Short Communication

Preliminary epitope mapping of Torque teno sus virus 1 and 2 putative capsid protein and serological detection of infection in pigs

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The aim of this work is to identify antigenic regions within the ORF1 protein of *Torque teno sus virus 1* (TTSuV1) and *Torque teno virus sus 2* (TTSuV2) that could be used as antigens to detect virus-specific antibodies following infection in pigs. Protein sequences of TTSuV ORF1 genes were analysed to predict linear antigenic epitopes. Synthesized peptides were analysed for serological reactivity with swine sera. Such an antigenic region was identified at the C terminus of the ORF1 protein of both viruses and showed serological reactivity with 78 % (TTSuV1) and 88 % (TTSuV2) of swine sera. An ELISA with an immunodominant peptide as antigen was used to examine the sera of piglets, aged 4–20 weeks, and adults. Results indicated that TTSuV1- and TTSuV2-specific antibodies were detectable at 4 weeks. Antibody titres increased from week 10 and peaked at week 20. A relatively high antibody titre persisted to adulthood.

Torque teno (TT) viruses are small, non-enveloped viruses containing a single-stranded, negative-sense circular DNA genome enclosed within an icosahedral nucleocapsid (Okamoto *et al.* 2002). *Torque teno sus virus 1* (TTSuV1) and *Torque teno virus sus 2* (TTSuV2) belong to the genus *Iotatorquevirus* within the family *Anelloviridae* (ICTV, 2010). The first TT virus isolate was detected in a human Japanese patient with post-transfusion hepatitis of unknown aetiology (Nishizawa *et al.* 1997). Since then, TT viruses have been described in pigs, dogs, cats, bovines, ovines and chickens, as well as wild animals, such as tupaias and primates (Cong *et al.* 2000; Leary *et al.* 1999; Okamoto *et al.* 2001; Verschoor, *et al.* 1999).

The role of swine TT viruses in the pathogenesis of swine diseases is currently being investigated. Although it seems that TTSuV infection is not directly responsible for any disease, the possibility remains that they play a role in different co-infections (Cornelissen-Keijsers *et al.*, 2012; Taira *et al.*, 2009).

Porcine anelloviruses have been found in pig populations worldwide with high prevalence ranging from 24 % to 100 % (McKeown *et al.*, 2004; Bigarré *et al.*, 2005; Kekarainen *et al.*, 2006; Martelli *et al.*, 2006; Taira *et al.*, 2009; Gallei *et al.*, 2010; Huang *et al.*, 2010; Cortey et al., 2012). It seems that the infection in pigs is ubiquitous (Kekarainen & Segalés, 2009). TTSuVs have been also found in biological fluids such as semen, colostrum, nasal cavity secretions and faeces (Kekarainen *et al.*, 2007; Martínez-Guino *et al.*, 2009; Sibila *et al.*, 2009a), which suggests that transmission may occur by both horizontal and vertical routes (Martínez-Guino *et al.*, 2009; Pozzuto *et al.*, 2009; Sibila *et al.*, 2009a, b; Aramouni *et al.*, 2010). Viral prevalence increases with age and in most animals the infection can be persistent (Sibila *et al.*, 2009a, b; Taira *et al.*, 2009). New porcine TT virus species (named TTSuV1a and TTSuV1b) have been proposed based on new sequence data (Cornelissen-Keijsers *et al.*, 2012).

The role and significance of the serological response induced by porcine anelloviruses remain unknown. The first test allowing detection of specific antibodies in pigs was developed recently and is based on recombinant ORF1 protein of the TTSuV virus expressed in *E. coli* cells (Huang *et al.* 2011). The first antibody survey revealed a relatively high rate of seropositivity to TTSuV2 in conventional pigs from different sources but not in gnotobiotic pigs. This result correlates with the ubiquitous nature of TT viruses and their high prevalence as detected by PCR and quantitative PCR techniques (Gallei *et al.*, 2010; Huang *et al.*, 2010; Kekarainen *et al.*, 2006; Jarosova *et al.*, 2011). The main structural protein of the virus is the nucleocapsid protein (thought to be coded by ORF1), the largest structural protein of the virus particle. Thus far nothing is known about the nature and presence of B- and T-cell receptors responsible for the induction of immune response in infected animals.

The aim of this work was to identify linear antigenic regions within the ORF1 protein of TTSuV1 and TTSuV2, which could be used as serological markers to detect TT-virus-specific antibodies following natural infection in the pig population.

