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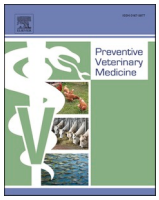

Cattle exposure to bubaline herpesvirus (BuHV-1) in Southern Italy: A hidden threat for IBR eradication?

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ABSTRACT

There is sufficient evidence that both bovine herpesvirus (BoHV-1) and bubaline herpesvirus (BuHV-1) can overcome the species barrier represented by their respective hosts, cattle and buffalo. Although several studies have focused on the impact of BoHV-1 on buffalo, little is known about the impact of BuHV-1 on cattle. In this work, we evaluated the seroprevalence of BuHV-1 in the cattle population in an area where intensive buffalo farming is highly developed (Campania region, Italy). BuHV-1 seroprevalence of cattle sampled in this study was estimated to be 21.4% using a specific commercial ELISA for the detection of antibodies against glycoprotein E of the virus. Risk factor assessment by univariate analysis revealed a correlation between housing type and higher prevalence. Similarly, cattle housed with buffalo and adult animals had a higher likelihood of being seropositive. BoHV-1 vaccination did not prove to be a protective factor against BuHV-1 exposure. The role of age, grazing, and co-living with buffalo in influencing BuHV-1 exposure was also confirmed by multivariate analysis. All BuHV-1 positive animals were also tested with cross-serum neutralization aimed at evaluating the specific antibody titers against BoHV-1 and BuHV-1. We, therefore, assessed the potential cross-reaction between BoHV-1 and BuHV-1, the co-infection rate, and the agreement of the assays used. This study described the presence of BuHV-1 in the cattle population of the Campania region (Italy) and indicated the requirement to take BuHV-1 into consideration for any measures and control and/or eradication plans to be applied against BoHV-1.

1. Introduction

Most herpesviruses in nature have a close relationship with a single host species as a result of millennia of co-evolution with their host, and virtually all animal species are infected by at least one herpesvirus (van der Kolk, 2016). On the other hand, multiple related herpesviruses can infect numerous related host species. Although bovine herpesvirus 1 (BoHV-1) is the best known among ruminant alphaherpesviruses, closely related viruses also include bovine herpesvirus 5, caprine herpesvirus, bubaline herpesvirus (BuHV-1), cervid herpesviruses, and elk herpesvirus (Egyed et al., 2000; Montagnaro et al., 2019; Rola et al., 2017; Thiry et al., 2007). These viruses share genomic and antigenic features with prototype BoHV1, the etiological agent of infectious bovine rhinotracheitis (IBR), a bovine disease subject to eradication and preventive measures in several European countries (Castrucci et al., 2005; Iscaro et al., 2021; Thiry et al., 2007). The circulation of BoHV-1-related alphaherpesviruses among ruminant species raised in IBR-free regions or in regions under an eradication plan may impose unnecessary restrictions (Nogarol et al., 2014; van der Kolk, 2016). To date, there is no evidence in literature whether these viruses should also be included in control and eradication plans. BuHV-1 is a significant issue given the number of water buffalo (*Bubalus bubalis*) in the Campania region (Southern Italy), due to the potential of this virus to infect other ruminants (Carlo et al., 2004). Since a large number of water buffalo are raised in this area (300,000 animals in total in 2023, according to the National Livestock Database - B.D.N.), they can easily come into contact with cattle and, in some cases, even live on the same farm. BuHV-1 is antigenically and genetically related to bovine herpesvirus 1 (BoHV-1) and bovine herpesvirus 5 (BoHV-5) (Hedayat et al., 2020). In fact, it shares a nucleotide identity of 88.9% with BoHV-1% and 95.9% with BoHV-5, respectively (Ros et al., 2002; Nogarol et al., 2014). The virus was first reported in Australia in 1971 and later has been described in

