Seroprevalence of St-Valérien-like caliciviruses in Italian swine

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The family *Caliciviridae* is composed of small non-enveloped viruses approximately of 35 nm in diameter with positive-polarity, ssRNA genomes of 7.4–8.3 kb. The family includes the genera *Vesivirus, Lagovirus, Norovirus, Sapovirus* and the new genus *Nebovirus* (Green *et al.*, 2000; Carstens, 2010). In addition, a number of unclassified caliciviruses including rhesus enteric calicivirus (*Recoverivirus*), St-Valérien-like viruses in swine and chicken caliciviruses (Cubitt & Barrett, 1985; Farkas *et al.*, 2008; L’Homme *et al.*, 2009; Wolf *et al.*, 2011) have been identified recently. Noroviruses (NoVs) and sapoviruses (SaVs) have emerged as the leading agents of food- and waterborne, acute, non-bacterial gastroenteritis in humans worldwide (Green *et al.*, 2001).

Viruses genetically and antigenically closely related to human NoVs and SaVs have also been isolated from pigs and has raised public health concerns of potential cross-species transmission (Wang *et al.*, 2005; Mattison *et al.*, 2007; Martella *et al.*, 2008). Recently, novel porcine caliciviruses, designated St-Valérien-like viruses (SVLVs), have been detected from finisher pigs in Canada and USA (L’Homme *et al.*, 2009; Wang *et al.*, 2011). By phylogenetic and pairwise distance analyses on complete genomic sequences, SVLVs are segregated on a unique branch within the genera *Norovirus* and *Sapovirus* and to investigate the seroprevalence of SVLV in pigs.

A total of 614 serum samples collected between December 2010 and March 2011 from swine in North and Central Italy were tested for antibodies against SVLVs. Sixty sera (six farms) were collected from post-weaning pigs (3–6 months), 162 (29 farms) from grower pigs (7–10 months), 246 (two slaughterhouses) from finisher pigs (9–12 months) and 146 (15 farms) from sows (10–24 months). VLPs were synthetized using the RNA of the SVLV strain 25A/ITA. The recombinant protein self-assembled in virus-like particles (VLPs) that were used to set up an ELISA kit to assess the seroprevalence of these novel caliciviruses in swine. Antibodies specific for St-Valérien-like virus were detected in 63 (10.3%) of 614 serum samples tested with titres ranging from 1 : 50 (28.6%) to 1 : 800 (40.7%). These results indicate that St-Valérien-like infections are common among domestic pigs, Italy.

St-Valérien-like viruses are newly recognized porcine caliciviruses recently detected in North America and Europe. In this study, baculovirus-expressed virus-like particles of the St-Valérien strain 25A/ITA were generated and used for the development of an antibody-detection ELISA kit to assess the seroprevalence of these novel caliciviruses in swine. Antibodies specific for St-Valérien-like virus were detected in 63 (10.3%) of 614 serum samples tested with titres ranging from 1 : 50 (28.6%) to 1 : 800 (40.7%). These results indicate that St-Valérien-like infections are common among domestic pigs, Italy.

However, reliable diagnostic assays and structured surveillance investigations are required to fill in the information gaps inherent for the prevalence and epidemiology of these novel porcine caliciviruses. In this study, we expressed in the baculovirus system the capsid protein of the SVLV strain 25A/ITA. The recombinant protein self-assembled in virus-like particles (VLPs) that were used to set up an ELISA kit and to investigate the seroprevalence of SVLV in pigs.

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promoter. The correct orientation of the insert was analysed by PCR and restriction enzyme digestion. The construct containing the full-length coding sequence capsid protein was co-transfected with linearized baculovirus DNA (BD Biosciences). The recombinant baculovirus was selected using X-Gal blue/white screening and plaque purified on Spodoptera frugiperda (Sf21) cells (King & Possee, 1992), as described previously (Di Martino et al., 2007).