Peptides were selected according to properties increasing the probability that the structure will be exposed on the surface of the virus protein. For peptide analysis, TTSuV1 (AY823990, GQ120664, GU450331, GU456384) and TTSuV2 sequences (AY823991, GU456385, GU456386, GU188046), accessible in GenBank, were used. The ORF1 genes of both TT swine viruses were analysed by software provided on the 'Immune epitope database' website based on previously published algorithms for the prediction of linear epitopes from protein sequences. The following protein properties were analysed: surface accessibility (Emini et al., 1985), antigenicity (Kolaskar and Tongaonkar 1990), hydrophilicity (Parker et al., 1986) and the presence of linear epitopes (Larsen et al., 2006). Following software processing, all predicted epitopes were aligned with existing sequences of porcine TT viruses to reveal peptides localized in highly variable regions of the ORF1 gene. The sequences of peptides used in the present study are shown in Table 1; all peptides were synthesized by GeneCust Europe (Luxembourg), dissolved in distilled water (1 mg ml^{-1}) and stored at -20 °C. Selected and synthesized peptides covered more than 40 % of the length of the ORF1 protein.

To compare immunoreactivity of all analysed peptides, a peptide-based-ELISA was performed on a panel of 100 randomly selected swine sera. Sera with optical density (OD) equal to or higher than double the unrelated control peptide were considered positive. Sera that did not react with any of the tested peptides were considered as negative. The number of sera displaying serological reactivity with individual peptides is shown in Table 2.

The most strongly reacting peptides were identified as peptide TT1-9 (for TTSuV1) and peptide TT2-10 (for TTSuV2) indicating the presence of an immunogenic structure on the virus surface near the carboxy-terminal part of the protein. Peptides TT1-9 and TT2-10 were then used for all subsequent serological analyses.

Three hundred and seven pig serum samples were collected from 16 herds. For sampling, the following age categories of animals were chosen: 4 weeks ($n=17$), 7 weeks ($n=65$), 10 weeks ($n=37$), 13 weeks ($n=36$), 16 weeks ($n=40$), 18 weeks ($n=18$), 20 weeks ($n=15$) and adults ($n=79$).

Serological prevalence in different age categories of animals was determined by ELISA using the identified immuno-dominant peptides as the antigens. The antibody detection results for different age categories are shown in Fig. 1.

An optimized ELISA was used to examine the sera of piglets aged 4–20 weeks and in adult sows.

Detection of IgG antibodies indicated that TTSuV1 and TTSuV2 specific IgG antibodies were detectable in piglets at 4 weeks of age. After an initial decrease at week 7, antibody titres started to increase substantially at week 10 and peaked at week 20. A relatively high antibody titre persisted to adulthood. There were almost no differences between the dynamics of titres between the two viruses.

Wells of Nunc Maxisorp ELISA plates were coated overnight with peptides dissolved in carbonate buffer, pH 9.6 at 3 µg per well (as determined by chequerboard titration). After peptide coating, the wells were rinsed with PBS, then blocked with T-PBS (PBS, 0.1 % Tween 20, with 2 % skimmed milk) at 37 °C for 60 min. Sera were diluted 1 : 50 in T-PBS with 2 % skimmed milk. One hundred microlitres of serum was added to peptide-coated wells and incubated for 60 min at 37 °C. Subsequently, the plates were washed three times with T-PBS and 100 µl of anti-pig IgG Peroxidase Conjugate (Sigma) diluted 1 : 30 000 in T-PBS with 2 % skimmed milk was added to each well. The plates were incubated for 60 min at 37 °C. After washing the plates, 100 µl per well of the TMB-Complete substrate (3,3′,5,5′-tetramethylbenzidine; Test-Line, Prague, Czech Republic) was added. The OD was measured at 450 nm.

Table 1. Primary amino acid sequence of analysed peptides (left side, TTSuV1; right side, TTSuV2)

Immunodominant peptides are in bold.

Peptide	TTSuV1 ORF1 amino acid sequence	Peptide	TTSuV2 ORF1 amino acid sequence
TT1-1	DVTLEGLYQEHLLW	TT2-1	DIPCRPLPYQNLHPLLMLLKKQHKIVLSQQ
TT1-2	LVRYFGCVVYLYPLKD	TT2-2	VTDNPGNMATTFKASGGQHPDAI
TT1-3	RVPPQGDSELPKKKEFSTGT	TT2-3	KSEQDIKKEAHSAEISREYTRDPKSK
TT1-4	DNPNYNVQDNEEKN	TT2-4	SSNYTTTGSDQNSGGSTSAIQ
TT1-5	RKADQENPKVSTWPIEGTWNTQDT	TT2-5	QGGYVAYAGSGVIGAG
TT1-6	KLTLPKSPHDLDFG	TT2-6	QQGWPSNQNWPNTNRDKTNF
TT1-7	GEYQPPTGIRDPCVDTPAYP	TT2-7	TMLTLFGPFVEKA
TT1-8	VPQSGSITHPKFAGK	TT2-8	GGHGTERFKTNIGDPSTIPC
TT1-9	DSEAKGEETEETASSSSITS	TT2-9	PFEPGDRFHSGIQDPSKVQNT
TT1-10	AESSTEGDGSSDDEETIR	**TT2-10**	GQKTEKEPLSDSDEESVISS
		TT2-11	TSSGSSQEEETQRRRHHKPS

Table 2. Serological reactivity of analysed peptides with a panel of positive swine sera

Numbers indicate percentage of sera displaying reactivity with particular peptide, absorbance value intervals of positive sera and percentage of peptide similarity between strains.

