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Detection and genome characterization of bovine polyomaviruses in beef muscle and ground beef samples from Germany

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ABSTRACT

Polyomaviruses are small, non-enveloped, circular double-stranded DNA viruses. Some polyomaviruses can induce tumors and cancer under certain circumstances. The bovine polyomaviruses (BPyV) 1–3 have been only scarcely analyzed so far. It was hypothesized that the consumption of beef meat containing polyomaviruses could contribute to the development of cancer in humans. In order to assess the distribution of the BPyV genome in meat from Germany, 101 beef muscle samples and 10 ground beef samples were analyzed here. A specific sample preparation method combined with or without rolling circle amplification (RCA), and BPyV-specific PCRs were developed and applied. BPyV-1 DNA was detected in 1/101 (1%) samples from beef meat and in 2/10 (20%) ground beef samples. BPyV-2 DNA was detected in 3/10 (30%) ground beef samples, whereas BPyV-3 was not detected in the samples. Application of RCA did not increase the detection rate in ground beef samples. Sequence analysis of the PCR products indicated the presence of BPyV-1, BPyV-2a and BPyV-2b. The whole genome of a BPyV-1 strain from ground beef meat showed 97.8% sequence identity to the BPyV-1 reference strain and that of a BPyV-2a strain from ground beef meet showed 99.9% sequence identity to strain 2aS11. It can be concluded that BPyV genomes can be frequently detected in ground beef samples, although higher sample numbers should be investigated in future to confirm this finding. Further studies should focus on the infectivity, tumorigenicity and heat resistance of the contained viruses in order to assess the risk of cancer induction through consumption of BPyVs present in beef products.

1. Introduction

Polyomaviruses are non-enveloped icosahedral viruses, which contain a circular genome consisting of approximately 5000 base pairs (bp) of double-stranded DNA. They are classified into the virus family *Polyomaviridae*. Within the family, >70 species are assigned to the genera *Alpha-*, *Beta-*, *Gamma-* and *Deltapolyomavirus* according to their phylogenetic relationship (Polyomaviridae Study Group et al., 2016). The polyomavirus genome encodes structural proteins (VP1 to VP3) forming the viral capsid and regulatory proteins designated as large and small tumor antigens. Additional proteins such as the agnoprotein and the ALTO protein are found in some polyomaviruses only.

A considerable high number of polyomaviruses is known that infect several animal species and humans. Most of the polyomaviruses cause only subclinical infections in their non-immunocompromized host. However, some of them are known to induce tumors, e.g. the Merkel cell polyomavirus is involved in the development of the Merkel cell carcinoma, a malignant skin tumor of humans (Oram et al., 2016). Also, inoculation of laboratory rodents with the monkey polyomavirus SV-40 leads to growth of tumors (Giardi et al., 1962). A transforming activity of the large tumor antigen of these viruses, which may result in unrestricted host cell division and growth, is considered as the major mechanism for tumor induction by polyomaviruses (Cheng et al., 2009). Based on epidemiological evidence suggesting a correlation between the consumption of beef and the occurrence of colorectal cancer in humans, zur Hausen (2012) supposed that a bovine infectious factor, e.g. a bovine polyomavirus, might be involved in cancer induction in humans.

The first polyomavirus of cattle (BPyV-1) was identified in 1974 in monkey kidney cell cultures (Rangan et al., 1974) and later shown to originate from the fetal bovine serum added to the cultures (Schuurman et al., 1990). Although cases of human cancer caused by this virus were not reported, a transforming potential of its large tumor antigen has been demonstrated by transfection experiments in cell cultures using plasmids encoding this gene under control of Rous sarcoma virus promotor sequences (Schuurman et al., 1992). BPyV-1 shows only low sequence identities to polyomaviruses from other

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mammalian hosts and is currently not assigned to a genus within the family *Polyomaviridae*.

