



Received for publication, February, 3, 2017

Accepted, December, 18, 2017

*Original paper*

## ***Torque teno virus was misidentified in meat products from Romania by different methods of detection***

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### **Abstract**

**Objectives:** Torque teno viruses (TTVs) are newly discovered infectious agents, highly prevalent in healthy individuals world-wide, as well as in farm and wild animals. The purpose of this study was detection of human TTVs in meat products.

**Methods and Results:** One hundred sixty meat products were purchased from retailers or Romanian producers, and DNA was extracted. Real-time PCR was used for detection of beef, pork, chicken and turkey DNA. Human TTVs were identified by PCR using sets of primers spanning the translated and untranslated regions in the viral genome. The specificity of the amplicons was checked by sequencing. No specific amplicons were obtained using primers targeting coding regions. Employing primers targeting 5'UTR, TTV and TTMV were detected in a high number of samples; however, the viral origin was not confirmed by sequencing. The sequences obtained were similar to regions from pig, chicken, turkey and bacterial genomes. The primers used in our study may not specifically detect TTVs when food-purified DNA is used as template.

**Conclusions:** Further investigations are needed to determine whether TTVs could be reliable viral markers for foods quality assessment.

### **Keywords**

Food quality assessment, meat products, PCR primers, Torque teno virus, viral bio-indicator.

**To cite this article:** SPANDOLE-DINU S, BERCA LM, ADASCALULUI M, NICULAE OM, NICA R, TOMA M, ZOANI C, RADU I, CIMPONERIU D. Torque teno virus was misidentified in meat products from Romania by different methods of detection. *Rom Biotechnol Lett.* 2020; 25(3): 1581-1586. DOI: 10.25083/rbl/25.3/1581.1586

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## Introduction

Torque teno viruses (TTVs) are emergent viral infectious agents discovered starting 1997 by a group of Japanese scientists (Nishizawa, 1997). The isolates infecting humans belong to *Torque teno virus* (TTV), *Torque teno midi virus* (TTMDV) and *Torque teno mini virus* (TTMV), and were included in the *Anelloviridae* family in three different genera. In the same family were also designated a series of anelloviruses genera that comprise viruses which infect animal hosts, such as pigs (*Torque teno sus virus*, TTsuV), cats (*Torque teno felis virus*), dogs (*Torque teno canis virus*) and tupaia (*Torque teno tupaia virus*) (International Committee on Taxonomy of Viruses, 2012).

The morphology of TTVs is simple, the virions are small (approximately 30 nm in diameter), nude and their genome consists of a circular single-stranded molecule of DNA. Although the resistance to thermal treatment of anelloviruses was only speculated (2009), it has been demonstrated that two related viruses (porcine circovirus 2 and chicken anemia virus) were extremely resistant to thermal treatment (WELCH, 2006). The most striking feature of TTVs, however, is their high prevalence worldwide and multiple transmission routes (saliva, blood, fecal-oral) (SPANDOLE, 2015). The blood prevalence of TTVs in humans ranges between 73% in Brazil (MASSAU, 2012) and 93% (VASILYEV, 2009) in Russia for TTV, 48% in Norway (MOEN, 2002) and 72% in Brazil (DEVALLE, 2004) for TTMV and approximately 40% for TTMDV (NINOMIYA, 2007). TTV was also identified in several others biological samples such as bile, cord-blood, saliva, semen, tears, breast milk, hair and skin (SABACK, 1999; GOTO, 2000; MARTINEZ, 2000; OSIOWY, 2000; HAYAKAWA, 2001). To our knowledge, it is unclear whether there are animals (other than primates) infected with human TTVs.

The animal anelloviruses are under-investigated, except for TTsuV, TTsuV genogroup 2 has been associated with post-weaning multisystemic wasting syndrome in pigs (ARAMOUNI, 2011). Animal anellovirus prevalence in pigs varies between 24% in Italy (MARTELLI, 2006) and 100% in Quebec and Saskatchewan, Canada (MCKEOWN, 2004) depending on geographic regions, animal care conditions and genogroup (MARTINEZ, 2006).

