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ATYPICAL BOVINE PESTIVIRUSES

(review)

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Abstract

Increasingly frequent outbreaks of atypical viral infections, detection of new viruses, modified isolates and quasispecies with a confirmed or potential emergence have become a worrying feature of the last decades characterized by extremely close international dealings. For the cattle industry, they pose real and serious threat because of a tendency to spread widely and quickly due to globalization and the use of standardized zootechnical and veterinary protocols. The *Flaviviridae* family comprises several genera of which the genus *Pestivirus*, including four viruses, i.e. the cattle viral diarrhea — mucosal disease (VD-MD) virus types 1 and 2, swine fever virus and sheep border disease virus, are important to farm animals (http://ictvonline.org/virusTaxonomy.asp). The characteristics of a new group of viruses genus *Pestivirus* of the *Flaviviridae* family, allocated in the period from 2000 to 2014 from the buffalo and cattle, as well as fetal calf serum used for cell cultures and vaccines production harvested in Australia, Canada, Mexico, Brazil and the United States and packaged in Europe (H. Schirrmeier et al., 2004; A. Cortez et al., 2006; E. Bianchi et al., 2011; B. Rodrigues et al., 2011; H. Xia et al., 2011; H. Xia et al., 2012; S. Peletto et al., 2012) are submitted in the review. The virus has been isolated in Thailand, Bangladesh and China (L. Liu et al., 2009; L. Mao et al., 2012; N. Haider et al., 2014). Messages on the isolation of the agent in other European countries, North America, Russia, India and Australia are absent (F.V. Bauermann et al., 2013). The widespread use of contaminated biological products can facilitate the penetration of the virus in different regions of continent causing their potential emergence for cattle. Strains of viruses presented cytopathogenic and non-cytopathogenic biotypes not officially classified and have a variety titles in literature: a third type of viral diarrhea-mucosal disease in cattle (BVDV), an atypical pestivirus (HoBi-like), the fifth type of *Pestivirus* genus (N. Decaro et al., 2012). Based on phylogenetic analysis were identified two genetic groups: Brazilian and Thai, which differ from the prototype member of the genus - the BVD virus but having a great similarity in the manifestation of clinical signs, the ability to infect the foetus of cattle and buffalo (F.V. Bauermann et al., 2013). In cattle a spontaneous or experimental infection caused by HoBi-like virus is very similar to the cattle VD-MD and manifests as diarrhea, abortion, respiratory syndrome, persistent infection (F.V. Bauermann et al., 2013). The situation is aggravated by the fact that they like the BVDV are able to induce persistent infection and forms permanent epizootic foci (M.N. Weber et al., 2014). The discovery of this group of viruses requires a critical assessment of the diagnostic tools and vaccines against the BVDV. To date, the there are no tests for the detection of ruminants' pestiviruses or their antibodies, particularly due to high variability of this virus group. That is why their laboratory diagnosis should not rely on the use of a single test. The best approach would be serological diagnosis of the herd followed by the identification of persistently infected animals, the virus isolation and molecular analysis (F.V. Bauermann et al., 2013). Given the lack of HoBi-like infection diagnostics, these viruses can remain unnoticed and, presumably, compromising the effectiveness of control or eradication programs of BVDV realized in certain European countries and the United States (K. Stehl et al., 2004; J.F. Ridpath, 2010).

Keywords: pestiviruses, viral diarrhea-mucosal disease, atypical viruses, sequencing, genetic subgroups, fetal serum, buffalo, molecular diagnostics, control programs.

The increasingly frequent outbreaks of atypical viral infections, detection of new viruses, modified isolates and quasispecies with a confirmed or potential emergence for animals, including bovine cattle, have become a worrying feature

of recent decades, which are characterized by almost non-limited expansion of international trade relations. For the herding, they pose a real and serious threat because of the tendency to a wide and rapid spread between regions and continents due to globalization and international shipments of large batches of animals.

