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Short communication

Genetic characterization of ovine herpesvirus 2 strains involved in water buffaloes malignant catarrhal fever outbreaks in Southern Italy

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ABSTRACT

Ovine herpesvirus 2 (OvHV-2) was responsible for two outbreaks of malignant catarrhal fever (MCF) on two water buffalo farms in Southern Italy. In this study, the presence of this virus in the nasal swabs from sick animals as well as in the organs of dead buffaloes was ascertained by a Real-time PCR assay. Positive samples also underwent a relative quantitative analysis of the viral DNA in them. All the dead animals had the highest relative viral quantities, while buffaloes recovering from the virus had intermediate quantities, and asymptomatic OvHV-2-positive sheep had the lowest relative quantities (as compared with the calibrator). The strains involved in the MCF outbreaks underwent genetic characterization by sequencing segments of their ORF50, ORF75 and Ov9.5 genes. The results showed that the outbreaks were caused by two specific genetic variants of OvHV-2, and that these variants exhibit nucleotide differences at the loci analysed. Sheep living in the surrounding farms, as well as sheep kept with buffaloes, were also investigated as possible transmitters of the virus. In this regard, local strategies for the control of MCF should consider separating reservoir species from susceptible animals.

1. Introduction

Malignant catarrhal fever (MCF) is a severe systemic viral infection affecting ruminants like cattle, deer, bison, and water buffalo (Russell et al., 2009). The disease is caused by six viruses belonging to the MCF group within the *Macavirus* genus of the *Gammaherpesvirinae* subfamily. The six viruses are ovine herpesvirus-2 (OvHV-2), alcelaphine herpesvirus-1 (AlHV-1), caprine herpesvirus −2 (CpHV-2), ibex malignant catarrhal fever virus, alcelaphine herpes virus-2- like virus and a virus responsible for MCF in deer (Modesto et al., 2015). These viruses cause subclinical infections in their reservoir hosts, but induce a very severe disease in susceptible species. The typical clinical signs of MCF in water buffalo include depression, anorexia, pyrexia, corneal opacity, inflammation, ulceration, diarrhoea and neurological deficiencies (Stahel et al., 2013). Other than by direct animal-to-animal contact, the virus is transmitted by exposure to aerosols and nasal discharge from goats and sheep, both animals of which are subclinical reservoirs for CpHV-2 and OvHV-2, respectively. In this report we describe two outbreaks of MCF occurring recently on two water buffalo farms located in two regions of Southern Italy, Campania and Calabria. In both farms, the animals showed clinical signs ascribable to MCF and some of them died within a few days after the disease manifested itself. Because the animals showed clinical signs peculiar for MCF, samples from them were analysed by Real-time polymerase chain reaction (PCR) for the detection of ovine hepresvirus-2 and caprine herpesvirus-2, which are the viruses responsible for most of the episodes of MCF in bovine and water buffalo animals (Decaro et al., 2003; Martucciello et al., 2006; Dettwiler et al., 2011). Supplementary investigations were also carried out on both of the farms to identify the potential reservoir(s) of the disease. Therefore, Real-Time PCR analysis was also conducted on nasal discharge samples from sheep living with the buffaloes on the Calabria farm and from sheep and goats living in the areas surrounding the Campania farm. Positive samples from this supplementary investigation underwent DNA sequencing analysis to genetically characterize the OvHV-2 strains. The results showed there was marked genetic variation in the viruses from the two farms, especially with respect to one of the three loci we analysed.

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2. Materials and methods

2.1. Animals

During the course of the study we investigated water buffaloes from two farms located in two different regions (Calabria and Campania) of Southern Italy.

Farm 1. This farm is located in Calabria and houses water buffaloes (around 200) and sheep (around 80), and in some cases these animals share the same paddock.

Farm 2. The herd is located in Campania and houses only water buffaloes (around 650); it is surrounded by three farms (FA, FB, FC) housing sheep and goats (located at distances of less than 5 km from the buffalo farm). Water buffaloes never move from their farm while sheep and goats living on the surrounding farms graze in a range 800 m from the buffalo farm.

2.2. Clinical samples

We sampled and analysed organs (liver, lungs, intestine, brain, heart, kidney and spleen) from dead water buffaloes and nasal discharge from live water buffaloes with clinical signs ascribable to MCF. Swabs from healthy animals (buffaloes, sheep and goats) were also investigated. Thirty healthy buffaloes from each of the two farms underwent nasal discharge analysis. We also sampled and analysed the nasal swabs from 22 healthy sheep sharing the paddock with sick buffaloes on Farm 1. For the Campania outbreak, we collected nasal swabs from sheep and goats living on the surrounding farms (31 samples from FA), (8 samples from FB) and (22 samples from FC). All the samples were analysed for the presence of viruses generally and MCF viruses specifically (caprine herpesvirus-1, CpHV-2 and ovine herpesvirus-2, OvHV-2) in water buffalo and bovine animals.

