yellow fever can be easily overlooked unless in the routine testing of dengue fever, yellow fever is also tested at the same time. Although no cases of yellow fever have occurred in Asia, there are still sporadic cases reported in Taiwan each year. *Aedes aegypti* mosquitoes are around in all parts of Asia. *Aedes aegypti* is also a key vector of dengue fever. Practically everyone is susceptible.

Along with the development in commerce and industries, traveling by air has become common; monitoring of yellow fever has become a major concern of the disease control authorities. Laboratory testing is the front line of disease control; development of a rapid and precise laboratory testing method for yellow fever has become more urgent.

Materials and Method

Sources of Samples

Samples of the present study came from eight voluntary acceptors of yellow fever vaccine. Blood specimens were collected from them before immunization, and also seven, 14 and 48 days after immunization. Two of them, however, declined to have blood taken on the 7^{th} and 48^{th} days after immunization. All sera were tested by ELISA, HI, PRNT and virus isolation methods. Positive cases were tested once again to be sure.

Preparation of HI and ELISA Antigens

17D (yellow fever), HAWAII (dengue 1) and JaGaro1(Japanese encephalitis) viruses were inoculated on the brains of 1-3 days old mice. The brains were taken from the dying mice for collecting antigens with sugar and acetone to test for the titers of antigens. Testing should be carefully conducted to prevent splinter of needle.

1. ELISA-IgM[5,6]
   1) 100 μl dilution containing 5 μl of goat anti-human IgM was added to the 96 well plate and placed overnight under 4℃ or for two hours under room temperature;
   2) washed with washing buffer five times and dried;
   3) added 200 μl of blocking buffer and placed under 37℃ for 30 minutes;
   4) diluted with blocking buffer at 1:100;

5) adjusted the antigen concentration to 20HA, diluted with HB112 (single strain antibody) at 1:1,000; placed under room temperature;
6) washed with washing buffer five times and dried;
7) added 100 μl/well of specimen saved for step 17, using positive and negative sera of dengue fever and Japanese encephalitis as contrast, placed under 37℃ for one hour;
8) washed with washing buffer five times and dried;
9) added 100 μl/well of disinfectant saved for step 18, placed under 37℃ for one hour;
10) washed with washing buffer five times and dried;
11) added 100 μl/well of AP-goat anti-mouse IgG (diluted at 1:1,000), placed under 37℃ for one hour;
12) added 100 μl/well of PNPP, placed in room temperature for about 30 minutes, tested for light absorption at wavelength 450 nm; light absorption value for the negative is about 0.3; for the positive, about 2.0.

2. Virus Isolation[7]

C6/36 or other cell strains were used for the isolation of the yellow fever virus and then tested for the existence of virus by the indirect fluorescent antibody test. 0.005 mL of serum was added to each well of the 96 well tissue culture plate. Specimens were diluted for 50 to 200 times with RPMI containing 2% of fetal calf serum. 0.1mL of C6/36 cells ($1x10^6$/mL) were added to each well, placed in incubator under 30℃ for culturing for seven days. The cells on the plate were removed, dried under room temperature, and fixed in –20℃ acetone. Monoclonal antibodies against yellow fever were added, placed in incubator under 37℃ for dyeing for 30 minutes. Excess antibodies were washed away with PBS(Phosphate-buffered saline), dried. Goat of anti-mouse globulin was added. Observation was made with fluorescent microscopes.

3. HI[5,8]

Testing was conducted by the current routine laboratory testing procedures. Antigen titers were first adjusted to 16HAU. Sera were extracted with acetone and absorption processed with 100% RBC. The supernatant was collected. Specimens were diluted at this point at 1:10. It was inactivate-processed under 56℃ for 30 minutes. Lastly, it was HI-tested at 2x dilution (HI<10, 80, 640 for the positives and negatives of dengue fever and Japanese encephalitis respectively).

4. PRNT[5,8]

Titers of the neutralizing antibodies of yellow fever, dengue fever and Japanese encephalitis were tested respectively with 17D (vaccine strain), HAWAII (DEN1) and Nakayama.

