

Laboratory diagnosis of Ebola and Marburg hemorrhagic fever.

A. Grolla (1), A. Lucht (1), D. Dick (1), J. E. Strong (1) & H. Feldmann (1, 2)*

(1) National microbiology laboratory, Public health agency of Canada, Winnipeg, Manitoba, Canada.

(2) Department of medical microbiology, University of Manitoba, Winnipeg, Manitoba, Canada.

*Correspondance : Heinz Feldmann, 1015 Arlington Street, Winnipeg, Manitoba R3E 3R2, Canada,

Tél. : +1-204-789-6019, Fax : +1-204-789-2140, E-mail: Heinz_Feldmann@phac-aspc.gc.ca

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Résumé : Diagnostic au laboratoire de la fièvre hémorragique due aux virus Ebola et Marburg

Les résultats de laboratoire confirmés à temps ou l'identification des différentes étapes de la maladie peuvent améliorer grandement le contrôle des épidémies causées par des Filovirus. Actuellement, les diagnostics de laboratoire concernant les infections dues aux virus Ebola et Marburg se posent en terme de dosages disponibles et de leur interprétation ; s'y ajoute le rôle du laboratoire de terrain, ses limites et ses capacités à répondre en cas d'épidémie, notamment lorsqu'il s'agit de la PCR en temps réel et des analyses d'immunofiltration.

Summary:

The control of Filovirus outbreaks can be greatly enhanced by timely laboratory confirmation of infection or the identification of alternative disease processes. The status of current laboratory diagnostics for Ebola and Marburg virus infections is discussed in terms of the assays available and their interpretation. In addition, the role of field-based laboratory support and its limitations and capabilities in an outbreak response setting, especially in regards to real-time PCR and immunofiltration assays, is presented.

**virus Ebola
virus Marburg
diagnostic de terrain
RT-PCR en temps réel
laboratoire de terrain
ELISA
immunofiltration
Afrique intertropicale**

**Ebola virus
Marburg virus
field diagnostic
real-time RT-PCR
field laboratory
ELISA
immunofiltration
Sub Saharan Africa**

Introduction

Identification of viral hemorrhagic fever (VHF) caused by Ebola virus (EBOV) and Marburg virus (MARV), family *Filoviridae* (5) may be difficult due to a wide variety of infectious diseases causing similar clinical symptomatology. The most common causes of severe, acute, febrile diseases in *Filovirus* endemic areas are malaria and typhoid fever. A wide range of infectious diseases must be considered next, such as shigellosis, meningococcal septicaemia, plague, leptospirosis, anthrax, relapsing fever, typhus, murine typhus, yellow fever, Chikungunya fever, Dengue fever and fulminant viral hepatitis. Travel, treatment in local hospitals, contact to sick persons or wild and domestic animals, particularly monkeys, are useful historical features, especially in returning travelers from Africa. Diagnosis of single cases is extremely difficult, but the occurrence of clusters of cases with prodromal fever followed by cases of hemorrhagic diatheses and person-to-person transmission are suggestive of VHF, and containment procedures have to be initiated. In filoviral VHF prostration, lethargy, wasting,

and diarrhoea seem to be more severe than observed in patients with other VHFs. If present, the macular rash is characteristic and useful in narrowing the differential diagnosis.

Current diagnostics

Despite all the achievements in laboratory diagnostics in the past decades, it should be kept in mind that the diagnosis of EBOV and MARV infections will initially have to be based on clinical assessment. For this purpose contingency plans should be developed that are still missing in many, particularly developing countries. As clinical microbiology and public health laboratories are not generally equipped for diagnosis of VHF, particularly *Filovirus* VHF, it is necessary that samples are sent to national and/or international reference laboratories capable of performing the required assays. In addition, many nations encounter difficulties in sample transport, which can cause substantial delays in laboratory response. Once samples are received, laboratory response is fairly efficient with results expected within 24 to 48 hours.

