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Establishment of the 1st WHO International Standard for anti-EV71 serum (Human)

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ABSTRACT

Enterovirus A71 (EV71) is the major causative agent of severe and fatal hand, foot and mouth disease. There is plenty of evidence that EV71 has circulated widely in the Western Pacific Region for the last twenty years. Vaccines against EV71 are already available or under development. A collaborative study to establish the 1st WHO International Standard for anti-EV71 serum (Human) was conducted to ensure that methods used to measure the serum neutralizing activity or antibody levels against EV71 are accurate, sensitive and reproducible. Two candidate samples as well as a third candidate reference containing low anti-EV71 antibody titre were produced from plasma samples donated by healthy individuals. All three serum samples exhibited good levels of neutralizing antibodies against a wide range of EV71 strains of various genotypes. The study showed that between laboratory variations in neutralization titres were significantly reduced when values were expressed relative to those of either of the two candidate sera. Sample 14/140 was established as the WHO 1st International Standard for anti-EV71 serum (human), 14/138 as its potential replacement and 13/238 as a WHO Reference Reagent, with assigned unitage of 1,000, 1090 and 300 International Units (IU) of anti-EV71 neutralizing antibodies per ampoule, respectively.

1. Introduction

EV71 is one of the major causative agents of severe and fatal hand, foot and mouth disease (HFMD) [1,2]. Since EV71 was first isolated from patients in California in 1969, it has been associated with sporadic cases and outbreaks of a wide spectrum of diseases, including HFMD, herpangina, aseptic meningitis, encephalitis, cerebellar ataxia, poliomyelitis-like syndrome [3]. From the late 1990s, outbreaks and epidemics caused by EV71 have occurred more frequently with increasing severity of the onset of HFMD, the incidence of severe HFMD cases and the number of mortalities [4–7]. In the Western Pacific Region, wide spread epidemics have been reported in many countries, including China, Malaysia, Japan, Singapore, Vietnam, Korea and Australia. For example, three large HFMD outbreaks associated with EV71 infection were reported in Malaysia in 1997, Taiwan in 1998 and China in 2008, respectively. There were 29, 78 and 22 deaths caused by HFMD, involving 2,628, 129,106 and 6049 reported cases. In China, according to the national enhanced surveillance system, there were approximately 2 million cases of HFMD, resulting in hundreds of deaths each year since 2009 [8]. HFMD and neurological complications induced by EV71 have become a serious public health problem in the Asia-Pacific Region.

Based on the successful experience with inactivated Polio and Hepatitis A virus vaccines and encouraging early immunogenicity results with inactivated EV71 candidate vaccines, the development of inactivated EV71 vaccines has advanced rapidly in the Western Pacific Region. Three EV71 vaccine products have been approved by the China Food and Drug Administration (CFDA) since December 2015 [9], having shown good immunogenicity and more than 90% protective efficacy in more than 30,000 infants and children [10–12].

Measuring neutralizing antibodies against EV71 virus in human sera

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is critical for evaluating the immunogenicity of EV71 vaccines and the seroprevalence of human populations against EV71 [13]. Different assays are in use for this purpose that employ different cells and virus challenge strains, which has resulted in difficulties in comparing analysis results of the quality and efficacy of different EV71 vaccines and in interpreting research results on virus antigenic and immunogenic properties. For this reason, a proposal to establish the 1st WHO International Standard (IS) for anti-EV71 serum (Human) was endorsed by the WHO Expert Committee on Biological Standardization (Sixty Third Report. Technical Report Series 980, page 40). To this end, a collaborative study was organized by the National Institute for Biological Standards and Control (NIBSC, UK) and the National Institutes for Food and Drug Control (NIFDC, China). The aim of the study was to characterize two candidate anti-EV71 sera in virus neutralization assays to assess their suitability to be used as the 1st IS for anti-EV71 serum (Human). A third serum preparation containing low anti-EV71 antibody titres was also evaluated as a possible WHO Reference Reagent. All three reference materials are intended to help laboratories to standardize virus neutralization methods. The candidate samples were produced from plasma samples donated by healthy individuals in China. The study involved seventeen laboratories from six countries and included national control laboratories, manufacturers of EV71 vaccines and public health laboratories. All participants used their own in-house virus neutralization method and a common challenge EV71 strain. The study samples comprised the two serum candidates, namely 14/138 and 14/140, the low titre sample, 13/238, the Chinese National Standard [13], four clinical sera from naturally infected individuals and a sample prepared with a mixture of sera from volunteers who had received EV71 vaccine as part of a clinical trial. Both candidates were included as coded duplicates and also as a single vial each in liquid format. The real time stability of samples maintained for 6 months at different temperatures was also assessed.

