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Detection of adenovirus, rotavirus, and hepatitis E virus in meat cuts marketed in Uruguaiana, Rio Grande do Sul, Brazil

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ABSTRACT

The aim of this study was to investigate the presence of adenovirus (AdV), rotavirus (RV), and hepatitis E virus (HEV) in beef, pork, and chicken meat cuts in retail trade in the city of Uruguaiana, RS, Brazil. A total of 131 meat products were collected (beef, $n = 55$; chicken, $n = 47$; pork, $n = 29$) from 18 commercial establishments (supermarkets, $n = 7$; butchers, $n = 7$; markets/grocery stores, n = 4). All samples were evaluated for AdV, RV, and HEV. The genomes of RV and AdV were identified in 29% ($n = 38$) and 5.34% ($n = 7$) of the samples, respectively. HEV was not identified in any of the samples. Chicken cuts had a higher frequency of AdV and RV isolates compared to beef and pork ($P < 0.05$). Among the categories of commercial establishments evaluated, all revealed at least one positive sample for AdV and RV; however, supermarkets showed a higher frequency of RV than others ($P < 0.05$). The genetic material of AdV and RV was identified simultaneously in 2.29% ($n = 3$) of samples from supermarkets ($n = 2$) and grocery stores ($n = 1$). This is the first report on detection of enteric viruses in meat cuts in the western region of the state of Rio Grande do Sul, Brazil, and the presence of AdV and RV in these products may indicate flaws during the process of handling these foods, especially in places where commercialization provides important public health issues.

1. Introduction

Foodborne diseases are classified as a global public health issue, directly affecting the economic situation of developed and underdeveloped countries [1,2]. Currently, official data show an increase in cases of gastroenteritis and more serious illnesses related to the consumption of contaminated food. Among the reports on outbreaks, bacterial pathogens have been identified as the main etiological agents [3–5].

However, despite underreporting and underdiagnosis, foodborne diseases caused by viruses are a common cause of outbreaks and gastroenteritis in humans and animals [6–8]. Between 2009 and 2018, 6809 outbreaks were reported in Brazil, involving 120,584 individuals, causing 99 deaths. The viral etiologic agents associated with these outbreaks included 3.9% norovirus (NoV), 3.1% rotavirus (RV), and 1.2% hepatitis A virus (HAV) [3]. Over the same period, these etiological agents were responsible for 18,156 outbreaks in the US, involving 620,853 individuals, resulting in 765 deaths [4]. The main foods associated with the transmission of human enteric viruses are raw fish, meat, fruits, and vegetables grown or handled in contaminated environments [9–12].

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Food-borne viruses are usually unenveloped and more resistant to heat, pH variations, drying, UV exposure and natural light [13]. Unlike bacteria, they are not free-living entities, relying on living host cells for their replication. Another relevant issue in food safety is that viruses are more resistant to environmental stresses, as well as to cleaning and sanitation programs, especially non-enveloped viruses, thus hampering the effectiveness of hygiene programs [10,14,15].

Among the viruses related to foodborne diseases, adenovirus (AdV), rotavirus (RV), and hepatitis E virus (HEV) are pathogens that have been studied frequently, and were found associated with cases of zoonotic transmission [8,16,17]. AdV is a non-enveloped DNA virus that belongs to the *Adenoviridae* family and can cause respiratory or gastrointestinal disorders due to its transmission via the oro-fecal route [18]. RV belongs to the *Reoviridae* family, has a double-stranded RNA genome and triple-layered external capsid, which confers resistance to environmental stresses, and is frequently involved in gastrointestinal problems [19]. HEV, on the other hand, is composed of a small non-enveloped virion, which encloses an infectious RNA. It belongs to the *Hepeviridae* family and is responsible for hepatitis cases associated with precarious basic sanitation [20]. Symptoms related to these diseases can vary depending on intrinsic factors related to the etiologic agent and the host's immune status [16,21].

The transmission of these microorganisms via food is worrying from a public health perspective because of the low infectious dose required to establish an infection, and the high viral excretion load through the feces, even in asymptomatic cases, thus facilitating the spread of these pathogens in the environment [22,23]. Despite the relevance of food-borne diseases caused by viruses to public health, there have only been few studies carried out in Brazil for evaluating the presence of these pathogens in meat cuts of animal origin [24,25], and data reporting the epidemiological situation of these meat product-associated pathogens are scarce in the state of Rio Grande do Sul, Brazil. Thus, the present study aimed to evaluate the presence of AdV, RV, and HEV in beef, pork, and chicken meat cuts in retail trade in the city of Uruguaiana, Rio Grande do Sul, Brazil.

