Short Paper

Detection of *Torque teno midi virus/Small anellovirus* (TTMDV/SAV) in the sera of domestic village chickens and its vertical transmission from hen to eggs

Bouzari, M.[1] and Salmanizadeh, Sh.[2*]

[1]Division of Microbiology, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran; [2]MSc Student, Division of Microbiology, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

[*]***Correspondence:*** *Sh. Salmanizadeh, MSc Student, Division of Microbiology, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran. E-mail: sharare.salmanizade@yahoo.com*

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Summary

Although the infection of different animals and non-human primates with other members of *Anelloviridae* have already been reported there is no report about infection of animals with *Torque teno midi virus/Small anellovirs* (TTMDV/SAV). The aim of this study was to detect the virus in domestic village chickens. Blood samples were collected from 79 domestic village chickens in Isfahan. Blood samples of five adult laying hens and one cockerel were collected in three consecutive weeks (days 1, 8 and 14) as experimental chickens. Ten eggs were randomly collected from the eggs laid during days 12 to 17 and thin and thick egg whites and yolk samples were collected aseptically. After DNA extraction Nested-PCR was performed using SMAs/SMAr primers. In PCR, 431 bp and 441 bp products were detected. The detected bands were extracted and sequenced. Totally 26 out of 79 (32.9%) of the blood samples were positive for the virus. The frequency of the infection of the different parts of the eggs tested was 76%. For the first time TTMDV/SAV was detected in domestic village chickens which also vertically transmitted to eggs.

Key words: Torque teno midi virus/Small anellovirus (TTMDV/SAV), Domestic village chickens, Vertical transmission

Introduction

In 2005, two circular single-stranded DNA viruses were identified and due to the size of their genomic DNA (smaller than *Torque tenovirus* (TTV) and *Torque teno mini virus* (TTMV), they were named *Small anellovirus 1* (SAV1) and *Small anellovirus 2* (SAV2) (Jones *et al*., 2005; Chung *et al*., 2007) and placed in the unclassified genus of the family *Anelloviridae* (Biagini and De Micco, 2010). Ninomyia *et al*. (2009) identified *Torque teno midi virus* (TTMDV) as an intermediate between TTV and TTMV and showed that SAV1 and SAV2 are deletion mutants of TTMDV. In subsequent studies, these viruses were referred to as TTMDV/SAV viruses and placed in a third genus of the family *Anelloviridae* (*Gamma torquevirus*) (ICTV, 2009; Ninomyia *et al*., 2009; Burian *et al*., 2011; Salmanizadeh *et al*., 2012).

TTMDV/SAV DNA has been detected in individuals in Italy and France (Andreoli *et al*., 2006; Biagini *et al*., 2006) and also in serum samples in the Republic of Korea (Chung *et al*., 2007) and in chronic cervicitis and cervical tumor samples of humans in Iran (Salmanizadeh *et al*., 2012).

Although the infection of different animals (tupaias, cats, dogs and pigs) and non-human primates with other members of *Anelloviridae* have already been reported (Leary *et al*., 1999; Verschoor *et al*., 1999; Okomoto *et al*., 2001; Okomoto *et al*., 2002; Catroxo *et al*., 2008), there is no report about the infection of animals with this virus. Considering this and also the importance of possible pathogenicity of this virus for chickens and possible transmission of these viruses from animals to humans and vice versa, the aim of this study was to detect the presence of TTMDV/SAV virus in domestic village chickens and also possible vertical transmission of it from hen to eggs for the first time.

Materials and Methods

Blood samples

Blood samples were collected from brachial vein of 79 domestic village chickens from different villages around Isfahan city in Iran. The sera were collected and stored at -20°C till tested.

Experimental chickens

Five adult laying hens and one cockerel were collected from one village (Kabootar-Abad) and were kept in a room on concrete floor with rice husk as litter. Blood samples were collected from brachial vein in three consecutive weeks (days 1, 8 and 14). The study was approved by the local ethics committee.

Egg collection

Ten eggs were randomly collected from the eggs laid during days 12 to 17. They were washed with tap water

and then floated in 70% ethanol in three separate containers in three steps and dried with sterile cotton. Thick and thin egg whites and yolk samples were collected (1000 µl each) aseptically and were placed at -20°C till tested.

DNA extraction of serum samples

Serum DNA

Serum DNA was extracted according to Karimi-Rastehkenari and Bouzari (2010).

