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SELECTED ASPECTS OF *FILOVIRUSES* IN THE VIEW OF EBOLA VIRUS DISEASE EPIDEMIC

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ABSTRACT

Ebola virus disease (EVD) is a zoonosis of high virulence in humans. Current epidemic in West Africa is the largest EVD epidemic reported so far, exceeding the number of cases notified and geographical regions affected. This article discusses selected aspects of Ebola virus biology and ecology which are of significance for the processes of primary infection in humans and the spread of epidemic in population. A special attention was drawn to the issues essential for the diagnosis of infection and safety of testing.

Key words: *Ebola virus, structure and genetic variability of virus, EBOV circulation in environment, transmission of infection, inactivation of Ebola virus and clinical material, diagnosis*

CHARACTERISTICS OF *FILOVIRIDAE*

Epidemic caused by Ebola virus resulted in intensifying studies on the biology of the family *Filoviridae*, consequently leading to the discoveries of many interesting biological features of this systematic group. *Filoviridae* belongs to the order of *Mononegavirales,* including viruses with characteristic filamentous nucleoprotein structure and genome - non-segmented, negative-stranded RNA. Order of *Mononegavirales* also comprises other free families: *Rhabdoviridae* (e.g. rabies virus), *Paramyxoviridae* (e.g. RSV, measles virus etc.) and *Bornaviridae* (e.g. *Avian bornavirus*). Compared to other viruses, *Filoviruses* have distinctive morphology. Filamentous particles of length up to 14,000 nm and uniform diameter of 80 nm are frequently observed on electron microscopy. Other viruses of the order *Mononegavirales* have various morphological features, including both spherical and short filamentous form. All viruses belonging to this order have similar genome structure, ss(-)RNA, where after initial noncoding region, nucleoprotein (NP) gene appears as the first and large RNA-dependent RNA polymerase gene (L) as the last (1).

Currently, *Filoviruses* consist of three genera: *Ebolavirus, Marburgvirus* and *Cuevavirus.* Genus *Ebolavirus* (EBOV) is composed of 5 species - *Zair ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV/SUDV[1]), *Cote d'Ivoire ebolavirus* (CIEBOV) – since 2009 referred to as *Tai Forest ebolavirus* (TEBOV/TAFV), *Bundibugyo ebolavirus* (BEBOV/BDBV) and *Reston ebolavirus* (REBOV/RESTV). The remaining genera of *Filoviruses* are represented by single species: *Marburgvirus* by *Marburg marburgvirus* (MARV) and *Cuevavirus* by *Lloviu Cuevavirus* (LLUV) (1).

In case of these viruses, bats are natural Ebola virus hosts, presenting mild or asymptomatic course of infection. In case of Ebola virus, fruit bats belonging to the species: *Hypsignathus monstrosus, Epomops franqueti, Rousettus aegyptiacus* and *Myonycteris torquata* are the hosts. For Marburg virus, *Rhinolophus eloquens* and *Miniopterus inflatus* are the hosts, while for Lloviu virus – an European bat *Miniopterus schreibersii* (2).

Integration of a fragment of cDNA transcribed from RNA of *Filoviruses* into the genome of hosts was indicated. Most commonly, this fragment is homologous to the matrix protein (VP35) or nucleoprotein (NP). Based on the integration of filovirus gene fragment into animal genome, it is estimated that this family exists for at least 50 million years (3).

1 Literature provides different abbreviations. Currently, there are no recommendations which of them should be used. In this article, two most common abbreviations were presented.

EBOLA VIRUS STRUCTURE

Filovirus genome is a negative-sense, single-stranded RNA with approximately 19,000 nucleotides in length. The genome encodes eight basic mRNA, of which 7 encode virus structural proteins: nucleoprotein (NP), VP35 protein (polymerase cofactor), VP40 (matrix protein), glycoprotein (GP), VP30 - transcription activator, VP24 - the second matrix protein and RNA-dependent RNA polymerase (L) and one non-structural protein (sGP) (Fig. 1) (4).

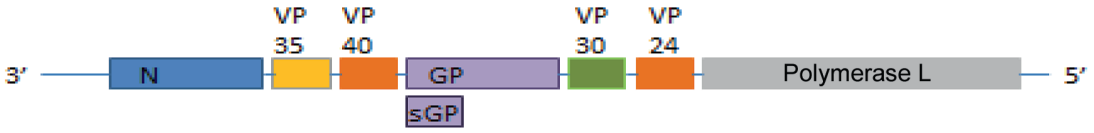

Figure 1. Scheme of Ebola virus genome

Ebola virus envelope is a host-derived lipid membrane, where specific viral proteins are located, i.e. VP40, VP24 – at the inner side and glycoprotein at the outer side of the membrane. Matrix proteins - VP40 and VP24 take part in binding of ribonucleoprotein and the inner surface of virus envelope. They participate in the processes of virion and virus assembling and budding. Furthermore, they have an influence on the capability of virus replication in different hosts and in that way - on species specificity. Such proteins play an important role in the virulence of Ebola and Marburg viruses by inhibiting the synthesis of type I and II interferon (5-8).