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Italy and Argentina (Maidana et al., 2014; Scheffer et al., 2017). Previously, BuHV-1 was considered to be associated with subclinical disease in water buffalo. Although there is still insufficient evidence on its pathogenic potential, a pathogenic role has recently been assigned after being identified in aborted fetuses and buffalo calves with respiratory issues (Amoroso et al., 2013; Preziuso et al., 2018). Although buffalo are the primary host and reservoir of the virus, BuHV-1 can also infect other ruminants, including cattle (Camero et al., 2019, 2017; Maidana et al., 2016). Because conventional serologic tests do not distinguish between antibodies against BoHV-1 and BuHV-1, BuHV-1 infection in cattle has effects on diagnosis and eradication programs (Nogarol et al., 2014). As a result of the antigenic similarities of BuHV-1 and BoHV-1, cattle exposed BuHV-1 are typically antibody positive and antibody negative, to BoHV-1 glycoprotein B (gB) and glycoprotein E (gE), respectively. The gB cross reactivity is due to a high level of conservation of immunodominant epitopes for this antigen compared to gE epitopes (Keuser et al., 2004; Dubuisson et al., 1992; Nogarol et al., 2014). Unvaccinated cattle exposed to BuHV-1 result in gB positive and gE negative outcomes when tested with BoHV-1 antigen-based assays (similarly to animals vaccinated with the gE-deleted vaccine) (Nogarol et al., 2014). The lack of specific diagnostic tests capable of detecting specific alphaherpesvirus antibodies makes the differentiation between BoHV-1 and BuHV-1 exposure problematic. In addition to cross-serum neutralization, few attempts have been made to use enzyme-linked immunosorbent assays (ELISA) for this purpose (Nogarol et al., 2014; Iscaro et al., 2021; Scheffer et al., 2023a). With this in mind, the aim of the current study was to determine the cattle exposure to BuHV-1 from the Campania region (densely populated with water buffalo) in order to establish its seroprevalence and identify risk factors associated with a higher exposure. Another purpose of this work was to establish the serological pattern of BuHV-1 positive cattle in comparison to tests commonly used for IBR eradication, assessing the concordance with the virus neutralization assays.

2. Materials and methods

2.1. Sampling and study area

The sera used in this work were randomly selected from samples obtained during previous samplings. Samples had been collected between 2021 and 2022 for prevalence studies of other infectious diseases in the Campania region (Ferrara et al., 2022, 2023a, 2023b, 2023c). The Campania region is an area located in southern Italy overlooking the Mediterranean Sea and represents the Italian region with the highest number of water buffalo, mainly raised to produce buffalo mozzarella (a product of protected origin exported all over the world and contributing to the rural economy of the region). The region is also the area with the highest density of buffaloes in Europe. A recent study found that the prevalence of BuHV-1 in water buffalo in Italy was around 40% (Caruso et al., 2016). Due to the lack of similar research in cattle, we assumed an expected prevalence of 0.35 (i.e., 35%), an absolute precision of 0.05 (5%), and a confidence interval of 95%. The required sample size (n) was calculated using Thrusfield's formula, which was as follows:

$$n = 1{\cdot}96^2 \times P(1 - P)/\, d^2$$

A total of 364 cattle from 21 farms belonging to 19 districts were sampled.