1) 0.5 mL of BHK_{21} cells (150,000/cc) was placed in 24 well plate, cultured in 5% CO_2 incubator under 37℃ for three days;
2) specimens were inactivate-processed under 56℃ for 30 minutes, diluted with serum dilution (containing minimum essential medium + 5% FCS and 0.01 M Hepes);
3) virus already quantified was diluted with virus dilution (containing minimum essential medium + 5% fetal calf serum + 0.01 M Hepes), mixed with inactivate specimen of the same quantity and placed under 4℃ for 18-21 hours;
4) threw away the upper cell fluid of step 1), added 50 μl of specimen and virus mixture fluid, placed in 5% CO_2 incubator under 37℃ for 3-6 days;
5) threw away the overlay medium, added 0.5mL of 0.9% NaCl to wash away the overlay medium, used NBB for dyeing of cells;

reading: when plaques of sera were 50% lower than the plaques of pairing viruses (not containing sera), the result was considered positive (compared with

both the positives and the negatives of dengue fever and Japanese encephalitis respectively).

Results and Discussion

1. ELISA-IgM

The test reagent for ELISA-IgM developed by the laboratory was used. Yellow fever IgM antibodies of sera collected seven, 14 and 48 days after immunization were all negative; while 100% of IgM antibodies on the 14th and 48th days were positive (Figure 1). IgM antibodies of dengue fever and Japanese encephalitis were also tested. They were all negative (Figures 2, 3). This fact indicated that both the sensitivity and specificity of the ELISA-IgM test reagent developed by the laboratory were high. This was a rapid serological testing method[9,10], and can be used for either routine or mass screening. In either primary or repeated infections of yellow fever, IgM antibodies would appear. ELISA-IgM was therefore a good choice in the diagnosis of suspected cases or recent infection of yellow fever[11].

2. Virus Isolation

It is always not easy to isolate viruses. Generally, multiplication of viruses reaches a climax 2-4 days after onset, and viruses can be isolated within 8-9 days after onset. Chances of virus isolation decline with time thereafter. No viruses were isolated from the sera of the 7th and the 14th days. Whether there were in fact no viruses or the sensitivity of the present laboratory testing system was inadequate required further study. Any reported suspected cases of yellow fever should be imported cases, they would have missed the optimum period for the isolation of viruses, and therefore isolation of viruses from sera was difficult. More specimens were needed for testing. The time required for the isolation of virus, seven days, was long and time-consuming.

3. HI

Yellow fever antibodies tested with the HI method in all sera collected before immunization and 14 days after immunization, except one, showed a four-fold increase. HI antibodies declined slightly after 48 days. In dengue fever, antibodies in all sera except two collected before immunization and 14 days after immunization also showed a four-fold increase. The HI antibodies in Japanese encephalitis were the same as yellow fever, their titers, however, were much higher than the titers of either yellow fever or dengue fever (Table 1). By the titers of HI antibodies, dengue fever had the lowest titer, whereas Japanese encephalitis had the highest. This could be due to the original antigenic sin[11,12]. A large number of the population have gained antibodies against Japanese encephalitis either through immunization or natural infection, when they are immunized against yellow fever, the titers of their HI antibodies would naturally be higher than yellow fever. Though there had been several outbreaks of dengue fever in Taiwan in the past, most recent infections are imported, only a few people carry antibodies, their titers therefore are low. There are likely to have cross-reactions by using the HI method for the testing of yellow fever antibodies, and the specificity of HI is generally lower. Test results of cases previously infected by other viruses of the family Flaviviridae are hard to read[13,14]. The present study showed that there were likely to be cross reactions of other flaviviruses when HI was used for the testing of yellow fever antibodies, and the viruses that caused the infection could not be specified. The present study, however, showed a four-fold increase. The method was thus considered good for initial screening. In serological diagnosis, HI would give result in 2-3 days, and in particular, specificity was high for cases of primary infection or cases in the early phase. The method, however, required paired serum specimens, collected 14 days apart. The result could only be read when the antibody titers of the paired sera had either increased or declined

by four-folds.