Recent metagenomics analyses of meat samples using next generation sequencing techniques demonstrated BPyV-1-sequences in ground beef samples from the USA (Peretti et al., 2015). In this study and in an earlier study of Zhang et al. (2014), additional sequences originating from a novel bovine polyomavirus (BPyV-2) were identified in US ground beef samples. In a phylogenetic analysis, BPyV-2 clusters within the genus *Alphapolyomavirus*, which also contains the Merkel cell polyomavirus. Two variants of the virus, designated as BPyV-2a and BPyV-2b, were detected. In addition, Peretti et al. (2015) identified a third bovine polyomavirus (BPyV-3) in ground beef samples from the USA. This virus clusters within the genus *Deltapolyomavirus*. The tumorigenic potential of BPyV-1, -2, and -3 is yet unknown.

In order to sensitively detect and thereby assess the distribution of the BPyVs in food of bovine origin, specific sample preparation methods combined with rolling circle amplification (Johne et al., 2009) and BPyV-specific PCRs were developed here. The methods were thereafter applied to beef muscle and ground beef samples collected in Germany. Full-genome sequence analysis of the detected viruses was then conducted to characterize the viruses in more detail. The study aimed at giving first information on the occurrence of BPyVs in food outside the USA and provide initial data for future assessments on the risk of cancer induction by ingestion of bovine meat.

2. Materials and methods

2.1. Samples

A total of 101 muscle samples were derived from the shoulder piece of cattle (23 heifers, 28 young bulls, 4 steers and 46 cows, originating from 10 different herds) collected at a slaughterhouse in Germany. The muscle samples were sealed individually, frozen and stored at −20 °C until analysis. Ten packages of ground beef meat were purchased at different supermarkets from the region of Berlin, Germany. According to the mandatory origin labelling, the cattle was born, raised and slaughtered in Germany. The packages were frozen and stored at −20 °C until analysis.

2.2. Sample preparation and DNA isolation

Muscle and ground beef samples were thawed and 1.5 g portions were excised from the inner part of the samples and placed into 15 ml tubes. After adding 7 ml phosphate-buffered saline (PBS, PAN Biotech GmbH, Germany) and 1.5 g Zirkonia beads (Bio Spec Products, Oklahoma, USA), the samples were homogenized two times for 30 s at 5.5 m/s in a FastPrep®24 homogenizer (MP Biomedicals, Germany), interrupted by a 5 min break to cool down the samples. Thereafter, the samples were centrifuged at 10,000 × *g* for 20 min to pellet the debris. The supernatant was transferred (avoiding aspiration of fat in the case of ground beef) into a 1.5 ml tube. A total of 500 μl of the preparation was used for nucleic acid extraction using the NucliSENS® easyMAG® device (BioMerieux, The Netherlands) according to the manufacturer's protocol and 100 μl DNA were eluted for each sample. The DNA preparations were stored at −20 °C until further analysis.

2.3. Rolling circle amplification (RCA)

Sequence-unspecific amplification of circular DNA was performed by RCA (Johne et al., 2009) using the illustra TempliPHI 100 amplification kit (GE Healthcare Europe GmbH, Germany). Briefly, 5 μl of the sample buffer was mixed with 1 μl of sample DNA and subsequently denatured for 3 min at 95 °C. After placing the tube on ice, 5 μl of reaction buffer and 0.2 μl of the TempliPhi enzyme were added. The reaction was incubated for 16 h at 30 °C and thereafter the enzyme was inactivated by heating for 10 min at 65 °C.

2.4. PCR for BPyV detection

Primer sequences for detection of BPyV-1 were derived from Wang et al. (2005) and Hundesa et al. (2006) and slightly modified based on an alignment of actual BPyV-1 sequences using the MegAlign module of the DNASTAR software package (Lasergene, Madison, USA). Primer sequences for BPyV-2 and -3 were newly designed on the basis of an alignment of available BPyV-2 and BPyV-3 sequences and closely related polyomavirus genomes. All PCR primers are listed in Table 1. A synthetic DNA was used as a PCR positive control, which was synthetized as a gBlocks® gene fragment by an external provider (Integrated DNA Technologies, Belgium). The sequence of the synthetic DNA consisted on joint sequences of the PCR products for BPyV-1 (GenBank acc.-no. NC_001442), BPyV-2b (NC_025811) and BPyV-3 (KM496326). DNA of BPyV-2b from a ground beef sample (Zhang et al., 2014) served as an additional positive control for the BPyV-2-specific PCR. Positive (synthetic DNA) and negative (distilled water) PCR controls were tested alongside in all analyses. PCR was performed with the Ampli Taq Gold DNA Polymerase with buffer II and $MgCl_2$ kit (Life Technologies GmbH, Germany) using 5 μl sample DNA (or RCA product) in a 50 μl reaction volume. Initial denaturation for 5 min at 95 °C was followed by 40 cycles each with 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, and a final elongation at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on ethidium bromide-stained agarose gels. For assessment of the detection limits of the PCRs, the synthetized DNA positive control was quantified using a NanoDrop device (Thermo Fisher, Germany) and twofold dilution series were tested by the PCRs. The detection limit was defined as the lowest DNA amount (expressed as genome copies), which resulted in visible bands in 3 of 3 replicates.

