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Bovine leukemia virus: Experimental infection in buffaloes and evaluation of diagnostic test reliability

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1. Introduction

Enzootic bovine leukosis (EBL) is an infectious disease that primarily affects cattle (Burny et al., 1985). EBL is widespread in all continents, but although eradication plans were successfully applied in most Western European countries and Oceania, high prevalence rates are still documented in North and South America as well as in Asia (Rodríguez et al., 2011). The causative agent has been identified as bovine leukemia virus (BLV), an RNA virus that can cause fatal neoplastic lymphosarcoma after a long incubation period. BLV belongs to the genus Deltaretrovirus, which is included in the family Retroviridae. It is structurally and functionally related to other retroviruses, some of which are associated with species that infect humans (primate T-lymphotropic viruses one, 2, and 3) (Romero et al., 1981).

Although cattle are the most common hosts of BLV, clinical symptoms are not frequently observed. However, 30–70% of infected animals develop persistent lymphocytosis, which can be associated with subsequent development of lymphosarcomas (Burny et al., 1985). Lymphosarcoma lesions may develop in different organs; they are closely associated with age and can be observed after a variable latency period in animals older than 4–5 years (Romero et al., 1981). Although other species of buffaloes are susceptible to experimental infection by BLV (Enzootic bovine leukosis. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animal, 2012, chap. 2.4.11), natural infection by the BLV has only been reported in water buffalo (*Bubalus bubalis*) (Molnar et al., 2000; Romero et al., 1981).

Despite the moderate rates of EBL incidence, prevalence, and mortality, it is considered relevant from a social and economic point of view because it imposes movement restrictions on infected animals, reduces their productivity, and causes economic depreciation of other animals reared in the infected farm. Therefore, EBL is included in the list of diseases notifiable to the World Organization for Animal Health (OIE). Furthermore, the European Union also endorses national eradication programs for EBL (Scientific Opinion on Enzootic Bovine Leukosis. EFSA Panel on Animal Health and Welfare-AHAW, 2015).

Italy has been undergoing such an eradication program since 1996.This program is governed by two specific laws of the European Legislation (DM n. 358/1996 and Legislative Decree 196/1999), includes all bovine and buffalo herds, and is based on the (serological) test and removal strategy. The laboratory tests applied are agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA). Animals positive for these tests should be considered infected, and therefore should be slaughtered (Maresca et al., 2015). Despite rigorous and systematic serological testing, no positive results have been recorded for buffalos in Italy since 1996 (data not show). Therefore, this population should be considered free from BLV infection.

In contrast, the persistence of EBL in several central as well as southern regions (Lazio, Campania, Sicilia, and Puglia) has compelled continued serological surveillance of buffalo herds in these regions. The serological tests are performed by local laboratories affiliated with the Istituti Zooprofilattici Sperimentali, in accordance with the procedures of a certified quality system (ISO 17025). The National Reference Laboratory for the Study of Ruminant Retroviral Infectious Diseases (CEREL) is located in the Istituto Zooprofilattico Sperimentale Umbria e Marche, Perugia, and is responsible for standardization of these methods; it also provides reference reagents and systematically organizes a National Laboratory Comparison to check and verify diagnostic systems. It is noteworthy that this EBL diagnostic structure is 'calibrated' for cows and therefore lacks specific references for buffalo species. For example, the E5-positive reference serum has a bovine origin, whereas no standards are available for buffalo samples. Moreover, available commercial tests have been validated using either sera or milk samples collected from bovines. Therefore, these commercial tests are only adapted to check buffalo specimens.

The existing literature in this field of research is scant and rather outdated. Therefore, in this study, we performed a BLV experimental infection to develop knowledge on the infection dynamics of BLV, and to assess the effectiveness of available diagnostic methods for EBL. Another important goal of this study was to obtain a stock of reference samples to be used for primary and secondary validation.

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Table 1
Details of the animals included in the study.

Animal	Sex	Age	Enrollment	Inoculum	Remarks
Buffalo 1	Male	7 years	20 dbi	T0	–
Buffalo 2	Male	2.5 years	20 dbi	T0	Used as contact control
Buffalo 3	Female	7 years	20 dbi	T0	Last third of pregnancy
Buffalo 4	Female	7 years	20 dbi	T0	Last third of pregnancy
Buffalo 5	Female	11 years	20 dbi	T0	Second third of pregnancy
Buffalo 6	Female	11 years	20 dbi	T0	Second third of pregnancy
Buffalo 7	Female	6 years	20 dbi	T0	Second third of pregnancy
Buffalo 8	Female	9 years	20 dbi	T0	First third of pregnancy
Buffalo 9	Female	5 years	20 dbi	T0	First third of pregnancy
Buffalo 10	Female	5 years	20 dbi	T0	First third of pregnancy
Buffalo 11	Female	6 months	145 dpi	150 dpi	Used to verify the experimental infections
Buffalo 12	Male	5 months	145 dpi	150 dpi	
Buffalo 13	Female	4 months	145 dpi	150 dpi	
Ewe 14	Female	1 year	20 dbi	T0	
Ewe 15	Female	1 year	20 dbi	T0	
Ewe 16	Female	1 year	145 dpi	150 dpi	
Ewe 17	Female	1 year	205 dpi	210 dpi	
Ewe 18	Female	1 year	205 dpi	210 dpi	
Ewe 19	Female	1 year	295 dpi	300 dpi	
Ewe 20	Female	1 year	295 dpi	300 dpi	
Bovine 21	Male	6 months	20 dbi	Not inoculated	Used as contact control

T0 = day of inoculation.
dpi = day post infection.
dbi = day before infection.

