Short Communication

First report of natural BoHV-1 infection in water buffalo

G. Fusco, M. G. Amoroso, G. Aprea, V. Veneziano, A. Guarino, G. Galiero, M. Viscardi

Bovine herpesvirus 1 (BoHV-1) is a double-stranded enveloped DNA virus belonging to the family *Alphaherpesvirinae*, genus *Varicellovirus*. It is the causative agent of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), a worldwide disease that affects ruminants. BoHV-1 causes respiratory and reproductive symptoms, abortion, vulvovaginitis, encephalitis and fetal death (Rana and others 2011, Amoroso and others 2013). The virus is not always restricted to its natural host species. Animals such as goat, sheep, red deer and reindeer were successfully infected with BoHV-1 under experimental conditions (Thiry and others 2007). In addition, Mediterranean buffalo have been shown to be sensitive to BoHV-1 by experimental infection (Thiry and others 2007, Scicluna and others 2010), and seropositivity to BoHV-1 in water buffalo has been well documented (Peshev and Christova 2000). Nonetheless, the virus has never been identified in naturally infected buffaloes. To the authors' knowledge, this study reports the first isolation of BoHV-1 from water buffaloes.

Over the period September 2011 to December 2012, the authors received the samples of 65 buffaloes with pathologies possibly ascribable to herpesvirus: in detail, samples from 35 animals with rhinotracheitis (nasal swabs and organs), samples from 27 cases of abortion (which occurred during the third/fourth month of gestation), organs from one calf born with a hind limb deformity and euthanased when three weeks old, and organs from two calves that had died a few days (4–7) after birth. With respect to abortion, in 14 cases (which we defined complete cases) both mothers' samples (cervical–vaginal swab and blood) and the organs of the fetus were received, in one case only the aborted fetus, and in the remaining 12 cases only cervical–vaginal swab and blood samples of the aborted buffaloes. All the samples came from animals belonging to three herds in Southern Italy.

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G. Fusco,
M. G. Amoroso, PhD,
A. Guarino,
G. Galiero,
M. Viscardi, PhD,
Department of Animal Health, Experimental Zooprophylactic Institute of Southern Italy, Via Salute, 2, Portici NA 80055, Italy
G. Aprea,
Department of Food Hygiene and Technology, Experimental Zooprophylactic Institute of Abruzzo and Molise 'G. Caporale', via Campo Boario, Teramo 64100, Italy
V. Veneziano,
Department of Pathology and Animal Health, University of Naples 'Federico II', Via Delpino, 1, Naples 80137, Italy
E-mail for correspondence: giovanna.fusco@cert.izsmportici.it

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All the blood samples were screened for the presence of antibodies against BoHV-1 glycoprotein B (gB) and glycoprotein E (gE) by ELISA (respectively IBR gB IDEXX and IBR gE IDEXX ELISA antibody test kits; Herd Check Laboratories). Serum samples from female buffaloes that had had abortions and from the euthanased calf were positive for both gB and gE antibodies.

Ruminant α-herpesviruses related to BoHV-1 (Thiry and others 2006) were detected by real-time PCR by amplifying a region of a highly conserved gene of herpesviruses (gB gene) (Wang and others 2007). DNA was extracted from cervical–vaginal swabs and organs using the Nucleic Acid Purification Kit (Macherey-Nagel) following the manufacturer's instructions. Real-time PCR was carried out as described in the OIE Terrestrial Manual (2010) using a RotorGene Q PCR amplification system (Qiagen). Results showed a total of 14 animals positive to ruminant α-herpesvirus. In detail, samples collected from heart, lung, liver and spleen tissues from the two dead newborn calves were all positive for α-herpesvirus. From these samples, it was also possible to isolate the virus by cell culture. Viral isolation followed the procedures described in the OIE *Terrestrial Manual* (2010). α-Herpesvirus was also detected by real-time PCR in liver and brain samples from the euthanased buffalo calf, even though isolation of the virus by tissue culture gave negative results. As to abortion, we registered positivity to α-herpesvirus for 11 cases out of the 27 analysed: five positive cases among the 14 complete cases, one positivity for the fetus alone, and six positive results among the cases for which we could only analyse the vaginal swab. As to virus isolation, cell culture was positive for only two cervical–vaginal swabs. Samples from cases of rhinotracheitis were all negative to ruminant α-herpesvirus and were sent to other laboratories for further investigations (data not shown). All the described results are summarised in Table 1. A sequencing assay based on pyrosequencing technology was developed to classify the α-herpesviruses identified by both real-time PCR and cell culture (Fusco and others 2015). By sequencing a small polymorphic segment of the US8 gene (gE), we could distinguish, on the basis of the nucleotide sequence differences (Table 2), between *Bovine herpesvirus 1.1, Bovine herpesvirus 1.2, Bovine herpesvirus 5, Bubaline herpesvirus 1* and *Caprine herpesvirus*. The US8 gene was first amplified in a