2.5. PCR for determination of animal species

All samples were analyzed for the presence of bovine DNA according to Matsunaga et al. (1999). BPyV-positive samples were additionally tested using published primers and probes for the presence of bovine (Laube et al., 2007), ovine (Laube et al., 2007), porcine (Köppel et al., 2011), equine (Köppel et al., 2011), chicken (Köppel et al., 2009) and turkey (Laube et al., 2007) DNA by real-time PCR. Detection of the myostatin gene of mammals and birds was performed according to Laube et al. (2007) as an internal control. The assays were applied as multiplex real-time PCRs and a cocktail of 10,000 DNA copies of each species was used as an external standard for semiquantitative detection.

2.6. Genome sequencing

Primers for amplification of the polyomavirus genomes in overlapping fragments (between 500 and 1200 bp length each) were deduced from BPyV-1 and BPyV-2 genome sequences available in GenBank. The PCRs were performed using the QIAGEN® LongRange PCR kit (Qiagen). PCR products were separated by electrophoresis on ethidium bromide-stained agarose gels. Bands of the expected length were excised and purified using the QIAquick Gel Extraction kit (Qiagen). Generally, PCR products were sequenced directly with the PCR primers. If this approach was not successful because of low amounts of the PCR product, it was cloned using the TOPO TA Cloning kit for sequencing (Invitrogen) and sequenced with primers M13 Forward and M13 Reverse (Invitrogen). Sequencing was performed in an ABI 3730 DNA Analyzer (Applied Biosystems). Genome sequences were assembled from the sequence reads using the Seq-Builder module of the DNASTAR software package (Lasergene, Madison, USA). All sequences were submitted to the GenBank database with accession numbers KX455479 - KX455484 (PCR products), KX455485 (BPyV-1 genome sample H8) and KX455486 (BPyV-2a genome sample H7).

Table 1
Primers used in PCRs for detection of bovine polyomaviruses.

Designation	Sequence (5′–3′)	Product length	Specificity	Reference
BPyV1-VP2F	TGAGGATTTCAAAGCCCCCTA	270 bp	BPyV-1	Wang et al. (2005); Hundesa et al. (2006); modified
BPyV1-VP2R	GGGCCTACGCCATTCTCATC			
BPyV2-s	GTTGACTGAAACAGCTCCATCCA	236 bp	BPyV-2	This study
BPyV2-as	AGTGGGAAAACAAGCTTTGCTGC			
BPyV3-s	TTGAAGGACCAGGTGGACTCGG	187 bp	BPyV-3	This study
BPyV3-as	GTAGGAGGTCCAGATTCCTCTG			

2.7. Sequence analysis

Closely related sequences were identified by BLASTn search of GenBank and percentages of sequence identity were calculated using the MegAlign module of the DNASTAR software package (Lasergene, Madison, USA). For phylogenetic analysis, large T antigen amino acid sequences were aligned and regions with gaps removed from the alignment. A cladogram was constructed using the tree builder module of Geneious software 9.1.3. (Biomatters, Auckland, New Zealand).

3. Results

3.1. Detection limits of PCRs for BPyV-1, -2 and -3

PCR protocols for the detection of BPyV-1, -2 and -3 were established by modification of published protocols or by design of primer pairs based on multiple sequence alignments (Table 1). The detection limits as determined by limiting dilution of a synthetic DNA positive control were 398 genome copies for the BPyV-1-specific PCR, 795 genome copies for the BPyV-2-specific PCR and 398 genome copies for the BPyV-3-specific PCR.

