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Review Article

Immunological Diagnosis of *Enterovirus* 71 in Developing Countries

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Abstract

Hand, Foot and Mouth Disease (HFMD) is a commonly occurring mild febrile disease in young children (<6 years of age). Clinical symptoms are high fever, rash, ulcers in the mouth and vesicles on the hands and feet. The two most common pathogens causing HFMD are *Enterovirus* (EV-A71) and *Coxsackievirus* (CV-A16). In recent years, large HFMD outbreaks have occurred in Asia and instead of manifesting itself as a mild disease; HFMD caused by EV71 has been increasingly associated with severe neurological disorders and high fatalities. More than 7 million cases of HFMD have been reported with over 2000 fatalities in China. Other Human *Enteroviruses* (HEVs) such as CV-A6, 8, A10, A16, *Coxsackie B5*, *Echovirus* 4, *Echovirus* 19 and *Echovirus* 30 have also been isolated from HFMD outbreaks but they have not been associated with fatal infections. Since there is no effective vaccine or antiviral for the treatment of EV-A71, surveillance of the pathogen in the community and social distancing by isolation of infected patients provide prospects for control of large outbreaks. The control measures are highly dependent on rapid identification of EV-A71 from clinical specimens. RT-PCR, real-time RT-PCR and Reversed Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) are highly sensitive and specific in detecting EV-A71 but these molecular approaches require expensive equipment and molecular reagents, trained personnel and could not be readily adopted for use in rural and provincial hospitals in developing countries. This review provides an update of the immunoassays that have been developed for the rapid and accurate diagnosis of EV-A71 in developing countries.

Keywords: *Enterovirus* 71; Immunoassays; Immunological diagnosis

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Background

Human *Enteroviruses* are positive stranded RNA viruses belonging to the genus *Enterovirus* of the Picornaviridae family. The genus *Enterovirus* consists of 12 species: *Enterovirus A, Enterovirus B, Enterovirus C, Enterovirus D, Enterovirus E, Enterovirus F, Enterovirus G, Enterovirus H, Enterovirus J, Rhinovirus A, Rhinovirus B and Rhinovirus C* [1]. The species *Enterovirus* A comprises 25 serotypes and includes the *Enteroviruses* causing HFMD such as EV-A71, CV-A16, CV-A5, CV-A6, CV-A8 and CV-A10. Humans are the only natural host of *Enteroviruses* and the clinical manifestations caused by different *Enteroviruses* can range from mild to life threatening [2]. With the exception of polioviruses being isolated from a few countries, the virus has been eradicated from most of the developed and developing countries. However, in its place, *Enterovirus 71* (EV-A71) has emerged as the new 'polio-like' pathogen capable of causing serious infections such as brainstem encephalitis, acute flaccid paralysis and neurological disorders [3].

HFMD is commonly regarded as a mild febrile disease in infants and young children less than 6 years of age. Children usually develop high fever, sore throat and rash on the hands and feet. Blisters can develop in the mouth which tends to lead to ulcers (herpangina). The most common pathogens isolated from HFMD infections are EV-A71 and *Coxsackievirus CA-16* [4]. Instead of causing a mild disease, HFMD caused by EV-A71 has been increasingly associated with severe neurological disorders and high fatalities in recent outbreaks in Asia [5]. The surveillance registry of China reported 7,200,092 probable cases of HFMD, with 2457 deaths from 2008-2012, but only 267,942 cases (3.7%) were laboratory confirmed [6]. Besides EV-A71 and CV-A16, other HEVs such as *Coxsackievirus* types A8, A10 and A12, *Coxsackie B5*, *Echovirus* type 4, *Echovirus* 19 and *Echovirus* 30 were reported to be isolated as viral pathogens in HFMD outbreaks [7]. However, the other *Enteroviruses* have not been commonly associated with fatal infections. Thus, there is a need to identify the viral pathogen(s) in every major HFMD outbreak that had high fatalities.

