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Human Adenovirus, Mesophilic Bacteria and Fungi in Puppies' Food Marketed in Bulk in Southern Brazil

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ABSTRACT

***Background*:** The Brazilian domestic canine population are the second largest in the world and their feeding means 0.4% of the Brazilian gross domestic product. For maintaining the quality of the food, the companies use worldwide standards for technical prevention and control of contaminants and biological conservation. The packaging is part of this process, since it provides a barrier between food and environment. However, in Brazil, packagings are often opened in retail stores for bulk marketing. The objectives of this work were to develop a methodology to detect viruses in foods and to analyze the bacterial and fungal contamination in puppies' food sold in bulk in Ivoti and Estância Velha, cities in Southern Brazil. ***Materials, Methods & Results*:** Twenty samples collected between September and October 2016 were analyzed for most probable number of coliforms, *Salmonella* sp., mesophilic aerobic bacteria and yeast/mold following the regulation of the Brazilian Ministry of Agriculture, Livestock and Food Supply guidelines. They were also tested for *Human Mastadenovirus C* (HAdV), *Canine Mastadenovirus A* (CAdV), and *Carnivore Protoparvovirus 1* (CPV) genomes. Viral analysis were performed by polymerase chain reaction (PCR) detection. During the collection of the samples hygienic-sanitary conditions, storage of feeds, animals' access, dog grooming, and veterinary care were considered to evaluate the conditions of each store. A pilot study was carried out using one food sample marketed in bulk and one sample from the original package (closed package) and testing them for bacterial and fungal contamination for standardizing viral detection. Ten grams of food from the original package were mixed with 90 mL of Eagles' Minimal Essential Medium (E-MEM) in 100 mL sterile bottles. These bottles were kept in room temperature and shaken for 60 min. Subsequently, aliquots were obtained by sequentially diluting the sample (10^{-2} to 10^{-4}). All final specimens contained 10 mL and each diluted sample was spiked with HAdV-C prototype viral strain (AdV5). A standard solution of HAdV-C was diluted from $3.6x10^{6}TCID_{50mL}$, (50% tissue culture infective dose) to $3.6x10^{3}$ $TCID_{50mL}$, and DNA extraction was performed. Nested-PCR targeting AdV DNApol was performed to detect adenoviruses from different hosts. AdV-positive samples were submitted to nucleotide sequencing and phylogenetic analysis. Specific PCRs were also carried out for CAdV and CPV. Mesophilic aerobic bacteria were detected in all samples and *Aspergillus* sp. was found in five samples, among which one sample was co-infected with *Penicillium* sp. One sample was positive for AdV, which was identified as HAdV by sequencing; while coliforms and *Salmonella* sp. were not detected.
***Discussion*:** The presence of fungi with mycotoxigenic potential, such as *Aspergillus* sp. and *Penicillium* sp. represents a threat for canines, due to toxins that may persist for a long period even after the fungus is not viable. Moreover, dogs seem to be more susceptible to the effects of the toxins, which is probably because of low glutathione s-transferase activity. Some species of Genus *Penicillium* may produce ocrathoxin A, which nephrotoxic and immunosuppressive effects in dogs are widely reported. Mesophilic bacteria were detected in all samples (at 10^{4} CFU/g) and considered harmless. The detection of human viruses points to the presence of anthropic contamination; on the other hand, ingestion of contaminated feed, even if it is by a heterologous species, turns the dogs into carriers of the virus. In addition, manipulation of those feeds by children who share the same space with dogs can result in gastroenteritis episodes.

Keywords: *Salmonella* sp., *Aspergillus* sp., *Penicillium* sp., HAdV-C.

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INTRODUCTION

A microbiological analysis of the feed was carried out in order to prevent and control contaminants in companies' products. The packaging is part of this process, providing a barrier between food and environment. However, packages are often opened in retail places for bulk marketing, a common practice in Brazil due to economic factors [2,10].

Zoonotic diseases, which include salmonellosis, may be spread during handling and preparation [10]. *Salmonella* sp. is present in the intestinal tract of birds, reptiles, and domestic animals, which is its primary habitat. It is also present in fecal contaminated food [15]. Fungi are also undesirable in feeds due to their potential to produce mycotoxins [10], which can cause mutagenic, carcinogenic, and hepatogenic disorders [15].