Glycoproteins GP1 and GP2 are the products of enzymatic digestion of the precursor of viral glycoprotein GP (9). Glycoproteins play a role in virus binding to target cells of a host (endothelium and monocytes are the targets for GP1), viral entry, endothelial cells damage and cytotoxicity in vessel cells. Glycoprotein is also responsible for immunosuppression (in vitro). Contrary to MARV and other *Filoviruses,* EBOV produces high volume of non-structural glycoprotein (sGP), a protein derived from the initial product of glycoprotein gene (10). Recent publications suggest that this protein activates non-infected dendritic cells, leading to the secretion of pro-inflammatory and anti-inflammatory cytokines (TNFa, IL1b, IL6, IL8, IL12p40 and IL1-RA, IL10). Several authors state that sGP has also cytotoxic activity (9).

Polymerase complex, composed of polymerase, polymerase cofactor VP35 and nucleoprotein, participates in the process of replication while for transcription – VP30 protein is also required (2).

VARIABILITY OF EBOLA VIRUS

Taking into account the fact that Ebola virus is a RNA virus and RNA-dependent RNA polymerase does not have any repairing functions in the process of transcription, an expected number of substitutions in the genome of the next generations may be even 10^{-4} /nucleotide/year. Mutation rate for EBOV was determined at $0.8x10^{-3}$ per nucleotide/year (11).

Based on the analysis of glycoprotein sequencing, probable origin of ZEBOV causing epidemics in West Africa in 2014, was determined as single lineage of the Ebola virus from 2004. It was also observed that compared to the viruses studied in previous periods, the mutation rate for those analyzed in 2014 was considerably higher. Distribution of substitution rate in 2014 was closer to the normal distribution while in case of viruses analyzed earlier, the level of dispersion was exceptionally low. Authors suggest that this change was a result of the character of epidemic – forest epidemics present in small and closely related populations would be of lower virus variability (11). An attention should be also drawn to the differences in the methodology. During 2014 epidemic, virus genome was sequenced directly from the material of patients, so viruses were replicated in different cells of patients. In previous studies, viruses were first isolated in Vero cells and then analyzed. Thus, they were pre-selected which consequently sharpened the curve of distribution of substitution rate.

CIRCULATION OF VIRUS IN INFECTED ORGANISM AND PATHOGENESIS

Following the entry to an infected organism, monocytes, macrophages and dendritic cells are early targets for *Filoviruses* (12,13). Then, virus is replicated in these cells and concurrently transported to other regions of infected organism. Virus is also replicated in a number of different cell types, including endothelial cells, fibroblasts, hepatocytes, adrenal cortical cells and several types of epithelial cells (12). Authors suggest that viral non-structural glycoprotein (sGP) is one of the major factors of vessel and endothelial cell damage. Its concentration determines the level of cell damage (13).

Table I. Methods of EVD diagnosis and inactivation of infectious material by the stage of disease and testing objective (17-19,22)

Testing	Clinical material	Testing schedule	Testing objective	Inactivation of infectious material* - selected methods
PCR with Reverse transcriptase (RT-PCR)	blood, serum, urine, body fluids	since day 2 of infection, recommended since day 3	diagnosis of infection	-Buffer AVL or another suitable -70% alcohol -heating for 60 minutes at 60ºC
	semen	even up to week 6 from recovery	indication for quarantine	
Antigen	blood, serum, body fluids	since day 4 of infection, acute stage	diagnosis	-gamma radiation - solution with 0.1% SDS in 1% Tween + albumin
IgM	serum	since day 2-6 of infection, recommended since day 10 up to 2-3 months	diagnosis, retrospective examination	- solution with 0.1% SDS in 1% Tween + albumin
IgG ELISA	serum	since day 10 of infection	diagnosis, possible cross-reactions, no link with prognosis, retrospective examination	-solution with 0.1% SDS in 1% Tween + albumin
Neutralizing IgG (alive Ebola virus)	serum	since day 10 of infection	prognosis and diagnosis, no applicable in EVD fatal cases, retrospective examination	not applicable
Electrolyte level	blood, body fluids	-	analytical examination	heating for 60 minutes at 60ºC
White blood cell count in the peripheral blood	blood	-	analytical examination	3% acetic acid with pH=2.5
Preparations	various	-	analytical examination	70% ethyl or methyl alcohol

* in case of suspicion of EVD, all diagnostic examinations can be performed in biosafety level 2 laboratory only provided the infectious material is inactivated; inactivation method of infectious material should be adjusted as not to interfere with test result; it is possible to adopt other tests than the ones presented above, however, they cannot affect the correct test result.