Moreover, the use of contaminated biological materials in relevant standardized protocols in zootechnics and veterinary medicine may result in expansion of viruses, including atypical ones.

In this regard, viruses from the *Flaviviridae* family are of special interest as they have a wide range of obligatory hosts (due to a great mutagenic activity) and are capable to cross the species barriers and infect heterologous animals.

This family comprises several genera, with the *Pestivirus* genus being the most significantly harmful among them for farm animals; this genus includes four viruses, i.e. bovine viral diarrhea-mucosal disease (BVD-MD) virus type 1 and 2; classical swine fever virus and (sheep) border disease virus (http://ictvonline.org/virusTaxonomy.asp).

Several new types have been discovered recently. Thus, the giraffe virus H138 was isolated from a giraffe in Kenya, the Pronghorn virus from a blind prong-horned antelope in the USA, the porcine Bungowannah virus from swine in Australia during a natimortality outbreak; also, a «HoBi»-like virus has been described [1-5]. The last one is of greatest concern for epizootologists and virologists due to its potential emergence and similarity to BVDV. The established strains of this virus presented both with cytopathogenic and non-cytopathogenic biotypes have been allocated into a separate species, which is designated variously in the literature, namely an atypical pestivirus, a bovine viral diarrhea virus type 3 (BVDV3, «HoBi»-like), the fifth type of the genus *Pestivirus* [6-9]. The International Committee on Taxonomy of Viruses (ICTV) has not accepted their official classification yet.

Manifestation peculiarities of pestivirus infections as exemplified in BVD. In 90 % cases, the diseases caused by pestiviruses in ruminants occur as acute subclinical and persistent infection. The major characteristic of this group of viruses is the ability to induce immune suppression associated with leukopenia, decreased lymphocyte proliferation, depletion of the lymphoid tissue, decreased chemotaxis and phagocytic activity, increased production of prostaglandin E2 and altered inflammatory cytokine production, which may be transient (2 to 3 weeks) in acute forms or prolonged in persistently infected (PI) animals [10-13]. Rare clinical signs of acute forms of the disease include gastrointestinal, respiratory and reproductive effects, such as diarrhea, fever, leukopenia, nasal and ocular discharge, abortion at all stages of pregnancy, delivery of PI calves. The PI animals are diagnosed with mucosal disease [14].

The BVDV is considered to be a prototype member of the genus *Pestivirus*. The disease caused by this virus is common throughout the world. The incidence of the infection in cattle is 60-85 % and is found to be region-specific [1, 2, 12, 13, 15]. The presence of PI animals in the herd increases this figure up to > 90 %. The economical losses are estimated as US$ 88 per animal [16].

Pestiviruses have a single-stranded RNA(+) genome of 12.3 thousand nucleotides. It consists of a single open reading frame (ORF) of approximately 4,000 codons in length, which encode 12 structural and nonstructural polypeptides (Npro-C-Erns-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B) and flanked by 5′- and 3′- untranslated regions (UTR) (5′-UTR and 3′-UTR) [17].

The virus is prone to mutations caused by errors of RNA-dependent RNA polymerase and recombination [17-20]. Due to frequent mutations, which occur during RNA replication, the virus exists as a pool of different but closely related mutants (quasispecies) subjected to continuous selection. Therefore,

strain pathogenicity varies considerably [10, 20].

The nucleotide sequence of genomic RNA is considered to be the most reliable tool for species and generic identification of pestiviruses. Most often, a highly conserved region suitable for amplification 5'-untranslated region (5'-UTR) and the N-terminal auto-protease (N^{pro}) gene are investigated. For phylogenetic analysis, the sites of the E_2 (the most variable) and E^{rns} genes are additionally investigated. The need to investigate several gene sites is associated with genome recombination [20-23].