2. Materials and methods

2.1. Sample collection

During the period from January 2017 to June 2018, 131 meat products were collected in the retail trade of the city of Uruguaiana, RS, Brazil. Of these, 55, 47, and 29 samples were beef, chicken, and pork, respectively. These samples were obtained from 18 establishments, i.e., 7 butchers ($n = 36$), 7 supermarkets ($n = 69$), and 4 markets/grocery stores ($n = 26$). They were obtained by purchasing approximately 300 g of the different meat products that remained in the original packaging, and were kept frozen at −18 °C until the time of analysis. Right before analysis, the samples were defrosted and punch cuts with approximately 1 g were collected from each one.

2.2. Detection of AdV, RV, and HEV

2.2.1. Sample preparation, RNA extraction, and cDNA synthesis

The samples (1 g) were macerated in 1 mL of 1× Minimum Essential Medium (MEM, pH 7.0), vortexed for 10 s, and subjected to RNA extraction by the TRIzol™ reagent method (Invitrogen), according to the manufacturer's instructions, with minor modifications. After homogenization with 1× MEM, 250 μL of the homogenate were suspended in 750 μL of TRIzol™ reagent (Invitrogen), incubated for 5 min, and centrifuged at 11,000 ×*g* for 10 min at 4 °C. The supernatant was transferred to a tube containing 200 μL of chloroform, incubated at 21 °C for 5 min, and centrifuged again at 11,000 ×*g* for 10 min at 4 °C, after which the aqueous phase was transferred to a new tube. Proteins were precipitated with 500 μL of isopropanol and incubation at room temperature for 5 min. Subsequently, the samples were centrifuged at 12,000 ×*g* for 8 min, and the supernatant was discarded. At the end of the process, 1 mL of 75% (*v*/v) ethanol was added, and a final centrifugation at 9000 ×*g* was performed for 5 min. The ethanol was removed by inversion of the tubes, which were subsequently subjected to air-drying for 3 min. The RNA pellets were suspended in 60 μL of TE buffer solution and stored at −80 °C.

To prepare cDNA (RV and HEV), 10 μL of total RNA were added to 10 μL of the main mixture (High-Capacity cDNA Synthesis, Applied Biosciences), which was prepared according to the manufacturer's instructions. Each cDNA synthesis mixture consisted of 3.2 μL DNase/RNase-free water, 2 μL of buffer, 0.8 μL of dNTPs, 2 μL of random primers, 1 μL of RNase inhibitor, and 1 μL of RT enzyme. The samples were amplified in a thermocycler (10 min at 20 °C, 120 min at 37 °C, and 5 min at 85 °C), and then refrigerated at 4 °C.

2.2.2. Nested PCR for AdV detection

The amplification target for AdV was the DNA polymerase gene, common gene to the *Adenoviridae*, as described by Li et al. (2010) (Table 1). As a positive control, the HAdV-41 isolate was used. Both reactions had a final volume of 50 μL, containing 25 μL of GoTaq Green Master Mix (Promega), 18 μL of DNase/RNase-free water, 1 μL of each primer (20 pmol), and 5 μL of the DNA sample. The amplification was performed in thermocycler (Applied Biosystems ProFlex PCR System) and the conditions for the two reactions were as follows: 94 °C for 5 min, 40 cycles at 94 °C, 50 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min [26]. Next, the PCR products were added to a 2% agarose gel containing 0.001% ethidium bromide and electrophoresed at 70 V for 1 h. The results of the reactions were visualized under UV light.

2.2.3. PCR for RV detection

For the detection of RV, specific primers were used to amplify the VP6 region common to the *Reoviridae*, as described by Spilki et al. (2013) (Table 1). The reaction had a final volume of 50 μL, containing 25 μL of GoTaq Green Master Mix (Promega), 18 μL of DNase/RNase-free water, 1 μL of each primer (100 nM), and 5 μL of cDNA. RNA extracted from

Table 1
Primers used for the detection of adenovirus (AdV), rotavirus (RV), and hepatitis E virus (HEV).