DNA extraction from different parts of egg samples

DNA of the egg samples (thin/thick white and yolk) were extracted according to Rovero *et al*. (2012).

PCR amplification

The primer sets used for amplification of TTMDV/SAV DNA (441 bp (SAV1) and 431 bp (SAV2)) included SMAs1/SMAr1 and SMAs2/SMAr2 primer sets in a Nested-PCR (Biagini *et al*., 2006; Han and Chung, 2006). In the first round of PCR 3 µl of DNA and in the second round 1 µl of the PCR products were used in a 25 µl reaction mixture containing 1 U Smar Taq Polymerase (Cinnagen, Iran), 0.4 µM of each primer, 240 µM of each dNTPs, 20 mM of Tris-HCL, 3 mM $MgCl_2$, 50 mM KCL.

Thermal cycling conditions in the first round were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s and an extension step at 72°C for 50 s. The amplification program was followed by a final extension step at 72°C for 5 min. Thermal cycling conditions in the second round of PCR were a repeat of the first round. PCR products were electrophoresed and then DNA envisioned under UV light (Salmanizadeh *et al*., 2012).

Positive controls

Positive controls used in this investigation were TTMDV/SAV positive human cervical tissues obtained in previous study (Salmanizadeh *et al*., 2012).

DNA sequencing and sequence analysis

The detected bands of about 431/441 bp were extracted from the agarose gel using Fermentase DNA extraction Kit K0513 (Fermentas, Germany) according to the manufacturer's instructions. Three of the PCR products of serum samples and 1 of thin white, 1 of thick white and 1 of yolk were randomly selected and the extracted DNA sequenced by Applied Biosystem 3730 DNA Analyzer (Geneservice, UK). The sequences were submitted in GenBank with accession numbers of JQ734977-JQ734978-JQ734979-JQ734987-JQ734988-JQ734989. A WU-BLAST-2 search of the determined sequences against the nucleotide sequence database (EMBL, European Bioinformatics Institute) was performed.

Molecular evolutionary analysis

Phylogenetic trees were constructed using the neighbor-joining method in MEGA5 software version 5.05 (Tamura *et al*., 2011) against *Torque teno midi virus* species 1, 2, and 3 sequences of the genus *Gamma torquevirus* of *Anelloviridae* obtained from GenBank and also the sequences already obtained from human samples in previous experiment (Salmanizadeh *et al*., 2012).

Results

In PCR, 431 bp and 441 bp products were recovered by gel electrophoresis (Fig. 1). As the size of the sequences were very close, the differentiation of SAV1 and SAV2 was not possible. Of 79 tested chicken blood samples, 26 (32.9%) were positive for TTMDV/SAV DNA.

The frequencies of the infection of the experimental chickens in different time intervals are shown in Table 1.

The frequencies of the infection of different parts of the eggs tested are shown in Table 2. The virus was detected in yolk, thick white and thin white of the eggs tested with variable frequencies.

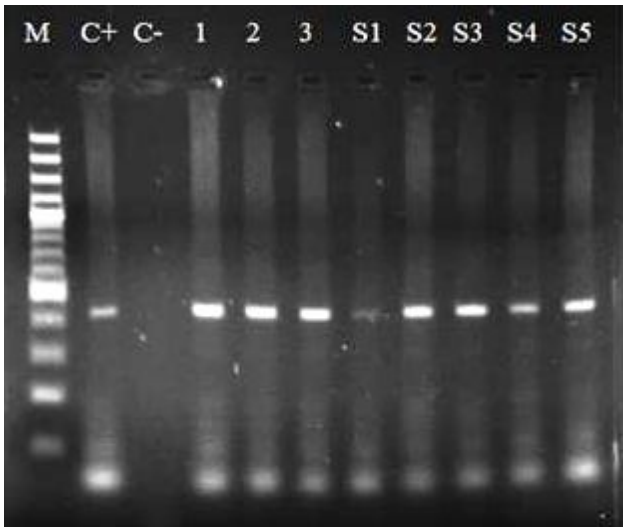

Fig. 1: Agarose gel electrophoresis showing PCR products of tested samples (1: Thin white, 2: Thick white, 3: Yolk, S1-S5: Serum samples, M: Marker; C^+, Positive control, and C^-: Negative control

Table 1: Frequency of the infection with the virus in experimental chickens

Infection	Frequency		
	Day 1	Day 8	Day 14
Positive	3	6	6
Negative	3	0	0
Percentage	50	100	100

Table 2: Frequency of infection of different parts of the eggs tested

Egg compartment	Frequency		Percentage
	Positive	Negative	
Yolk	5	5	50
Thick white	10	0	100
Thin white	8	2	80
Total	23	7	76

In Wu-BLAST-2 search, the sequences obtained showed high homology to TTMDV/SAV sequences already reported.