Infection with *Filoviruses* leads to extensive pathologic lesions, including hemorrhagic lesions which may manifest itself as ecchymoses on skin, mucous membranes and internal organs, accompanied by large effusions to body cavities. Multifocal necrotic lesions are reported in liver, spleen, kidneys, testes and ovaries. Lesions in the region of liver include necrosis and apoptosis of hepatocytes, steatosis and hyperplasia of Kupffer cells. Eosinophilic cytoplasmic inclusions present in these cells constitute the aggregates of virus nucleoprotein. Hemorrhagic lesions and diffuse injuries are observed in lungs (14). Laboratory findings of patients in the first phase of infection show leukopenia with lymphopenia and then leukocytosis and thrombocytopenia. Liver damage results in an increased serum concentration of aspartate and alanine aminotransferases. At the end of the first week of infection, kidney damage leads to oliguria and increased concentration of creatinine and urea in serum. Infected patients present the symptoms of disseminated intravascular coagulation. Hemorrhagic lesions may be associated with a decreased concentration of coagulation factors and other plasma proteins resulting from liver and adrenal cortical cell necrosis. As the adrenal cortex has an important role in the control of blood pressure, impaired function of this organ results in hypotension and sodium loss. Such manifestations are reported in all symptomatic cases of Ebola virus infection (13,14).

EBOLA VIRUS DISEASE

Clinical course of infection with Ebola virus may differ dependent on virus species. In general, Ebola hemorrhagic fever is present after the incubation period of 2-21 days (4-10 on average). It is characterized by fever, chills, malaise and muscle pain. Within a short time, subsequent multiple organ symptoms appear, including those referring to the following systems: gastrointestinal (anorexio, nausea, vomiting, abdominal pain, diarrhoea), respiratory (chest pain, dyspnoea, cough, rhinorrhoea), vascular (hypotension) and neurological (headache, confusion, coma). At the final phase of disease, external bleeding, ecchymoses, uncontrolled leakage from vein puncture sites and internal bleeding are reported. They are frequently accompanied by rash and abrupt weight loss. Such symptoms are present 5-7 days following the infection. In the successive days, a part of infected patients may experience shock, convulsions, severe metabolic and coagulation disorders which can be fatal. In case of convalescents, symptoms of days 5-7 gradually disappear. It is believed that if an

infected patient survive for more than 10 days after the onset of first symptoms, it may be indicative of positive prognosis (12,14).

In the *Filovirus* infection outbreaks, the average fatality rates were 78% and 53% for Zaire Ebola virus and Sudan Ebola virus, respectively. The only one reported case of infection with CIEBOV survived. The lowest fatality rate was reported in the Bundibugyo Ebola virus outbreak in 2007, i.e. 25% (15). Marburg virus infection is considered to be a disease of lower fatality rate, but approximately 82% persons infected with this virus die. It should be noted, however, that the percentage of fatal cases differs by regions and level of medical care. Only 24% of patients infected with MARV died in Europe and the United States while in Democratic Republic of the Congo and Angola, case fatality rates were 83% and 90%, respectively (14,15).

ROLE OF HUMORAL IMMUNE RESPONSE IN *FILOVIRUS* INFECTIONS

Fatality of EBOV infection is associated with the action of virus, reducing the innate immune response, thus, leading to uncontrolled release of inflammatory mediators and chemokines in the late stage of infection. Such process correlates with extensive apoptosis of T and B lymphocytes despite they were not infected. Survival and recovery of patients infected with EBOV is linked with neutralizing EBOV-specific immunoglobulin G (IgG) response, whose level in fatal cases is more than 100-fold lower or undetectable (16). IgG antibodies, other than neutralizing ones, are frequently detected, using ELISA or similar test while neutralizing IgG are tested only by neutralization assay in BSL-4 laboratory EBOV GP-specific neutralizing antibodies were exclusively found in the samples from convalescents. IgG antibodies directed against non-structural protein glycoprotein (sGP) do not have properties typical of neutralizing antibodies and they are detected only by ELISA tests. Simultaneously, IgG antibodies detected by ELISA in persons who have never had symptoms indicative of Ebola virus disease were mainly reactive to VP40 (16).