3.2. Detection of BPyV DNA in beef muscle and ground beef samples

A total of 101 bovine muscle samples from a slaughterhouse and 10 ground beef samples from retail were homogenized and the DNA was extracted. All samples were shown to contain bovine DNA using a cattle-specific PCR. One part of the sample DNA was pre-amplified by RCA and thereafter used in the polyomavirus-specific PCRs, another part was directly used in PCR. Table 2 summarizes the results of the PCR testings. Out of the 101 muscle samples, only one sample was positive for BPyV-1 and only after pre-amplification with RCA. This sample originated from a heifer. No muscle sample was positive in BPyV-2- or BPyV-3-PCR, either with or without RCA pretreatment. Out of the 10 ground beef samples, 2 samples tested positive for BPyV-1 (only one of them was also positive after RCA pretreatment). In the BPyV-2-specific PCR, 3 of the 10 samples turned out to be positive, none of them after RCA pretreatment. Two of these samples had a positive BPyV-1 test result indicating the presence of both virus genomes. No ground beef sample tested positive for BPyV-3.

3.3. Sequence analysis of PCR products

The PCR products were sequenced and the sequences were compared to known BPyV strains. For BPyV-1, nucleotide sequence identities between 95% and 98% to BPyV-1 sequences of the GenBank database were revealed. For BPyV-2, the identities to BPyV-2 sequences of the GenBank database ranged from 90.6% to 100%, where the sequences from ground beef samples H5 and H7 showed 100% identity to the BPyV-2a prototype 2aS11 (KM496324) and the sequence from ground beef sample H8 showed 100% identity to the BPyV-2b prototype 2bS5 (KM496325).

3.4. Genome sequencing and analysis

Determination of the whole BPyV genome sequences was attempted for all positive samples by amplification of overlapping fragments followed by Sanger sequencing. This approach was successful for one complete BPyV-1 sequence from ground beef sample H8 and one complete BPyV-2 genome sequence from ground beef sample H7, but not for the other samples. A phylogenetic tree based on large T antigen amino acid sequences confirms the designation of these viruses as BPyV-1 and BPyV-2a (Fig. 1).

The BPyV-1 full genome sequence from sample H8 showed 97.8% identity to BPyV-1 strain #7535 (KU170643) as well as to the BPyV-1 reference sequence (NC_001442). In comparison to these sequences, it exhibits two sequence duplications, one in the non-coding region near the origin of replication and one in the non-coding region at the polyadenylation site (Supplementary data S1). Both duplications do not alter the open reading frames (ORFs) for the structural or non-structural proteins. The deduced amino acid sequence of the large tumor antigen indicates the presence of all typical functional domains. Especially, the proposed binding site for the retinoblastoma protein (consensus sequence LxCxE) can be found at amino acid positions 60–64.

Comparison of the complete BPyV-2 genome sequence from sample H7 showed a very high identity of 99.9% (6 nucleotide exchanges among the whole 5091 nucleotide sequence) with the BPyV-2a strain 2aS11 (KM496324). A comparison of its genome organization with all known BPyV-2 strains shows the presence of all known ORFs in this strain (Supplementary data S2). Analysis of the deduced amino acid sequence of the large tumor antigen indicates an LxCxE sequence at amino acid positions 171–175.

Table 2
Detection of BPyV-DNA in bovine muscle and ground beef samples.

	BPyV-1 Positive/all samples (%)			BPyV-2 Positive/all samples (%)			BPyV-3 Positive/all samples (%)		
	RCA/PCR	PCR	Total	RCA/PCR	PCR	Total	RCA/PCR	PCR	Total
Muscle samples	1/101 (1.0)	0/101 (0.0)	1/101 (1.0)	0/101 (0.0)	0/101 (0.0)	0/101 (0.0)	0/101 (0.0)	0/101 (0.0)	0/101 (0.0)
Ground beef samples	1/10 (10.0)	2/10 (20.0)	2/10 (20.0)	0/10 (0.0)	3/10 (30.0)	3/10 (30.0)	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)

