Prevalence of antibodies against Bubaline herpesvirus (BuHV-1) among Mediterranean water buffalo (Bubalus bubalis) with implications in buffalo trade

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ABSTRACT
Background: Both Bovine herpesvirus (BoHV-1) and Bubaline herpesvirus (BuHV-1) have been reported to cross the species barrier. Antibody seroconversion in glycoprotein E (gE) blocking ELISA during BuHV-1 infection has been documented. Recent diagnostic efforts have focused on the development and application of discriminatory tests to distinguish between infections with BoHV-1 and BuHV-1.

Objective: To evaluate the impact and distribution of these two infections in water buffalo farms in two regions (Piedmont (\(n=3\)) and Campania (\(n=10\)), Italy) where infectious bovine rhinotracheitis control programs have been implemented.

Animals and methods: Sampling was carried out on 13 buffalo farms comprising 1089 animals using specific gE-indirect ELISA’s test able to discriminate among BoHV-1 and BuHV-1 infections.

Results: 59.0% of animals reacted positive to ELISA (irrespective of whether BoHV-1 or BuHV-1 antigen was used) and 86.4% of these were reactive to BuHV-1 only, whereas 11.8% showed absorbance values for both antigens and were classified as inconclusive. There was a statistically significant age-related difference in BuHV-1 infection rates but not in overall individual (47% vs. 58%) or herd prevalence (100% vs. 90%) of infection between the two regions.

Conclusion: The low percentage of sera reactive to BoHV-1 (1.8%, 12/643) indicates that BuHV-1 may be the main circulating alphaherpesvirus infection in Mediterranean water buffalo in the two study areas. Since Bubalus bubalis is included in Directive 64/432/EEC on animal health problems affecting intra-community trade in bovine animals, diagnostic testing with nonspecific ELISA for BoHV-1 infection in buffalo may yield false-positive reactions. This scenario could lead to economic losses and hamper buffalo trade and movement, particularly for reproduction purposes.

1. Introduction

Bovine herpesvirus 1 (BoHV-1), Bovine herpesvirus 5 (BoHV-5), and Bubaline herpesvirus 1 (BuHV-1) are classified in the same genus Varicellovirus and belong to the family Alphaherpesvirinae. BoHV-1 is the etiological agent of infectious bovine rhinotracheitis (IBR). Recognized as one of the major cattle diseases of economic importance, IBR control and eradication programs have been implemented in more and more European countries.

BoHV-5 infection induces either a subclinical infection or disease of moderate severity in adult cattle (Del Médico Zajac et al. 2006) and lethal encephalitis in young animals (Meyer et al. 2001). BuHV-1 has been associated with subclinical disease in water buffalo (Bubalus bubalis). Although its pathogenic potential is still unclear, it has recently been detected in an aborted water buffalo fetus (Petrini et al. 2012; Amoroso et al. 2013).

BoHV-1 and related ruminant alphaherpesviruses have been reported to cross the species barrier, making both cattle and water buffalo susceptible to heterologous infection. BoHV-5 infection has not been reported in Italy to date, whereas BoHV-1 has been recently isolated and characterized from an aborted buffalo fetus (Fusco et al. 2015), raising questions about the role of this animal in the epidemiology of BoHV-1.

Within the current scenario of livestock farming in Italy, buffalo and cattle are often raised together in mixed-farm operations, which creates a risk factor for cross infections. There is no compulsory national plan for IBR surveillance and control in Italy. Many regions
have established their own programs in which only cattle are considered as a reservoir of BoHV-1, despite documentation for specific antibody seroconversion against glycoprotein B (gB) and glycoprotein E (gE) in water buffalo (Peshev & Christova 2000; De Carlo et al. 2004; Scicluna et al. 2010).

As reported by Nogarol et al. (2014), cross reaction between BuHV-1 and BoHV-1 on serological tests can occur, including gold standard serum neutralization (SN). Because the SN titers are very similar, this test failed to discriminate between the two infections and produced results somewhat discrepant with the true infectious status. Moreover, gB/gE blocking competitive ELISA to define the infectious status of water buffalo has yielded conflicting results in a few cases (Scicluna et al. 2006) and leads to ‘vaccination-like’ behavior (Bertolotti et al. 2015).