Production of large amount of non-structural soluble glycoprotein (sGP) during an infection is considered to be an additional element of virus protection against elimination by host's immune system. This protein is homologous with GP in the virus envelope to more than 90%. It is a strong inducer of humoral immune response, however, the antibodies antisGP do not neutralize Ebola virus. In the process of co-immunization with sGP and GP in experimental animals, a shift of humoral response toward non-neutralizing antibodies was reported (17).

INFECTION TRANSMISSION AND VIRUS ECOLOGY

As it was stated earlier, bats are considered to be natural hosts of *Filoviruses*. As with human and apes, forest African antelopes are accidental virus hosts for whose fatality rate is very high. In case of forest epidemics, both epidemiological investigations and analyses of virus genetic material showed a considerable role of these animals as the major source of primary infection in humans (18). Having crossed the barrier between humans and animals, the infection is transmitted in humans via physical contact. However, during the epidemic in Sudan in 1976, it was noted that approximately 5% of cases neither had direct contact with Ebola cases nor carcasses of infected animals. During an urban epidemic in Kikwit (1995, Democratic Republic of Congo, formerly known as Zaire), the percentage of cases without known exposure amounted to 17.4%. Such observations suggested that virus could be transmitted not only through direct contact (maybe by droplets or contact with infected animals other than primates and forest antelopes). As other animals, including pets and domestic animals, may have contact with *Filoviruses*, a special attention was drawn to dogs. In African villages, dogs are not fed, thus, they eat carcasses of animals found in the vicinity of villages as well as internal organs and remainings of animals hunted by locals. Irrespective of the fact that transmission of *Filoviruses* in dogs has not been documented, dogs behaviour and feeding may suggest their role in infection spread. *Allela* and the team (18) examined three populations of dogs for the presence of Ebola virus–specific immunoglobulin (Ig) G. Dog populations were sampled from areas in which Ebola virus infections in humans were reported. Reference group comprised dogs (more than 100) living in France. Spectacular results were achieved. An increasing linear gradient of seroprevalence was reported from France (2% of dogs that had contact with *Filoviruse*s), through approximately 9% noted in African city, in which no EVD human cases were reported, then through more than 15% in Mekambo (a town in which sporadic cases were reported) to approximately 25% in dogs living in villages in which Ebola virus epidemic occurred (18). Results suggest that Ebola virus antibody prevalence in dogs reflects the virus activity. It may be indicative of a risk of infection with this virus in humans.

Olson et al. conducted an in-depth analysis of available data on *Filovirus* infections in animals documented by 2012 (19). Analysis included not only the methodology (virus detection or seroprevalence study), but also the material studied (alive animals or carcasses). In total, data on 13,440 animals representing 158 species, including 1,214 alive captured animals and 19 carcasses

(10 different species) were analyzed. In a group of alive animals, the most common were bats and rodents. EBOV genome was identified in 0.2% (13/5,309) of alive animals while EBOV-specific antibodies were found in 2.2% of animals (180/8,050). In a group of dead animals, the carcasses of non-human primates predominated. During epidemics in humans, Ebola virus was identified in more than 32% of studied dead cases of primates. Having considered the possibility of dog-to-human transmission of Ebola virus, it should be highlighted that antibodies were detected in 24.1% of studied animals, but neither alive nor dead dogs were infected. In case of apes studied, neither antibodies nor the virus were detected in alive animals, but 51.5% of dead animals were infected with EBOV (19).

Such data suggest the lack of symptomatic Ebola virus infection, leading to death in dogs. Asymptomatic infections in these animals are reported frequently during epidemics. There is a lack of data on the role of asymptomatic animals, other than bats, in the transmission of virus to humans. Specific route of disease transmission, i.e. through contact with excretions and secretions of infected persons, does not allow for clear defining their role in infections in humans. Simultaneously, the lack of such observations during outbreaks reported so far suggests that the role of asymptomatic animals in the transmission of infection in humans is very important for the first case (case 0) and marginal or even non-essential in the progression of epidemic.

During an epidemic in West Africa in 2014 (actually in Spain), concerns regarding the transmission of infection in pets and possible subsequent spread of infection from such animals to humans were raised. Studies carried out so far demonstrated that there is a possibility of Ebola virus infection in pigs. In 2008, infections with REBOV in pigs were reported in the Philippines. Furthermore, antibodies were detected in humans who had contact with these animals. It should be noted, however, that pigs presented mainly symptoms of respiratory system (atypical course of infection). It may indicate the possibility of infection via infected aerosol. Such route of transmission was reported in first REBOV infection identified in humans and spread from apes imported to the United States from the Philippines (12). Furthermore, infection described referred to virus which is non-pathogenic for humans. Antibodies were detected only in persons who had close contact with animals.

