DETECTION OF BOVINE FOAMY VIRUS IN MILK AND SALIVA OF BFV SEROPOSITIVE CATTLE

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Abstract

The aim of this study was to demonstrate the occurrence of bovine foamy virus (BFV) and BFV DNA in peripheral blood leukocytes (PBLs), milk cells, and saliva of cattle serologically positive to BFV. The virus was detected by co-cultivation technique with canine thymus cells (Ci2Th) and viral DNA was quantified by the real-time PCR. Out of co-cultures from 23 cattle, BFV was found in 19, 13, and 15 samples of PBLs, milk cells and saliva, respectively, while BFV-DNA was confirmed in DNA extracted from 14 PBLs and 8 milk cells samples. All 23 saliva samples were negative. The number of BFV DNA copies in milk cells was in average over 2.5 times lower than in PBLs. The presence of BFV and viral DNA in milk and saliva samples is discussed in the context of virus spread and possible human exposure to BFV through food products of cattle origin.

Key words: cows, bovine foamy virus, blood cells, milk, saliva, virus detection.

Foamy viruses (FVs) known also as spumaviruses are members of Spumaretrovirinae subfamily of Retroviridae family and constitute a distinct group of retroviruses. Natural infection with FVs is characterised by persistent humoral response to structural and non-structural proteins and continuous presence of viral DNA. Having a wide tissue tropism, these viruses can be easy propagated in vitro in a variety of cell types, causing typical cytopathic effect, but they appear to be entirely non-pathogenic under in vivo conditions. Nevertheless, data from rabbits experimentally infected with FVs and transgenic mice expressing human foamy virus proteins suggest that pathogenic potential of FVs cannot be excluded (14). It is supposed that FVs may be cofactors for other viral diseases, as was shown in vitro for primate foamy virus type 1, which enhances cell-to-cell transmission of HIV-1 (19). FVs can also cross species barrier and it is now generally accepted that all foamy virus infections of men were derived from non-human primates (4). For this reason, the zoonotic potential of FVs is extensively examined, especially in relation to foamy viruses isolated from farm animals.

Bovine foamy virus was first isolated from lymphocytes, lymph nodes, and milk sediment of cattle with lymphosarcoma (11). The molecular structure of BFV shows that its genome, of 12 kb length, harbours the same features as other known foamy viruses (17). Available data demonstrate that from 30% to 45% of cattle are seropositive for BFV and infection with BFV are worldwide distributed (8, 6, 16). Recently, BFV has been isolated from cattle from Poland and partially characterised (13). Like other spumaviruses, BFV can be transmitted by several routes and there are only few reports showing that milk and saliva of infected cattle contain active virus or cell-associated virus.

The present study was undertaken to estimate load of viral DNA in milk cells and saliva to detect BFV in naturally infected seropositive cattle. In addition, the presence of BFV and BFV DNA in peripheral blood leukocytes was examined. For virus isolation, traditional co-cultivation technique was used while DNA viral load was estimated by quantitative real-time PCR.

Material and Methods

Sample collection. Blood, milk, and saliva samples were taken from 23 cows selected as seropositive for BFV by GST-ELISA (11). Blood samples were collected from the jugular vein into tubes containing EDTA. Blood was haemolysed, centrifuged, and peripheral blood leukocytes (PBLs) were collected. Eighty millilitres of milk was centrifuged at 1,500 x g for 30 min and pelleted cells were washed and resuspended in 2 ml of PBS. Saliva was collected from the mouth using cotton swabs, which were then squeezed in 1 ml of PBS into a tube with the help of plastic rod.

Detection of BFV DNA by the real-time PCR. The QuantiTect SYBR Green Kit (Qiagen, Germany) was used as recommended, with addition of 0.2 μM of each primer: Int3 5’- TCCCGCC
cells (Cf2Th) in 25 cm² flasks (NUNC) in DMEM, were co-cultured with BFV-permissive canine thymus cultures to 10⁸ copies) was used as template for reaction, observed under light microscope. After five passages, the cultures were passaged at 1/3 ratios until cytopathic effect (CPE) appeared. Antibiotic-antimycotic solution (Sigma, Germany) was added and all the samples were tested in duplicates. DNA was extracted using DNeasy Tissue Extraction Kit (Qiagen, Germany), measured spectrophotometrically using GeneRay (Biometra, Germany), and stored at -20°C until usage. Real-time PCR was carried out in the LightCycler (Roche, Switzerland) under the following temperature conditions: initial incubation and polymerase activation at 95°C for 30 s, denaturation at 94°C for 30 s, annealing at 54°C for 45 s, and elongation 72°C for 1 min through 45 cycles. The quantification of amplified DNA fragments was based on the standard curve, which was generated using scale down standard plasmid DNA dilutions (10¹ - 10⁸ copies), each in triplicate. For standard preparation, the 241 bp fragment of integrase gene of Polish BFV isolate was amplified and directly cloned into pDRIVE vector (Qiagen, Germany). Additionally, three series of the following plasmid concentrations: 5, 10, 25, 50 and 100 copies were tested in a single run, in order to determine the detection limit of the assay. Assay specificity was confirmed by the analysis of the melting temperature of amplified products. Additionally, the resulted real-time PCR products, which gave a single melting peak of 81.26°C ± 0.12, were run on 1% agarose gel, to confirm the presence of one specific band of 241 bp. 