Early laboratory diagnosis of EBOV and MARV infections is most desirable because of current implications in case patient management and public health interventions. There is currently no therapeutic treatment for *Filovirus* infections but this may become available in future (2, 7). If treatment is to be implemented, rapid, sensitive, and quantitative laboratory assays will be needed especially to initiate and evaluate treatment options such as anticoagulant therapy (7).

Laboratory diagnosis of *Filovirus* infections can basically be achieved in two ways: Measurement of the host-specific immune responses to the infection and detection of viral particles or particle components in infected individuals (table I). Today, reverse transcriptase-polymerase chain reaction (RT-PCR) (3, 28, 29, 31) and antigen detection "Enzyme-linked Immunosorbent Assay" (ELISA) (12, 17, 18, 21, 25) are the primary test systems to diagnose an acute infection. For antibody detection the most commonly used assays are direct IgG and IgM ELISAs and IgM capture ELISA (1, 10, 11, 17, 19, 22).

Confirmatory tests include western blot (1, 4) and the indirect immunofluorescence assay (IFA) on acetone-fixed infected cells inactivated by γ -radiation (13, 15, 16, 32). Due to relatively high viremia levels in humans, electron microscopy has been particularly useful in diagnosis of *Filovirus* infections (8, 9, 14, 23, 30). Viral structures can be directly visualized in serum, in culture fluid from initial passage cell cultures by negative staining, and in thin sections of any infected material. Immunohistochemistry (IHC) on formalin-fixed material and paraffin-embedded tissues can be used for detection of filoviruses (14) as well as immunofluorescence (IF) on impression smears of tissues (26). For non-outbreak surveillance, IHC on formalin-fixed biopsy/autopsy material is available for filoviruses and has several advantages including its simplicity, specificity and the lack of any need for enhanced biocontainment (33).

Virus isolation from serum or other clinical material should always be attempted. The most commonly used cell lines for isolation are Vero cells (clone E6) and MA-104 cells (both monkey kidney cells). However, most filoviruses do not cause extensive cytopathic effect on primary isolation. Guinea pigs

can be used for primary isolation of those filoviruses that initially do not grow well in tissue culture. Several passages are usually required to produce a uniformly fatal disease.

Interpretation of diagnostic test results

Of the available techniques for diagnosis, antigen-capture ELISA and RT-PCR are today the most useful for making a diagnosis in an acute clinical setting. Viral antigen/nucleic acid can be detected in blood from day 3 up to 7 to 16 days post onset of symptoms (27). RT-PCR assays seem to be favored by many investigators since BSL-4 biocontainment is not necessary after proper inactivation as well as the sensitivity/specificity and rapidity of the technique. However, the diagnosis of index cases of outbreaks or of single imported cases should not be solely based on RT-PCR to avoid the problem of cross-contamination and, thus, false-positives. Confirmation by an independent assay such as antigen capture ELISA should always be attempted. When case confirmatory techniques and biocontainment (virus isolation) are not available, RT-PCR on an independent target gene and/or independent sample should be the minimum confirmation. In such instances it may be useful to seek confirmation through another reference laboratory.

Serology can be useful for confirmation but it should be kept in mind that negative serology is not exclusive since filovirus-infected individuals often die without a proper humoral immune response. Based on past investigations, IgM antibodies can appear as early as two days post onset of symptoms and disappear between 30 and 168 days after infection. IgG-specific antibodies develop between day 6 and 18 after onset and persist for many years (27). Rising IgM or IgG titer constitutes a strong presumptive diagnosis. However, a single positive result should be confirmed on a follow-up sample preferentially taken a week apart. Decreasing IgM and/or increasing IgG titers (four-fold) in successive paired sera are highly suggestive of a recent infection.

Standardization and evaluation of diagnostic procedures for EBOV and MARV is difficult because of the restricted

Table I.