2. Material and methods

2.1. Animal enrollment and accommodation

A panel of animals (including buffaloes, cows, and sheep; $n = 21$) was enrolled for the experiment (Table 1).A young buffalo was employed as a “control” and held in contact with inoculated animals; eight female buffaloes were enrolled and inoculated to verify the eventual prenatal transmission of infection at different pregnancy steps. Although the animals were obtained from farms located in the Campania Region, in the province of Salerno, and registered as officially free from EBL, they were again verified as free from BLV infection through the combined use of AGID and ELISA. The trial was conducted on approval of the Ministry of Health of two current research projects (RC IZSME 09/2011and RC IZSUM 06/2011) and authorized by art. 7 of D.L. 116/92, dated 03.21.2014. AGID was conducted at the CEREL according to the protocol described in the OIE Manual (Enzootic bovine leukosis. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animal, 2012, chap. 2.4.11), whereas ELISA was performed at the laboratory of Salerno (IstitutoZooprofilatticoSperimentale del Mezzogiorno), which is located at the CReNBuf (National Reference Center on Water Buffalo Farming and Production Hygiene and Technologies). ELISA was performed using a commercially available kit routinely used in laboratories, in accordance with the manufacturer's instructions.

All of the animals were housed in the experimental structure of CReNBuf as authorized by the Italian Ministry of Health with the Decree N° 246/2013-A. The animals were maintained in a paddock with 15 m^2 for each animal (Fig. 1). A total mixed ration was formulated according to the physiologic phase and was fed daily in a group pen situation. The buffaloes and cows in contact were allowed to roam in a wide outdoor paddock and in large indoor premises, whereas the ewes were housed separately in a dedicated pen. Facilities for containment were adequate to ensure safety of the animals, and specialized operators were present during sampling. The structure also included a modern milking room in addition to other quarters used by the operators.

The experimental infection was performed after 20 days of animal acclimatization. Buffaloes 1–10 (excluding number 2) were inoculated with 10 mL of whole blood (in ethylenediaminetetraacetic acid; EDTA) derived from BLV-infected cows at the CEREL. Buffalo 2 and Cow 21 were not inoculated and were thus used as a negative controls (Table 1); they were kept in close contact with the infected buffaloes to monitor the eventual natural transmission of the virus. Ewes14 and 15 were each inoculated with 10 mL of whole blood (EDTA) derived from BLV-infected cows at the CEREL. Starting on the day of inoculation (T0), serum and whole blood samples (10 mL each)were collected twice a week.

Placenta, colostrum, milk, blood, and serum samples were collected from Buffaloes 3–10 at the birth of their calves. Thereafter, milk samples were regularly collected twice a week. In cases of stillbirth, the fetuses were subjected to pathological examination and sampling of blood and organs.

At 150 dpi, Buffaloes 11–13 and Ewe 16 were inoculated with 10 mL of whole blood (EDTA) derived from a pool collected from Buffaloes one, 9, and 10 (these animals were already identified as seropositive by ELISA). Moreover, at 210 dpi, Ewes 17–18 were inoculated with 10 mL of whole blood (EDTA) derived from a pool collected from buffaloes 3, 4, 5, 6, 7, and 8 (these animals were still identified as seronegative by ELISA). Finally, at 300 dpi, Ewes 19–20 were inoculated with 10 mL of whole blood (EDTA) derived from a pool collected from buffaloes 5 and 8 (these animals were recently identified as seropositive by ELISA).

At the end of the experiment, animals were slaughtered, and samples such as the lymphoid organs and blood were collected. All of the samples were properly stored. The storage procedures were performed with utmost care to create a reference bank for EBL samples from buffaloes.

The trial was stopped after 13 months, i.e. clinical observations and samples collection were discontinued.

2.2. Laboratory tests

2.2.1. Serological tests

The serum samples were subjected to serological tests to detect the presence of specific antibodies induced by BLV (Table 2).

Three different commercial ELISA kits were used (kits 1–3) in accordance with the manufacturer's instructions. These were competitive ELISA tests, in accordance with the guidelines described in the OIE Manual (Enzootic bovine leukosis. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animal, 2012, chap. 2.4.11).The CEREL provided standardization of these kits using a dedicated panel of sera including the E5 international reference serum(as recommended by the national and European Union legislation). Kits 1 and 3 were two-fold-tested (two different batches for each kit). Kit 2 was tested once; therefore, the results of five analytical sessions were recorded. Additionally, the serum samples were also examined by AGID, which was performed in accordance with the procedures described in the OIE Manual (Enzootic bovine leukosis. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animal, 2012, chap. 2.4.11).

The milk samples were also subjected to ELISA; four commercial kits were used (kits A–D). As mentioned above, the ELISA kits were in accordance with the procedures described in the OIE Manual (Enzootic bovine leukosis. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animal, 2012, chap. 2.4.11) and were standardized by the CEREL. Kits A and B, respectively, were used for “screening” and “verification”, and were developed by the same manufacturer. Because little knowledge is available on milk testing for EBL diagnosis in