However, presence of anelloviral DNA was identified in farm animals (with prevalence of 19% in chickens, 20% in pigs, 25% in cattle, 30% in sheep (LEARY, 1999), 43% in cats, and 38% in dogs (OKAMOTO, 2002), as well as wild animals (wild boars (CADAR, 2013), bushpigs (BLOMSTROM, 2012), wood mice, field voles, bank voles (NISHIYAMA, 2014), treeshrews (OKAMOTO, 2002), camels (AL-MOSLIH, 2007), and non-human primates (OKAMOTO, 2000).

In two recent studies the presence and source of TTV DNA was tested in dairy products to assess if consumption of contaminated food might be a possible source of infection. TTV DNA was found in raw and pasteurized camel milk from the United Arab Emirates (AL-MOSLIH, 2007) and in raw buffalo milk, but not in pasteurized buffalo milk or dairy products from Italy (ROPERTO, 2009). To our knowledge, there is no available data on the presence of TTVs DNA in the food products, both in Romania and elsewhere, with the exception of the previously mentioned works.

The objective of this study was to test whether torque teno viruses DNA is present in meat products and could be used as a viral marker for food safety.

## Materials and Methods

### 1. Samples

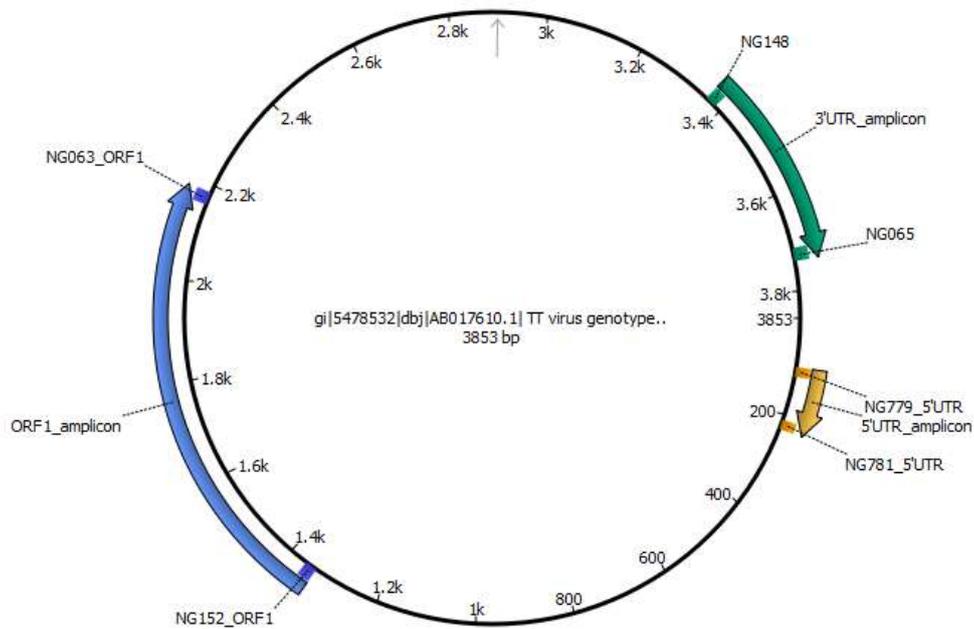
For this study, 160 meat products (i.e. hot dogs, ham, pastrami, bacon, salami, baloney, mortadella, sausages, fish, canned foods and unprocessed meats) were collected from retailers or Romanian producers. For each product, ingredients, expiration date, batch number, packaging and manufacturer were recorded.