4. PRNT

When the neutralizing antibodies of yellow fever were tested with the PRNT method, it was found that antibodies in sera collected before immunization and 14 days after immunization had shown a four-fold increase, and the titer of the neutralizing antibodies was more than 640; whereas the titers of the neutralizing antibodies of dengue fever and Japanese encephalitis had not shown a four-fold increase (Table 1). A large number of the population are immunized against Japanese encephalitis in their early ages or are naturally infected, they carry neutralizing antibodies of Japanese encephalitis, though the titers of these antibodies are low. In dengue fever, the neutralizing antibody of one case was 10, and his/her dengue fever IgM-ELISA antibody was positive. It was unknown whether he/she had been infected by dengue fever previously. In the rest seven cases, no neutralizing antibodies were detected, indicating that there could be cross-reactions in repeated infections, the neutralizing antibodies of yellow fever in most cases were still higher than those of other flaviviruses. Results revealed that by using the PRNT method to test for the neutralizing antibodies of the flaviviruses, the types of flaviviruses that caused infection could be clearly identified. Relevant studies also indicated the ability of the method in identifying the types of flavivirus infections[10,15,16]. Both the sensitivity and specificity were high, in particular, with cases of primary infection or cases in the early phase. The time required, however, was longer. Though there could be cross reactions in cases of super-infection, in most cases, the titers of the yellow fever neutralizing antibodies were higher than those of other flaviviruses, readings of the results could not be misleading. This method, however, requires paired serum specimens, takes longer time, and blood should be collected 14 days apart. The PRNT method can also be used

for the typing of dengue fever infections, though rather untimely, and takes longer time of 6-9 days. For its time-consuming, the method is considered not adequate for mass epidemiological investigations[17].

Conclusion and Recommendations

The present study found that, for the testing of yellow fever antibodies, the sensitivity and specificity of the ELISA-IgM method were higher than those of the HI and the virus isolation methods, and were close to those of the PRNT method. The ELISA-IgM testing system developed by the laboratory was high in its sensitivity and specificity, although more testing of specimens was necessary. The ELISA-IgM is a commonly used method in laboratories. It is, as compared to other methods, fast, simple, and of high sensitivity and specificity. Results obtained by this method are similar to those of the PRNT method[17]. The method is particularly good for early diagnosis and early control of diseases. It is equally applicable to either primary or repeated infections[10].

The HI is a conventional testing method for flaviviruses. The procedure is complicated and requires sera of cases in the acute and the recovery phases for comparison. Its sensitivity and specificity are not high, and there are cross-reactions of other flaviviruses[13,14]. It is not easy to identify from test result the types of flaviviruses that cause the infection. This is not considered a priority method for the diagnosis of yellow fever.

The PRNT method is the golden standard method for the testing of flaviviruses. It is a method of high sensitivity and specificity and is able to precisely identify the type of pathogenic agents that has caused the infection. Although cross-reactions may occur in cases of super-infection, the titers of the yellow fever neutralizing antibodies in most cases are higher than those of other

flaviviruses, readings of the test results will not be affected. The method can be used for the testing of various flaviviruses[10,15,16]. The PRNT though is an important technique in the testing of flavivirus infections, users should always be careful and experienced, or otherwise the instability of test findings can lead to misreading. Procedures of the PRNT are complicated, the method is rarely used for screening in mass epidemiological investigations[17], and not even in routine testing either. However, as the reported number of yellow fever infection is small, and when PCR is not established, the PRNT is a good method for the identification of yellow fever. Some cases of dengue hemorrhagic fever have been reported this year, prevention of yellow fever should be strengthened.

In the future, a rapid method for the identification of yellow fever, molecular biological method in particular, should be established to improve the sensitivity and specificity of testing methods, and to accurately identify families and types of viruses in the shortest possible time for accurate diagnosis and monitoring and control of diseases.