Test	Diagnostic procedures.			Remarks
	Target	Source		
Polymerase chain reaction (PCR)	viral nucleic acid	blood, serum, tissues		rapid and sensitive, but requires special equipment
Antigen ELISA	viral antigen	blood, serum, tissues		rapid and sensitive, but requires special equipment
Immunohistochemistry	viral antigen	tissues (e.g., skin, liver)		inactivated material, but requires time
Fluorescence assay (FA)	viral antigen	tissues (e.g. liver)		rapid and easy, but interpretation is subjective
Electron microscopy	viral particle	blood, tissues		unique morphology (immunostaining possible), but insensitive and requires expensive equipment
Indirect immunofluorescence assay (IFA)	virus-specific antibodies	serum		simple to perform, but prone to non-specific positives and subjective interpretation
Enzyme linked immunosorbent assay (ELISA)	virus-specific antibodies	serum		specific and sensitive, but initial response slower than IFA
Immuno blot (Westernblot)	virus-specific antibodies	serum		rotein-specific, but interpretation sometimes difficult
Virus isolation	viral particle	blood, tissues		virus available for studies, but needs biocontainment and requires time

availability of virologic and clinical material. Recently, the "European Network for Imported Viral Diseases" (ENIVD) provided an external quality assurance for *Filovirus* PCR diagnostic procedures (24). Though most laboratories in this study showed reasonable abilities to detect filoviruses by PCR technology, a small but significant fraction of laboratories demonstrated rather poor sensitivities. A quality assurance panel for EBOV diagnostics was also performed by the "International High Security Laboratory Network" (IHSLN) (IHSLN, unpublished data) and revealed similar results. Thus, continued and extended quality assurance studies are required to maximize the robustness of *Filovirus* diagnostic procedures.

Field diagnostics

The recent years have been marked by repeated outbreaks of EBOV in several countries of Central Africa (6). Often these outbreaks occur in remote sites where sophisticated medical support systems are limited and timely diagnostic services are extremely difficult to provide. Provision of a fieldable laboratory to provide basic diagnostics for filoviruses, and other agents that may be confounding to the diagnosis, could help aid in the management of patients specifically and the outbreak in general. The development of truly portable real-time thermocyclers and simple, fieldable immunological assays has made the provision of a field diagnostic laboratory a reasonable undertaking.

As it might not be possible to relocate an entire laboratory to remote settings it is necessary to bring the equipment essential to provide a safe work environment while completing the necessary testing. The initial and most important task is to minimize exposure for workers. If time and logistics permits, this can be ideally accomplished using a portable class III biosafety cabinet. This allows for safe handling of samples until the infectious agents are inactivated or packaged appropriately for shipping. Although portable, these units are often too large to travel as checked baggage on commercial flights and would need to be shipped cargo or by charter, which can cause delays. Alternatively, personal protective equipment (PPE) similar to that used by the isolation ward medical staff can be used to protect workers while handling infectious material. Clinical and other specimens can be safely inactivated for serological analysis by heat in the presence of appropriate ionic or non-ionic detergents. We have used a

final concentration of 0.2% sodium dodecyl sulphate (SDS)/0.1% Tween 20 and heat treatment of 60° C for 15 minutes to treat samples prior to ELISA based assays and 1% SDS prior to immunofiltration based antigen detection assays.

