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ORIGINAL ARTICLE

Broad reactive monoclonal antibodies for rapid identification of enteroviruses show cross-reactivity with chikungunya virus infected cells

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Abstract

In the past decade, enterovirus 71 (EV71) and chikungunya (CHIK) virus have re-emerged periodically causing serious public health problems in Malaysia, since their first emergence in 1997 and 1998 respectively. This study demonstrates that CHIK virus causes similar patterns of cytopathic effect in cultured Vero cells as some enteroviruses. They also show positive cross-reaction on direct immunofluorescence staining using monoclonal antibodies meant for typing enteroviruses. Without adequate clinical and epidemiological information for correlation, CHIK virus isolated from patients with acute febrile rash can be wrongly reported as untypeable enterovirus due to its cross-reactivity with commercial pan-enterovirus monoclonal antibodies. This is due to the diagnostic laboratory being unaware of such cross-reactions as it has not been reported previously. Final identification of the virus could be determined with specific antibodies or molecular typing using specific oligonucleotide primers for the CHIK virus.

Keywords: enterovirus 71, chikungunya virus, cytopathic effect, Vero cells, cross-reaction, viral diagnosis

INTRODUCTION

Human enterovirus 71 (EV71) was first isolated in the USA in 1969 from a child with aseptic meningitis.[1] In Malaysia, the virus was first isolated in 1997 following an outbreak of hand, foot and mouth disease with cases of fatal encephalomyelitis occurring first in east Malaysia which subsequently spread to Peninsular Malaysia.[2] Chikungunya (CHIK) virus, however, was first isolated in Tanzania, Africa in 1953 and in Malaysia following its first emergence in 1998 when it caused outbreaks of febrile rash illness with cases of arthralgia/arthritis.[3,4] Since then these two newly emerged viruses have re-emerged periodically causing serious public health problems.[5-8] Both types of these viruses may be isolated from the blood specimens of patients taken during the early acute phase of illness using the same Vero ATCC-81 cell-line. Unfortunately this can lead to confusion and wrong identification of the causative virus in an inexperienced diagnostic virology laboratory especially in situations when there is inadequate clinical and epidemiological information provided in the laboratory request form. This study describes the observation of cross-reactivity of commercial pan-enterovirus monoclonal antibodies with CHIK virus.

MATERIALS AND METHODS

From late April to May 2008, the National Public Health Laboratory, Malaysia received more than 100 blood specimens for virus study from clusters of patients with acute febrile illness in three separate locations in the state of Johore (southern Peninsular Malaysia). Less than fifteen percent of the affected patients were noted to develop maculopapular rash. Otherwise, there was gross lack of epidemiological and clinical information to suggest which of the likely virus, such as alphaviruses, rubivirus (rubella), enteroviruses, adenovirus or other viruses, were the likely cause of the acute illness. Besides running the usual available serological tests, such as anti-dengue

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IgM, anti-CHIK IgM, anti-rubella and anti-measles IgM to exclude the possible cause of the febrile illness, 100 serum specimens were inoculated into Vero cells seeded in JM-cell culture tubes for virus isolation.[9] The inoculated cell-cultures were observed daily for up to 10 days for evidence of virus replication by the occurrence of cytopathic effect (CPE). For culture cells showing CPE, 0.5 ml aliquot of the culture supernatant containing suspended infected Vero cells was harvested upon full CPE and placed in a microfuge tube. After two washings with sterile phosphate buffered saline (PBS), the cell pellet was resuspended in 150 μl of PBS and 10 μl of the suspended cells was carefully layered onto each well of a 12-well Teflon coated slide. After drying over a heated (40^0C) warm plate and fixed in cold acetone, identification of virus was made by indirect immunofluorescence test using a panel of commercial monoclonal antibodies for identification of human enteroviruses (Chemicon Inc., USA). Vero cells infected with human enterovirus 71 used as positive control were similarly processed in the immunofluorescence assay.

