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Analyzing the genetic diversity of teschoviruses in Spanish pig populations using complete VP1 sequences

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ABSTRACT

Porcine teschoviruses (PTVs) have been previously shown to be the most abundant cytopathic viruses found in swine feces. In the present study, the diversity of PTVs was studied, using PTV isolates collected between 2004 and 2009 in a wide territory in Spain. In order to characterize genetically the isolates, phylogeny reconstructions were made using maximum likelihood and Bayesian inference methods, based on the 1D (VP1) gene, and including sequences available in public databases. The phylogenetic trees obtained indicated that PTVs present 12 main lineages, 11 corresponding to the PTV serotypes described to date, and one lineage distinct from the rest.

The geographic distribution of the different lineages does not seem to be strongly associated to particular territories, and co-circulation of multiple lineages was found in the same geographic areas. Nevertheless, some spatial structuring of the viral populations studied is indicated by the differences found between Spanish samples with respect to other European countries. A coalescent-based approach indicated that mutation may have been the main factor in originating the genetic diversity observed in the VP1 gene region. This study revealed a high diversity of teschoviruses circulating in the pig populations studied, and showed that molecular analysis of the complete VP1 protein is a suitable method for the identification of members of the porcine teschovirus group. However, further analyses are needed to clarify the geographical structuring of the different PTV populations.

1. Introduction

Porcine teschoviruses (PTV, genus *Teschovirus*, family *Picornaviridae*) are non-enveloped RNA viruses that infect swine populations. PTVs were originally misclassified as "porcine enteroviruses", but modern molecular analytical tools revealed that they differ significantly from the enterovirus group (Kaku et al., 2001). Complete genome sequence studies defined 11 distinct porcine teschovirus serotypes (PTV1–PTV11) (Zell et al., 2001). Similarly to other members of the family *Picornaviridae*, the teschovirus genome consists of a single-stranded RNA of positive polarity approximately 7.0–7.2 kb long which is translated into a unique polyprotein. This polyprotein is processed to yield eight viral polypeptides: four structural polypeptides (1A or VP4, 1B or VP2, 1C or VP3 and 1D or VP1) and four non-structural polypeptides (2A, 2B, 2C and 2D). While VP1–3 are exposed to the surface of the virion, VP4 is internal, and interacts with the RNA molecule that is protected by the capsid shell.

Teschoviruses are transmitted by the fecal-oral route, and different studies indicate that PTVs are abundant and ubiquitous in healthy pig populations (La Rosa et al., 2006). In fact, PTVs are generally non-pathogenic, and infected swine most often remain asymptomatic (Knowles, 2006; OIE, 2008). However, some virulent variants can cause a variety of clinical conditions, the most severe of which is a non-suppurative viral encephalomyelitis known as Teschen disease. The first epizootic of this disease was recorded in the Czech city of Teschen in 1929 (Trefny, 1930), and during the 1940s and 1950s the disease spread throughout Europe and other continents and caused enormous losses to the pig production industry. Subsequently, milder forms of the disease were reported in Talfan, Wales and Denmark (Harding et al., 1957). In recent years, several outbreaks have occurred again in Asia (Wang et al., 2010; Yamada et al., 2004; Zhang et al., 2010), America (Bangari

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et al., 2010; Pinto et al., 2010; Salles et al., 2011), Africa and Oceania (OIE, 2008). Although most Teschen disease outbreaks are usually caused by strains of porcine teschovirus serotype 1 (PTV-1), no clear link has been found between pathogenicity and serotype so far (Wang et al., 2010; Zhang et al., 2010). Given that outbreaks only occur sporadically in different countries, global programs of surveillance, control and eradication of this infectious disease are not implemented, and vaccines are currently not being produced.

Porcine teschoviruses are frequently found in swine fecal samples, and they are highly stable in environmental conditions (Mahnel et al., 1977); which makes PTVs optimal markers of swine fecal contamination in environmental samples (Jimenez-Clavero et al., 2003). During a wide survey carried out in Spain in 2004–2005, Buitrago et al. (2010) found that 47% of the 97 cytopathic viruses isolated from 600 pig fecal samples were PTVs. These observations suggest that PTVs are highly prevalent in asymptomatic pig populations, and raise questions about their evolution, adaptation to their hosts and role in nature. An assessment of the genetic diversity is of utmost importance to define key evolutionary factors such as the effective mutation and recombination rates and the spatial distribution of the different PTV viruses. Furthermore, knowledge on the phylogenetic relationship between different PTV strains may be useful in order to infer deeper epidemiological links.

