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Laboratory Detection and Diagnosis of Filoviruses

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Abstract: Ebola virus (EBOV) and Marburg virus (MARV), belonging to the *Filoviridae* family, emerged four decades ago and caused severe viral hemorrhagic fever in human and other primates. As high as 50-90% mortality, filoviruses can cause significant threats to public health. However, so far no specific and efficient vaccine has been available, nor have other treatment methods proved to be effective. It is of great importance to detect these pathogens specific, rapidly and sensitively in order to control future filovirus outbreaks. Here, recent progresses in the development of detection and diagnosis methods for EBOV and MARV are summarized.

Key words: Filovirus; Ebola virus (EBOV); Marburg virus (MARV); Enzyme-linked immunosorbent assay (ELISA); Polymerase chain reaction (PCR)

Filoviruses could cause severe viral hemorrhagic fever in human and other primates leading to 50%-90% mortality in patients; however, so far no specific and efficient vaccine has been synthesized[25], nor have other treatment methods proved to be effective [10, 20, 24]. The pathogen belongs to the order *Mononegavirals*, family *Filoviridae*, which further divides into two genera: Ebola virus (EBOV) and Marburg virus (MARV). The first outbreak of Ebola virus was recorded in Zaire, sub-Saharan Africa and four species have been identified: *Zaire ebola virus*, *Sudan ebola virus*, *Ivory Coast ebola virus* and *Reston ebola virus*. Its counterpart, the MARV, has only one species, *Lake Vicoria marburg virus* isolated in 1967 (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB). Since EBOV and MARV cause serious diseases with high morbidity and mortality, these viruses are classified as the BSL-4 pathogens and can cause significant threats to public health and affect the economic growth on a global scale due to the increase of international trade and traveling as a consequence of globalization. Consequently the viral pathogen might be able to pass the borders and spread across different countries. Their existence as endemic disease threats and as potential biological warfare weapons suggests that it is of great importance to detect these pathogens specific, rapidly and sensitively. Here, we summary the recent progress in the development of detection and diagnosis methods for EBOV and MARV and mainly focus on virus isolation, electron microscopy, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR).

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VIRUS ISOLATION

As obligate intracellular parasites, viruses require host cells in order to replicate and complete its life cycle. Virus isolation plays a very important role in the process approach of diagnosis and detection of filoviruses. Although high-level containment is warranted and a BSL-4 laboratory is required because of the extreme hazard associated with handling filoviruses, virus isolation is still a basic and simple method for diagnosis of filoviruses. Acute sera or postmortem tissues are usually positive. Virus growth is detected by cytopathic effect or more usually by fluorescent antibody detection of antigen in cells. It is usually efficient. The virus isolation may require several days, multiple cell systems, and blind passage. The virus isolation is time consuming, expensive and dangerous, but probably most sensitive. EBOV and MARV grow well in a large variety of cell lines. Among them, Vero or Vero E6 cells have been commonly used. The shipment of infection specimens for virus isolation needs favorable conditions, such as a cold chain during the shipment until arrival, which is often difficult in developing countries. Therefore, diagnostic criteria based on virus isolation alone will not yield an ideal etiologic diagnosis [26].

ELECTRON MICROSCOPY

Electron microscopy (EM), which can visualize the virus particles in specimens directly, has been particularly useful in identification and detection of various viral infections. It works on the basis of the characteristic morphology of the viruses and is widely used in virus diagnosis because of its major advantages, including high-speed and the lack of requirement for viral viability. Thus it can be developed and used as a rapid diagnostic method. On the other hand, the main disadvantages of EM include its inability to examine multiple specimens quickly and simultaneously, the expensive cost, the complexity of maintaining an electron microscope, and requirement of highly skilled personnel. Also EM requires a minimum number of virus particles for visualization and usually needs a high concentration (10^5 to 10^6/L) of viral particles for detection [21].