For infected cultured cells that reacted with broad reactive monoclonal antibodies that covered all types of enteroviruses (Chemicon Inc., Cat No. 3360) but failed to be typed with specific enterovirus typing monoclonal antibodies (Chemicon Inc., USA), total RNA was extracted using High Pure Viral RNA Kit (Boehringer Mannheim, USA). Briefly, lysis of virus in the supernatant was accomplished by incubation of the culture fluid showing CPE (200 µl) in 400 µl of special lysis/binding buffer for 10 minutes. The lysate was transferred into a filter tube and passed through the glass fleece at the base of the tube by centrifugation at 8,000 × g for 15 seconds. Residual impurities in the tube were removed by a wash step using the supplied wash buffer and 30 µl of elution buffer was added. After allowing the elution buffer to equilibrate for a minute at room temperature, the RNA is eluted into a sterile RNase-free microfuge tube by centrifugation at 8,000 × g for 15 seconds. Molecular detection and characterization of the virus causing CPE in the culture was subsequently carried by reverse transcription-polymerase chain reaction (RT-PCR) in accordance with the methods by Hasebe *et. al.* for CHIK virus and Arola *et. al.* for all type of enteroviruses.[10,11] Known CHIK virus and human enterovirus 71 (EV71) were used as positive controls in the molecular identification.

RESULTS

After 5 to 8 days of incubation, similar pattern of cytopathic effect (CPE) was noted in Vero cells inoculated with serum specimens from 12 of the 100 patients (Figure 1c). No virus causing lytic CPE was isolated from the remaining 88 serum samples. The infected cells detached from the surface with "rounding-off" and distinct cell margins, followed by cell-shrinkage with irregular cell margins. This morphological feature of the infected cells showing CPE was indistinguishable from that of enterovirus-infected Vero cells (Figure 1b).

The infected cells stained positive with the blended pan-enterovirus monoclonal antibodies (Chemicon Inc., Cat No. 3360) (Figure 1e) that covered all known types of human enteroviruses inclusive of EV71 which was used as the positive enteroviral control (Figure 1d), but not with other type-specific monoclonal antibodies for identification of individual enterovirus type. The positive fluorescent staining was quite similar to that of EV71 infected Vero cells (Figure 1d) except the positive "speckled" fluorescence was more evenly distributed in the cytoplasm of the later. The infected cells also gave strong positive fluorescence by indirect immunofluorescence test using a convalescent human serum known to be reactive to CHIK virus (Figure 1f), supporting that the cells were infected with CHIK virus.

The diagnosis was further confirmed with the infected cells showing positive RT-PCR amplification with oligonucleotide primers specific for CHIK virus envelope protein 1 (E1) and non-structural protein 1 (nsP1) genes but negative with primers specific for the 5'non-coding genomic region of all known enteroviruses (Figure 2).

DISCUSSION

The identity of the virus isolated from the serum specimens of the 12 acute febrile illness patients was initially thought to be a type of enterovirus because it caused a similar type of CPE in Vero cells and reacted with the commercial pan-enteroviral blend monoclonal antibodies. However, when the infected Vero cells that failed to be stained with various type-specific enterovirus monoclonal antibodies were re-processed and stained with convalescent human serum that was known to be reactive with CHIK virus, positive fluorescence was detected supporting infection with CHIK virus. Final identification of CHIK virus was subsequently