**Virus detection.** PBLs, milk cells, and saliva were co-cultured with BFV-permissive canine thymus cells (Cf2Th) in 25 cm² flasks (NUNC) in DMEM, supplemented with 10% of foetal bovine serum and single (PBLs) or double (milk and saliva) dose of antibiotic-antimycotic solution (Sigma, Germany). The non-adherent cells were removed after 24 h and the cultures were passaged at 1/3 ratios until cytopathic effect (CPE) appeared. After five passages, the cultures were stained with May-Grünwald-Giemsa method and observed under light microscope.

**Results**

In order to detect and to quantify viral DNA of BFV, the real-time PCR was developed and validated. When wide range of plasmid DNA copies (from 10¹ copies to 10⁸ copies) was used as template for reaction, the standard curve with high correlation coefficient (r=0.99) was obtained from a plot of crossing points (Ct) against log concentrations of standard (Fig. 1). The slope of the line was -3.606 giving an assay efficiency of 1.9, which corresponds to the amplification of one full-length target copy per every template following each cycle. The curve clearly shows a linear relationship between the Ct values and cycle numbers indicating that the assay was quantitative, though had relatively low limit of detection of 10 copies/reaction. The same level of sensitivity was achieved by a single run of three independent series of different plasmid DNA dilutions, involving: 5, 10, 50 and 100 plasmid copies. Specificity of the real-time PCR was confirmed by the analysis of the melting temperature of amplified products, where only PCR products which showed a single melting peak of 81.26°C ± 0.12, were indicated as positive.

The newly developed real-time PCR was then used for the detection and quantification of viral DNA in PBLs, milk samples and saliva. BFV-DNA was confirmed in DNA extracted from PBLs and milk samples of 14 and eight cows, respectively, while all 23 saliva samples were negative. Number of DNA viral copies varied from 240 to 7,490 in PBLs and from 340 to 3,300 in milk cells per 0.5 µg of genomic DNA (Fig. 2). Average number of copies in milk cells was over 2.5 times lower than in PBLs and there was a strong positive correlation between copy numbers in PBLs and milk cells (Spearman’s correlation coefficient r=0.681, P=0.0013).

When susceptible Cf2Th cells were co-cultured with PBLs, milk cells and saliva, variable intensity of CPE was noted among co-cultures, allowing the differentiation within three-degree scale, from multigiant to single syncytia. The most advanced CPE was noted mostly in co-cultures of PBLs, while co-cultures of milk cells and saliva showed moderated changes characterised mainly by single syncytia. This corresponded to the mean times until detection of positive culture, which were 9, 14, and 18 days for PBLs, milk cells and saliva, respectively. When samples from 23 cows were analysed, typical CPE was observed in 19 (83%), 13 (68%), and 15 (65%) co-cultures from PBLs, milk cells and saliva, respectively (Fig. 3). The co-cultures with all other samples remained virus-free even after the fifth passage, thus they were considered as negative. The virus was isolated from PBLs, milk, and saliva of the same animal, from eight cows, and it could not be isolated from any co-culture from one cow. In addition, in two animals we were able to isolate the virus from saliva only. Cows with strong CPE in PBLs co-cultures had higher but statistically non-significant viral DNA load in blood cells (Mann-Whitney U-test, P = 0.197) and significantly higher viral DNA load in milk cells (Mann-Whitney U-test, P = 0.0012) than cows with weak CPE.