In cattle, the disease may be induced by two viruses, BVDV type 1 and 2. Today at least 15 subspecies of BVDV type 1 (1a to 2o) and no fewer than 5 subspecies of BVDV type 2 (2a to 2e) are distinguished [4, 17]. Both types of the pathogen cause the same pathology in animals; however, the strains of the virus type 2 are more virulent and less common [15, 24-27]. Both viruses are represented with cytopathogenic (CP) and non-cytopathogenic (NonCP) biotypes, and NonCP types are prevailing [26, 27].

«HoBi»-like viruses (atypical pestiviruses). The first strain of the HoBi virus (D32/00_HoBi) later accepted as the prototype has been isolated in Switzerland from fetal calf serum, imported from Brazil [7]. Thereafter several more isolates have been obtained, e.g. two isolates from fetal serum in South America, CH-Kaho/cont from cell culture, Brz buf from samples of buffalo biological material, two isolates from aborted fetuses in Brazil, Th/04_Khonkaen from calf blood serum in Thailand [28]. In Italy in 2010, the virus was isolated from calves during a respiratory disease outbreak and also in the persistent form of infection [29, 30]. There was a report from Brazil in 2014 on the first case of the disease resembling the mucosal disease in BVD MD, with associated recovery of the CP strain of the virus from calves [31].

These viruses have also been detected in fetal serum, which was packaged in Europe though harvested in Australia, Canada, Mexico and the United States. According to some estimates, more than 30 % fetal calf serum batches shipped to Europe from South America are contaminated with the HoBi virus [9, 32]. Increased demand for fetal bovine serum contributes to the penetration of the virus in different regions.

The disease has been recorded in Bangladesh [33]. There were no reports on the isolation of virus from this group in other countries of Europe, North America, in Russia, India or Australia.

The origin of «HoBi»-like viruses is unknown. Currently there are two proposed anticipations. The first says that they are newly evolved or historically existed in South America and were brought to other countries and continents with biological products (fetal serum and vaccines). Another explanation is that these viruses have passed to cattle from buffalo and adapted as a result of multiple transspecies transmission. This anticipation explains the occurrence of these viruses in the regions with significant populations of buffaloes, such as Brazil or Thailand [9].

The phylogenetic analysis results demonstrate that all isolates of «HoBi»-like viruses identified at the moment have a great similarity and are grouped together. Considering the high genetic variability of pestiviruses, it is suggested that the emergence of «HoBi»-like viruses in South America and their subsequent spread to other regions is quite a recent event from the point of view of evolution. In the meantime, the way the isolate Th/04_KhonKaen from Southeast Asia differ from other «HoBi»-like viruses may suggest the independent evolution of at least two genetic subgroups of «HoBi»-like viruses (Brazilian and Thai), which has been confirmed by phylogenetic analysis of the strains [9, 28, 34].

The discovery of this group of viruses requires a critical reassessment

of existing diagnostic tools and vaccines against the BVD-MD and other viral diseases. As long as there are no routine lab tests of HoBi virus infection in most countries and available diagnostic tests lack specificity, these viruses may remain unnoticed and supposedly exist in other countries. The situation is aggravated by the fact that they like the BVDV are able to induce persistent infection and form permanent epizootic foci [35].

In Brazil, Italy and Thailand, in the herds, where these viruses might have already spread, they may lead to economic losses associated with the clinical manifestation of infection, reduced productivity and decrease in immunity (independently from the BVD virus circulation or concurrently).

The HoBi-positive status of countries may present a challenge in the international trade of animals and biological products derived from them (sperm, fetal serum, embryos) with countries free from these viruses.

As long as «HoBi»-like viruses were isolated from many species of ruminants on several continents and tend to the global spread, they represent the greatest threat for the herding among all new pestiviruses detected in 2000-2014 [9, 36].

Clinical manifestation of «HoBi»-like virus infection. In cattle, a spontaneous or experimental infection caused by «HoBi»-like viruses is very similar to the BVD-MD and manifests as diarrhea, abortion, respiratory syndrome, and persistent infection [9].