TABLE 1: Detection of BoHV-1.1 by real-time PCR, sequencing and tissue culture

Case	Animals analysed (n)	Real-time PCR and sequencing: Presence of BoHV-1.1 (number of animals)	Real-time PCR and sequencing: Type of sample with positivity	Virus isolation results
Dead newborn calf	2	2	Heart, lung, liver, spleen	Isolation from all the samples
Calf with hind leg malformation	1	1	Liver, brain	No isolation
Abortion (fetus +vaginal swab)	14	5	Organs of the fetus (heart, lung, liver, spleen) vaginal swab	No isolation
Abortion (fetus)	1	1	Heart, lung, liver, spleen	No isolation
Abortion (cervical-vaginal swab)	12	5	Vaginal swab	Isolation from 2 samples
Rhinotracheitis	35	0	–	–

TABLE 2: Strains of α-herpesviruses identifiable by the developed pyrosequencing assay

Virus strain name	Host species	GenBank glycoprotein E reference strain sequence	Polymorphic site
Bovine herpesvirus 1.1	Bovine	*Bovine herpesvirus 1* strain Lam (1.1) glycoprotein E (US8) gene (EF624467.1)	GAGGCCG
Bovine herpesvirus 1.2	Bovine	*Bovine herpesvirus 1* strain K22 (1.2) glycoprotein E (US8) gene (EF624466.1)	GAGGCCT
Bovine herpesvirus 5	Bovine	*Bovine herpesvirus 5* strain N569 glycoprotein E (US8) gene (EF624468.1)	GCGGCCGCC
Bubaline herpesvirus 1	Bubaline	*Bubaline herpesvirus 1* strain B6 glycoprotein E (US8) gene (EF624469.1)	GAGGCCGCG
Caprine herpesvirus 1	Caprine	*Caprine herpesvirus 1* strain Ba-1 glycoprotein E (US8) gene (EF624470.1)	GAGGCCGCGGG

Sequencing of the polymorphic site allows unambiguous classification of the herpesvirus identified.

RotorGene Q Real Time PCR amplification system (Qiagen) using the PyroMark PCR kit (Qiagen) as indicated by the manufacturer. Samples were pyrosequenced by a PyroMark Q24 instrument (Qiagen) programmed with cyclic dispensation of deoxynucleoside triphosphates (8× (ACGT)). The results were elaborated by Geneious v7.1 software (Biomatters). The pyrosequencing technique allowed us to classify the α-herpesvirus from the specific polymorphic site sequenced (Fig 1 and Table 2). In all analysed samples, we identified the nucleotide polymorphic sequence specific for BoHV-1.1 (GAGGCCG). Pyrosequencing results were confirmed by sequencing as previously reported (Amoroso and others 2013). Sequences showed 100 per cent identity with the BoHV-1 gE (GenBank accession number EF624466.1).

The assay created represents a valid alternative to the conventional Sanger sequencing method because it requires much less time for processing of the samples but offers the same specificity. PyroMark can efficiently detect subtle differences in sequences, allowing identification of very closely related organisms. This technique has already been successfully employed for the detection of herpes simplex virus types 1 and 2 (Adelson and others 2005) and for the determination of *Equine herpesvirus* nucleotide polymorphism (Tewari and others 2013). In all samples analysed by pyrosequencing, we identified and classified the isolated α-herpesvirus as BoHV-1 and not as BuHV-1. Buffalo sensitivity to BoHV-1 has been widely demonstrated by experimental infection and serological surveys (Peshev and Christova 2000, Scicluna and others 2010). However, the virus has never been isolated from naturally infected buffaloes. The present results show that the samples collected from both dead newborn and euthanased buffalo calves were positive for BoHV-1 and negative for all the other pathogens investigated (*Bovine viral diarrhoea virus*, *Bovine coronavirus*, *Schmallemberg virus*, *Bluetongue virus*, *Border disease virus*, *Chlamydophila* species, *Coxiella burnetii*, *Leptospira* species, *Neospora caninum*, *Toxoplasma gondii*, *Ovine herpesvirus-2*). It has been largely reported that neonatal bovine calves infected with BoHV-1, either before or early after birth, usually die within a few days (Muylkens and others 2007). The present findings seem to indicate that BoHV-1 exhibits the same level of pathogenicity, including death, in newborn buffaloes, as both aborted fetal tissue samples, and cervical swabs analysed were positive for the virus. Fetal abortion as a result of BoHV-1 infection has been broadly described for cattle (Thiry and others 2006, Muylkens and others 2007, Nandi and others 2009). The present results suggest that BoHV-1 may be responsible for the abortions observed in buffaloes. As to the calf with hind limb deformity, viruses other than BoHV-1 have been described as possible causes of malformation (Roger 2015), and BoHV-1 has never been associated with cases of limb deformity. Since liver and brain of the euthanased calf tested positive for BoHV-1 in real-time PCR, we could ascribe responsibility for the malformation to the virus, speculating that the infection had occurred during pregnancy at the time of limb genesis, probably engendering a teratogenic effect on the fetus. However, this assumption needs to be investigated further for corroboration. Through experimental infection with BoHV-1 in buffaloes, Scicluna and others (2010) reported no clinical symptoms characteristic of a BoHV-1 infection and therefore concluded the