2. Materials and methods

2.1. Plasma samples

Sixty-two plasma samples (600 g each) donated by healthy unvaccinated individuals with glutamic-pyruvic transaminase (ALT) < 25U and confirmed to be negative for HBsAg, HIV, HCV and syphilis antibodies were available for identifying those with suitable anti-EV71 neutralizing antibody titres to prepare the candidate serum samples.

2.2. Study samples

A total of thirteen coded samples were provided to participants as follows:

2.2.1. Sample A (13/238), low titre candidate reference for anti-EV71 antibodies

Freeze-dried preparation form a pool of 5 plasmas from Chinese donations that had similar low anti-EV71 serum antibody neutralization titres and tested negative for Coxsackivirus A16 (CA16) antibodies.

2.2.2. Samples B and D (14/138), candidate for 1st WHO IS for anti-EV71 serum (Human)

Freeze-dried preparation form a pool of 5 plasmas from Chinese donations that had similar high anti-EV71 serum antibody neutralization titres and tested negative for CA16 antibodies.

2.2.3. Samples E and F (14/140), candidate for 1st WHO IS for anti-EV71 serum (Human)

Freeze-dried preparation form a pool of 5 plasmas from Chinese donations that had similar high anti-EV71 serum antibody neutralization titres and tested negative for CA16 antibodies.

2.2.4. NIFDC National anti – EV71 antibody standard, sample G

Freeze–dried preparation produced from a single plasma from a naturally infected donor and validated in a collaborative study in 2010: code 2010/No 0024 with assigned 1000 units of anti-EV71 neutralizing antibodies per ml. The custodian is NIFDC and long term storage is −20 °C.

2.2.5. Sample H (14/138), candidate for 1st WHO IS for anti-EV71 serum (Human) in liquid form

Sample 14/138 was reconstituted in 0.5 ml sterile distilled water and frozen at −20 °C prior to the study.

2.2.6. Sample I (14/140), candidate for 1st WHO IS for anti-EV71 serum (Human) in liquid form

Sample 14/140 was reconstituted in 0.5 ml sterile distilled water and frozen at −20 °C prior to the study.

2.2.7. Sample T, human serum from EV71 vaccinees

Ten sera from Chinese donors with anti-EV71 neutralizing antibody titer higher than 1:32 were selected, pooled and distributed into 0.5 ml aliquots.

2.2.8. Samples W, X and Z, clinical sera from naturally infected individuals

Sera from healthy Chinese adults naturally infected with EV71.

2.2.9. Sample Y, low titre anti-EV71 serum

Serum with low anti-EV71 neutralizing antibody titer from an individual from a different geographical region.

2.3. Defibrination and pooling

Plasma samples were thawed at +4 °C prior to defibrination and pooling. Once thawed the samples for each reference were pooled and aliquoted in 300 ml volumes into 500 ml sterile blood bottles. They were treated with 10% CaCl2 (0.125 M) and incubated for 30 min at 37 °C. The bottles were then placed at +4 °C overnight to form a clot. The liquid was removed and the clot was squeezed to release any retained liquid. The clot was then returned to +4 °C overnight and the process of squeezing was repeated over several days. The serum was spun at 4000 rpm for 30 mins and the supernatant removed. The resulting sera were stored at +4 °C prior to filling and freeze drying.

2.4. Filling, freeze-drying and sealing

Filling was completed for all three candidates from homogenous stirred bulks maintained at +4 °C throughout the filling using a Bausch and Strobel AFV5090 machine. 3 ml ampoules were filled with 0.5 ml of material each. For every 90 ampoules filled, 3 ampoules (4–5% of total filled ampoules) were taken for measurements of the fill volume. Freeze-drying was carried out directly after filling using a 2 day cycle. After completion of the freeze–drying the candidate materials were put on long term storage at −20 °C. Ampoules were sealed under boil-off gas from high purity liquid nitrogen (99.99%) and measurement of the mean oxygen head space after sealing served as a measure of ampoule integrity. The mean oxygen head space was measured non-invasively by frequency modulated spectroscopy (FMS 760, Lighthouse Instruments, Charlottesville, USA). Residual moisture content was measured using the colorimetric Karl Fischer method in a dry box environment (Mitsubishi CA100, A1 Envirosciences, Cramlington, UK) with total moisture expressed as a percentage of the mean dry weight of the ampoule contents.

2.5. Virus strains

EV71 strains from known B and C genotypes, representative of viruses that have been widely circulating globally in recent years