Peptide TTSuV1	Reactivity (%)	Absorbance value intervals	Lowest similarity among TTSuV1* strains (%)	Peptide TTSuV2	Reactivity (%)	Absorbance value intervals	Lowest similarity among TTSuV2 strains (%)
TT1-1	30	0.135–0.253	86 (50)	TT2-1	76	0.112–0.468	87
TT1-2	17	0.133–0.174	88 (50)	TT2-2	60	0.126–0.240	61
TT1-3	39	0.126–0.263	93 (79)	TT2-3	60	0.117–0.211	73
TT1-4	60	0.136–0.496	80 (35)	TT2-4	80	0.111–0.239	58
TT1-5	17	0.126–0.157	43 (30)	TT2-5	56	0.112–0.205	50
TT1-6	34	0.130–0.362	63 (0)	TT2-6	25	0.105–0.231	70
TT1-7	26	0.122–0.219	90 (60)	TT2-7	64	0.118–0.0239	93
TT1-8	13	0.130–0.295	94 (54)	TT2-8	56	0.113–0.258	95
TT1-9	78	0.122–0.605	75 (40)	TT2-9	68	0.103–0.303	77
TT1-10	47	0.130–0.154	95 (78)	TT2-10	88	0.113–0.488	95
				TT2-11	72	0.103–0.265	95

*Numbers in parentheses refer to TTSuV1b strains.

Serum samples that were non-reactive with any of the tested peptides were considered negative and these sera were used to calculate the cut-off value of the ELISA.

The cut-off value was determined by defining the upper prediction limit based on the upper tail of the t-distribution of negative control OD readings, at a confidence level of 99.5 %. For this calculation, all negative sera were included. The cut-off was calculated as follows:

$$\text{cut-off} = X + \text{SD} \, t \sqrt{1 + \frac{1}{n}}$$

where X is the mean of independent control sera readings, SD is the standard deviation, t is the $(1 - \alpha)$th percentile of the one-tailed t-distribution with $n - 1$ degrees of freedom and n is the number of independent controls. Positive serum with an absorbance corresponding to the calculated cut-off was included in all test plates.

The absorbance of the calculated weak positive serum was considered to be titre 1. Titres of examined sera were then calculated as a ratio of the sample absorbance : absorbance of weak positive serum.

Intra- and inter-assay variabilities were checked to assess the reproducibility of the ELISA. Intra-assay variability was determined on a set of 20 duplicated samples on the same plate (a total of 40). The intra-assay coefficient of variation was calculated as follows: (mean of the SD of the duplicates/ means of the duplicates) × 100. A precision of 10 % or less was considered as satisfactory. Inter-assay variability was determined using one serum on duplicate samples in ten different runs. The inter-assay coefficient of variation was calculated from the formula: (SD of the means of the duplicates/mean of the duplicates) × 100. A precision of 10–15 % was considered as satisfactory.

Repeatability of the ELISA test was compared using control sera. The intra-assay variability was calculated to be 9.75 % (for TTSuV1 assay) and 9.5 % (for TTSuV2 assay), and the inter-assay variability between runs was 9 % (for TTSuV1 assay) and 13 % (for TTSuV2 assay). These results confirmed that the repeatability of ELISA tests is sufficient for diagnostic tests.

TT viruses in pigs as well as in another animal species represent an interesting group of viruses with unknown pathogenesis and clinical significance. Recent research in the field of TT viruses in pigs has brought new information about the spread of TT viruses in the pig population and levels of viraemia determined by the quantitative PCR technique (Brassard et al., 2010; Huang et al., 2010; Nieto et al., 2012). Only recently was the recombinant protein derived from the putative capsid protein of TTSuV2 developed and used in the detection of virus-specific antibodies (Huang et al., 2011).