Epidemiology

Although EV-A71 was first isolated from a child in California, USA, in 1969 [8], there is phylogenetic evidence to show that it was present in the Netherlands as early as 1963 [9]. Subsequently, smaller outbreaks with neurological infections caused by EV-A71 were reported in Australia, Japan, Sweden and the USA [10]. High fatalities were reported for two large outbreaks caused by EV-A71 in Bulgaria in 1975 and in Hungary three years later. There were 44 fatalities amongst 451 children presenting with non-specific febrile illness or neurological disease in Bulgaria [11] and 47 deaths amongst 1550 children (826 aseptic meningitis and 724 encephalitis) in Hungary [12]. Smaller outbreaks and sporadic clusters had occurred in Hong Kong in 1985 and in Australia in 1986 before another large outbreak involving 2618 HFMD cases and 34 deaths were reported for Sarawak in 1997 [13]. Taiwan reported the largest HFMD outbreak in 1998 involving 1.5 million cases with 78 deaths [14]. In 2000, a large HFMD outbreak occurred in Singapore involving 3790

patients and there were 5 deaths, 3 due to HFMD and 2 to non-HFMD [15]. Since then, HFMD is recognised as an endemic mild disease in both Malaysia and Singapore. China was the next country to report a large HFMD outbreak involving 490,000 infections with 126 deaths in 2008. Since 2009, the number of HFMD infections in China had steadily increased and there were 2,819,581 HFMD cases being reported with 394 deaths in 2014. Vietnam reported 4265 HFMD cases with two deaths in 2015 [16]. Outside the Asia Pacific region, smaller outbreaks or sporadic infections with no fatality or low fatalities have been reported in Europe [17].

The EV-A71 genome is about 7.4 Kb and has a 5' Non-Translated Region (5' NTR), a long Open Reading Frame (ORF) and a short 3' NTR followed by a polyadenylated (poly A) tail. The 5' NTR contains an Internal Ribosome Entry Site (IRES) which allows viral protein translation in a cap-independent manner [18]. The ORF is translated into a single large polyprotein of approximately 2100 amino acids, which is divided into three regions (P1-P3). The polyprotein undergoes a series of processing events, culminating in the maturation cleavage of the polyprotein, giving rise to structural and non-structural viral proteins [19]. The four structural proteins, VP1, VP2, VP3 and VP4, are encoded by the P1 region, which constitutes the virus capsid. Proteins derived from the non-structural P2 ($2A^{pro}$, 2B, 2BC, and $2C^{ATPase}$) and P3 (3A, 3AB, 3B, $3C^{pro}$, $3CD^{pro}$, and $3D^{pol}$) regions are most directly involved in virus replication [20] (Figure 1).

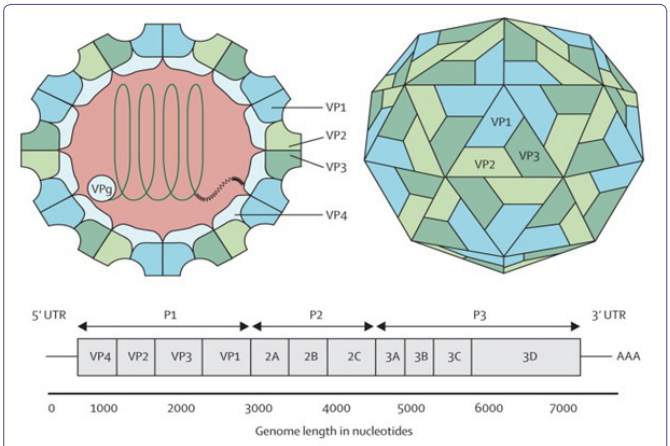

Figure 1: Structure and genome of *Enterovirus* 71.

The capsid consists of 60 protomers, each consisting of four polypeptides that comprise the structural proteins: VP1, VP2, VP3, and VP4 and are encoded by the P1 region of the genome. The P2 and P3 regions encode for seven non-structural proteins: 2A-2C and 3A-3D (the EV-A71 genome is represented by the green line, followed by poly-A residues at the 3' UTR). Reproduced from viral zone, with permission from Swiss Institute of Bioinformatics.