2.2. Serological analysis

Each serum was obtained from a coagulated blood sample by centrifugation at 1000 g for 10 min and stored at − 20 °C until testing. Each sample was tested for the presence of antibodies against BuHV-1 gE, BoHV-1 gB, and BoHV-1 gE in order to determine serological status against BuHV-1 and BoHV-1, as well as to differentiate between BoHV-1 infected and vaccinated cattle (Flores et al., 1993). Three commercial assays were used for this purpose, namely Eradikit BuHV1 (IN3 Diagnostic, Italy), IDEXX IBR gB X3 Ab Test (IDEXX, US), and IDEXX IBR gE Ab Test (IDEXX, US). Eradikit BuHV1 (IN3 Diagnostic, Italy) is an indirect ELISA for the detection of antibodies against gE of BuHV-1. IDEXX IBR gB X3 Ab Test (IDEXX, US) is a competitive ELISA for the detection of antibodies against gB of BoHV-1 and IDEXX IBR gE Ab Test (IDEXX, US) is a competitive ELISA for the detection of antibodies against gE of BoHV-1. All samples that tested positive for BuHV-1 gE were evaluated for the presence of neutralizing antibodies against BuHV-1 and BoHV-1 using a cross-virus neutralization test (VNT) protocol described in the literature and capable of distinguishing co-infection and eventual false positive ELISA reactions. Briefly, each sample was incubated for 1 h at 56 °C before being serially diluted twofold (1:2 to 1:128) on 96-well microtiter plates. Each dilution was incubated for 90 min at 37 °C with 100 $TCID_{50}$ of BuHV-1 b6 strain and, separately, BoHV-1 Cooper strain. After this incubation, the serum-virus mixture was placed in corresponding wells of the microtiter plates (where 20,000 MDBK cells were cultured in Dulbecco's Modified Eagle Medium) and incubated for three days to observe the cytophatic effect (CPE) and read the titre (expressed as the maximum serum dilution that neutralized the virus) (Forte et al., 2021; Scheffer et al., 2023b). Serum was considered positive when the inhibition of viral cytopathic effect (CPE) was observed at a dilution 1:2.

3. Statistical analysis

A bivariate study of potential risk variables for BuHV-1 seropositivity at the animal level was performed using the chi-square test. The BuHV-1 ELISA result (positive or negative) was considered the dependent variable, whereas the information obtained for each animal was considered the independent variable. The independent factors investigated were province, age (considering as adults the animals older than 24 months), type of housing, IBR vaccine (some farms applied vaccination with a marker vaccine based on a gE-deleted BoHV-1 strain), contacts with water buffalo, number of water buffalo in municipalities, and BoHV-1 exposure. The relationships of the two variables (dependent and independent) were evaluated with Chi-square. A p-value of less than 0.05 was considered significant, and factors included in the multivariate logistic regression were chosen using a 0.2 criterion. The forward elimination approach was used to conduct a multivariable logistic regression study of putative risk factors for BuHV-1 seropositivity. Odds ratios (OR) were used to assess the degree of connection between independent variables and BuHV-1 seropositivity. The Akaike Information Criterion (AIC) was used to evaluate fit models, and those that best fit the data were chosen. To test for collinearity, the variance inflation factor (VIF) was used. MedCalc Statistical Software version 16.4.3 (MedCalc Software, Ostend, Belgium; www.medcalc.org) and JMP version 14.1.0 (SAS Institute Inc.) were used for statistical analysis.

4. Results

In this study, a total of 364 serum samples from cattle belonging to four different Italian provinces were tested for the detection of BuHV-1 and BoHV-1 antibodies. All samples were collected from dairy cattle in production, most of which were adults (86%). Only a small percentage of samples belonged to grazing animals (22.2%) and cattle living in the same herd with buffalo (17.3%). Approximately half (48.9%) of the cattle had been immunized against BoHV-1, while 18.4% had been exposed to the field strain (resulted positive in gE-ELISA). A total of 102 of the 364 cattle (28%) originated from municipalities with a high concentration of buffalo.

We observed an individual seroprevalence of 21.4%, demonstrating the wide distribution of BuHV-1 in the Campania region. At the herd level, at least one positive animal was found in 12 out of 22 farms (suggesting that BuHV-1 was circulating in more than 50% of farms).

Prevalence was shown to differ by province. In fact, the prevalence observed in the province of Salerno (50%) was statistically higher than that assessed in the province of Benevento (5%). The univariate analysis indicated housing type was a main factor capable of influencing BuHV-1 seroprevalence. In fact, we found a large number of positive cattle when grazing cattle were tested. The same was applicable to age, with adult animals showing a much higher seroprevalence compared to younger cattle (Table 1).