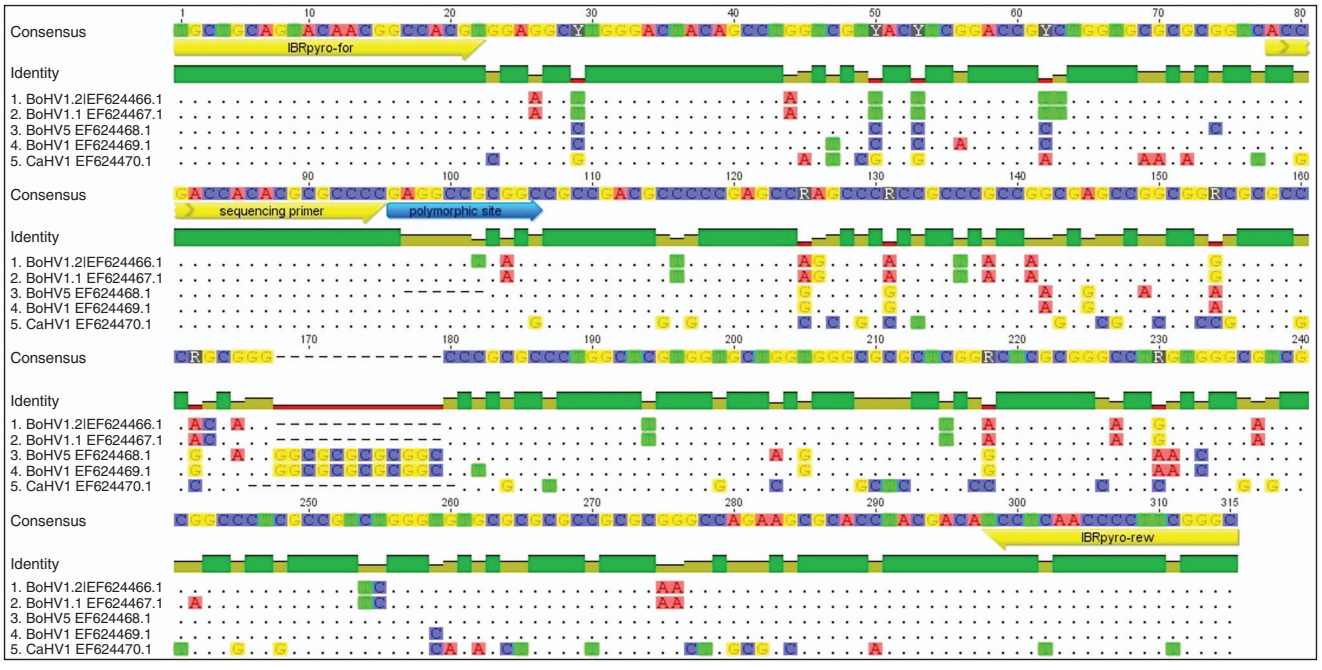

FIG 1: Geneious alignment of the five herpesvirus glycoprotein E sequences used to create the PyroMark assay. Yellow arrows indicate the sequences of the primers used: (1) forward primer, (IBRpyro-for) 5′-TGCTGCAGTACAACGGCCACGT-3′; (2) biotinylated reverse primer, (IBRpyro-rev) 5′-GCCCGAAGGGGTTGAGGA-3′; (3) sequencing primer, 5′-ACCGACCACACGCGCCCC-3′. The blue arrow indicates the polymorphic site where the sequence allows unambiguous identification of the herpesvirus.

infection to be subclinical. The present results are in contrast with those of Scicluna and others. Naturally, there are many factors that can influence the severity of BoHV-1 infection, including virulence of the virus strain, host resistance factors, animal age and potential concurrent bacterial infections (Muylkens and others 2007). The present study suggests that BoHV-1, similarly to other ruminant α-herpesviruses, can adapt to other related species (Thiry and others 2006) and cause the same symptomatology present usually in the species of origin. One of the most important features of BoHV-1 is that it has the capacity to persist in infected animals long term, becoming latent, periodically being re-activated, and readily transmitted between animals (Raaperi and others 2014). BoHV-1-infected buffaloes could therefore become permanent carriers of BoHV-1 and a potential source of infection for the rest of the herd. Considering that in southern Italy it is common to find farms with cattle and buffaloes living together (Scicluna and others 2010), any systematic IBR eradication plan to be applied to these farms should involve not only the cattle but also the water buffaloes. At the same time, in the authors' opinion, any national surveillance/eradication programme implemented for cattle should mandatorily also include buffalo herds.

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