Adenovirus (AdVs) is DNA virus found in gastroenteric and respiratory infections in different species [3]. *Canine Mastadenovirus A* (CAdV-A) is an important pathogen for canines, being divided in CAdV-1 and CAdV-2. The former is responsible for canine infectious hepatitis [14], whereas the latter is among the multiple pathogens involved in kennel cough syndrome [3,14]. *Carnivore Protoparvovirus 1* (CPV) is a major pathogen that causes severe diarrhea in infected puppies [6,8,24].

The presence of viruses in dog food had not yet been tested in Brazil, therefore no regulations are available for this purpose in the country. Furthermore, the objective of this study is to standardize a tool to detect viral genome in feeds and to evaluate the contamination by AdV, CAdV, and CPV viruses, as well as by coliforms, mesophilic bacteria, *Salmonella* sp., and fungi in canine feed sold in bulk in Southern Brazil.

MATERIALS AND METHODS

Samples

Twenty samples of canine food sold in bulk were collected between September and October 2016 in Ivoti and Estância Velha, cities in Rio Grande do Sul (RS), the southernmost state in Brazil. During the collection of the samples hygienic-sanitary conditions, storage of feeds, animals' access, veterinary care and clinic were observed and registered for each seller. These were named from A to F, as seen in Table 1.

Table 1. Hygienic-sanitary characteristics in establishments with bulk counties in Ivoti and Estância Velha, Southern Brazil.

Commercial establishments	Number of samples collected	Tulha's material[a]	Cover container	Tulha's hygienic conditions	Tools' collected	Veterinary proceeds[b]
A[c]	5	Natural wood with a coat of matt varinish	Yes	Without residues	Stell shell	No
B[c]	3	Natural wood with a coat of matt varinish	No	Without residues	Stell shell	No
C[c]	1	Feeds expose in original package	NA	NA	Stell shell	No
D[d]	3	Feeds to transfere at plastics package	NA	NA	ND	Yes
E[d]	6	Product in plastic box and fractionated for plastic packaging.	NA	NA	Stell shell	No
F[d]	2	Natural wood with a coat of matt varinish	Yes	Contaminatted products with other foods and presence of insects	Stell shell	No

[a]Tulha: container where the food is exposed in bulk for sale; [b]only to care for bathing and grooming; [c]Ivoti; [d]Estância Velha; NA: Not applicable; ND: Not detected.

The bacterial analysis were performed following the guidelines of the Brazilian Ministry of Agriculture, Livestock, and Supply [4]. Fungi analysis were performed following the protocols by Copetti *et al*. [7] with little modifications and the fungal genera identification was performed through analysis of macroscopic and microscopic characteristics [17]. Viral analysis were performed using viral genome detection.

Before the testing of the sample, a pilot study was carried out using one food sample marketed in bulk and one from the original package (closed package) aiming at testing the bacterial and fungal analysis techniques, as well as creating a standard for the viral detection technique.

Viral analysis

Ten grams of feed from the original package were mixed with 90 mL of Eagles' Minimum Essential Medium (E-MEM)[1] in a 100 mL sterile bottle. This bottle was kept in room temperature and shaken for 60 min. Subsequently, aliquots were obtained with sequential dilution (10^{-2} to 10^{-4}). All final samples contained 10 mL and each of them was inoculated with HAdV-C. The dilution of HAdV-C was from $3.6x10^6 TCID_{50mL}$, (50% tissue culture infective dose) to $3.6x10^3$ $TCID_{50mL}$, and DNA extraction from each sample was performed using kit following the manufacturer's instructions[2].

A nested polymerase chain reaction (PCR) was performed using the following primer pairs: Pol-F 5´ CAGCCKCKGTTRTGYAGGGT 3´, Pol-R 5´ GCHACCATYAGCTCCAACTC 3´, Pol-nF 5´GGGCTCRTTRGTCCAGCA 3´, and Pol-nR 5´ TAYGACATCTGYGGCATGTA 3´. These aim to amplify the conserved region of DNA polymerase (DNA pol) gene from different AdVs. The final volume of the reactions was 50 µL: 25 µL from Master Mix[3], 18 µL of water RNAse and DNAse free, 20 pmol of each primer[3], and 5 µL of DNA (at approximately 100 ng/µL). Negative and positive control samples, containing water and HAdV-C, respectively, were used. Reactions were run at 94°C for 5 min for initial denaturation. Subsequently, 40 cycles were carried out at 94°C for 30 s, at 50°C for 30 s (-0.5ºC per cycle), and at 72°C for 1 min. A final cycle at 72°C for 10 min was included as final extension.