Among factors linked to buffalo breeding, the density of buffalo raised in the municipality and coexistence with buffalo within the same herd have been shown to affect BuHV-1 exposure. Cattle vaccinated against BoHV-1 did not have higher prevalences of BuHV-1, while BoHV-1 gE-positive cattle revealed higher exposure rates. Multivariate risk analysis and calculation of odds confirmed age, co-living with buffalo, and type of housing as risk factors associated with greater prevalence (Table 2). In particular, the factor most implicated in influencing BuHV-1 seroprevalence was co-living with buffalo (OR= 12.77; 95% CI 6.4–25.6). All cattle positive in BuHV-1 gE ELISA (67/364) were subjected to double virus neutralization to exclude any doubt of cross-reactivity (the results were compared with the outcomes obtained with the various ELISAs used). Positive cattle were divided into both BoHV-1/BuHV-1 gE positive (34), and BuHV-1 only animals (43). In the first category, four gE +BuHV-1 cattle showed no neutralizing antibodies to BuHV-1 (considered BuHV-1 gE false positive). In 8 cows we found a BuHV-1 antibody titer higher than that for BoHV-1, while in 5 cows, the titer was equal. Finally, 18 cows had more neutralizing antibodies to BoHV-1 than to BuHV-1 (all were considered potentially co-exposed cattle) (Fig. 1). Thirty of the 43 cattle positive for BuHV-1 only in the gE ELISA (confirmed in the VTN) were also positive for gB, of which 20 were not vaccinated (Fig. 1).

Table 1
Univariate analysis of potential risk factors for BuHV-1 exposure.

Factor	n	Positive	%	95%CI	χ^2	p-value
Total	364	78	21.4	17.2–25.6		
Age						
Adult (>24 months)	313	74	23.6	18.9–28.3		
					6.5	0.011
Young (≤ months)	51	4	7.8	0.5–15.2		
Location						
Avellino	102	17	16.7	9.4 –23.9		
Benevento	80	4	5	0.2–9.8	63.1	<0.001
Caserta	93	12	12.9	6.1–19.7		
Salerno	89	45	50.6	40.2–60.9		
Housing						
Partly grazed	81	26	32.1	21.93–42.3		
					7	0.008
Fully stallfed	283	53	19.4	14.2–23.3		
Coexposure with BoHV-1						
Yes	78	35	44.8	33.8 –55.9		
					26.6	<0.001
No	286	48	16.2	12.45–21.1		
BoHV-1 vaccination						
Yes	178	45	25.3	18.9–31.7		
					3.1	0.08
No	186	33	17.7	12.25–23.2		
Co-living with buffalo						
Yes	63	34	54	41.7–66.3		
					47.9	<0.001
No	301	44	14.6	10.63–18.6		
Buffalo in the municipality						
≤1000	262	44	16.8	12.3–21.32		
					11.9	<0.001
>1000	102	34	34.3	24.2–42.5		

Table 2
Multivariate analysis of potential risk factors for BuHV-1 exposure.

Factor	Coefficient (β)	OR	95% CI	p-value
Co-living with buffalo (Yes)	2.57	12.77	6.4–25.6	<0.001
Housing (Stallfed)	-1.79	0.16	0.1-0.3	<0.001
Age (Young)	-1.39	0.24	0.1-0.7	0.014
Buffalo in the municipality (≤1000)	-1.74	0.17	0.1-1.3	0.095

5. Discussion

The presence of BuHV-1 in the Campania region not only causes economic losses to the farm industry due to its pathogenic potential, but also poses a challenging diagnostic issue that is difficult to resolve using the ELISAs commonly employed for BoHV-1 eradication (Caruso et al., 2016; Lecchi et al., 2023). In this study, we estimated the frequency with which cattle in the Campania region were exposed to BuHV-1, considering several risk variables. The findings revealed a considerable and widespread BuHV-1 exposure in all provinces, which was more frequent than BoHV-1 exposure. There is no data in the literature on the prevalence of BuHV-1 in cattle with which to compare our results. A similar approach, applied to the Italian buffalo population, established a prevalence of more than 50% for BuHV-1, while BoHV-1 infection appeared marginal (just above 1%) (Caruso et al., 2016).