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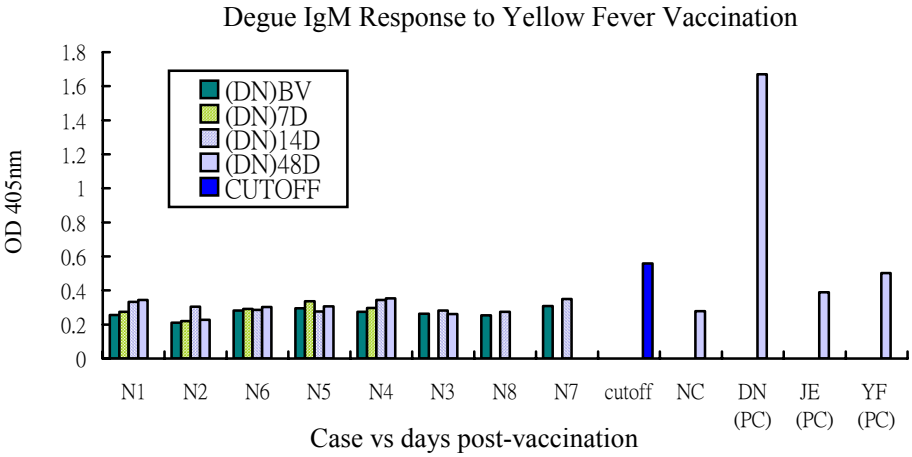
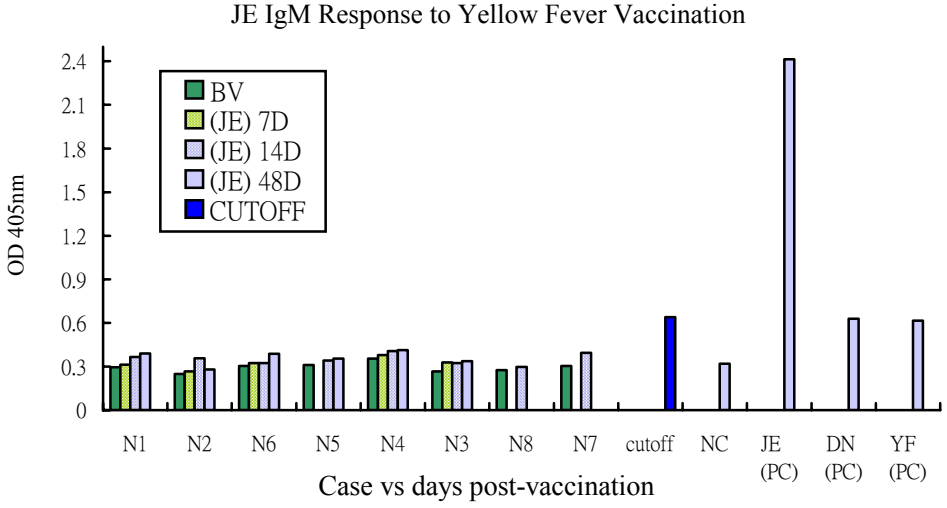
Table 1. The HI Antibody Titers among Yellow fever 、Japanese encephalitis and Dengue fever