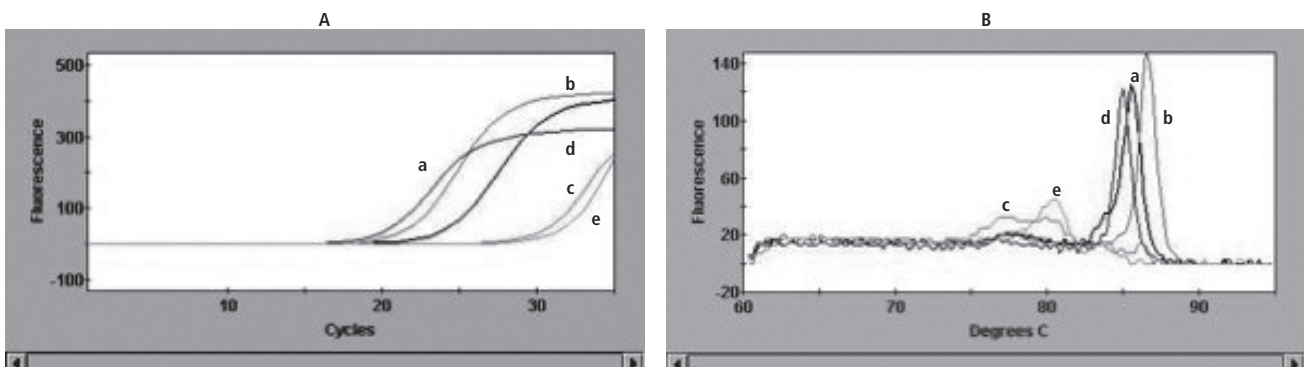
For molecular diagnostics, samples are commonly inactivated using a guanidinium thiocyanate buffers supplied with commercial RNA isolation kits. Real-time PCR based assays run on machines designed to be portable are well suited to these environments and can provide extremely sensitive tests in a very short time. In addition to confirming the presence of a virus in patient samples, real-time tests also provide a quantitative assay that may be of use when viral load determination is necessary as in trials of antivirals or other treatment regimes. Tests for other agents that may be confounding and mistaken originally as the causative agent (e.g. *Plasmodium* sp.) should also be included in the reagent inventory. The introduction of real-time RT-PCR allows assays to be carried out with minimal manipulation and equipment and can provide results in less than two hours.

The first step of molecular diagnostic techniques requires the extraction/purification of the RNA from the sample, which can then serve as a template for conversion from RNA to DNA by the action of a reverse transcriptase. This is followed by the specific amplification of viral sequences in the PCR assay. Several methods and chemistries are available for the amplification reaction, which also allow monitoring the accumulation of product for real-time. Our approach is using two primers directed against well-conserved regions of the *Filovirus* genome and monitoring for the accumulation of amplification products using SYBR Green I, a dye which upon binding non-specifically to double-stranded DNA, results in an increase in fluorescence at 530 nm. As this dye will non-specifically bind to all double-stranded DNA produced during the amplification process, the presence of specific *Filovirus* product must be determined by melt point analysis of the products carried out at the end of the amplification (figure 1A, 1B). The primers (EBsp5 – 5' TTYCCTAGCAAYATGATGG, EBsp3 – 5' TATAA-TAATCACTGACATGCAT) detect an approximately 250 bp region of the glycoprotein gene of EBOV species. The electrical power demands of the realtime PCR and laboratory equipment is small and can easily be met by small electrical generators, or directly from a vehicle (picture 1). Preservation of reagents in the field by refrigeration is also desirable and a

Figure 1.

Panel A and B show the amplification plot (A) and melting point analysis (B) for Zaire Ebolavirus (a), Sudan Ebolavirus (b), Reston Ebolavirus (c), Ivory Coast Ebolavirus (d) and mock (e) using a EBOV virus specific real-time RT-PCR. The amplification plot (A) shows an increase in fluorescence indicating an amplification product has been generated in all test samples. The melting point analysis (B) shows that the EBOV species all produced melting peaks in the range of 84 to 88°C, whereas the mock control produced a peak at a lower temperature, characteristic of primer/dimer formation, and not an EBOV specific product.

Les tableaux A et B indiquent le point d'amplification (A) et l'analyse de point de fusion (B) pour l'Ebolavirus (virus Ebola) du Zaïre (a), l'Ebolavirus du Soudan (b), l'Ebolavirus de Reston (c), l'Ebolavirus de Côte-d'Ivoire (d) et le témoin blanc (e) utilisant une PCR-TR spécifique en temps réel du virus Ebola (EBOV).



Picture 1.