Three genotypes (genogroups) of EV-A71 were established based on the phylogenetic analysis of the VP1 gene. They are designated as A, B and C and each genotype differ from each other by at least 15% at the nucleotide level [21]. Genotype A consists of only one member and is represented by the prototype BrCr strain which was not reported outside of the USA until 2008 when it was isolated from five children presenting with HFMD from the Anhui province in China. The B genotype group can be further subdivided into 5 sub-genotypes (B1-B5) and were predominant in Malaysia and Singapore. The C genotype group is represented by 5 sub-genotypes. Since 2000, the sub-genotype C4 was the predominant sub-genotype circulating in China. There were reports of shifts of sub-genotype dominance in Taiwan and Japan, from B-sub-genotypes to C-sub-genotypes [22,23]. In more recent years, three new genotypes were discovered; one in India (designated as genotype D), one in Central Africa (designated as genotype E) and another from Madagascar (designated as genotype F) by sequencing the VP1 and VP2 regions of clinical isolates [24].

The frequency and size of HFMD outbreaks in Asia present a challenging public health issue. Most countries in Asia have made HFMD a notifiable disease and outbreak control measures are targeted at interrupting person to person transmission or removing EV-A71 from contaminated surfaces of inanimate objects. The usual route of transmission is through the oral-faecal route and distancing measures were effective in reducing the rate of transmission [25]. Since there is neither a vaccine to prevent nor antivirals to treat HFMD due to EV-A71, empirical supportive treatment using fluid replacement and pain relieving is the current option [26]. Expensive treatment involving administration of Intravenous Immunoglobulins (IVIG) appears to be useful for severe disease in some studies but due to the lack of randomized, placebo-controlled phase 2 trials, there is no clear evidence to support the claim that it is an effective treatment strategy [27].

Diagnosis of *Enterovirus* 71

EV-A 71 can cause highly fatal pulmonary oedema and serious neurological syndromes in the very young in large outbreaks. Laboratory diagnosis must be rapid, accurate and efficient to identify this pathogen from all other *Enteroviruses* in order to treat seriously ill patients and implement urgent public health interventions. Tissue culture is the gold standard but is time-consuming and laborious as there is a need to grow the virus followed by micro-neutralization tests using the Lim-Benyesh-Melnick A-H equine antiserum pools (WHO, Statens Seruminstitut, Copenhagen, Denmark) [28]. Neutralization test is highly dependable on the supply of available antisera from government agencies. Immunofluorescence assay is a common diagnostic assay but requires a commercially available fluorescent tagged monoclonal antibody and an expensive fluorescent microscope [29]. However, the sensitivity and specificity of indirect fluorescent antibody assay has not been evaluated.

Molecular techniques such as real-time qRT-PCR has the advantage of speed and accuracy of diagnosing EV-A71 and other *Enteroviruses* [30] but the requirement for a sophisticated real-time PCR instrument, expensive reagents and the need to sequence the RT-PCR products tend to hinder its diagnostic applications in massive outbreak situations. Rapid identification of EV-A71 in rural clinics and hospitals is needed to identify patients who require hospitalizations. An improvement in molecular diagnosis of EV-A71 based on the Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay has received good evaluation in terms of its sensitivity and specificity [31]. Although the requirement for an expensive real-time PCR can be dispensed with, there is still the need for an expensive and specific commercial loop amp RNA amplification kit and a fluorescent detection reagent [32] which may prohibit laboratories from the rural hospitals from the developing countries to adopt a molecular approach for the rapid diagnosis of EV-A71 from large outbreaks in the field. It has been developed for research and has not been commercialised.