For detection of CAdV-A a set of primers, which had the hexon protein as the target gene, was used. The forward was CAV-F1 5´-CACGATGTGACCACTGAGAG-3´ and the reverse was CAV-R1 5´-GGTAGGTATTGTTTGTGACAGC-3' [20]. The final volume of the reaction was 50 µL: 25 µL from Mix at PCR solution[3], 18 µL of water RNAse and DNAse free, 20 pmol of each primer[3], and 5 µL of DNA (at approximately 100 ng/µL). Negative and positive control samples containing water and DNA extracted from a commercial vaccine (Vanguard®)[4] respectively, were used. Difference of height from amplicons in agaroses gel can be classified in CAdV-1 with 300 bp and CAdV-2 with 350 bp, compared to a 100-pb DNA Ladder[5]. Reactions were run at 98°C for 7 min for initial denaturation. Afterwards, 35 cycles composed of three stages, at 94°C for 1 min, at 54°C for 1 min, and at 72°C for 1 min, were carried out. A final cycle at 72°C for 7 min was carried out as final extension.

The detection of canine parvovirus (CPV) was performed using the following set of primers: CPV-555-for 5'- CAGGAAGATATCCAGAAGGA-3' and CPV-555-rv 5'-GGTGCTAGTTGATATGTA-ATAAACA-3' [6]. The reactions were carried out in a final volume of 50 µL: 25 µL from Mix at PCR solution[3], 18 µL of water RNAse and DNAse free, 20 pmol each one primer[3] and 5 µL of DNA (at approximately 100 ng/µL). Negative and positive control samples containing water and DNA extracted from a commercial vaccine[4], respectively, were as used. Reactions were performed at 95°C for 5 min for initial denaturation. Subsequently, 35 cycles were carried out at 95°C for 30 s, at 50°C for 30 s, and at 72°C for 1 min. A final cycle at 72°C for 7 min was included as final extension.

The resulting PCR products were found by electrophoresis in 2% agarose gels stained with ethidium bromide (0.5 mg/mL) and visualized under UV light. The amplicon was compared to a 100-bp DNA Ladder[5].

Sequencing and molecular phylogenetic analysis

Nucleotide sequencing was performed in PCR amplicons. These were purified using a Quick Gel Extraction and Purification Combo Kit and following the manufacturer's instructions[5]. Sequencing was carried out in an ABI Prism® 3700 Genetic Analyzer[3].

The quality of the nucleotide sequences was checked and overlapping fragments were assembled using the BioEdit 7.0.5 software [13]. Assembled sequences with high quality were aligned using Clustal

X [23] with default gap penalties. The phylogenetic relationship between these protein sequences was assessed with MEGA7 [16]. Neighbor-joining trees were constructed based on Kimura-2 parameters and calculated using pairwise deletion. This protocol was chosen after being tested for the model that best fit the nucleotide substitutions patterns found in the nucleotide alignments, using MODELTEST (http://www.molecularevolution.org/software/phylogenetics/modeltest). Bootstrap was resampled as a test of phylogeny using 500 replications.

Protocols for bacterial analysis

Twenty-five grams of animal feed were added to 225 mL of 0.1% peptone water, which corresponds to a 10^{-1} dilution. Subsequently, serial dilutions (10^{-2} and 10^{-3}) were prepared in 9 mL of 0.1% peptone water.

For culturing, 1 mL of each dilution (from 10^{-1} to 10^{-3}) was inoculated into three tubes containing Lauryl Sulphate Tryptose (LST) broth[6] and which had inverted Durham tubes in them. The tubes were kept at 35ºC for 48 h. Tubes showing turbidity and Durham gas inside were considered positive.

Each positive tube in the presumptive test had an inoculum of the culture transferred using a sterile 10 µL loop into inverted Durham tubes containing 2% Brilliant Green Bile broth[7], which were kept at 35ºC for 48 h. Those presenting turbidity and gas production were considered positive. The most probable number (MPN) of total coliforms per gram of feed was determined according to the number of positive tubes and based on Hoskins' table for three-tube dilutions.