2.3. Real-time PCR and relative quantitation experiments

DNA was extracted from all the samples using the MagMax automated system (Applied Biosystems). Swabs were incubated at room temperature in 600 μl of phosphate-buffered saline (PBS) for 30 min with shaking. After incubation, nucleic acid extraction was carried out from a 300 μl aliquot of each sample. For the organs, 25 mg of a tissue sample was suspended in 2 ml of PBS and homogenized with the help of glass beads in a Tissue Lyser (Qiagen). Samples were clarified by centrifugation and 300 μl of each supernatant was loaded into a MagMAx extraction sample plate. Nucleic acids were eluted in 90 μl of elution buffer and then stored at −20 °C until use.

Real-time PCR was carried out for the simultaneous detection of OvHV-2 and CpHV-2 in the samples with the primers and probes already described in the literature (Hussy et al., 2001; Cunha et al., 2009). The OvHV-2 primer set was used together with a β-actin set (Wernike et al., 2011) for the relative quantitation experiments with the samples that were OvHV-2- positive. The primer and probe sequences are available upon request. Relative quantitation of OvHV-2 DNA was performed using the 2(-Delta Delta Ct) method ($\Delta\Delta C_t$) described previously (Amoroso et al., 2004; Adur et al., 2003). Specifically, for each sample, the quantity of virus was calculated, normalized against the endogenous reference sequence (β-actin), recorded relative to a calibrator according to the following expression: target relative quantity = $2^{-\Delta\Delta Ct}$. The calibrator was a sample from sheep that had the lowest Ct value during the Real-time PCR screening. All the Real-time PCR experiments were performed using a 7500 Fast Real time PCR system (Applied Biosystems) with the following thermal profile: 95 °C for 15 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

2.4. OvHV-2 gene segment sequencing

DNA from all the OvHV-2-positive samples (18 samples from water buffalo and 10 samples from sheep) was further investigated by end-point PCR, followed by, in case of positivity, DNA sequencing. The following gene segments from the virus were analysed: *ORF50, ORF75* and *Ov9.5* (Russell et al., 2014). Each sample underwent the nested PCR protocol described by Russell et al. (2014). The amplicons were analysed by capillary electrophoresis (Agilent) and then bi-directionally sequenced as described previously (Amoroso et al., 2013). Sequence data generated by the genetic analyser (3130 Genetic Analyzer; Applied Biosystems) were analysed by Sequence Scanner software 2 (Applied Biosystems) and Jalview (java multiple alignment editor) v.2.9. The sequences were aligned using MAFFT, and phylogenetic analysis was conducted using MEGA version 7.0 (Kumar et al., 2016).

2.5. Statistical analysis

Statistical analysis was conducted using IBM® SPSS® Statistics 21 software (IBM Corp., Armonk, NY, USA). Pearson's chi-squared test, with one degree of freedom, was performed to evaluate whether the different relative viral concentrations observed among the dead water buffaloes, recovered water buffaloes, and sheep were statistically significant.

3. Results

3.1. OvHV-2 was identified as the virus responsible for MFC disease outbreak

We registered 12 cases of MFC on Farm 1. The MFC-affected water buffaloes were of different ages and varied from suckling calves to 7 year-old animals. Of these animals, nine died as a consequence of MFC disease, while three recovered from it. All 12 animals had positive results when analysed for the presence of OvHV-2 DNA (and were CpHV-2-negative), but their quantitative PCR assays generated different threshold cycles. In particular, the dead animals showed lower Ct (<27) values compared with the animals that recovered from the disease ($28 < Ct < 33$). All the results are summarized in Table 1. The clinical signs were very severe (i.e., very high fever, corneal opacity and anorexia) in the buffaloes that died from the disease. In contrast, the clinical signs were milder (nasal and ocular discharge, lower high fever, anorexia) for the animals that recovered.

We registered six cases of buffaloes with clinical signs ascribable to MFC on Farm 2. The animals were all around 18 months old and showed the following symptoms: persistent and very high fever, nasal and ocular discharge, keratitis conjunctivitis, corneal opacity (Fig. 1), lack of appetite, rumen block, and

Table 1
Results of the Real time PCR for the detection of OvHV-2.

	Animals positive to OVHV-2 by Real time PCR		
BUFFALOES	With MFC symptoms		Asymptomatic
	Dead	Recovered	
Farm 1	9/12	3/12	0/30
Farm 2	6/6	0/6	0/30
OVINES			
Farm 1	0/22	0/22	4/22
Farm 2	0/61	0/61	6/61

We carried out the analysis respectively on 6 MFC affected buffaloes in Farm 1 and 12 MFC affected animals in Farm 2. Also 30 healthy buffaloes were examined in both the farms. As to the ovines the analysis was carried out on 61 asymptomatic animals living in the surroundings of the Farm 2 and on 22 asymptomatic animals belonging to the Farm 1.