As it is shown in Fig. 2, except for sequence for thin white the other sequences obtained in this study were all placed in the same cluster with variable homologies. The virus isolated from thin white (SR1-THW) was placed in the same cluster with TTMDV1, TTMDV2 and TTMDV3. The highest homology was observed with TTMDV3.

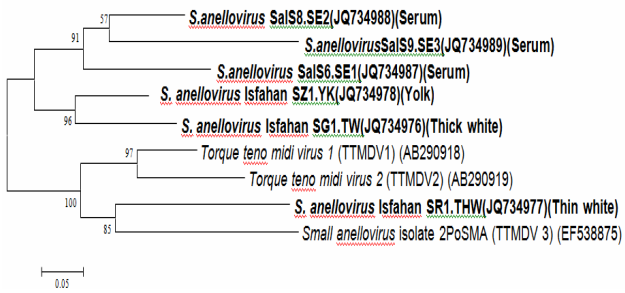

Fig. 2: Phylogenetic tree constructed against Torque teno midi virus species 1, 2, and 3 sequences of the genus *Gamma torquevirus* of *Anelloviridae*. The sequences obtained in this study are shown in bold text

The phylogenetic tree constructed to compare the chicken sequences and the human ones is shown in Fig. 3. Five of the chicken sequences were placed in the same cluster along with one of the human sequences which was somehow distant from the chicken ones. On the other hand, three of the human sequences along with one of the chicken sequences were placed in another cluster. The chicken sequence in this cluster was the same as the human one.

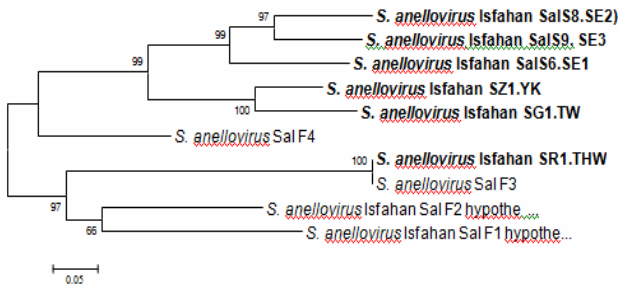

Fig. 3: Phylogenetic tree constructed against the sequences already obtained from human samples in our laboratory. The sequences obtained in this study are shown in bold text

Discussion

In this study, detection of TTMDV/SAV infection in the sera of domestic village chickens and its vertical transmission from hen to egg is reported for the first time.

Torque teno virus (TTV) of the *Anelloviridae* family has rarely been reported in chickens (Leary *et al*., 1999; Catroxo *et al*., 2008; Bouzari and Shaykhbaygloo, 2013). As there is no information about the infection with TTMDV/SAV in other animals, comparison with other data was not possible. Due to high diversity in TTMDVs reported, using different primer sets, different prevalences have been reported for the virus. So, the frequency of the virus reported in this study may differ if other primer sets designed for detection of the virus are used.

On day one, only 50% of the experimental chickens were positive for TTMDV/SAV but after seven days the rest of the chickens were infected, this indicated the horizontal transmission of the virus from infected to uninfected chickens most probably through fecal-oral route.

Considering the different stages of the egg development in chickens after ovulation, when the follicle moves through different parts of the oviduct, the source of contamination of the yolk with the virus might be before entering the oviduct or after moving through infandibulum (fertilization) and magnum where the albumen (egg whites) are added. So, the detection of the virus in both white and yolk shows that the ovary and also the oviduct can be the source of the virus detected (Table 2). Also, in phylogenetic analyses (Fig. 2) the isolates from the sera and different parts of the eggs are placed in the same cluster which can be considered as evidence of the vertical transmission of the virus from hen to egg.

The results showed that most of the viruses circulating in chickens are genetically distant from the human ones, but some of them are circulating in both human and chickens (sequence identified in thin white) in a low frequency (Fig. 3). To confirm this more sequencing data should be tested.