Our results showed how the presence of buffalo in certain areas (such as, for example, in the province of Salerno) greatly influenced exposure to BuHV-1 for cattle. As demonstrated in susceptibility experiments, sentinel cattle exposed to experimentally infected buffalo developed antibodies against BuHV-1 and shed the virus with their own secretions (Maidana et al., 2014). Furthermore, inoculation of buffalo calves with BuHV-1 resulted in intermittent viral excretion until the 18th day post-infection and mild respiratory clinical signs (nasal secretions and rhinitis) (Martucciello et al., 2023). However, in our study, we observed cattle seropositive to BuHV-1 in herds where buffalo were not present, supporting the hypothesis that the dynamics of transmission of this virus in the bovine population, as in other herpesviruses, are complex and not simply limited to coexistence with the main host (however considered a bad practice that promotes pathogen exchange between these two species, as highlighted in previous studies) (Ferrara et al., 2023a). We can, therefore, assume that possible sources of infection are exactly those described for BoHV-1, such as food and water sources, vehicles, personnel and other driving factors (Ostler and Jones, 2023; Hay, . et al., 2016)). In fact, in our study, grazing proved to be one of the most important risk factors for higher exposure rate (together with the age of the animal). Grazing can play an important role in the spread of various infections, because other susceptible ruminants (both domestic and wild) could share the same pasture and contaminate it with infected excreta. For example, goats have been shown to be susceptible to BuHV-1 infection (including latent infection) and to spread the pathogen (Camero et al., 2019, 2017).

On the other hand, among the risk factors evaluated in the buffalo species in previous studies, only age appeared to be a factor significantly correlated to greater exposure possibilities (Caruso et al., 2016). This is also confirmed in our study: cattle older than 12 months showed higher prevalence. In fact, increasing age could increase the chance of exposure to pathogens (more time to come into contact).

The absence of correlation between BuHV-1 positivity and BoHV-1 vaccination that our data suggest is a very interesting finding, even if vaccination with the BoHV-1 marker vaccine results in cross-protection against BuHV-1 in water buffalo (Montagnaro et al., 2014). Previous studies have extensively evaluated the protection provided by heterologous vaccines against various alphaherpesviruses infections in ruminants. In particular, vaccination with BoHV-1 has been shown to be effective and safe, and it also protects buffaloes against BuHV-1 infection