### 2. Nucleic acid purification and quantification

DNA was extracted with SureFood® PREP Animal X (Congen Germany) from 50 mg product. DNA fragmentation was assessed by agarose gel electrophoresis (1% TBE 0.5X, 5V/cm) and its purity by spectrophotometry on Beckman DU170. SureFood® Animal QUANT (Congen Germany) was utilized for real-time quantitative detection of beef, pork, chicken and turkey DNA (Rotor-Gene 6000 instrument, Corbett research). The amplification program, according to the manufacturer's instructions, consisted of initial denaturation at 95°C for 5 min, followed by 45 cycles of 5 s denaturation at 95°C, 10 s annealing at 62°C and extension 15 s at 65°C. The fluorescence was detected on the green channel (517 nm). Positive and negative controls were included in each experiment. A sample was considered positive for the DNA of the tested species when it showed amplification in the green channel and negative when it showed no amplification. If one of the two controls showed aberrant amplification patterns the experiment was invalidated.

### 3. Viral DNA detection

The viral DNA of human anelloviruses (TTV, TTMDV and TTMV) was detected using PCR primers targeting 5'UTR, as described by Ninomiya *et al.* (NINOMIYA, 2008), PCR primers targeting the 3'UTR of only TTV, as described by Okamoto *et al.* (OKAMOTO, 1999), and TTV ORF1 primers as described by Nishizawa *et al.* (NISHIZAWA, 1999) with slight changes. Amplicons were resolved in 1.5% or 2% agarose gels and stained with ethidium bromide. Primers and amplicons positioning on the viral genome of TTV are depicted in Figure 1.



**Figure 1.** Positions of the selected primers and of the amplicons obtained compared to the TTV genome, isolate TA278 (GenBank acc. no. AB017610.1).

Seven random samples that tested positive for TTV DNA were amplified with primers NG148 and NG065 spanning approximately 400 nucleotides of 3' UTR of TTV genome, as described by Okamoto et al (OKAMOTO, 1999). The resulting amplicons were subjected to Sanger sequencing on an Applied Biosystems 3130 Genetic Analyzer.

#### 4. Statistical analysis

Statistical analysis was performed using non-parametric tests enclosed in SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA). For all statistical tests,  $p < 0.05$  was considered significant.

## Results

The DNA extracted from all food samples showed little degree of fragmentation after agarose gel electrophoresis and the A260/A280 ratio ranged between 1.7 and 2. Quantitative detection of pork, chicken, turkey and beef DNA by real-time PCR confirmed the ingredients listed on product labels.

Using primers targeting 5'UTR, viral DNA was found in approximately 74% of the samples. The highest detection rate was recorded for TTMV DNA with a value of 55% of all samples, followed by TTV with 37.5% and TTMDV with 16% (Table 1).

**Table 1.** Distribution and detection rate of TTVs viral DNA by type of product using Ninomiya PCR primers targeting 5'UTR

Product	No. of samples	TTV DNA	TTMDV DNA	TTMV DNA
hot dogs	27	10	7	14
Fish	18	7	1	11
ham/pastrami/bacon	33	11	8	15
Salami	20	9	4	10
baloney/mortadella	8	1	3	7
Sausages	22	11	0	16
canned foods	9	5	1	7
unprocessed meats	23	9	1	10
<b>Total</b>	<b>160</b>	<b>63 (39.37%)</b>	<b>25 (15.62%)</b>	<b>90 (56.25%)</b>

The type of product with the highest rate of viral DNA detected was canned foods, while the lowest were unprocessed meats with no significant difference in the distribution of the three types of viral DNA ( $p=0.18$ ).