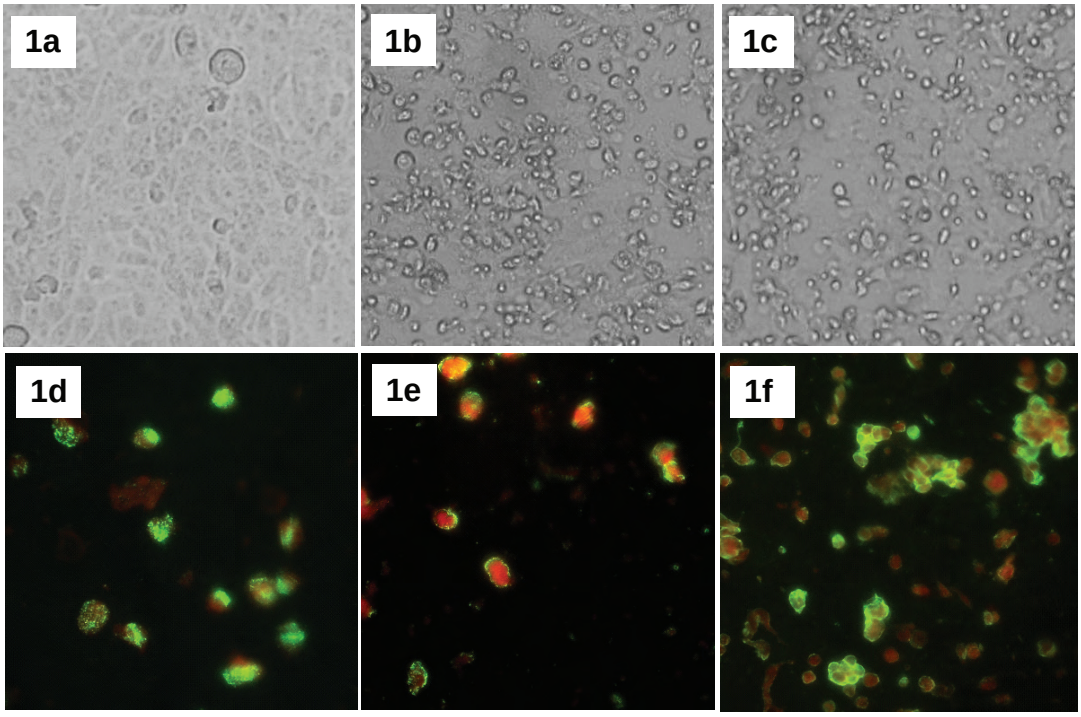

FIG. 1: A composite photograph of Vero cells: normal and those infected with chikungunya (CHIK) virus or enterovirus 71 (EV71). Fig. 1a: monolayer of tissue culture normal Vero cells. Fig. 1b: Vero cells showing cytopathic effect following infection by EV71. Fig. 1c: Vero cells showing cytopathic effect following infection with CHIK virus. Fig. 1d: EV71 infected cells showing distinctive positive green fluorescent stain with pan-enterovirus monoclonal antibodies. Fig. 1e: CHIK virus infected cells showing weak and inapparent green fluorescent stain with pan-enterovirus monoclonal antibodies. Fig. 1f: CHIK virus infected cells showing strong positive green fluorescent stain with human convalescent serum reactive to CHIK virus.

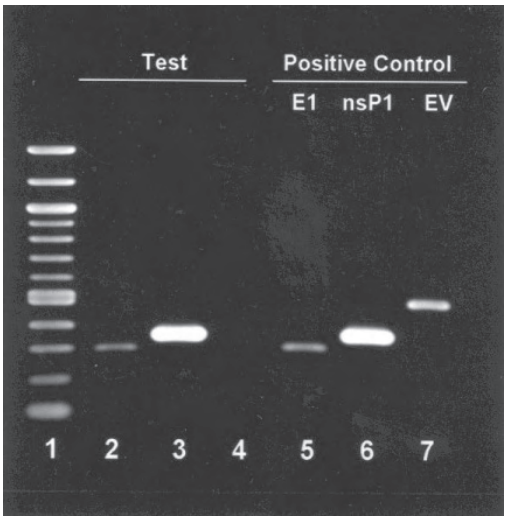

FIG. 2: Photograph of RT-PCR amplification products after electrophoresis in 1.2% agarose gel. Lane 1: molecular ladder size marker. Lane 2: Test virus amplified with primers specific for chikungunya (CHIK) virus E1 protein gene. Lane 3: Test virus amplified with primers specific for CHIK virus nsP1 protein gene. Lane 4: Test virus amplified with primers specific for 5'-non coding region of enteroviruses. Lane 5: CHIK virus amplified with primers specific for CHIK virus E1 protein gene. Lane 6: CHIK virus amplified with primers specific for CHIK virus nsP1 protein gene. Lane 7: Enterovirus 71 amplified with primers specific for 5'-non coding region of enteroviruses.

made by reverse transcriptase-polymerase chain reaction (RT-PCR) using known CHIK virus and EV71 as positive control. The infected cells gave positive RT-PCR amplification with oligonucleotide primers specific for CHIK virus envelope protein 1 (E1) and non-structural protein 1 (nsP1) genes but were negative with primers specific for the 5'non-coding genomic region of all known enteroviruses.

This study shows that without adequate clinical and epidemiological information, CHIK virus which gave similar type of CPE as of some enteroviruses in culture cells and also cross-reacted with the pan-enterovirus blend monoclonal antibodies could be wrongly reported as non-typeable enterovirus by the virus diagnostic laboratory. This may subsequently lead to implementation of wrong control measures for outbreaks caused by the CHIK virus. Final identification of the virus can be determined with specific antibodies or molecular typing using specific oligonucleotide primers for the CHIK virus.

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