Virus	Name	Sequence	Reference
AdV	DNA Polymerase pol-F	5′ CAGCCKCKGTTRTGYAGGGT 3′	[26]
	DNA Polymerase pol-R	5′ GCHACCATYAGCTCCAACTC 3′	
	DNA Polymerase pol-nR	5′ GGGCTCRTTRGTCCAGCA 3′	
	DNA Polymerase pol-nF	5′ TAYGACATCTGYGGCATGTA 3′	
RV	ROTAFEEVALE F	5′ GATGTCCTGTACTCCTTGT 3′	[45]
	ROTAFEEVALE R	5′ GGTAGATTACCAATTCCTCC 3′	
HEV	HEVORF1con-s1	5′ CTGGCATYACYCTACTGCYATTGAGC 3′	[24]
	HEVORF1con-a1	5′ CCATCRARRCAGTAAGTGCGGTC 3′	
	HEVORF1con-s2	5′ CTGCCYTKGCGAATGCTGTGG 3′	
	HEVORF1con-a2	5′ GGCAGWRTACCARCGCTGAACATC 3′	

Table 2
Number and frequency (%) of hepatitis E virus (HEV), adenovirus (AdV), and rotavirus (RV) in samples of meat products from Uruguaiana, Brazil.

Samples	N	Virus		
		HEV	AdV	RV
By product				
Beef	55	0 (0)	1 (1.81)	4 (7.27)
Chicken	47	0 (0)	6 (12.76)	32 (68.08)
Pork	29	0 (0)	0 (0)	2 (6.89)
P value[1]		–	0.017	0.001
By establishment				
Supermarket	69	0 (0)	4 (5.79)	28 (40.57)
Butcher	36	0 (0)	2 (5.55)	7 (19.44)
Market/grocery store	26	0 (0)	1 (3.84)	3 (11.53)
P value[1]		–	0.929	0.007
Total	131	0 (0)	7 (5.34)	38 (29.0)

[1] A *P* value <0.05 indicates a statistically significant association for the presence of viruses with regard to the category assessed.

cultured cells treated with the RV vaccine served as positive control. The amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, and a final elongation at 72.8 °C for 7 min (Applied Biosystems ProFlex PCR System). Electrophoresis and visualization of the PCR products followed the same protocol used for AdV detection.

2.2.4. Nested PCR for HEV detection

For the detection of HEV, specific primers for amplifying the ORF1 region of HEV were used, according to Heldt et al. [24] (Table 1). A RNA isolate from HEV-positive monkey feces was obtained under ethics approval [27] and kindly provided by Dr. Marcelo Alves Pinto to be used as positive control. The reaction had a final volume of 50 μL, containing 25 μL of GoTaq Green Master Mix (Promega), 18 μL of DNase/RNase-free water, 1 μL of each primer (100 nM), and 5 μL of cDNA. The amplification conditions were as follows: initial temperature of 95 °C for 5 min, followed by 45 cycles at 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 1 min, and 72 °C for 7 min for final elongation (Applied Biosystems ProFlex PCR System). Electrophoresis and visualization of PCR products followed the same protocol used for AdV detection.

2.3. Data analysis

Obtained data were tabulated, and the results were expressed as positive frequency for each virus analyzed. Then, they were subjected to Chi-square testing to verify the association of the product type and category of establishment with the detection of the pathogen. The corresponding odds ratios (ORs) were calculated to estimate the likelihood of the presence of the genetic material of the virus in the products analyzed. Analyses were performed using the IBM® SPSS® Statistics Version 20 statistical program, with a significance level of 0.05.

3. Results

Of the 131 samples evaluated for the detection of enteric viruses, 38 (29%) contained the RV genome, and 7 (5.34%) were positive for AdV. Genetic material of HEV could not be detected in any of the samples analyzed (Table 2). Chicken cuts showed a higher frequency of RV and AdV isolates compared to beef and pork ($P < 0.05$). Among all of the categories of commercial establishments evaluated, at least one of the samples tested positive for AdV or RV. Supermarkets showed a higher frequency of RV compared to other establishments ($P < 0.05$). For AdV, there was no significant association with the type of establishment evaluated ($P > 0.05$).

Table 3 shows the ORs for the detection of AdV and RV listed by product and establishment categories. Based on the corresponding *P* values and confidence intervals, significant OR values were obtained for RV in chicken meat compared to beef (OR 27.2; 95% CI 8.29–89.24) and pork (OR 9.87; 95% CI 2.55–38.13). Regarding the category of establishment, a significant OR was observed for the detection of RV in supermarkets, both when compared with butcher shops (OR 2.82; CI 95% 1.08–7.35) and markets/grocery stores (OR 5.23; 95% CI 1.43–19.12).

Among the 18 commercial establishments evaluated, 14 were positive for AdV and RV in at least one sampling. AdV was detected in 4 samples from three supermarkets, and RV was present in 35 samples from seven supermarkets ($n = 28$) and five butchers ($n = 7$). Of the 131 samples analyzed, 3 (2.29%) simultaneously contained the genetic material from AdV and RV, which were obtained from two commercial establishments, this being a supermarket (n = 2) and a grocery market ($n = 1$).