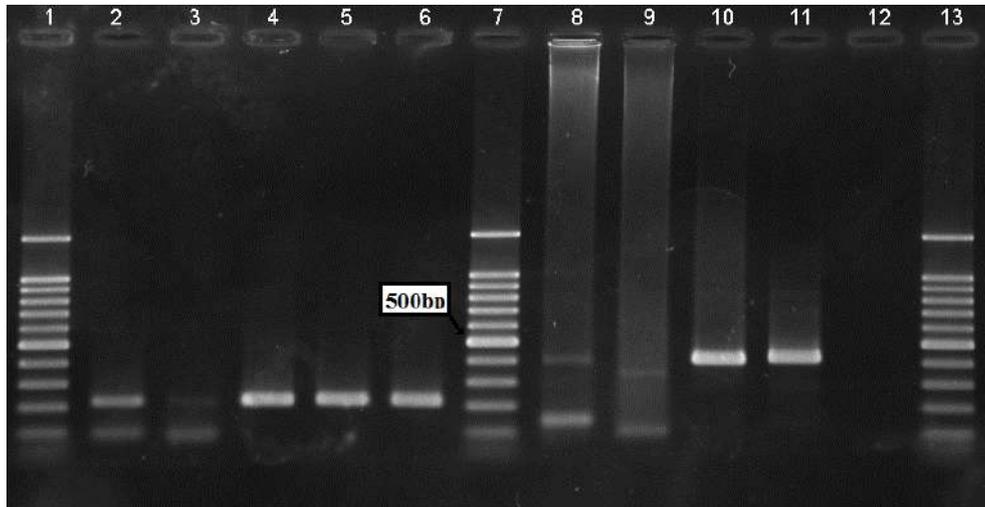
Of the selected products, 72 contained pork meat, 45 poultry, 21 meat blend, 18 fish, 3 beef and 1 mutton. The composition described on the labeling of products was confirmed by real-time PCR.

TTV and TTMV DNA were mainly found in meat blend products, while TTMDV in pork-based products. Nonetheless, the distribution of viral DNA was similar regardless the type of meat contained by the product ( $p=0.56$ ).

Amplification of food-derived DNA with primers complementary to the HVR of TTV ORF1 failed to

result in specific amplifications.

Sanger amplicon was employed initially on three samples amplifying 3' UTR region that yielded sufficient templates for the technique (lanes 4-6, Figure 2) and, later on, two more samples were sequenced. The sequences were similar to fragments of pig, turkey, and *Pseudomonas sp.* genomes.



**Figure 2.** Random amplification of seven samples of food-extracted DNA with NG065 and NG148 primers. 1.5% agarose gel in 1X TAE; lanes 1, 7, 13 – molecular mass marker (100 bp Step Ladder, Promega); lanes 10-11 – positive control, lane 12 – PCR no template control.

## Discussions

The untranslated region of TTV genome (3' and 5' UTR) is conserved among divergent isolates (Takahashi, 1998) and primers derived from this region were shown to detect more isolates compared to the coding region-derived primers (BIAGINI, 2001). For our study we selected primers derived from both untranslated region, (Ninomiya primers for TTV, TTMDV and TTMV from the 5'UTR and NG065 and NG148 from the 3'UTR of TTV) (OKAMOTO, 1999; NINOMIYA, 2008) and coding region (NG063 and NG152 from the HVR in TTV's ORF1) (NISHIZAWA, 1999). The amplification product of the coding region primers were expected to span ~850 nt of ORF1. Even though the quality and fragmentation degree of the nucleic acid appeared low, DNA purified from food characterized by high heterogeneity and by a certain degree of fragmentation may explain the lack of specific amplification of 850 nt.

Amplicons obtained with the Ninomiya protocol had a diffuse aspect in agarose gel. Moreover, the number of food samples found positive for TTVs was higher than expected, thus there were doubts whether the amplification was specific. Since the amplicons resulted were too short (70-110 nt) for Sanger amplicon sequencing, we resorted to the amplification of a larger region (~400 nt) of 3'UTR of the most common human anellovirus, TTV, using NG065 and NG148 primers.

Three of the amplified samples yielded enough templates for the sequencing reaction, even though the

molecular mass was lower than expected (lanes 4-6, Figure 2). Intragenomic rearrangements of TTV were speculated before (LEPPIK *et al*, 2007), thus we continued to sequence, considering it may be the case of an insertion/deletion polymorphism or even a new viral isolate.

Sequences obtained from the three samples of pork-based foods (i.e. pork salami, pork kaiser and, respectively, pork smoked sausage) proved to be derived from a non-coding region of the swine X chromosome.