For large-scale production of VLPs, 100 ml Sf9 cells (1 × 10^6 cell ml^{-1}) suspension culture (King & Possee, 1992) was infected with the recombinant baculovirus at an m.o.i. of 3 p.f.u. per cell. Assembled VLPs were separated from the culture medium of infected cells at 48 h post-infection by centrifugation at 2683 g for 20 min. The recombinant capsid protein was concentrated by ultracentrifugation through a 17% sucrose cushion in TEN-buffer (100 mM NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM EDTA) and purified on a discontinuous 20–60% (w/v) sucrose gradient, as described previously (Di Martino et al., 2010). The gradients were fractionated by bottom puncture and aliquots of each fraction were analysed by SDS-10% PAGE, and visualized by Coomassie brilliant blue staining. Gradient fractions containing a band of approximately 55 kDa, corresponding in size to the predicted molecular mass of the capsid protein of SVLV (L’Homme et al., 2009), were selected.

The antigenicity of the recombinant capsid protein was assessed by Western blotting (WB) (Fig. 1), using sera (~20) collected from a farm with animals found to shed SVLV in the stools (Di Martino et al., 2011).

VLP morphology was assessed by negative staining electron microscopy (EM) observation. Gradient fractions containing the purified protein were prepared using the Airfuge method (Lavazza et al., 1990). Briefly, the fractions (80 µl) were ultracentrifuged (Airfuge Beckman) for 15 min at 82,000 g using a rotor holding six 175 µl test tubes with specific adapters for 3 mm carbon-coated Formvar copper grids. Grids were then stained using 2% sodium phosphotungstic acid, pH 6.8, for 1.5 min and observed with a Philips CM-10 electron microscope operating at 80 kV, at ×19–25,000 (Fig. 2a and b). Immunoelectron microscopy (IEM) was used to assess VLP-specific antigenicity. Drops (50 µl) of each gradient fraction were incubated (37 °C for 1 h) with equal amounts of a WB-positive swine serum, diluted 1:20 (Fig. 2c).

Protein concentration of VLP preparations was determined by measuring the optical density at 280 nm (OD_{280}) and by running aliquots on SDS-10% PAGE containing BSA standards.

For the development of the antibody-detection ELISA, purified VLPs were coated onto 96-well EIA plates (Costar)

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**Fig. 1.** WB analysis of the recombinant capsid protein of the SVLV strain 25A/ITA. Lane 1, Precision Plus protein standards (Bio-Rad); lane 2, Sf9 mock-infected cells; lane 3, capsid protein of the SVLV 25A/ITA purified from the supernatants of Sf9 insect cells.

**Fig. 2.** EM analysis of the SVLV VLPs (a and b), IEM analysis of VLPs after incubation with a positive serum for SVLVs (c). Bar, 100 nm. The arrows indicate VLPs with typical morphology of caliciviruses.
at 100 μl per well (final concentration of 8 μg ml⁻¹) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and incubated at 4 °C overnight, as described previously by Di Martino et al. (2010). After blocking with 1 % BSA in PBS buffer at room temperature for 2 h, the VLP-coated microplates were incubated with 100 μl swine serum samples (diluted to 1:50 in PBS) at 37 °C for 1 h. Plates were washed three times in PBS with 0.1 % Tween 20 (PBST) and then incubated with HRP-conjugated anti-swine goat IgG (1:10 000) (Sigma-Aldrich) for 30 min at 37 °C. Plates were washed three times in PBST before adding 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate; ABTS) substrate and incubating at room temperature for 20 min. Absorbance was measured at 405 nm. The cut-off point of the test was established as the mean of the OD₄₀₅ readings of 50 WB-negative swine sera plus 2 SD (OD₄₀₅ >0.5). A positive/negative ratio (OD₄₀₅ on VLPs/OD₄₀₅ on mock-infected cells) >1.7 was used to evaluate background binding. In order to assess the antibody titres in SVLV-positive sera, all the sera with OD₄₀₅ >0.5 (at dilution of 1:50) were serially diluted twofold in PBS. Mean ELISA antibody titres were calculated and expressed as the reciprocal of the highest serum dilution with a positive absorbance. A χ² Fisher’s exact test was used to determine significant differences among different age groups. A P-value of <0.05 was considered statistically significant.