Possible complications caused by TTMDV/SAV without causing any clinical manifestation or disease during embryonic development and also after hatching should be investigated.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Andreoli, E; Maggi, F; Pistello, M; Meschi, S; Vatteroni, M; NelliL, C and Bendinelli, M (2006). Small Anellovirus in hepatitis C patients and healthy controls. Emerg. Infect. Dis., 12: 1175-1176.

Biagini, P and De Micco, P (2010). Anellovirus. In: Mahy, BWJ and VanRegenmortel, MHV (Eds.), *Desk encyclopedia of human and medical virology*. San Diego, Academic Press Linacre House. PP: 17-22.

Biagini, P; De Micco, P and De Lamballerie, X (2006). Identification of a third member of the anellovirus genus (‘‘small anellovirus’’) in French blood donors. Arch.

Virol., 151: 405-408.

Bouzari, M and Shaykh Baygloo, N (2013). Detection of Torque teno virus (TTV) in domestic village chickens in Iran. Vet. Res. Forum. 4: 55-58.

Burian, Z; Szabo, H; Szekely, G; Gyurkovits, K; Pankovics, P; Farkas, Tn and Reuter, G (2011). Detection and follow-up of Torque tenomidi virus (''small anello-viruses'') in nasopharyngeal aspiratesand three other human body fluids in children. Arch. Virol., 156: 1537-1541.

Catroxo, MHB; Nishiya, A; Sabino, E; Teixeira, PS; Petrella, S; Milanelo, L; Vieira, JCF and Diaz, RS (2008). Torque teno virus (TTV) is prevalent in Brazilian nonhuman primates and chickens (*Gallus gallus-domesticus*). Int. J. Morphol., 26: 363-372.

Chung, JY; Han, TH; Koo, JW; Kim, SW; Seo, JK and Hwang, ES (2007). Small anellovirus infections in Korean children. Emerg. Infec. Dis., 13: 791-792.

Han, T and Chung, JY (2006). Detection of small anellovirus DNA from blood products. Korean J. Blood Transfus., 17: 126-133.

ICTV virus toxomomy: 2014 release. Available at: http:// ictvonline.org/virusTaxonomy.asp

Jones, MS; Kapoor, A; Lukashov, VV; Simmonds, P; Hecht, F and Delwart, E (2005). New DNA identified in patients with acute viral infection syndrome. J. Virol., 79: 8230-8236.

Karimi-Rastehkenari, A and Bouzari, M (2010). High frequency of SEN virus infection in thalassemic patients and healthy blood donors in Iran. Virol. J., 7: 1-7.

Leary, TP; Erker, JC; Chalmers, ML; Desai, SM and Mushahwar, IK (1999). Improved detection systems for TT virus reveal high prevalence in humans, non human primates and farm animals. J. Gen. Virol., 80: 2115-2120.

Ninomyia, M; Takahashi, M; Hoshino, Y; Lchiyama, K; Simmonds, P and Okamoto, H (2009). Analysis of the entire genomes of Torque teno midi virus variants in chimpanzees: infrequent cross-species infection between humans and chimpanzees. J. Gen. Virol., 90: 347-358.

Okamoto, H; Nishizawa, T; Takahashi, M; Asabe, SH; Tsuda, F and Yoshikaw, A (2001). Heterogeneous distribution of TT virus of distinct genotypes in multiple tissues from infected humans. Virology. 288: 358-368.

Okamoto, H; Takahashi, M; Nishizawa, T; Tawara, A; Fukai, K; Muramatsu, U; Naito, Y and Yoshikawa, A (2002). Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaias. J. Gen. Virol., 83: 1291-1297.

Rivero, ERC; Neves, AC; Silva-Valenzulela, MG; Sousa, SOM and Nunes, FD (2006). Simple salting-out method for DNA extraction from formalin-fixed, paraffin-embedded tissues. Pathol. Res. Pract., 202: 523-529.

Salmanizadeh, SH; Bouzari, M and Talebi, A (2012). Detection of Torque teno midi virus/Small anellovirus (TTMDV/SAV) in chronic cervicitis and cervical tumors in Isfahan, Iran. Arch.Virol., 157: 291-295.

Tamura, K; Peterson, D; Peterson, N; Stecher, G; Nei, M and Kumar, S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.

Verschoor, EJ; Langenhuijzen, S and Heeney, JL (1999). TT viruses (TTV) of non-human primates and their relationship to the human TTV genotypes. J. Gen. Virol., 80: 2491-2499.