An inoculum of the culture from each positive sample found in the presumptive test was transferred into inverted Durham tubes containing EC broth[7]. These were incubated at 45 ± 0.2°C for 24 h. The tubes showing turbidity and gas production were considered positive. The MPN of total coliforms per gram of animal feed was determined according to the number of positive tubes and using Hoskins's table for three-tube dilutions.

Twenty-five grams of each food sample were conditioned in flasks containing 225 mL of 1% buffered peptone water, being homogenized vigorously and incubated at 37°C/24 h.

Two mL aliquots from each of the pre-enrichment cultures were inoculated in 20 mL of Cystine Selenite broth[7], in 20 mL of Rappaport-Vassiliadis broth[7], and in 0.2 mL of a 0.4% novobiocin solution, respectively, resulting in a concentration of 40 µg/mL of medium incubated at 37°C for 24 h.

With the aid of a sterile loop, each culture in enrichment broth was seeded by the spread plate method on a Bright Green Agar[7] and on a MacConkey Agar[8] and incubated at 37ºC for 24 h. The bacterial inoculations were carried out in Triple Sugar Iron Agar (TSI)[9], LIA (Lysine Iron Agar)[9], and SIM[9] media for presumptive identification of *Salmonella* sp.

One mL of each decimal dilution (from 10^{-1} to 10^{-3}) was transferred to empty sterile Petri dishes. The Standard Agar for Counting (PCA)[8] was added at 45°C (molten) for homogenizing with a figure-eight motion. After solidification, samples were incubated on plates at 35°C for 48 h. Plates containing between 25 and 250 colonies were used for counting. The results were expressed in CFU/g of animal feed.

Fungal analysis

The 10^{-1} dilutions were kept at room temperature for 1 h under shaking condition every 10 min. Subsequently, the successive decimal dilutions (up to 10^{-3}) were prepared. From each dilution, 100 µL were plated on 18% Dichloram Glycerol Agar (DG 18)[6] plates by the spread plate technique. The colonies were quantified after incubation at 28°C for 7 days. The results are expressed in CFU/g of animal feed. In addition, macroscopic and microscopic evaluation techniques were used for the taxonomic identification of fungi by their structures [21].

RESULTS

All samples analyzed in this study tested positive for mesophilic bacteria, with concentrations ranging from 10 to 4.1 x 10^4 CFU/g. Twenty-five percent (5/20) of the samples were positive for fungi (10^2 at 2.3 x 10^4 CFU/g), being the *Aspergillus* sp. detected in 5 samples. In one case, *Aspergillus* sp. and *Penicillium* sp. were detected co-contaminating a sample. Coliforms, *Salmonella* sp., CAdV, and CPV were not detected; however, in one sample, after having the positive PCR amplicon submitted to purification, sequencing, and analysis (Table 2), HAdV was identified.

Table 2. Microbiological results in twenty samples of puppies feeds marketed bulk in Ivoti and Estância Velha, Rio Grande do Sul State, Southern Brazil.

Commercial establishment	Sample	Virus	Mesophlic (CFU/g)	Fungal	
				(CFU/g)	genus
A[c]	A1	Neg	9 x 10	10^2	*Aspergillus*
	A2	Neg	2 x 10^2	5 x 10^3	*Aspergillus*
	A3	Neg	1.8 x 10^3	<10^{2b}	
	A4	**Pos**[a]	10^2	<10^2	
	A5	Neg	5 x 10	<10^2	
B[c]	B1	Neg	2 x 10^2	<10^2	
	B2	Neg	3.4 x 10^2	2.1 x 10^4	*Aspergillus*
	B3	Neg	1 x 10	2.3 x 10^4	*Aspergillus & Penicillium*
C[c]	C1	Neg	3.4 x 10^2	10^2	*Aspergillus*
D[d]	D1	Neg	7.1 x 10^2	<10^2	
	D2	Neg	<10^2	<10^2	
	D3	Neg	<10^2	<10^2	
E[d]	E1	Neg	10^2	<10^2	
	E2	Neg	4.1 x 10^4	<10^2	
	E3	Neg	10^2	<10^2	
	E4	Neg	4 x 10^2	<10^2	
	E5	Neg	1.1 x 10^3	<10^2	
	E6	Neg	1.1 x 10^3	<10^2	
F[d]	F1	Neg	8 x 10^2	<10^2	
	F2	Neg	<10^2	<10^2	

CFU/g: colony forming unit/gram; Neg: negative; Pos: positive; [a]Positive sample by PCR at DNApol gene from AdV - nucleotides sequence signed in HAdV-C; [b]The detectable limits of the test; [c]Ivoti; [d]Estância Velha.