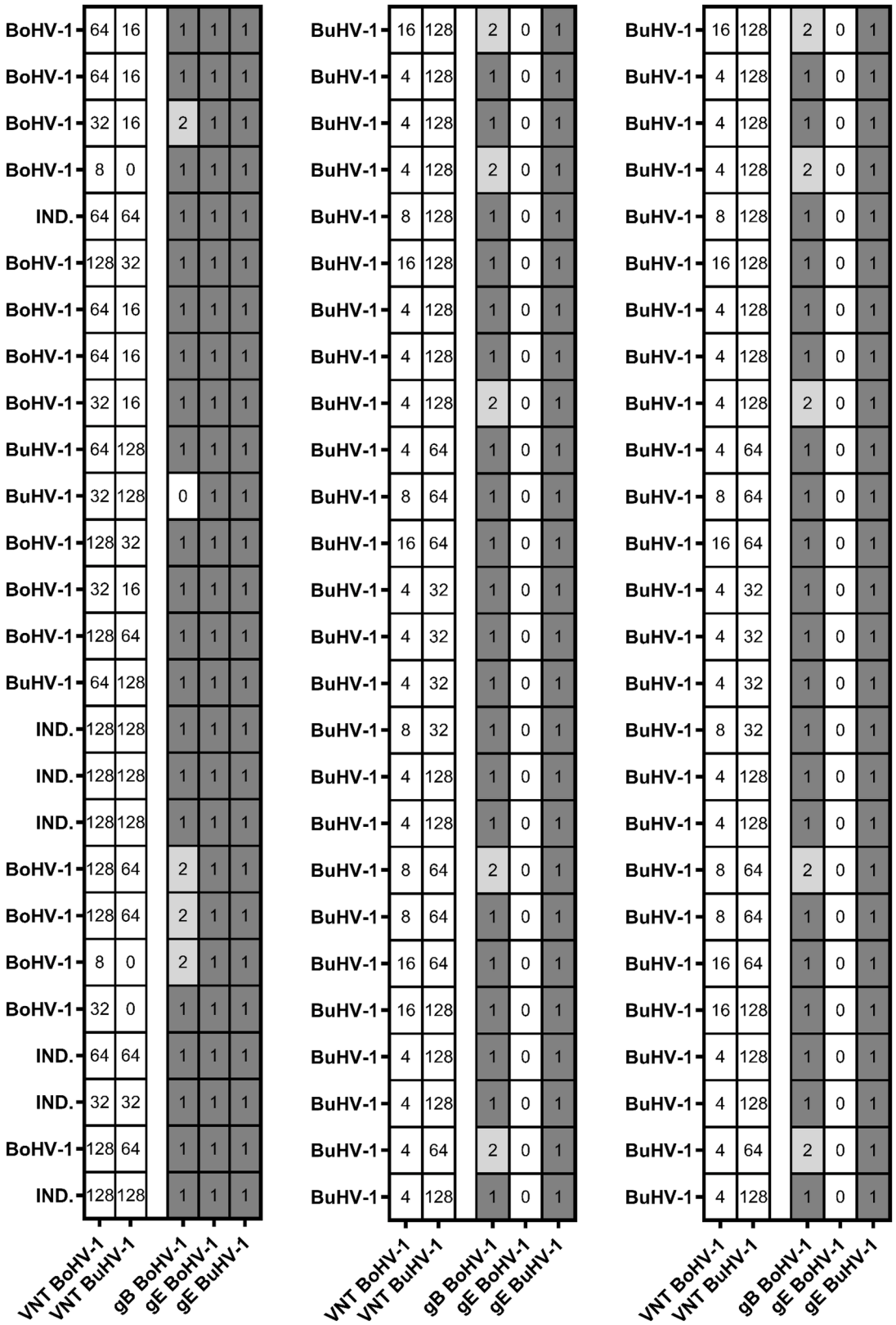

Fig. 1. ELISAs outcome and viral titers obtained from the various serological tests (virus neutralization and results in gB/gE BoHV-1 and gE BuHV-1 ELISAs) in animals tested positive for BuHV-1 gE antibodies. VNT BoHV-1 = viral titer against BoHV-1 (expressed in a range between 0 to 128); VNT BuHV-1 = viral titer against BuHV-1 (expressed in a range between 0 to 128); gB BoHV-1 = outcome in gB BoHV-1 ELISA (0 = negative, 1 = positive, 2 = doubtful); gE BoHV-1 = outcome in gE BoHV-1 ELISA (0 = negative, 1 = positive); gE BuHV-1 = outcome in gE BuHV-1 ELISA (0 = negative, 1 = positive). Serum was considered positive when the inhibition of viral cytopathic effect (CPE) was observed at a dilution 1:2. Row labels represent the outcome of the diagnostic assays for either BoHV-1 or BuHV-1 or indeterminant (IND.).

(although virus shedding is only reduced and not completely limited) (Martucciello et al., 2023; Petrini et al., 2021). The same result was obtained when goats were vaccinated with BoHV-1 and challenged with CpHV-1. However, there are no experimental tests in cattle. Even if a similar outcome is conceivable (i.e., that vaccination for BoHV-1 protects animals from BuHV-1), our results would still be justified since the BuHV-1 infection could have occurred prior to vaccination and, furthermore, there is no information on the cross-protection offered by vaccination over a long period of time (therefore the BuHV-1 infection in cattle vaccinated with BoHV-1 could still occur when the neutralizing antibodies decay). Furthermore, in our work, we have not considered other alphaherpesviruses that could cross-react with BoHV-1 or BuHV-1, which would make this diagnostic "enigma" even more complicated. These difficulties are not limited to BuHV-1 but also to all other related BoHV-1 alphaherpesviruses. A recent study, for example, describes the occurrence of serological cross-reactivity between BoHV-1 and BoHV-2, solvable only using VTN and plaque reduction assay (Petrini et al., 2020).