The SVLV VLPs were not recognized by genogroup (G) I and GII NoV-specific antibodies in a commercial antigen detection ELISA (Ideia Norovirus; Oxoid). In addition, they were not recognized in ELISA plates coated with the SVLV VLPs (OD₄₅₀ <0.5) and in WB, by rabbit hyperimmune sera raised against the prototype lion GIV.2 strain Pistoia/387/06/ITA (Martella et al., 2007) and against the vaccine feline calicivirus strain F9 (Di Martino et al., 2007), using antigen concentrations of 0.1–1 μg ml⁻¹.

Out of 614 serum samples tested, 63 (10.3 %) contained antibodies against SVLVs with titres ranging from 1:50 (28.6 %) to 1:800 (40.7 %). The seroprevalence rate in slaughtered pigs (13.4 %) was higher than in sow (8.9 %) and grower pigs (9.2 %), while the prevalence in post-weaning pigs (3.3 %) was significantly lower than in any other group.

The age-related prevalence of antibodies against SVLV was further examined. Seropositive rates gradually increased with age from 3.3 % (2/60) in the 3–6 months age group to 8.4 % (18/214) in the 7–10 months age group, to 12.6 % (43/340) in pigs more than 10 months old (Supplementary Table S1, available in JGV Online). The difference in estimated prevalence was statistically significant when percentages of seropositive pigs younger or older than 10 months were compared (P=0.0433).

Similarly, the titre of antibodies against SVLV increased with age and peaked (1:800) after 10 months of age (Fig. 3). ELISAs based on VLPs have been used successfully to gather information on the epidemiology of non-cultivatable enteric caliciviruses in humans and animals (Jiang et al., 2000; Glass et al., 2000; Peasey et al., 2004; Farkas et al., 2005). In this study, using baculovirus-generated VLPs of the SVL strain 25A/ITA, the seroprevalence for SVLVs was investigated in pigs of various ages during the productive cycle. SVLV-specific antibodies were detected at various prevalence rates and in several porcine herds, suggesting firmly that these novel caliciviruses circulate in swine populations in Italy.

Age-related differences in the prevalence of SVLV-specific antibodies were observed, with the highest rates in adult pigs, suggesting age-restricted patterns of susceptibility to infection. In a similar fashion, the prevalence of NoV-specific antibodies in humans seems to follow age-related patterns (Gray et al., 1993; Jing et al., 2000). The seroprevalence rates usually drop by 4–9 months of age, when maternal antibodies decrease, and then gradually increase to 100 % in the adult population (Jiang et al., 2000; Peasey et al., 2004). Similar age-related patterns of seroprevalence variations have been observed for recoviruses in macaques (Farkas et al., 2010).

Of interest, seroprevalence was higher in slaughtered animals than in sows, although the difference was not statistically significant. A possible explanation for this could rely in differences in housing and management conditions between the two animal groups or in the sampling inclusion criteria and the relatively small number of samples analysed.

Noteworthy, the overall prevalence rate for SVLV observed in this study (10.3 %) was markedly lower than the rates reported for other calicivirus infections in humans and animals, with prevalence peaking to 100 % in some studies (Jiang et al., 1995; Deng et al., 2003; Farkas et al., 2006). Several hypotheses could be suggested as possible explanations, chiefly geographical or temporal variations, or, again, difference in sampling inclusion criteria and size.

In conclusion, the findings of this study provide insights into the epidemiology of SVLVs in swine and collect further evidence that infections of these novel viruses occur mainly in adult animals. Future investigations are necessary to assess the pathogenic potential of SVLVs by experimental
infections in germ-free pigs. In addition, since these novel caliciviruses appear to be genetically more related to recoviruses and NoVs, SVLV-specific ELISAs could be used to assess whether these viruses may pose a zoonotic risk for humans.

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