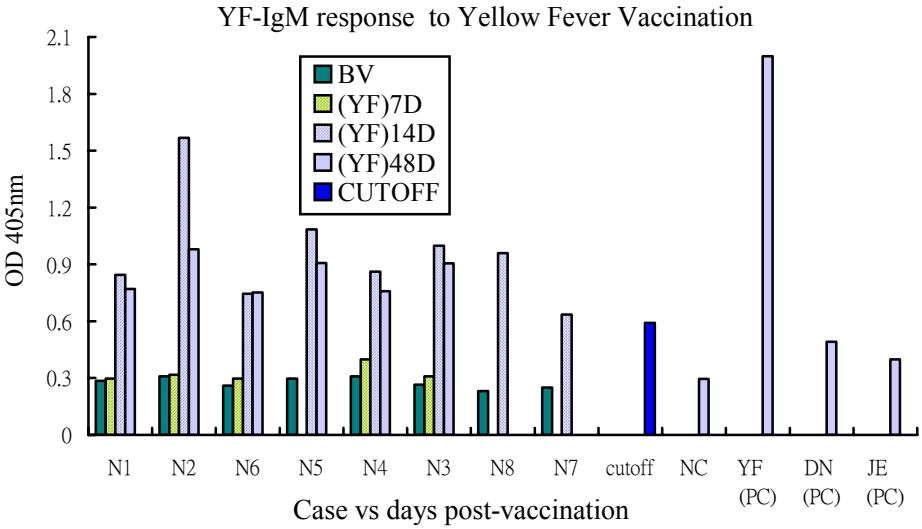
number	Unvaccinated	7days	14 days	48 days
	HI-titer	HI-titer	HI-titer	HI-titer
Yellow fever				
N1	<10	<10	320	320
N2	ND	<10	5120	2560
N3	<10	<20	20	20
N4	<10	ND	40	80
N5	<10	<10	40	40
N6	<10	<10	320	320
N7	<10	ND	80	ND
N8	<10	ND	80	ND
Japanese encephalitis N1	<10	<10	2560	1280
N2	ND	40	≥10240	≥10240
N3	<10	<10	<10	<10
N4	<10	<10	80	40
N5	<10	ND	320	80
N6	<10	<10	640	1280
N7	40	ND	320	ND
N8	<10	ND	80	ND
Dengue fever				
N1	<20	<20	160	80
N2	ND	<20	1280	640
N3	<20	<20	<20	<20
N4	<20	<20	40	<20
N5	<20	ND	80	<20
N6	<20	<20	80	80
N7	<20	ND	40	ND
N8	<20	ND	20	ND

Table 2. The Neutralizing Antibody Titers among Yellow fever 、Japanese encephalitis and Dengue fever

number	Unvaccinated	7days	14 days	48 days
	Nt-titer	Nt-titer	Nt-titer	Nt-titer
Yellow fever				
N1	<10	<10	10240	5120
N2	<10	<10	≥20480	5120
N3	<10	<10	2560	2560
N4	<10	<10	2560	1280
N5	<10	ND	2560	1280
N6	<10	<10	10240	5120
N7	<10	ND	640	ND
N8	<10	ND	2560	ND
Japanese encephalitis N1	10	10	10	10
N2	20	20	20	20
N3	10	10	10	10
N4	<10	ND	<10	<10
N5	10	10	10	10
N6	10	10	10	10
N7	20	ND	20	ND
N8	20	ND	20	ND
Dengue fever				
N1	<10	<10	<10	<10
N2	<10	<10	≥10	≥10
N3	<10	<10	<10	<10
N4	<10	ND	<10	<10
N5	<10	<10	<10	<10
N6	<10	<10	<10	<10
N7	<10	ND	<10	ND
N8	<10	ND	<10	ND


Degue IgM Response to Yellow Fever Vaccination
OD 405nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(DN)BV
(DN)7D
(DN)14D
(DN)48D
CUTOFF
N1
N2
N6
N5
N4
N3
N8
N7
cutoff
NC
DN (PC)
JE (PC)
YF (PC)
Case vs days post-vaccination


JE IgM Response to Yellow Fever Vaccination
OD 405nm
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
BV
(JE) 7D
(JE) 14D
(JE) 48D
CUTOFF
N1
N2
N6
N5
N4
N3
N8
N7
cutoff
NC
JE (PC)
DN (PC)
YF (PC)
Case vs days post-vaccination


YF-IgM response to Yellow Fever Vaccination
OD 405nm
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
BV
(YF)7D
(YF)14D
(YF)48D
CUTOFF
N1
N2
N6
N5
N4
N3
N8
N7
cutoff
NC
YF (PC)
DN (PC)
JE (PC)
Case vs days post-vaccination