Natural infection. The first report on natural infection with Brazilian buffalo virus was received in 1990. The «HoBi»-like viruses isolated from samples of biological material from two aborted fetuses in Southeast Brazil were characterized in 2006 [34]. Three isolates of the virus were identified and sequenced in the same country in 2011. The isolate SV713/09 was obtained from a sample of a bull-producer's sperm, the use of which in herds resulted in multiple cases of blind calves born. The isolates SV241/10 and SV311/10 were identified from white blood cells of cattle and buffaloes with reproductive disturbances in the southern part of the country. In the mid-west region of Brazil the «HoBi»-like virus was identified from a calf spleen during a gastrointestinal disease [6, 35].

During an outbreak of a respiratory disease in 6-7-month old calves in southern Italy in 2009-2010, fever (39.4-40.1 °C), cough, serous nasal discharge, leukopenia, high heart and respiratory rates were reported. The autopsy of two animals revealed tracheitis and bronchopneumonia affecting apical lobes of the lungs. The virus was detected in nasal swab samples from six calves and in the lungs of the dead animals by quantitative RT-PCR (reverse transcription—polymerase chain reaction) and isolated from lungs in the MDBK cow calf kidney cell culture (Italy-1/10-1 and Italy-1/10-2 strains) [29].

In mass abortions in cattle, it was found possible to identify virus RNA and antigen in the tissues of aborted fetuses. The molecular research to identify other possible etiologic agents of the pathology produced negative results. The detected virus demonstrated a close affinity to the Italian, Australian and South American strains, though differed from the Thai one [29, 37, 38].

The site of the gene encoding the glycoprotein E2 and the 5′-UTR region in the Thai isolate revealed 99 % affinity to the Italian strain Italy-1/10-1. Titers of the relevant neutralizing antibodies were considerably higher than titers to BVDV type 1 and 2. The non-cytopathogenic «HoBi»-like virus was isolated in biological material samples of two aborted fetuses. Moreover, the first CP virus isolate was also identified in this herd, and it was obtained from the lungs of a heifer that died after a respiratory disease [39].

M.N. Weber et al. in 2014 reported clinical signs caused by the «HoBi»-like virus in cattle in Brazil, similar to the mucosal disease [31]. Sequencing and phylogenetic analysis of 5′-UTR, N^{pro} and E_2 regions revealed circulation of

four different strains of the virus in the herd. The main clinical signs and conditions involved respiratory and gastrointestinal tracts. Moreover, skin effects and corneal opacity were reported. The mucosal disease symptoms were identified in one cow calf, with subsequent isolation of the CP virus isolate. This paper gives the first case report on a condition resembling mucosal disease, which was associated with natural infection with a «HoBi»-like pestivirus [31].

Experimental infection. After the administration of the strain HoBi_D32/00, calves and pigs developed clinically asymptomatic seroconversion. Furthermore, mild fever and slight leukopenia were recorded in calves. The virus was detected in leukocytes on day 5, with its recovery over days 3 to 5 after infection [34].

In 1-month old cow calves, the Italian strain (Italy-1/10-1) caused moderate hyperthermia, serous nasal discharge and lymphocytopenia, while in lambs nasal discharge and minor lymphocytopenia only. Pigs displayed no clinical symptoms in response to virus challenge. All animals are reported to develop virus seroconversion on day 21 after infection; however titers of specific antibodies were higher in calves [40].

A comparative pathogenicity study of the Thai isolate and a highly virulent strain of BVDV revealed that the disease caused by the HoBi virus occurred in a milder form and manifested as bilateral conjunctivitis, serous nasal and ocular discharge, coughing, and thrombocytopenia on day 7, and lymphocytopenia on days 2 to 5, which returned to physiological values on day 14 after infection [41].