The objective of this study was to characterize the genetic diversity found in PTVs of different provinces of Spain by introducing a new approach based on the analysis of the complete VP1 protein-coding gene, including the C-terminal region. A large collection of field samples from a wide geographic area during the years 2004–05 and 2009 were included in the analyses. The VP1 sequences obtained were compared to other VP1 sequences available in GenBank, and phylogenetic analysis were performed to assess the relationship between them in order to infer the evolutionary history of this group of viruses, which would in turn eventually clarify their still unclear epidemiology. Finally, the study aimed at defining whether the genetic diversity observed in PTVs mainly results from mutation or recombination events.

2. Materials and methods

2.1. Source of prototype virus and field samples

A total of 82 field PTV isolates from pig fecal samples were analyzed in this study. Field samples consisted of viral isolates from porcine feces collected in farms during an epidemiological surveillance program for swine diseases carried out in Spain. Most of the samples ($n = 74$) were collected during 2004–2005 and had been investigated in an already published study, where details on sample collection, virus isolation and RT-PCR analysis, were described (Buitrago et al., 2010). In the present work, these isolates were analyzed together with a small number of additional PTV isolates ($n = 8$) obtained in 2009 and processed as in (Buitrago et al., 2010). Field isolates were identified as PTV by a real-time RT-PCR method as described (Cano-Gomez et al., 2011). Prototype strains of porcine teschovirus 1–7 were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy. Porcine teschovirus 8–11 prototype strains were obtained from the Institut für Virologie und Antivirale Therapie, Jena, Germany (Table 1).

Table 1
Porcine teschovirus prototype strains used in this study.

Virus	Serotype	Strain	GenBank accesión n°
Teschovirus	PTV-1	PS34	AF296105
	PTV-2	O 3b	ND
	PTV-3	O 2b	AF296088
	PTV-4	PS 36	AF296088
	PTV-5	F 26	AF296090
	PTV-6	PS 37	AF296091
	PTV-7	WR2	GQ293237
	PTV-8	UKG 173/74	AF296093
	PTV-9	Vir 2899/84	AF296094
	PTV-10	12/15 Ge	AY392547
	PTV-11	Dresden	AF296096

2.2. Cell culture and propagation of field viruses

Field virus samples were propagated in the IB-RS_2 cell line (De Castro, 1964) following standard procedures. Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% (v/v) fetal bovine serum. Cells were supplemented with 2% fetal bovine serum for viral propagation. Cell monolayers (25 cm^2 flasks) were inoculated with 1 ml of each isolate and incubated at 37 °C, 5% CO_2 until cytopathic effect was developed (24–72 h post-infection). After three freeze–thaw cycles, each isolate was clarified prior to storage at −70 °C until used.

2.3. RNA extraction and RT-PCR amplification of VP1

Total RNA was automatically extracted from 100 μl of virus-infected cell culture fluid as described (Sotelo et al., 2009). As the sequence encompassing the whole VP1 and flanking regions is highly variable between PTV serotypes and strains, a strategy based on the sequential application of four different RT-PCR methods (numbered 1–4), all aimed at the amplification of the whole VP1, but using different target regions, was adopted (Table 2). Each method comprised two external primers designed to amplify the whole VP1 sequence, and in three of them, two internal primers enabled sequencing of the central portion of the VP1 region when necessary. Seven oligonucleotide primer pairs were designed specifically for this work (Table 2) using aligned nucleotide sequences from 71 strains representing all teschovirus genotypes. A first screening of the viral isolates was carried out with RT-PCR 1 (primers I2, V2, II2 and VI2). When the first RT-PCR reaction failed, then RT-PCR 2 was attempted, and so on. All RT-PCR reactions were performed using One Step RT-PCR Kit (Qiagen) following the manufacturer's instructions. Reaction tubes contained 2 μl template RNA, 1 μl enzyme mix and 22 μl reaction mix (1× Q solution containing 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 U RNAse inhibitor and 0.6 μM of each primer). Reverse transcription and polymerase chain reactions were performed sequentially in one step, consisting of a first reverse transcription at 50 °C for 30 min, followed by 15 min at 95 °C, and 40 cycles of 94 °C for 30 s, 54–58 °C (depending upon the primers used) for 1 min, and 72 °C for 1 min, with a final extension step of 7 min at 72 °C. The resulting amplified DNAs were visualized by electrophoresis in 2% agarose gel stained with ethidium bromide, next to negative (RNase-free water) and positive (reference strains) controls.

2.4. Nucleotide sequencing and phylogenetic analysis

Amplified cDNAs were purified using ExoSAP-IT kit (GE Healthcare), and then bidirectionally sequenced by automatic dideoxy cycle sequencing techniques, Big Dye Terminator (version 3.1) Cycle Sequencing Kit, in an ABI 3730 XL DNA Analyzer (Applied Biosystems), using the same primers sets of the RT-PCR assays. All the VP1 nucleotide sequences obtained in this work were deposited in GenBank (Accession numbers: JF23984–JF24047). Sequences were assembled using SeqMan program (DNASTAR, Lasergene)