Under Council Directive 64/432/EEC (Directive on animal health problems affecting intra-community trade in bovine animals and swine), *Bison bison* and *B. bubalis* are considered bovines. This has implications for the restriction of gB+/gE+ animals in IBR control plans; BuHV-1 infection can lead to misdiagnosis of IBR, resulting in unjustified restrictions on buffalo trade. This has been demonstrated in France, where the only virus that could be responsible for IBR misdiagnosis is CpHV-1, since goats and cattle can be in close contact in some field situations (Thiry et al. 2008).

Recent diagnostic efforts have focused on the development and application of discriminatory tests for alphaherpesvirus infection. A new indirect ELISA based on BuHV-1 and BoHV-1 gE antigen developed by our research group has shown that specific epitopes in the BoHV-1 and BuHV-1 gE ectodomain can be used to detect differential reactivity based on the specific infectious status.

Here we report the results of field application of this new discriminatory ELISA test. Since water buffaloes are susceptible to both BoHV-1 and BuHV-1 infection, our aim was to evaluate the impact and distribution of these two infections in buffalo farms in two regions where IBR control programs have been implemented. We also conducted a preliminary analysis of animal age and herd size as risk factors for infection.

2. Materials and methods

2.1. Animal sampling

Between September 2013 and August 2015, a total of 1089 serum samples were collected from buffaloes from farms in Piedmont (*n* = 3) and Campania (*n* = 10). Information at the herd level (breeding, management type, herd size, etc.) was obtained from the National Animal Registry Office during routine annual census. The number of farms to be sampled was set to detect 50% interherd prevalence with 10% precision and 95% confidence interval (95% CI). The farm sampling frame was derived from the National Database for the Identification and Registration of Livestock (http://statistiche.izs.it/portal/page?_pageid=73,129188&dad=portal). Table 1 presents the age distribution of the animals.

In Piedmont (Northern Italy), 254 serum samples from animals from three different farms were tested. Two farms were classified as medium (100–499 animals), and one as large (≥500 animals). All farms produce both meat and milk and are mixed-farm operations where cattle and buffaloes were reared together. Twenty-four of the 254 serum samples were from animals <7 months, 30 from animals 7–12 months, and 200 from animals >12 months of age. All farms were classified as IBR positive according to the regional control plan. Indeed, during the annual official veterinary control, the cattle on all three farms tested negative on gE blocking ELISA (IDEXX gE Ab test kit, IDEXX, Westbrook, MA, USA). No vaccination against IBR was applied.

In Campania (Southern Italy), 835 serum samples were collected from 10 farms. Two were classified as large, seven as medium, and one as small (≤50 animals). All farms produce buffalo milk: nine are single-species farms (buffalo) and one is a mixed-operations farm where cattle are also bred. Forty-four of these serum samples were from animals <7 months, 93 from animals 7–12 months, and 698 from animals >12 months of age. All farms were classified as IBR positive according to the regional control plan. No vaccination against IBR was applied.

2.2. Serological analysis

An indirect ELISA test was employed. Briefly, microplates were coated with BoHV-1 gE recombinant protein (even number wells) and an equal amount with BuHV-1 gE recombinant protein (odd number wells) (Nogarol et al. 2014). Both antigens were previously expressed in HEK293T cells. Serum samples were diluted 1:20, then added to the plates and incubated for 1 hr at room temperature. After washing, ruminant peroxidase-labeled Ab anti-IgG was added and the plates incubated as described above. Reaction was developed with tetramethylbenzidine substrate and stopped with H2SO4. Reactivity of each serum sample against both wells was recorded: the antigen for which the serum showed increased reactivity (>40% of the same samples in the other well) indicated the

| Age          | Number | %  |
|--------------|--------|----|
| <7 months    | 68     | 6.2|
| 7–12 months  | 123    | 11.3|
| >12 months   | 898    | 82.5|
| Total        | 1089   | 100.0|

Table 1. Age distribution (in months) of buffalo (*Bubalus bubalis*) tested for BoHV-1 and BuHV-1 infection.
circulating infection (Figure 1). A result showing high absorbance values for both wells was classified as doubtful (inconclusive). Test performance is reported according to a previous report (Nogarol et al. 2014).

2.3. Statistical analysis

For the epidemiological study, the percentage (with 95% CI) of the ELISA results (BoHV-1, BuHV-1, and inconclusive) was estimated at both the herd and the individual levels (Rogan & Gladen 1978; Reiczigel et al. 2010). Multinomial logistic regression was fit to investigate the effect of potential risk factors for reactivity to BuHV-1 gE antigen: herd size (<50, 50–100, 100–500, >500 animals) and age (<7, 7–12, and ≥12 months).