Due to the high mortality in infected patients, electron microscopy has been particularly useful in diagnosis of filovirus infections in the past several decades [2-6, 9, 12]. Viral structures can be directly visualized in serum, in culture fluids form initial passage cell cultures by negative staining, and in thin sections of any infected material [9]. The immuno-EM method in conjunction with standard transmission EM provided consistent results and is simple to perform for the detection of a filovirus serologically related to EBOV in MA-104 cells. Thus, immuno-EM can be potentially used in direct immunological diagnosis of EBOV and related filoviruses in clinical samples [3]. An indirect immuno-electron microscopy method was successfully applied in identification of Ebola-R (EBOV-R) particles in serum and tissue culture fluid specimens with infectivity titres of 300 plaque forming units (pfu) per ml or more. It can differentiate antigenically distinct filoviruses in less than three hours and should be valuable in rapid detection of potential filoviral infections [6]. In 1995, systematic research on the ultra-structural details of morphology and morphogenesis of filoviruses using electron microscopy was reported. MA-104, Vero 76, SW-13 and DBS-FRhL-2 cells, infected with Marburg, Ebola-Sudan (EBOV-S), Ebola-Zaire (EBOV-Z), and Ebola-

Reston viruses, were examined by electron microscopy. Results demonstrated that intermediate MARV inclusions were morphologically different form EBOV-S, EBOV-Z, and EBOV-R inclusions, while there was no structural difference of viral inclusion material being observed among EBOV-S, EBOV-Z, and EBOV-R. These details facilitate the efficient and definitive diagnosis and identification of filoviruses by electron microscopy [4]. Case studies demonstrated that electron microscopy was a valuable tool for assisting in diagnosis and detection of filoviruses, which also provided insight into the pathogenesis of these agents [2].

ELISA ASSAY

The high mortality in patients infected with EBOV or MARV, is because patients usually die before the antibody response can be mounted. This fact suggests that serological diagnostics are suitable for the diagnosis of patients who survive but not for those who succumb to the infection [26]. High titers of filovirus particles are present in the blood and tissues of patients at the early stage of illness and several ELISA systems have been developed for rapid, sensitive and specific detection of EBOV and MARV antigens.

Ksiazek *et al* developed the ELISA method using a mixture of mouse antibodies for antigen capture and polyclonal hyperimmune rabbit anti-EBOV serum for antigen detection. Through the evaluation on the tissues of monkeys naturally or experimentally infected with strains of EBOV-Z and EBOV-S, the assay was found to be both sensitive and specific [13]. Afterwards, virus antigen, as well as Ig G and Ig M antibody was found in EHF patients in Kikwit and the Democratic Republic of Congo [14], and an ELISA for Ig G and Ig M antibodies directed against Ebola viral antigens was developed [15]. This works gave great promise that, as a diagnostic tool, ELISA would be useful in field investigations of EBOV.

Monoclonal antibody prepared by immunization with recombinant nucleoprotein of EBOV-Z also reacted to the nucleoprotein derived from EBOV-R and EBOV-S [22]. This made the preparation of a mixture of monoclonal antibodies with different specificities necessary. Two novel monoclonal antibodies, Res2-6C8 and Res2-1D8, specific to the nucleoprotein of EBOV-R partly solved the problem [11]. The ELISAs using the two antibodies were useful for the rapid detection of the nucleoprotein in EBOV-R infected cynomolgus macaques and might be a promising tool for the diagnosis of EBOV-R infection, especially in monkey quarantine and field studies. More polyclonal and monoclonal antibodies against glycoprotein (GP) [34], viral protein 40 and the aglycosyl sub-fragment of glycoprotein (GP1) [29] brought more candidates for developing ELISA methods for detection and diagnosis of EBOV. These antibodies also have potentials for identifying and differentiating EBOV strains.

Highly sensitive and specific monoclonal antibodies against the viral protein 40 (VP40) [18] and the glycoprotein (GP) of EBOV were obtained by Lucht *et al*[19]. Recently, an immunofiltration assay for the detection of EBOV antigen using the monoclonal antibodies against to the EBOV VP40 mentioned above was developed by the same group [17]. The system was able to provide the results in 30 min without a need for electricity or sophisticated equipment. It would therefore be a good choice for

field detection in outbreak areas in remote regions, especially in Africa.

Compared to EBOV, much fewer antibodies against MARV were reported. The monoclonal antibodies against the nucleoprotein of MARV [27, 36] and single-domain antibodies [30] for rapid diagnosis of MARV have been developed by others and our group. It is generally considered that MARV antigen detection ELISAs are useful for accurate and rapid diagnosis of MHF. However, the availability and efficacy of such methods should be further evaluated and validated by using authentic patient specimens in a clinical setting.