Table 3
Odds ratios (ORs) and confidence intervals (CIs) for the presence of adenovirus (AdV) and rotavirus (RV) in samples of meat products from Uruguaiana, Brazil.

Category	AdV			RV		
	P[1]	OR	CI (95%)	*P*[1]	OR	CI (95%)
By product						
Beef × Chicken	0.029	7.92	0.915–68.22	< 0.001	27.2	8.29–89.24
Beef × Pork	> 0.05	0.98	0.47–1.01	> 0.05	0.94	0.162–5.49
Pork × Chicken	nc*	nc	nc	< 0.001	9.87	2.55–38.13
By establishment						
Supermarket × Butcher	0.003	0.90	0.18–6.0	0.029	2.82	1.08–7.35
Supermarket × Market/grocery store	> 0.05	1.53	0.16–14.44	0.007	5.23	1.43–19.12
Butcher × Market/grocery store	> 0.05	0.68	0.05–7.92	> 0.05	0.54	0.12–2.32

[1] A *P* value <0.05 indicates a statistically significant association for the presence of viruses by assessed category; *nc - not calculated.

4. Discussion

The presence of AdV and RV in many meat cuts from several commercial establishments of Uruguaiana, Brazil, demonstrates the risk associated with the consumption of these products. The fact that AdV and RV were detected in various establishments, at different product sampling time points, indicates that inadequate practices of obtaining/manipulating are routine at those places. The most frequent sources of foodborne viral diseases worldwide are fish, fruits, and vegetables, and are often associated with water transmission [10,12,28]. However, there are few studies on the presence of viruses in other products of animal origin in Brazil, mainly in fresh meat [24,25].

It is already stated that AdV, RV and HEV can be shared both by animals and humans and might pose a risk to the last ones by consumption of contaminated food [16,23,29,30]. While infections caused by them are usually self-limited, studies of these foodborne viruses on animal products are relevant and beneficial on the One Health concept in order to clarify epidemiological aspects and molecular characteristics.

The presence of these pathogens in food may indicate a public health problem, since these microorganisms have the ability to survive on different surfaces [31,32] and at low temperatures, such as during storage of food [33]. RV is considered an important pathogen associated with neonatal diarrhea worldwide. In the last few decades, this agent was responsible for 3.1% of foodborne outbreaks in Brazil [3]. AdV is associated with diarrhea in adults, and HEV is considered an important etiologic agent of hepatitis in humans [18,20].

Although there are no reports of a direct relationship between contaminated products and outbreaks of AdV and RV in the population, infections by these viruses occur through the fecal-oral route, through person-to-person contact, or contaminated food. Enteric viruses, such as AdV and RV, can be used as indicators of environmental contamination in the food handling process, and to assess the sanitary quality of the water used in these procedures [18,19]. Thus, the spread of these pathogens can occur in food-handling environments, mainly through handlers or contaminated water [34].

In addition, some viruses may be resistant to cleaning and sanitizing processes [10,15,35] and can serve as a source of cross-contamination in commercial and domestic environments. The viruses investigated in this study exist extracellularly as small and non-enveloped virions; that is, they are devoid of superficial lipid bilayers forming an envelope, whereas outer protein layers, so-called capsids, provide them with greater resistance to environmental conditions, disinfectants, and common sanitizers [10,35]. Additionally, RVs are covered by a triple-layered capsid, forming very stable and resistant viral particles in the environment [19].

In Brazil, there is no specific legislation to evaluate these pathogens in food; however, there are environmental monitoring studies for the occurrence of enteric viruses [24,36,37]. The presence of these pathogens in the samples evaluated in the present study demonstrates poor hygiene conditions in places where products are handled and marketed; however, it does not allow for inferring their infectious capacities. In this study, it was not possible to quantify or verify the infectivity of the viral particles, but rather the genetic material was amplified. However, there are reports on low numbers of infectivity viral particles being sufficient to develop gastroenteritis, especially in children and immunocompromised patients [38–40].

The results of this study are important to assist health inspection and surveillance agencies, as they indicate that meat products sold in the city of Uruguaiana, RS, may pose a risk to consumers' health. In a previous study, [25] Pereira et al. (2018) reported the presence of HAV and RV in fresh and processed products from Argentina and Uruguay, demonstrating the risk of viral circulation through these products in this border region.