3. Results

Serum samples from 12 of the 13 farms tested positive on ELISA (irrespective of whether BoHV-1 or BuHV-1 antigen). A total of 643 of 1089 (59.04%) serum samples were positive on ELISA, 555 (86.4%) of which were reactive to BuHV-1 and 76 (11.8%) showed high absorbance values for both wells and were classified as inconclusive; 12 (1.8%) were reactive to BoHV-1 antigen only (Table 2). Thirty-one of the 555 sera reactive to BuHV-1 were from animals <7 months, 13 from animals 7–12 months, and 511 from animals >12 months of age. All sera reactive for BoHV-1 were from animals older than 12 months.

Table 3 presents the results of serological testing. Briefly, at the individual level in Piedmont, 117/249 (47.0%, 95% CI 40.9–53.2) sera were reactive for BuHV-1 antigen and 1/133 (0.75%, 95% CI 0.13–4.1) for BoHV-1. In Campania, 438/752 (58.2%, 95% CI 54.7–61.7) sera were reactive for BuHV-1 antigen and 11/325 (3.4%, 95% CI 1.9–6.0) for BoHV-1. At the herd level in Piedmont, the BuHV-1 prevalence was 100% (3/3 farms, 95% CI 43.9–100) and 33.3% for BoHV-1 (1/3 farms, 95% CI 6.1–79.2). In Campania, the herd prevalence of BuHV-1 infection was 90.0% (9/10 farms, 95% CI 59.6–98.2) and the prevalence of BoHV-1 was 60.0% (6/10 farms, 95% CI 31.3–83.2). Overall, the percentage of BuHV-1 positive sera by age group was 45% in animals <7 months, 10.5% in animals 7–12 months, and 56.9% in animals >12 months. The logistic regression model showed a statistically significant age-related difference in infection rates but no association with herd size (Table 4).

4. Discussion

In recent decades, Mediterranean water buffalo farming has expanded widely throughout the Mediterranean area. After the introduction of EU milk quotas (EU Regulations 856/84 and related documents, definitely repealed after 1 April 2015), buffalo breeding spread to other EU countries (Romania, Bulgaria, Greece, Germany, the Netherlands, and Hungary) and Switzerland. As underlined by Maidana et al. (2014), these regulations brought about important changes in the

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**Figure 1.** Indirect ELISA based on BoHV-1 and BuHV-1 gE antigen. BoHV-1 gE recombinant protein (even number wells) and an equal amount of BuHV-1 gE recombinant protein (odd number wells) are coated to the plate.

**Table 2.** Overall number of buffalo (Bubalus bubalis) reactive to the ELISA test. Positive sera were also classified for reactivity against BoHV-1 antigen/BuHV-1 gE antigen/both wells.

| Results          | Number | %   |
|------------------|--------|-----|
| Negative         | 446    | 41.0|
| Positive         | 643    | 59.0|
| Total            | 1089   | 100.0|

**Reactivity of positive sera**

|               |       |
|----------------|-------|
| BoHV-1 antigen| 12    |
| BuHV-1 antigen| 555   |
| Inconclusive  | 76    |
| Total         | 643   |

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**Table 3.** Overall number of buffaloes (Bubalus bubalis) tested positive for BoHV-1 and BuHV-1 infection distributed by region.

| Infection | Piemonte | Campania | Total |
|-----------|----------|----------|-------|
| BoHV-1    | 1        | 11       | 12    |
| BuHV-1    | 117      | 438      | 555   |
| Inconclusive| 4      | 72       | 76    |
| Negative  | 132      | 314      | 446   |
| Total     | 254      | 635      | 1089  |

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**Table 4.** Analysis of risk factors considered in the study. Age (months) and herd size of buffaloes (Bubalus bubalis).

| Result          | Odds Ratio | 95% Conf. Interval |
|-----------------|------------|-------------------|
| Age in months   |            |                   |
| <7 months       | Used as reference for calculation of odds ratio |
| 7–12 months     | 0.054      | 0.023             | 0.126 |
| >12 months      | 1.114      | 0.596             | 2.080 |
| Herds size      |            |                   |
| <100 herds      | 0.136      | 0.004             | 4.911 |
| 100–499 herds   | 0.202      | 0.012             | 3.329 |
| >500 herds      | Used as reference for calculation of odds ratio |

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distribution of reared animals, with intensification of buffalo farming, a shift to mixed (buffalo and cattle) production systems, and changes in circulating pathogens and increased risk of cross infections.