Immunoassays

Immunoassays have served as the mainstay of diagnosis of infectious pathogens for more than 50 years. Immunoassays are based on the detection of specific antigens from the infecting pathogen or antibodies produced by the host in response to the pathogen. Enzyme-Linked Immunosorbent Assay (ELISA) is an enzyme-facilitated colorimetric assay to detect specific antigen-antibody interaction. ELISA is commonly used in clinical laboratories and is relatively inexpensive when compared to molecular techniques. They can be offered as a high throughput technology for monitoring antibody response in large outbreak situations. A rapid μ-capture indirect ELISA assay based on the detection of IgM to EV-A71 genotype B or C was first developed by Tsao et al. [33]. Evaluation of the ELISA assay using purified whole virus as the antigen and 213 serum samples established a sensitivity of 91.5% and a specificity of 93.1%. This assay was able to detect IgM as early as the second day of disease onset. However, there was some cross-reactivity (23.0%) with CV-A16 positive sera. The high performance of the IgM μ-chain capture ELISA kit produced by Beijing Wantai (China) against EV-A71 was confirmed by Xu et al. (2010) Sensitivity of early detection was achieved at 90% with 20 patients in their first day of illness and increased to 95% to 100% after that. The cross-reactivity with other non-EV-A71 *Enteroviruses* was reported to be 11.4% [34]. Good sensitivity for early stage EV-A71 IgM detection at 95.7% in an 'in house' IgM-capture ELISA assay was again confirmed in the study by Yu et al. (2012) However, for the sera derived from 134 EV-A71 infected patients, significant cross-reactivity towards *Coxsackievirus* CV-A 16 IgM was observed in 28.2% of the 206 clinical samples. For the 119 sera derived from 16 infected patients, CV-A16 IgM was detected only in 69.7% of clinical samples, while cross reactivity towards EV-A71 IgM was present in 30.3% of samples. The EV-A71 IgM was also detected in 14 of 49 sera infected with other *Enteroviruses* [35]. Despite the cross-reactivity being reported by several studies, EV-A71 IgM ELISA kits have been marketed by a few commercial companies. One of the commercial EV-A71 IgM ELISA kits was reported to have a detection rate of 88.5% one day after disease onset and has a specificity of 95.2%. However, there is still a need for laboratory setting to run the ELISA assay and interpret the data. A rapid test based on a lateral flow device for detecting IgM from patient's sera to EV-A71 antigens and colloidal gold conjugated with antibody specific to EV-A71 was recently introduced to the market by several companies. With a small sample size of 282, one of the companies reported an in-house sensitivity of 98.1% (51/52 samples) and a specificity of 99.1% (228/230) which were confirmed with the RT-PCR assay. Huang et al. (2013) evaluated a commercial Immunochromatography (ICT) kit but reported a much lower sensitivity at 84%, specificity at 77% and accuracy of diagnosing EV-A71 at 80.8%. This could be due to the differences of ICT kits being produced by different manufacturers. The ICT kit was based on the capture of IgM using immobilized anti-human μ-chain antibodies and the subsequent detection of the captured EV-A71 VP1 antigens using mouse anti-VP1 antibodies conjugated to latex. The kit was designed to detect IgM response to EV-A71 sub-genotypes C4 and B5 and it is unknown if the ICT kit will be able to detect other EV-A71 sub-genotypes. They raised the problem of low detection rate as the sensitivity was <50% on the first day of the onset of symptoms. In the non-EV-A71 infection group, there was some cross-reactivity with *Coxsackievirus*, adenovirus and respiratory syncytial virus which should be addressed in future studies [36].

The commercial immunological assays relied mainly on the use of purified whole virions and the epitope present can be cross-reactive due to the high genome homology shared by some *Enterovirus* serotypes such as CV-A16 and human *Echovirus* 6 with EV-A71. Pozzetto et al. (2010) reported that IgM positive anti-*Enterovirus* antibodies were not serotype specific [37]. An immunodominant VP1 linear epitope bearing the core sequence LEGTTNPNG was identified by Foo et al. (2008) The GST-fusion protein carrying the epitope was showing significant immunoreactivity in the Western blot assay but was non-reactive with anti-EV-A71 IgG in ELISA [38]. Routsias et al. (2014) designed *Enterovirus* serotype specific synthetic peptides spanning the amino-terminal 1-15 residues of VP1 of the 10 most common *Enterovirus* serotypes reported to the Centers for Disease Control and Prevention in USA from 1970 to 2008. They showed that majority of the IgM positive sera were reactive with a single serotype specific peptide, thus establishing the homotypic nature of the peptide-ELISA assay. The specificity of peptide recognition was assessed in competitive inhibition studies. Homologous peptides were able to inhibit the binding of IgM to their target antigens from 67-95%, thus supporting specific peptide recognition [39]. Alignment analysis of amino acid sequences of EV-A71 showed that amino acid residues from 6-43 was highly conserved among all genotypes and sub-genotypes of EV-A71 and are different from strains of CV-A16 and *Echovirus 6*. When a VP1 truncated protein carrying the N-terminal antigenic epitope expressed as a GST-$VP1_{6-43}$ fusion protein was used in an indirect ELISA assay to capture anti-EV-A71 IgM in human sera, it showed a sensitivity of 77.8% and 100% specificity for early diagnosis of EV-A71 [40]. The sensitivity of this EV-A71-specific assay could perhaps be further improved by employing a direct IgM capture format. The authors speculated that the lower sensitivity could be due to the presence of specific IgG in the test sera which competed with the EV-A71 specific IgM for antigen binding. A cross-neutralizing epitope within residues 136-150 of VP2 which was highly conserved among EV-A71 genotypes and sub-genotypes was identified to be a good surrogate biomarker in potency testing of EV-A71 vaccine candidates. A synthetic peptide VP2-28 which corresponded to amino residues 136-150 of VP2 was employed to develop an epitope specific sandwich enzyme-linked Immunosorbent assay (Q-ELISA). However, the VP2-28 peptide specific Q-ELISA was found to recognize neutralizing antisera only from rabbits and was non-reactive with antisera from mice and rats immunized with formalin-inactivated whole EV-A71 virion. It is unknown whether the synthetic peptide VP2-28 will be able to elicit neutralizing antibodies in humans and this need to be assessed before the Q-ELISA can be further developed for immunoassays with human sera [41].