In the present study, there was a higher frequency (40.57%) of RV being detected in samples of chicken cuts, when compared to beef and pork, a fact of concern due to the high consumption of this animal product in Brazil [41]. Thus, the risk of inappropriate handling of these products in the home environment must also be considered. Although there is no preference for the consumption of this meat cut in a raw way, it has already been reported that consumers have difficulties handling and storing chicken meat properly, which, in this context, becomes a risk factor for the dissemination of RV in a home environment [42].

Supermarkets are one of the main places for people to buy food products. In this study, there was a significant association between the presence of RV in samples from supermarkets, when compared to butchers and markets/grocery stores. Inadequate adherence to good manufacturing practices can compromise food safety. Studies by Robertson et al. [43] and Silva et al. [44] [43,44]reported that inadequate installations, flaws in hygiene processes, and lack of knowledge of workers are among the main factors that can interfere with the quality and safety of the products sold in these places. In addition, Maunula et al. [31] evaluated the presence of RV and AdV on industrial surfaces and the hands of manipulators, thus identifying the handling of products as a contaminating factor. In this context, we emphasize that, due to the multiplicity of factors involved in the viral contamination of food (environment, animals, and humans), it is essential to choose generic primers with focusing on the detection of the viral family or multiples genotypes to detect genomes from different sources like those used in this study (DNA polymerase – AdV, VP6 region – RV, and ORF1 region – HEV) because the food contamination can occur in several stages and is directly related to how they are handled during the various steps of preparation as well as the environmental conditions in which food is obtained and processed.

Although pigs are known to have a high seroprevalence of HEV and its RNA was already reported in processed pork products [29], none of the samples evaluated contained genetic material of HEV. This result may indicate a limited circulation of this virus in the study region, or that the pathogen is present at undetectable levels in these products. Heldt et al. [24] described the occurrence of HEV in 36% of swine pâté samples sold in the city of Novo Hamburgo, RS. Boxman et al. [45] evaluated the sanitary hygienic quality of pig liver products marketed in supermarkets in the Netherlands, obtaining a prevalence of 68% HEV-positive samples. However, Jones and Muehlhauser [46] did not detect HEV in industrial plants for the slaughter and processing of pigs, or in samples in retail trade in Canada, suggesting an effective adherence to processes and guidelines of good manufacturing practices.

4.1. Study limitations and further research

The present study was restricted to the detection of some viruses, and it is important to increase the scope of the research to verify the presence of other pathogens, however, due to the difficulties imposed for the funding of research currently in our country, we were limited to the detection of the mentioned viruses. In future studies, we intend to expand the search for the detection of other viruses, such as Norovirus and Hepatitis A Virus. In addition, our study evaluated foods from a single region of the state, which does not allow an assessment of the broader epidemiological situation, however, despite this limitation, we emphasize that this is the first study in the sampled city. In future studies, we intend to expand the evaluation to other cities in the Uruguaiana region to obtain a better scenario regarding the presence of viruses in foods.

5. Conclusions

This study is the first to report on the detection of enteric viruses in meat cuts in the western region of Rio Grande do Sul, Brazil. The presence of AdV and RV in these products could indicate failures during the process of handling these foods, especially in commercial places, providing important insights relevant to public health. Detection of viruses identified in this study is useful as they share both animals and humans as hosts and can be also found in food chain, being able to pose a

risk to the health of consumers by cross-contamination. Moreover, it is important to clarify the status of these pathogens in the food chain to impose better control measures in the environment, since this kind of information is not available in Brazilian inspection bodies.

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CRediT authorship contribution statement

Vanessa Mendonça Soares: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Emanoelli Aparecida Rodrigues dos Santos:** Methodology, Writing – original draft, Writing – review & editing. **Leonardo Ereno Tadielo:** Methodology, Writing – original draft, Writing – review & editing. **Camila Koutsodontis Cerqueira-Cézar:** Methodology, Writing – original draft, Writing – review & editing. **Aryele Nunes da Cruz Encide Sampaio:** Methodology, Writing – original draft, Writing – review & editing. **Ana Karolina Antunes Eisen:** Methodology, Writing – review & editing. **Kelen Gras de Oliveira:** Methodology, Writing – review & editing. **Matheus Beltrame Padilha:** Methodology, Writing – review & editing. **Maria Eduarda de Moraes Guerra:** Methodology, Writing – review & editing. **Raíssa Gasparetto:** Methodology, Writing – review & editing. **Mário Celso Sperotto Brum:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. **Carolina Kist Traesel:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. **Andreia Henzel:** Methodology, Writing – original draft, Writing – review & editing. **Fernando Rosado Spilki:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. **Juliano Gonçalves Pereira:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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