Here we report the results of the first field application of ELISA based on specific BuHV-1 and BoHV-1 gE antigen that can discriminate between the two infections in Mediterranean water buffalo. Our serological results indicate that BuHV-1 infection is widespread on water buffalo farms in both Northern and Southern Italy: 86.4% of sera tested positive to BuHV-1 infection. There was no difference in overall individual seroprevalence (47.0% vs. 58.2%) and herd prevalence (100% vs. 90%) between the two study areas. BuHV-1 was found to be endemic in all sampled farms, which could be explained by the lack of diagnostic tests specific for BuHV-1 infection and by the possibility that infected animals are introduced into farms. Indeed, when a panel of 150 BuHV-1 positive sera was tested with a commercial gE blocking ELISA (bovine rhinotracheitis virus gE Ab Test, IDEXX), used as a routine test for IBR diagnosis, 108 (72%) resulted negative or doubtful (data not shown).

Only one farm (in Campania) was found to be completely free of both herpesvirus infections. Though they make up the same epidemiological unit, the adult buffaloes (>12 months) are kept in an area about 10 km completely separated from other animals. Our hypothesis is that, as for IBR, segregation of animals by age and management, in which contact between adult and young animals is reduced, prevents the spread of BuHV-1 infection on the farm.

Analysis of BuHV-1 prevalence by age group showed that the infection rate was 45% (31/68) in animals <7 months, 10.5% (13/123) in animals 7–12 months, and 57% (511/898) in animals >12 months of age (Table 4). A multilevel logistic regression model to investigate the effect of age as a potential risk factor showed that the buffaloes in the lowest age group (<7 months) are more at risk of being positive to BuHV-1 than those 7–12 months old (odds ratio [OR] 0.053) but no statistically significant difference in risk for infection between the oldest and the youngest age groups (OR 1.11) was detected. The high BuHV-1 prevalence among the youngest animals (<7 months) is likely due to colostrum-derived passive antibodies (Abs). As the Abs titer decreases with age (lower prevalence in the middle age group), the possibility of infection and seroconversion in animals older than 12 months increases. In all, 12 of 643 sera were reactive against BoHV-1 antigen. Importantly, these serum samples were all from adult animals and nearly all (11/12) from the Campania herd. Furthermore, many of these samples (4/12) were from the only mixed-operations farm in Campania where cattle are also reared. This herd also had the highest number of doubtful (inconclusive) samples (14/72, prevalence of 19%) as compared with an average of less than 10% in the other herds. This could represent an important aspect in the epidemiology of BoHV-1 in water buffalo, since the introduction of cattle potentially infected with IBR could be an associated risk factor for the co-circulation of both viral strains, which may be the reason for the indeterminate serological test results.

In this respect, a limitation of our study is that, because serum samples from water buffalo experimentally co–infected with BoHV-1 and BuHV-1 were not available, we are unable to explain the high reactivity of 76 sera against both recombinant proteins (gE BuHV-1/gE BoHV-1). An attempt to isolate and characterize the virus in these 76 reactive but inconclusive animals was also conducted without dexamethasone treatment, as described in Maida’s study (2014): DNA from vaginal and nasal swabs was extracted and real-time PCR was carried out (OIE, Chapter 2.4.13) to detect both BoHV-1 and BuHV-1 glycoprotein B. Unfortunately, no samples tested positive.

In conclusion, despite this limitation, our results indicate that BuHV-1 circulates in Mediterranean water buffalo in the two study areas and that, at the individual level, the percentage of cases of BoHV-1 infection is low (1.8% equivalent to 12/643 sera). Furthermore, our findings demonstrate that nonspecific ELISA for diagnostic testing of BoHV-1 infection in buffalo sera could produce false-positive results. In such instances, misdiagnosis can lead to economic loss from restriction of buffalo trade and movement, particularly for reproduction purposes.

A future area of focus will be the wider application of an indirect ELISA test, specific for BuHV-1 infection, for the development of a homologous vaccine and BuHV-1 DIVA (differentiating infected from vaccinated animals) vaccination strategies based on the deletion of gE as for IBR.

Disclosure statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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