Our study also observed a high rate of co-exposure (more than half of the BuHV-1-gE+ animals also tested positive for gE BoHV-1). In a previous study, a coinfection rate of 28% was observed (Scheffer et al., 2023b). To remove any doubt of a cross-reaction, we carried out a cross virus neutralization assay, which highlighted 8 animals with antibody titres for BuHV-1 higher than those for BoHV-1, while instead 18 animals had higher neutralizing antibodies for BoHV-1 than for BuHV-1 and 5 animals had equal titers for BoHV-1 and BuHV-1 (all can be considered as co-exposed or vaccinated animals exposed to BuHV-1). Only four animals did not detect antibodies against BuHV-1, considered false positives (out of a total of 78 positive animals, a specificity of about 95% was obtained for the gE BuHV-1 ELISA). Our data demonstrated that currently available ELISA tests are quite sensitive and specific and, at the same time, showed how complicated was to discern the two exposures in the absence of some critical elements. A previous study highlighted the response to competitive ELISAs, also used in our study, in buffalo experimentally infected with BoHV-1 (Scicluna et al., 2010). The seroreactivity against gB appeared earlier (seventh or tenth day) than that of gE (fourteenth day), while the appearance of the neutralizing titer was variable (mainly directed against gD and observed starting from 12 to 40 days after infection). However, there is no information on the decay of seroreactivity following natural infections (and therefore whether the response to gB or gE decays first). This information on the dynamics of seroreactivity to specific proteins, if also applicable to BuHV-1 infections in cattle, would justify the presence of some discordant results (such as the absence of reactivity for gB in some sera presenting antibody titers in VTN) (Dubuisson et al., 1992).

The exchange of herpesviruses that often occurs between different ruminant species and the cross-reactivity involving the ELISAs used during eradication campaigns indicate the need for further development of discriminatory tests (Sarangi et al., 2021). In the past, a panel of monoclonal antibodies against glycoprotein C and D (available for ELISA and immunofluorescence assay) has been proposed to discriminate the most common cross-reactions (Keuser et al., 2004). A recent study evaluated the diagnostic performances of mAg ELISAs able to differentiate antibodies against BoHV-1, BoHV-5, and BuHV-1 (Scheffer et al., 2023a). Although the laboratory results were promising when compared to seroneutralization, application as a routine test is still premature. Since genomic sequence identity also affects the specificity of molecular assays, protocols able to distinguish the nucleic acids of different species of ruminant herpesviruses have recently been validated (Oberto et al., 2023).

Until now, BoHV-1-related alphaherpesvirus infection has not been a concern in the Campania region (in contrast to other Italian regions) because there is no eradication plan and the regional control plans are voluntary. The resolution of these issues is critical for areas where significant numbers of buffalo are bred, such as Campania, especially if BuHV-1 is included in eradication plans or considered for health attestations for movement of animals. Although the influence of BuHV-1 infection on BoHV-1 eradication is difficult to quantify, we carried out a preliminary assessment of the difficulty in differentiation between BuHV-1 and BoHV-1 exposure.

Since the pathogenic potential of BuHV-1 in buffalo is of current interest (BuHV-1 has been associated with abortion and respiratory signs), it would be interesting to understand the abortigenic potential in cattle.

6. Conclusions

In this work, we defined the BuHV-1 exposure of cattle raised in an area highly populated by buffalo, assessed the risk factors associated with a higher risk of infection, and discussed some of the concerns regarding the distinction between BuHV-1 and BoHV-1 exposure. Our findings suggest including the BoHV-1-like alphaherpesviruses in eradication programs, as well as warning against the co-living of cattle and buffaloes due to the risk of infection exchange between the two species.

CRediT authorship contribution statement

Improda Elvira: Methodology, Resources. **Moje Nebyou:** Data curation, Resources. **Iovane Valentina:** Resources, Visualization. **Montagnaro Serena:** Formal analysis, Supervision, Writing – review & editing. **Pagnini Ugo:** Supervision, Validation, Writing – review & editing. **Iovane Giuseppe:** Project administration, Supervision, Validation. **Ferrara Gianmarco:** Conceptualization, Data curation, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

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