Diagnostic approaches in the identification of «HoBi»-like viruses. *Fetal serum testing*. Fetal calf serum is widely used in cell cultures and often found to be contaminated with pestiviruses. To obtain it, serum from many fetuses is used. The increased number of fetuses in a commercial batch contributes to the risk of inclusion any persistently infected fetus. The use of serum even with low-level virus contamination may result in cell culture infection and growth of a non-cytopathogenic strain in it. To rule out contamination, it is necessary to develop and use antigen-binding types of ELISA (enzyme-linked immunosorbent assay), qualitative and quantitative PCR. To determine the virus extension, all fetal serum batches should be tested, and not just those shipped from regions with documented cases of infection with viruses of this group [42-44].

Robust, highly sensitive test systems for the virus detection in animals and products of animal origin are warranted. This is particularly important for international trade. Currently there are no diagnostic tests for the detection of all ruminant pestiviruses or antibodies to them. Their development is particularly difficult due to high variability of this virus group, and therefore the laboratory diagnosis should not rely on the use of a single test only [9].

The «HoBi»-like virus antigen or genome can be detected in leukocytes, serum and nasal secretions of persistently infected animals. For virus isolation according to standard protocols, it may be appropriate to use free of pestivirus contamination primary trypsinized and finite septum cell lines (M-17) and the MDBK cell lines. The phylogenetic analysis of virus strains is recommended at the final stage of laboratory test [9, 21, 45, 46].

Identification of infected animals. Commercial diagnostic test kits for the detection of antibodies to BVDV using ELISA method produce false-negative results on the calf blood serum samples infected with «HoBi»-like viruses. A comparative study of BVDV-1, BVDV-2 and the HoBi virus using a commercial ELISA test kit and a serum neutralisation test found that cross reactions between epitopes of E^{rns} and NS2/3 proteins were higher than glycoprotein E_2. These results suggest that diagnostic tests for the detection of all three viruses should be

based on E^{rns} and NS2/3 epitopes, with the variability of the E_2 gene used for their differentiation [18, 47].

An additional problem is the time needed to achieve a detectable antibody titers concentration in infected animals. Furthermore, these test kits do not allow differentiating between antibodies to both viruses and the immune response to them. The neutralization test using CP strains of two viruses may be promising. Although the neutralization test is quite expensive in terms of time and results calculation, requires trained personnel and cell line stock in the laboratory, currently it is considered to be the most appropriate method for the detection and/or differentiation of animals exposed to infection with BVDV and/or «HoBi»-like viruses. However, the method requires validation.

The best diagnostic approach would be a serological survey in the herd, identification of PI animals, with virus isolation and subsequent molecular diagnostics [9].

Atypical pestiviruses and effectiveness of BVD-MD control programs. The BVD control or eradication programs, which are brought into effect in a number of countries, rely on three principles, and these are the identification and removal of PI animals from the herd; the prevention of including infected animals in the herd along with monitoring; and vaccination, the use of which depends on the disease prevalence in a region [48-50]. The successful implementation of such programs and maintenance of the free of BVDV status in the herd demand reliable diagnostic tests capable of differentiating persistently and transiently infected animals and identifying all viral quasispecies.

The existence of «HoBi»-like viruses requires special attention. They were isolated from commercial pools of blood serum used for cell culture and production of biological products, and pose a risk in view of the potential spread into new regions, possible reduced effectiveness of vaccines, diagnosticums and consequently the BVD-MD eradication programs. Moreover, animals intended for sale may also pose a threat.

Thus, the ability to infect calves, the severity of the disease, and the development of respiratory distress, intermittent fever, leukopenia, and lymphopenia caused by strains of «HoBi»-like viruses give evidence of significant similarities with the symptoms attributable to a typical bovine viral diarrhea-mucosal disease (BVD-MD). Given that all currently known strains of «HoBi»-like viruses were isolated from cattle or buffalo (if not adapted to other animal species in case of experimental infection), it suggests that they are obligate hosts of the virus. These novel, not yet fully characterized pestiviruses may affect the BVD-MD control and eradication programs and pose a hazard as being emergent pathogens for the cattle worldwide. The occurrence of the «HoBi»-like and other pestiviruses in ruminants, animal products and biopharmaceuticals should be considered and controlled.

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