Picture 1 shows the set up of the real-time PCR workstation in Mbomo, Congo. In the foreground is the thermocycler and its accessories; power is being supplied by means of a 700 watt inverter attached to the battery of the vehicle present. The clinic used in the management of the outbreak is visible in the background, demonstrating the ease at which samples could be collected and handed to laboratory support for testing.

La photo 1 montre l'installation du poste de travail où se réalise la PCR en temps réel à Mbomo, Congo.



reliable power source is preferred. However, we have found the reagents to be still useful after 10 days without refrigeration and thus, reliable re-supply of perishable reagents can also allow continuous operation of a field lab in remote areas where constant power is not available.

As mentioned before, confirmatory assays are extremely important and fieldable antigen and antibody detection assays are powerful tools. An immunofiltration assay for the direct detection of EBOV, subtypes Zaïre, antigen in sera and other body fluids will soon be available in column format (20). These assays have a number of features that make them suitable for rapid tests that can be used in a field or outbreak setting. Refrigeration is not required for any component nor is there any need for electrical power sources other than a battery driven photometer. A simple protocol consisting of rehydration of the column and sequential loading of the sample followed by test reagents and substrate provides a colorimetric determination in approximately 30 minutes (picture 2). Assays for the detection of *Filovirus* specific antibodies based on this system are currently under development (LUCHT et al., unpublished data). This method should be applicable to most species of filoviruses and should lend itself very well to field applications.

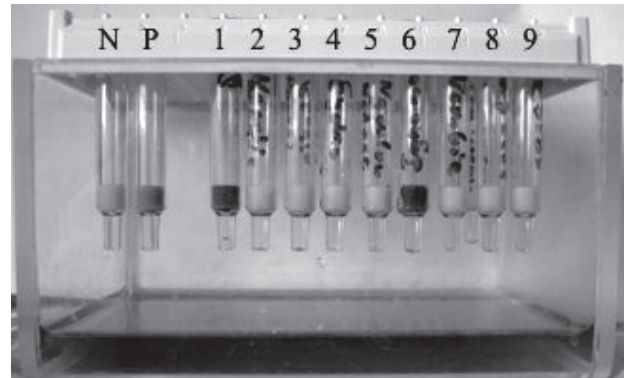
Conclusions

Rapid, sensitive and quantitative laboratory diagnosis for *Filovirus* infections is available in national and international reference laboratories. In this setting, delays and other problems are generally due to improper collection, storage and transport of sensitive samples. In contrast, local laboratory diagnosis has not been standard during EBOV and MARV outbreaks. The Centers for Disease Control and Prevention operated a laboratory during the EBOV outbreak in Gulu (31). The Public Health Agency of Canada provided field laboratory support for an EBOV outbreak investigation in Mbomo, Democratic Republic of the Congo (picture 1).

Picture 2.

Picture 2 shows the EBOV antigen detection assay on the basis of immunofiltration. The assay can be performed in approximately 30 minutes without the need of power. N, negative control; P, positive control; lanes 1-9, clinical specimens (samples in lanes 1 and 6 are positive for EBOV antigen).

La photo 2 indique le dosage de recherche d'antigène EBOV par immunofiltration.



Field diagnosis in remote areas such as Mbomo poses many problems for molecular based techniques mainly in the area of power supply and refrigeration. However it was possible to provide timely and accurate testing with a minimum of equipment to provide aid that could be of significant benefit to those affected in the outbreak and those assisting in its management.