REAL-TIME QUANTITATIVE RT-PCR

The polymerase chain reaction (PCR) has been used for detecting a wide variety of pathogens across a range of research fields, including virology. In recent years, real-time PCR is widely used in the fields of pathogen detection and diagnosis because of its advantages of speed, simplicity and high sensitivity. It has revolutionized molecular diagnostic technologies. In the past decade, many PCR detection systems have been developed for diagnosis of EBOV and MARV (Table 1).

The use of traditional RT-PCR to detect acute EBO-Z virus disease in humans and animals were developed by Sanchez *et al* for identifying outbreaks and supporting epidemiologic investigations. RT-PCR assays were proven effective for detecting viral RNA in body fluids and tissues of EBO-Z infected individuals [28]. The first field evaluation of RT-PCR for EBOV disease was reported by Leroy[16]. They used the same primer set as Sanchez *et al* to detect EBOV viral RNA in peripheral blood mononuclear cells[28]. Twenty-six laboratory confirmed patients of EBOV hemorrhagic fever were studied. The results were compared with ELISA antigen capture, and EBOV specific IgM and IgG antibody. The one-step, real-time RT-PCR method were developed by Drosten *et al* with the utilization of the DNA-interacting dye SYBR green I using the primer set of Sanchez *et al* [1]. It demonstrated that RT-PCR was the most sensitive method and able to detect the viruses from early acute disease through early recovery.

In order to address the challenge of diversity of filoviral genomes in PCR-based assays, Zhai *et al*. [35] developed a consensus PCR method similar to Sanchez *et al* [28], which utilized a cocktail of specific primers in a one-step RT-PCR. It yields a long product that could be sequenced for automated phylogenetic analysis. This allowed a more accurate placement of newly identified filovirus positive samples relative to existing species, lineages, and strains.

The introduction of real-time RT-PCR allows detection of filoviruses to be carried out with minimal manipulation and equipment, and can provide results in less than two hours [9]. It may prove to be a useful diagnostic tool for control and management of future outbreaks. Highly sensitive and specific primers and probe sets were reported by Gibb *et al* [7, 8] for Real-time RT-PCR amplification of MARV and EBOV sequences and targeted genes, respectively. The newly designed primer and probe sets for MARV was equivalent to, or 10 to 100-fold more sensitive than previously designed primer sets. It was able to detect all stains of MARV [7]. Meanwhile, one-tube real-time RT-PCR assay for identification of EBOV-Z and EBOV-S was developed. One common primer set and two differentially labeled fluorescent probes were used to simultaneously detect and differentiate the two