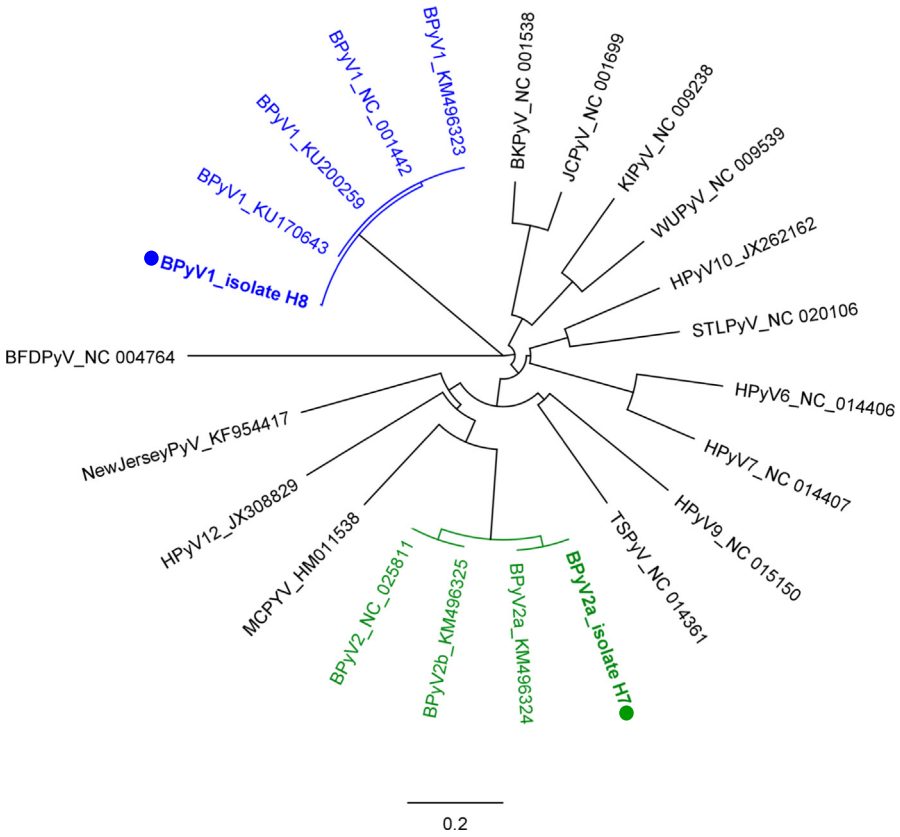

Fig. 1. Phylogenetic relationship of the detected BPyV together with other bovine and human polyomaviruses. BPyV-1 strains are shown in blue and BPyV-2 strains in green. Human polyomaviruses are given in black; the budgerigar fledgling disease virus 1 (BFDPyV_NC004764) is used as an outgroup virus. The viruses detected in this study are in bold and marked with full circles. The tree is based on complete large T antigen amino acid sequences and was generated using the tree builder module of Geneious software 9.1.3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Determination of animal species origin of BPyV-positive ground beef samples

The ground beef samples H5, H7 and H8 were analyzed for the presence of bovine, ovine, porcine, equine, chicken and turkey DNA using specific PCR assays. In all samples, bovine DNA was detected in high amounts. No DNA from other animal species could be identified, with the exception of sample H7, in which traces of porcine DNA (<1%) were found.

4. Discussion

In the study, the presence of bovine polyomaviruses in bovine meat samples was investigated. As the detection of BPyVs in meat samples has been done so far only by laborious and expensive next generation sequencing techniques (Zhang et al., 2014; Peretti et al., 2015), laboratory assays for routine analysis had to be developed first. A protocol using Zirkonia bead-based homogenization of the samples followed by nucleic acid extraction using silica beads is relatively easy to perform and results in DNA preparations suitable for PCR analysis. The subsequent application of previously described (Wang et al., 2005; Hundesa et al., 2006) or newly developed PCR protocols resulted in the sensitive detection of BPyV-1, -2 and -3-DNA using a control DNA and enabled the detection of BPyV-1 and -2 in field samples indicating the suitability of these assays. A development of real-time PCRs may be desirable in future in order to allow quantification of the viral DNA in the samples.