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Références bibliographiques

1. BECKER S, FELDMANN H, WILL C & SLENCZKA W - Evidence for occurrence of filovirus antibodies in humans and imported monkeys: do subclinical filovirus infections occur worldwide? *Med Microbiol Immunol*, 1992, **181**, 43-55.
2. BRAY M & PARAGAS J - Experimental therapy of filovirus infections. *Antiviral Res*, 2002, **54**, 1-17.
3. DROSTEN C, GÖTTIG S, SCHILLING S, ASPER M, PANNING M et al. - Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, Dengue virus, and yellow fever virus by real-time reverse transcription-PCR. *J Clin Microbiol*, 2002, **40**, 2323-2330.
4. ELLIOTT LH, BAUER SP, PEREZ-ORONNOZ G & LLOYD E - Improved specificity of testing methods for filovirus antibodies. *J Virol Methods*, 1993, **43**, 85-99.
5. FELDMANN H, GEISBERT TW, JAHRLING PB, KLENK HD, NETESOV SV et al. - *Filoviridae*. In: FAUQUET CM, MAYO MA, MANILOFF J, DESSELBERGER U & BALL LA, (Eds), *Virus Taxonomy, VIIIth Report of the ICTV*, Elsevier/Academic Press, London, 2004, pp. 645-653.
6. FELDMANN H, JONES S, KLENK HD & SCHNITTLER HJ - Ebola virus: from discovery to vaccine. *Nat Rev Immunol*, 2003, **3**, 677-685.
7. GEISBERT TW, HENSLEY LE, JAHRLING PB, LARSEN T, GEISBERT JB et al. - Treatment of Ebola virus infection with a recombinant inhibitor of factor VIIa/tissue factor: a study in rhesus monkeys. *Lancet*, 2003, **362**, 1953-1958.
8. GEISBERT TW & JAHRLING PB. Use of immunoelectron microscopy to show Ebola virus during the 1989 United States epizootic. *J Clin Pathol*, 1990, **43**, 813-816.
9. GEISBERT TW & JAHRLING PB - Differentiation of filoviruses