SENSITIVITY OF EBOLA VIRUS

Recent studies suggest that Ebola virus transmission via aerosol cannot be excluded. It was found that drying of clinical material (blood, body fluids, secretions), containing Ebola virus, serum proteins and cellular elements, provides protection and leads to a considerable increase of virus resistance, i.e. approximately by 3-5%, to physical and chemical environmental conditions such as UV radiation, gamma radiation, temperature, chlorine-based disinfectants etc. Then, aerosol, containing infectious virus particles, may be produced (20,21). Protective action of serum proteins and blood morphotic elements was also demonstrated in studies, using liquid media – infectious Ebola virus was identified even after more than a month. Temperature is an important factor, affecting Ebola virus. In the temperature +4ºC, virus survival is considerably prolonged, while heating for at least 30 minutes at 60°C totally inactivates Ebola virus (22).

Irrespective of the fact that Ebola virus in an enveloped virus, it is recommended to use virucides also against non-enveloped viruses and those of higher resistance compared to enveloped viruses in accordance with PN-EN 14476:2013-12 (23). Furthermore, such disinfectants, beside virucidal formulations, should also contain detergents as to remove all possible contaminations that could act as protection for Ebola viruses (22).

EVD DIAGNOSIS

In the course of Ebola virus disease, the symptoms, especially in the first stage of disease are non-specific and may be also observed in other infections, including malaria, typhoid fever, hemorrhagic fevers caused by other viruses like Lassa virus. Thus, it is required to confirm the etiological agent of infection. For the purpose of microbiological diagnosis of Ebola virus infection, molecular diagnostics is applied (polymerase chain reaction with reverse transcriptase) or tests for the presence of antigens (rapid tests or ELISA) or IgM and IgG antibodies (Tab.I) (24,25). As it was stated earlier, neutralizing antibodies may be detected exclusively by neutralization assay - test performed in biosafety level 4 laboratory. IgM and IgG antibodies, other than neutralizing ones, are detected by widely available commercial tests such as ELISA (13,25).

As with other infections, the methods should be adapted to the stage of infection. At the beginning - since ca 2-3 days, virus genome is detected (PCR), while in case of some patients also IgM. From the day 4 and 10, virus antigen and IgG may be identified, respectively. Taking into account the fact that the majority of patients die around the day 10 of disease, IgG titres are mainly determined in retrospective examinations.

Contrary to a number of other viral pathogens, the presence of EBOV in specimens may be demonstrated following the onset of symptoms. Furthermore, EBOV is detected in the majority of body fluids up to ap-

proximately day 20 after the infection, while in faeces, material collected from vagina and semen up to weeks 4, 9 and 13, respectively (8).

Samples collected from patients suspected of Ebola virus infection should be considered as highly infectious. Tests in biosafety level 2 laboratory may be performed only after the inactivation of sample infectivity. Until the sample is inactivated, it should be managed as a potentially very dangerous material and proper protective measures should be undertaken (26). It applies for both analytical examinations (white blood cell count, blood glucose level, electrolyte level etc.) and microbiological tests (Tab.I). It is assumed that the number of diagnostic tests in case of patients suspected of EVD should be restricted to a minimum.

SPECIFIC THERAPY AND VACCINATION

Currently, EVD cases are treated, using immunological products, including convalescent serums and provisionally - ZMapp. ZMapp is a product, containing three different monoclonal antibodies against GP of Ebola virus. Furthermore, studies are conducting on the use of another product (by Tekmira), employing short interfering RNA (ribonucleic acid interference; iRNA). Another product, which may be applied in the future, is Favipiravir – RNA-dependent RNA polymerase inhibitor (8,12).

Other products that are now under phase I/II clinical trials are vaccines. The most advanced works focus on two vaccines: the first vaccine containing chimpanzee adenovirus vector with inserted Ebola virus gene (ChAd3-ZEBOV) and the second vaccine, including recombinant vesicular stomatitis virus with inserted EBOV gene (rVSV-ZEBOV). Animal studies demonstrated their effectiveness and lack of toxicity (12,27). Furthermore, there are also other vaccines in development. Probably, phase I clinical trial for these vaccines will start in 2015. These are: Russian product containing recombinant influenza and Ebola viruses and 2-dose vaccination with two different vaccines referred to as Ad26-EBOV and MVA-EBOV (Johnson&Johnson and Bavarian Nordic) (27).

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