Table 1. RT-PCR amplification systems for the detection of EBOV and MARV

Assay method	Primer and probe	Target gene	Reference
Traditional RT-PCR	EBO-GP1 AATGGGCTGAAAATTGCTACAATC	EBOV GP	[28]
	EBO-GP2 TTTTTTTAGTTTCCCAGAAGGCCCACT		
	FILO-A ATCGGAATTTTTCTTTCTCATT	Filovirus L	
	FILO-B ATGTGGTGGGTTATAATAATCACTGACATG		
	RES-NP1 GTATTTGGAAGGTCATGGATTC	EBOV-R NP	
	RES-NP2 CAAGAAATTAGTCCTCATCAATC		
	ZAI-NP1 GGACCGCCAAGGTAAAAAATGA	EBOV-Z NP	
	ZAI-NP2 GCATATTGTTGGAGTTGCTTCTCAGC		
TaqMan Real-time PCR	MBGGP3 TTCCCCTTTGGAGGCATC	MARV GP	[7]
	MBGGP3 GGAGGATCCAACAGCAAGG		
	MBGGP3Prb CGATGGGCTTTCAGGACAGGTGT		
	EBOGP-1D TGGGCTGAAAAYTGCTACAATC	EBOV-S GP	[8]
	EBOGP-1D CTTTGTGMACATASCGGCAC		
	EBOGP-1DSPrb TTACCCCCACCGCCGGATG		
	ENP1 GAAAGAGCGGCTGGCCAAA	EBOV-S NP	[32]
	ENP2 AACGATCTCCAACCTTGATCTTT		
	ENPP TGACCGAAGCCATCACGACTGCAT		
	ENZ FP ATGATGGAAGCTACGGCG	EBOV-Z NP	[33]
	ENZ RP AGGACCAAGTCATCTGGTGC		
	ENZ P CCAGAGTTACTCGGAAAACGGCATG		
	ENS FP TTGACCCGTATGATGATGAGAGTA	EBOV-S NP	
	ENS RP CAAATTGAAGAGATCAAGATCTCCT		
	ENS P CCTGACTACGAGGATTCGGCTGAAGG		
	MN FP CAATTCCACCTTCAGAAAACTG	MARV NP	
	MN RP GCTAATTTTTCTCGTTTCTGGCT		
	MN P CACACACAGTCAGACACTAGCCGTCCT		
	MN FP GGACCACTGCTGGCCATATC	MARV VP40	[31]
	MN RP GTCGGCAGGAGGIGAAATCC		
	MN P CTCTGGGACTTTTTCIACCCTCAGTTGATGA		
	FiloA2.4, AAGCATTTCCTAGCAATATGATGGT		[23]
	FiloA2.2, AAGCCTTTCCTAGCAACATGATGGT		
	FiloA2.3, AAGCATTCCCTAGCAACATGATGGT	EBOV-Z L	
	Filo B, ATGTGGTGGGTTATAATAATCACTGACATG	EBOV-S L	
	Filo B-R,GTGAGGAGGGCTATAAAAGTCACTGACATG	EBOV-IC L	
	FAMEBOSu, CCGAAATCATCACTIGTITGGTGCCA	MARV L	
	FAMEBOg, CCAAAATCATCACTIGTGTGGTGCCA		
	FAMMBG, CCTATGCTTGCTGAATTGTGGTGCCA		

subtypes of EBOV. They were unique in their abilities to simultaneously detect and differentiate EBOV-Z and EBOV-S [8]. Rapid detection protocol for filoviruses using TaqMan RT-PCR for detection MARV and EBOV were developed after the series of studies mentioned above [33]. Three primer and probe sets were designated into blocks of nucleoprotein gene conserved sequences of MARV, EBOV-Z and EBOV-S. All three assays were highly sensitive and specific and able to detect and identify filoviruses at an early stage

of a suspected filovirus disease.

In the largest outbreak of Ebola hemorrhagic fever to date that occurred in Uganda from August 2000 to January 2001, a nested RT-PCR, combining with two-step real-time RT-PCR and one-step real-time RT-PCR was used in field diagnosis [32]. The greatest value of early case identification in the real-time RT-PCR-based assay was clearly demonstrated by its ability to identify patients. It gave earlier identification than any other available tests. Recently, in the outbreak of a large hemorrhagic fever which was investigated by the Centers for Disease Control and Prevention in northern Angola, MARV was confirmed as the cause of the outbreak. The primer and probe set was newly designed specifically for the VP40 gene sequences in the outbreak [31]. As well as in the field diagnosis in Uganda mentioned above, real-time RT-PCR assay outperformed all other assays designed to detect acute MARV infection, including the "gold standard" virus isolation assay. It was also sufficiently robust to allow deployment into a field setting in Angola. The extensive virus genomic analysis in this study also confirmed that the VP40 gene target of MARV was an excellent choice for a broadly reactive MARV detection assay. The performance of real-time RT-PCR in such outbreaks suggests that it should be a highly useful assay for detection of potential naturally occurring or terrorist activities outbreaks in the future.

The first industry-standard molecular assay for all filoviruses species was designed by Panning *et al* [23]. They developed a diagnostic real-time RT-PCR reaction kit for filoviruses based on the strain collections of all participants in the network of European biosafety level 4 laboratories. The kit facilitated reliable detection or exclusion screening of filovirus infections. Meanwhile, they also stated that any kit like this can never be guaranteed that a PCR assay will detect unknown filovirus strains that may emerge in the future.

To conclude, there exist many methods for detection and diagnosis of filoviruses. Among them, PCR methods have shown their values for early detection of the viruses due to their high sensitivity and fast speed. However, due to continuous mutation of the viruses, it is expected that the more traditional methods, such as virus isolation and electron microscopy, would also need developing for better identification of filoviruses during future outbreaks.

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