**Discussion**

The isolation of foamy viruses mainly from blood and lymphoid tissues is generally considered indicative of current foamy virus infection in humans and animals (9). This assumption was largely confirmed by the isolation attempts of BFV performed in this study. BFV was more consistently isolated from PBLs than from milk cells and saliva of seropositive cows, despite the fact that isolation was attempted only once per animal. Presumably, repeating the procedure over the time would have lead to a higher isolation rate. BFV isolation from milk cells of seropositive cows has been achieved once, thus confirming previous data showing that the virus is shed through this way (11, 18). In fact, the isolation of BFV from 68% milk samples from BFV-seropositive cows suggests that in the nature nursing may be the most important route of virus spread and can explain relatively high prevalence of BFV infection noted in dairy herds (10, 12).
**Fig. 1.** Standard curve generated as a plot of crossing points (Ct) against log concentrations of the standard (10^1 copies to 10^8 copies).

**Fig. 2.** Load of BFV DNA in PBLs, milk cells and saliva tested by real-time PCR.

**Fig. 3.** Results of BFV isolation from co-cultures of Cf2Th cells with PBLs, milk cells, and saliva.
Successful isolation of BFV from PBLs and milk cells was justified by quantitative measurement of BFV-specific DNA. In our estimation of BFV DNA, we specifically did not isolated chromosomal DNA, thus the extracted DNA could possibly contain both integrated proviral DNA and viral genomic DNA. Our data indicate that BFV DNA, in either a proviral or a viral form, is present in blood leukocytes and milk cells. Interestingly, we show that level of BFV DNA copies in milk cells was 2.5 times lower, and strongly correlated to that noted in PBLs. This can be explained by the fact that milk cells are more heterogeneous population than peripheral blood lymphocytes in ruminants (3) and perhaps the cells with lower BFV burden traffic selectively into bovine milk. The good candidate to support this assumption would be CD8+ T cells, which are predominantly present in bovine milk (20). However, contrary to this expectation, CD8+ lymphocytes were shown to carry the highest amount of FV DNA in African green monkey infected with SFV-3 (15). It is also likely that lymphocytes with low amount of viral DNA, which are present in bovine milk, can emigrate from compartment other than peripheral blood, as was shown for jejunal lamina propria of rhesus macaques infected with SFV (15). Since blood and milk cell counts were not determined in this study, it is unknown if lower load of BFV DNA in milk cells can be explained by the existence of specific subpopulation of lymphocytes susceptible to infection with BFV.

Our most unexpected finding is the relatively high isolation rate of BFV from saliva samples with lack of concomitant possibility to detect BFV DNA. Transmission of FVs is thought to occur through saliva in naturally and experimentally infected nonhuman primates and cats (1, 2). In infected humans, FV transmission often followed contact with saliva of infected monkey (5). In only few studies, the transmission of BFV through saliva has been investigated, as was shown by seroconversion of calves inoculated with saliva from BFV seropositive cattle (7). However, in other study, the isolation of saliva-associated BFV was possible from only one naturally infected steer (8). Thus, our findings revealing isolation of BFV from saliva of 15 out of 23 cows were unexpected; however, isolation rate was considerably less frequent than virus isolation from PBLs and milk cells. In the context of the virus isolation, our main objective was to determine the presence of infectious BFV in whole saliva, without distinction between cell-associated and cell-free virus. Since viral DNA has not been detected in any of saliva samples tested by real-time PCR, which employs DNA as template, one reasonable explanation is that the infection in saliva was caused by free viral particles but not cell-associated virus. We also cannot exclude the inability of real-time PCR to detect minute amount of BFV DNA, since the detection level was minimum10 copies of viral DNA. Relatively high rate of virus isolation from saliva runs the question about the main source of the virus. Data from experimentally SFV-infected macaques clearly showed foamy virus entering saliva from infected cells, mainly from buccal cavity and pharynx epithelium (15).

This study showed also that SFV RNA was not detected in PBMC of any of the infected animals; however, it was consistently detected at high level in saliva and cellular material from the oral cavity. Therefore, it is likely that the isolation of BFV from saliva of two cows, and lack of possibility to rescue the virus from co-cultures of PBLs and milk cells from the same animals noted in our study, account for the preferential expansion of BFV to the oral cavity.

Our data let us assume that milk and saliva of cattle serologically positive to BFV can be considered as real route of BFV spread among cattle. Other aspect concerns public health in relation to BFV in products of cattle origin. Even if no risk of transmission to humans is evident, the retroviral nature of BFV may evoke concern in part of public and it might become necessary to assess whether cattle are BFV free. Therefore, it seems that detection of BFV, especially in blood and milk, remains an important concern.

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References