He et al. (2013) developed an epitope blocking ELISA (EB-ELISA) which was able to detect specific serum antibodies to purified EV-A71 virus and differentiate it from serum antibodies to other *Enterovirus* subtypes such as CVA4, CVA6, CVA10 and CV-A16. In EB-ELISA, antibodies from human sera specific for EV-A71 could be detected by the ability to block the binding of a specific Mab IC6 to the target epitope present in the EV-A71 virion. The EB-ELISA was found to be more sensitive than the virus neutralization and the Immunofluorescent test (IFA). It has a specificity of 100% in detection of EV-A71 viruses from 100 samples of human sera with positive neutralization titer [42]. Five monoclonal antibodies which specifically reacted with EV-A71 and did not cross-react with CV-A16 or *Echovirus* type 6 (ECHO6) were produced by Xu et al. (2013) When the five monoclonal antibodies were evaluated in a Capture ELISA assay format, they were highly specific for EV-A71 particles. The binding specificities of four

of the monoclonal antibodies varied with different EV-A71 sub genotypes but not for MAb 27. A linear epitope DVIESSIGDSVSRAL located at the N-terminus (aa 6-20) of EV-A71 VP1 was identified to be highly conserved in all EV-A71 sub-genotypes by peptide ELISA [43]. Thus, MAb 27 may have specific applications in diagnosis of EV-A71 in Capture-ELISA assays but will need further confirmation using clinical specimens.

Conclusion

EV-A71 is commonly associated with a self-limiting febrile illness but its propensity to cause brainstem encephalitis, pulmonary edema, acute flaccid paralysis and serious neurological disorders in young children in large scale HFMD outbreaks in Asia is of great concern. Currently, there is no vaccine to prevent or antivirals to treat EV-A71 infections. Rapid diagnosis of EV-A71 infection can help clinical management and implementing public health interventions. Virus isolation and neutralization test remain the gold standard but are laborious and time consuming. Molecular techniques are fast and accurate but require sophisticated equipment, expensive reagents and trained personnel which hinder their usefulness in rural clinics and hospitals in the developing countries in Asia. Immunoassays based on IgM capture ELISA and rapid IgM detection are more amenable as diagnostics in the rural clinics and hospitals in developing countries. IgM capture ELISA using the whole EV-A71 virion as antigen generally has high sensitivity (>90%) but lacked specificity. Improved IgM capture ELISA assays employing a specific VP1 region and epitope blocking ELISA using a specific monoclonal antibody both showed higher specificity. Rapid tests which have been commercialized as bedside immunochromatographic kits demonstrated lower sensitivity at <90% and showed cross-reactivity with other *Enteroviruses* and some non-*Enteroviruses*. Careful evaluations of commercial IgM capture ELISA and rapid test kits with large numbers of the same clinical specimens and with different EV-A71 sub-genotypes will need to be conducted in the future to assess sensitivity and specificity.

Competing Interests

The authors declare that they have no competing interests.

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