Our work demonstrates that peptides derived from the sequence of TTSu viruses can be used in serological detection of this swine infection. All peptides tested in our work were designed following in silico analysis and peptides localized in the proximity of the C-terminal part of the
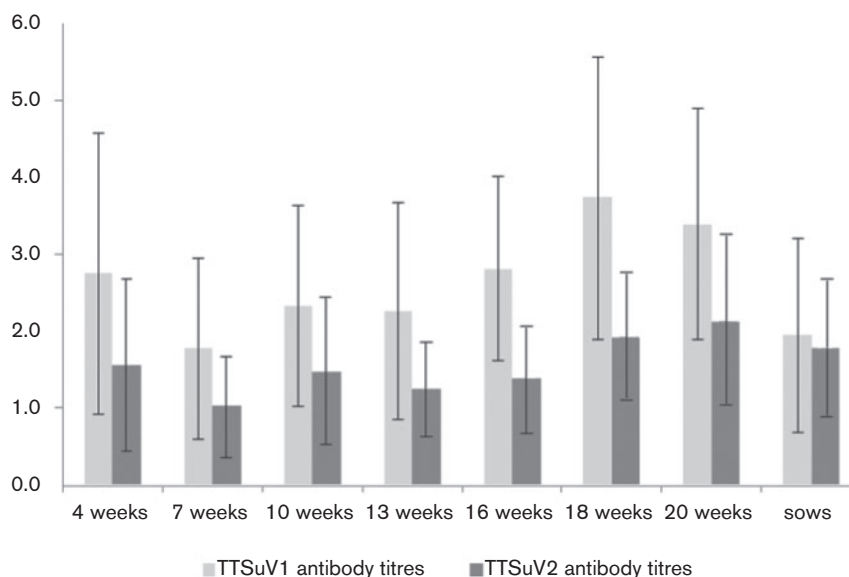
Fig. 1. Prevalence of TTSuV1 and TTSuV2 seropositive animals and antibody titres in different age categories. Cut-off value is equal to 1. Bars represent variability of titres (standard deviations).

nucleocapsid protein were shown to exhibit serological reactivity with highest percentage of swine sera and represent antigenic structure on the protein surface. This immunoreactive domain was found in both TT virus species.

Although remaining computed and tested peptides were also predicted to be localized on the ORF1 protein surface, they displayed weaker serological reactivity probably either due to the variability of a particular part of the protein or a partial sterical hindrance. Remarkably, some of the peptides were recognized by few pig sera. However, it is questionable whether these sequences can be considered as relevant antigenic epitopes.

The identified immune-dominant structures in both viruses displayed the most favourable combination of calculated values determining surface localization, and were situated in the most conserved part of the protein structure. Peptides covering this antigenic structure were used as an antigen in an indirect ELISA test to evaluate serological reactivity of different age categories of pigs.

Because control negative or positive sera were not available, we established sera showing no reactivity with any of the tested peptides as negative control sera. These sera were then used to determine the cut-off value for our diagnostic ELISA.

Levels of TTSuV specific antibodies were detected in all age categories of animals, which is in agreement with results obtained using a recombinant ORF1 antigen (Huang et al., 2011) in which TTSuV2 specific antibodies were detected in pigs from conventional farms. Also recently published serological data (Xiao et al., 2012) have shown that the proportion of seropositive pigs increased with age at least in the case of TTSuV2.

Another study by Huang et al. (2012) demonstrated correlation of IgG antibody levels between anti-TTSuV1a and -TTSuV1b indicating potential antigenic cross-reactivity. Analysis of the amino acid sequence of antigenic peptide (TT1-9) has shown the presence of a conserved area of seven amino acids (GEETEEG) between TTSuV1a and TTSuV1b, which could be responsible for observed cross-reactivity.

Serum examination of some individual weaned piglets showed an IgG antibody decrease in the first two weeks of the study. This initial decrease can be considered as a decrease in residual maternal antibodies, which could be normally present in weaned piglets for several weeks.

After week 7, the IgG antibody titre started to increase, probably as a result of immune system stimulation caused by replicating virus. The increase in the IgG antibody titre was evident up to week 20. Similar dynamics of antibody titres in conventional piglets have been observed by Huang et al. (2011). The subsequent decrease of antibody titre in adult animals is likely due to gradual virus neutralization by virus-specific antibodies together with the activation of cell-mediated immunity. This concept of TTSuV2 control by virus-neutralizing antibodies has been suggested by comparing antibody titres and virus load in experimentally exposed pigs (Huang et al., 2011). Antibodies were also detected in sows indicating the continued presence of viral replication despite the presence of neutralizing antibodies.

In all serum samples, antibodies to both viruses were detected, indicating co-infection by TTSuV1 and TTSuV2. Again, similar co-infection was previously diagnosed by the PCR virus detection method in recent work (Jarosova et al., 2011).

By studying the antibody response in naturally infected pigs over a period of 20 weeks, we have established that the TT1-9 and TT2-10 peptides could be considered as serological markers for natural TTSuV infection since all pigs had detectable antibodies to the end of the observation period and maintained it to adulthood. Although we have revealed the presence of immune-dominant epitope in the ORF1 virus protein, the localization of the neutralizing epitope still remains to be determined.

Acknowledgements

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