Application of RCA prior to the PCR was intended to pre-amplify the circular genomes of polyomaviruses and thus increase the sensitivity of the assays (Johne et al., 2009). However, our study shows that a higher detection rate after use of RCA was only evident in the muscle samples, but not in the ground beef samples. An efficient RCA is mainly dependent on the presence of circular DNA (Johne et al., 2009). Therefore, the negative effect in ground beef samples may be explained by a higher degree of DNA fragmentation in this type of sample. Alternatively, factors may be present in ground beef extracts, which inhibit the RCA. Generally, it can be concluded from the results of this study that an RCA pretreatment does not increase the BPyV detection rate in ground beef samples and can therefore be omitted in future studies. As only DNA was detected, no conclusion on the infectivity of the viruses of origin can be made.

Only little is known about the presence of BPyVs in muscle samples, and the distinct tissue tropism and transmission routes are not known so far. However, the virus was frequently detected in fetal bovine serum (Schuurman et al., 1991; Kappeler et al., 1996; van der Noordaa et al., 1999; Wang et al., 2005) and in sewage (Hundesa et al., 2006; Hundesa et al., 2010; Wong and Xagoraraki, 2011) indicating a significant viremia and shedding of bovine polyomaviruses. Specific diseases in cattle have not been associated with bovine polyomavirus infection. However, BPyV-1 has been recently detected in aborted cattle (Van Borm et al., 2014) and BPyV-2 in cattle with non-suppurative encephalitis (Wüthrich et al., 2016). Our investigation indicates that BPyVs can be present in muscle samples of cattle, however with a relatively low prevalence (<1%). In contrast, the detection rates of BPyV-1 (20%) and BPyV-2 (30%) were markedly higher in the investigated ground beef samples. Although investigations of higher sample numbers will be necessary to confirm this finding, this is in concordance with the reported high detection rates of bovine polyomaviruses in ground beef samples from the USA (Zhang et al., 2014; Peretti et al., 2015). The use of meat from a higher number of animals for production of ground beef may explain the higher prevalence in this type of food. Alternatively, a virus contamination may take place during the production process of ground

beef. The almost exclusive detection of bovine DNA in the ground beef samples should exclude other animal species as origin of the viruses.

Analysis of the amplified sequences indicated the presence of BPyV-1, BPyV-2a and BPyV-2b in the samples. Whereas the BPyV-1 sequences showed significant differences to other published sequences, the BPyV-2 sequences were almost identical to that detected in the USA. Laboratory contamination could be excluded, at least for the BPyV-2a sequences, as only BPyV-2b-positive samples were present in the laboratory which were used as PCR positive controls. A very high degree of genome nucleotide sequence identity (99.4%) has also been documented recently between BPyV-2b sequences from San Francisco (California, USA) and Bethesda (Maryland, USA) indicating a very low variability of BPyV-2 sequences (Peretti et al., 2015). The analysis of the genome sequences of the BPyV-1 and BPyV-2a strains detected in our study indicated the presence of the complete set of genes and functional regions, thus suggesting that replication-competent genomes were present in the samples. In addition, conserved functional domains, which have been connected to the transforming activity in other polyomaviruses, could be identified in the ORF encoding the large tumor antigen of the BPyV-1 and BPyV-2a sequences. However, the transforming and tumorigenic activity of BPyVs has been only scarcely investigated. For BPyV-1 a transforming activity could be demonstrated, but only by using a very artificial experimental system, in which sequences of another tumor virus were also present (Schuurman et al., 1992). Further studies are needed including BPyV-2 and BPyV-3 and applying more realistic conditions in order to assess the risk of tumor induction in humans by bovine polyomavirus infection.

In conclusion, this study showed the presence of BPyV-1- and BPyV-2-DNA in ground beef samples from Germany. The developed methods should be used in future to determine the distribution of the viruses in a higher number of samples, different types of food and in different geographical regions. As only viral DNA was detected here, further research is necessary in order to assess the infectivity of the viruses. The suitability of cell culture techniques, which have been developed for BPyV-1 isolation from bovine serum (Nairn et al., 2003), should be tested for virus isolation from meat samples. Investigations on the thermal stability of the viruses may indicate, which heating procedures could reduce the risk of BPyV transmission to the consumer. In addition, the tumorigenic potential of the BPyVs has to be investigated in more detail in future. Those studies in combination with a systematic testing of human cancer samples are necessary to assess the risk of cancer induction in humans through consumption of beef products containing bovine polyomaviruses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ijfoodmicro.2016.10.024.

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