DISCUSSION

The presence of fungal contamination in feeds may be associated with seasonal spread. In September, the weather, which is in transition from winter to spring in Brazil, may aid the development and spreading of fungal propagules, such as *Aspergillus* [12]. On the other hand, the samples collected in store F (seen in Table 2) showed no fungi and were infested with grain insects. Some reports have shown that insects, such as *Liposcelis* sp. [18] and *Cryptolestes ferrugineus* [19] are of the Myxophaga suborder (insects that eat fungi), which can explain why there is no fungi in these samples.

Samples with *Aspergillus* sp. and *Penicillium* sp. detected in this study were also found in other study carried out in the city of Santa Maria (central region of the state of Rio Grande do Sul, Brazil). Those fungi were co-infecting, along with *Cladosporium* and *Fusarium*, 76% of the samples of dogs and cats feeds sold in bulk. *Aspergillus* was the most detected fungus in that study [7]. The presence of fungi that have potential to produce mycotoxins is a risk to the animals' health because of the toxins that may persist for a long period after the fungi are not viable [5]. Dogs seem to be more susceptible to the effects of the toxins probably due to low glutathione s-transferase activity, which is present in the detoxification process of mycotoxins [25]. Dogs exposed to 500-1000 g/kg of mycotoxins will suffer from an acute form of intoxication that may lead to death after a few days with clinic symptoms, which include hepatic hyperplasia, bleeding, and disseminated intravascular coagulation [11].

Toxins frequently produced by *Penicillium* sp. genus are: rubratoxin, patulin, citrinin, and penicillic acid [10]. Some species may produce ocrathoxin A [15], which nephrotoxic and immunosuppression effects in dogs are well known [9]. Studies have reported

that the ocrathoxin A was lethal in beagles with diary doses of 200 g/kg for 2 weeks or with one dose of 7.8 mg/kg [22]. This study has evaluated only the presence of fungi with potential to produce toxins, the mycotoxin production by the fungi, which means a health risk for dogs, was not subject of research.

Mesophilic bacteria were detected in all samples, with countings < 4.1 x 10^4 CFU/g (as seen in Table 2). Although there are no reference values for these pathogens, some studies have reported that countings below 10^6 CFU/g are not considered dangerous or compromising of the food quality [1]. Moreover, potentially pathogenic bacteria, such as *Salmonella* sp. or coliforms were not detected.

On the other hand, detection of viruses from human species, such as HAdV-C, in samples of canine food marketed in bulk demonstrates how poorly hygienic conditions are maintained. HAdV-C is a pathogen of respiratory tract diseases. It is spread from infected hosts through fecal route [21]. Ingestion of feed contaminated by heterologous virus may turn the dogs into carriers. Furthermore, manipulation of these feeds by children in places shared with dogs can result in gastroenteritis episodes, even though they may be more significant in adults.

Infections from heterologous species have been described in free-living pampas foxes (*Lycalopex gymnocercus*) and crab-eating foxes (*Cerdocyon thous*). One study by our team has reported these infections in the city of São Francisco de Paula, Rio Grande do Sul. That study detected HAdV DNA in 82.3% (14/17) of stools samples and CAdV in five specimens [20]. In addition, enteric viruses can be easily spread from humans to domestic animals due to the non-enveloped virus environmental resistance and its capacity to spread by contaminated feeds.

CONCLUSION

The samples analyzed in this study showed mesophilic bacteria, some fungi with potential to produce mycotoxin, and viruses of human origin (HAdV-C), the most interesting result. The presence of fungi that can produce mycotoxin is a threat to the health of canines because there are many reports of severe health complications in these animals. Market places that sell feeds in bulk should follow the guidelines of the Brazilian Ministry of Agriculture, Livestock and Food Supply. The regulatory standards recommend, in order to minimize the presence of infectious agents, the following: air-conditioning system, two-door system to diminish contact of the feeds with external factors, restriction of animal movement; appropriate feed storage; and standard operating protocols for locations and personnel.

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***Declaration of interest*.** The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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