Further, we tested all the samples with these primers to verify our initial findings. All samples yielded multiple non-specific amplifications, thus bands of anticipated molecular mass (~400 nt) were excised from gels, purified and re-amplified in the same conditions. From the ten samples subjected to purification and re-amplification, only two yielded the expected-size PCR product suitable for sequencing. The sequenced samples originated from turkey breast ham and, respectively, boneless skinless chicken breast fillets. The sequence obtained from the turkey breast ham sample resembled a fragment of *Meleagris gallopavo* (turkey) and *Gallus gallus* (jungle fowl) spinster homolog 3 mRNA (sequence ID XM\_003211673.2, XM\_004946447.1), while the sequence obtained from the chicken breast fillets resembled a fragment of *Pseudomonas sp.* genome (sequence ID CP003961.1). These organisms (with the exception of bacteria) were found on the labeling of the products from which the amplicons were obtained and were confirmed by real-time PCR.

**Table 2.** Properties of TTV primers in relation with pig, chicken and turkey genomes

Primer code	Primer sequence 5'-3'	Similarity with <i>Sus scrofa</i> sequences			Similarity with <i>Gallus domesticus</i> sequences		Similarity with <i>Meleagris gallopavo</i> sequences	
		Score; length	Acc. no.	Score; length	Acc. no.	Score; length	Acc. no.	
NG065	GCCGACGGTTTTTGGCGC CTTTTTTC	95%; 19/27	CU207411.5	100%; 15/27	XM_004941331.1	100%; 13/27	XM_003209971.2	
		93%; 15/27						
		100%; 11/27						
NG148	CGAAAGTGAGTGGGGCCA GACTTC	100%; 16/24	FN677326.1	100%; 17/24	XM_004947880.1	100%; 17/24	XM_010723521.1	
NG054	TTTGCTACGTACTAACCAC	94%; 17/20	CU695181.11	100%; 14/20	XM_004949244.1	94%; 17/20	XR_795955.1	
		100%; 10/20						
		100%; 10/20						
NG147	GCCAGTCCCGAGCCGAAT TGCC	100%; 14/23	XM_001926 939.4	100%; 14/23	XM_417823.4	100%; 15/23	XM_010715153.1	
NG133	GTAAGTGCACCTCCGAATG GCTGAG	100%; 14/25	XR_300020.1	100%; 15/25	NM_001007892.2	94%; 18/25	XM_010726125.1	
NG132	AGCCCGAATTGCCCTTGAC	100%; 15/20	CU855649.11	100%; 15/20	AC192285.3	100%; 14/20	JF275060.1	

Considering that our attempt to detect TTV DNA misresulted due to low specificity of the primers chosen, we matched them against the genomes of organisms most abundant in our food products. In table 2 we summarized the first hit obtained by performing BLAST against the genome of pigs, chickens and turkeys with the primers used in our study, as well as the other two studies reporting TTV DNA in food products, more specifically in dairy products (AL-MOSLIH, 2007; ROPERTO, 2009). All primer sequences showed regions of complementarity in the tested genomes, especially towards the 3' end. However, the difference in the results obtained in our study compared to the others may be due to the complex mixture of DNA purified from highly processed meats (e.g. baloney, sausages, mortadella) compared to milk or cheese.

These primer pairs may not amplify with high specificity the viral DNA in certain circumstances, such as those when complex mixtures of DNA are used as template and further investigations are needed in order to assess whether TTVs DNA is present in meat products and could be used as a viral marker for food safety. However, this study raise questions about the presence of TTV in the food chain and highlights the need for investigations of viral markers to ensure the safety of animal-sourced foods for human consumption.

## Conclusions

The methods of detection of human torque teno viruses DNA in food samples are not yet tuned in for high precision. The primers used in our study may not amplify with high specificity viral DNA in certain circumstances, such as those when complex mixtures of DNA are used as template (e.g. DNA purified from meat products). Further investigations are needed for determining whether TTVs are viral markers for the quality assessment of animal-sourced foods for human consumption.

## Acknowledgments

This paper was written in the frame of Core Programme PN 16 46 05 01.

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