- by electron microscopy. *Virus Res*, 1995, **39**, 129-150.
10. GONZALEZ JP, NAKOUNE E, SLENCZKA W, VIDAL P & MORVAN JM - Ebola and Marburg virus antibody prevalence in selected populations of the Central African Republic. *Microbes Infect*, 2000, **2**, 39-44.
 11. GROEN J, VAN DEN HOOGEN BG, BURGHORN-MAAS CP, FOOKS AR, BURTON J *et al.* - Serological reactivity of baculovirus-expressed Ebola virus VP35 and nucleoproteins. *Microbes Infect*, 2003, **5**, 379-385.
 12. IKEGAMI T, NIIKURA M, SAIJO M, MIRANDA ME, CALAOR AB *et al.* - Antigen capture enzyme-linked immunosorbent assay for specific detection of Reston Ebola virus nucleoprotein. *Clin Diagn Lab Immunol*, 2003, **10**, 552-557.
 13. IKEGAMI T, SAIJO M, NIIKURA M, MIRANDA ME, CALAOR AB *et al.* - Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. *Microbiol Immunol*, 2002, **46**, 633-638.
 14. JAHRLING PB, GEISBERT TW, DALGARD DW, JOHNSON ED, KSIAZEK TG *et al.* - Preliminary report: isolation of Ebola virus from monkeys imported to USA. *Lancet*, 1990, **335**, 502-505.
 15. JOHNSON KM, ELLIOTT LH & HEYMANN DL - Preparation of polyvalent viral immunofluorescent intracellular antigens and use in human serosurveys. *J Clin Microbiol*, 1981, **14**, 527-529.
 16. JOHNSON BK, OCHENG D, GICHOGO A, OKIRO M, LIBONDO D *et al.* - Antibodies against haemorrhagic fever viruses in Kenya populations. *Trans. R Soc Trop Med Hyg*, 1983, **77**, 731-733.
 17. KSIAZEK TG - Laboratory diagnosis of filovirus infections in non-human primates, *Lab Animal*, 1991, **20**, 34-46
 18. KSIAZEK TG, ROLLIN PE, JAHRLING PB, JOHNSON E, DALGARD DW & PETERS CJ - Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. *J Clin Microbiol*, 1992, **30**, 947-950.
 19. KSIAZEK TG, WEST CP, ROLLIN PE, JAHRLING PB & PETERS CJ - ELISA for the detection of antibodies to Ebola viruses. *J Infect Dis*, 1999, **179 Suppl 1**, S192-S198.
 20. LUCHT A, FORMENTY P, FELDMANN H, LEROY E, BATABOUKILA P *et al.* - Development of an immunofiltration-based antigen detection assay for rapid diagnosis of Ebola virus infections. *J Clin Microbiol*, 2005, revision.
 21. LUCHT A, GRUNOW R, OTTERBEIN C, MOLLER P, FELDMANN H & BECKER S - Production of monoclonal antibodies and development of an antigen capture ELISA directed against the envelope glycoprotein GP of Ebola virus. *Med Microbiol Immunol*, 2004, **193**, 181-187.
 22. MIRANDA ME, KSIAZEK TG, RETUYA TJ, KHAN AS, SANCHEZ A *et al.* - Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. *J Infect Dis*, 1999, **179 Suppl 1**, 115-119.
 23. MURPHY FA, VAN DER GROEN G *et al.* - Ebola and Marburg virus morphology and taxonomy, In: PATTYN SR (Eds), *Ebola virus haemorrhagic fever*, 1st edn, Elsevier/North-Holland, Amsterdam, 1978, pp. 61-84.
 24. NIEDRIG M, SCHMITZ H, BECKER S, GUNTHER S, TER MEULEN J *et al.* - First international quality assurance study on the rapid detection of viral agents of bioterrorism. *J Clin Microbiol*, 2004, **42**, 1753-1755.
 25. NIIKURA M, IKEGAMI T, SAIJO M, KURANE I, MIRANDA ME & MORIKAWA S - Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. *J Clin Microbiol*, 2001, **39**, 3267-3271.
 26. ROLLIN PE, KSIAZEK TG, JAHRLING PB, HAINES M, PETERS CJ - Direct detection of Ebola-like viruses by immunofluorescence (IF) in impression smears from macaque tissues. *Lancet*, 1990, **336**, 1591.
 27. ROWE AK, BERTOLLI J, KHAN AS, MUKUNU R, MUYEMBE-TAMFUM JJ *et al.* - Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. Commission de Lutte contre les Epidemies a Kikwit. *J Infect Dis*, 1999, **179 Suppl 1**, S28-S35.
 28. SANCHEZ A & FELDMANN H - Detection of Marburg and Ebola virus infections by polymerase chain reaction assays. In: BECKER Y & DARAI G (Eds), *Frontiers of Virology - Diagnosis of human viruses by polymerase chain reaction technology* (2nd ed.), Springer Verlag, Berlin, Heidelberg, New York, 1996, pp. 411-418.
 29. SANCHEZ A, KSIAZEK TG, ROLLIN PE, MIRANDA ME, TRAPIER SG *et al.* - Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. *J Infect Dis*, 1999, **179 Suppl 1**, S164-S169.
 30. SIEGERT R, SHU HL, SLENCZKA W, PETERS D & MULLER G - [On the etiology of an unknown human infection originating from monkeys] (German), *Dtsch Med Wochenschr*, 1967, **92**, 2341-2343.
 31. TOWNER JS, ROLLIN PE, BAUSCH DG, SANCHEZ A, CRARY SM *et al.* - Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. *J Virol*, 2004, **78**, 4330-4341.
 32. VAN DER WAALS FW, POMEROY KL, GOUDSMIT J, ASHER DM & GAJDUSEK DC - Hemorrhagic fever virus infections in an isolated rainforest area of central Liberia. Limitations of the indirect immunofluorescence slide test for antibody screening in Africa. *Trop Geogr Med*, 1986, **38**, 209-214.
 33. ZAKI SR, SHIEH WJ, GREER PW, GOLDSMITH CS, FEREBEE T *et al.* - A novel immunohistochemical assay for the detection of Ebola virus in skin: Implications for diagnosis, spread, and surveillance of Ebola hemorrhagic fever. Commission de Lutte contre les Epidemies a Kikwit. *J Infect Dis*, 1999, **179 Suppl 1**, S36-S47.7.