Virus Research 251 (2018) 1–5

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Seroprevalence of sapovirus in dogs using baculovirus-expressed virus-like particles

Irene Melegari[a], Fulvio Marsilio[a], Federica Di Profio[a], Vittorio Sarchese[a], Ivano Massirio[b], Andrea Palombieri[a], Anna Rita D'Angelo[c], Gianvito Lanave[d], Georgia Diakoudi[d], Alessandra Cavalli[d], Vito Martella[d], Barbara Di Martino[a,*]

[a] Faculty of Veterinary Medicine, Università degli Studi di Teramo, Italy
[b] Azienda USL di Reggio Emilia, Reggio Emilia, Italy
[c] Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale", Teramo, Italy
[d] Department of Veterinary Medicine, Università Aldo Moro di Bari, Valenzano, Italy

ARTICLE INFO

Keywords:
Canine sapoviruses
Virus like-particles
IgG antibodies
Dogs

ABSTRACT

Caliciviruses of the *Sapovirus* genus have been recently detected in dogs. Canine sapoviruses (SaVs) have been identified in the stools of young or juvenile animals with gastro-enteric disease at low prevalence (2.0–2.2%), but whether they may have a role as enteric pathogens and to which extent dogs are exposed to SaVs remains unclear. Here, we report the expression in a baculovirus system of virus like-particles (VLPs) of a canine SaV strain, the prototype virus Bari/4076/2007/ITA. The recombinant antigen was used to develop an enzyme-linked immunosorbent assay (ELISA). By screening an age-stratified collection of serum samples from 516 dogs in Italy, IgG antibodies specific for the canine SaV VLPs were detected in 40.3% (208/516) of the sera. Also, as observed for SaV infection in humans, we observed a positive association between seropositivity and age, with the highest prevalence rates in dogs older than 4 years of age.

1. Introduction

Sapoviruses (SaVs) are small non-enveloped viruses of approximately 30–35 nm in diameter, classified in the *Sapovirus* genus, within the *Caliciviridae* family (Carstens, 2010). The SaV icosahedral capsid surrounds a 7.3–7.5 kb positive-sense single-stranded RNA genome organized in two open reading frames (ORFs). ORF1 encodes a polyprotein of ~2300 amino acids (aa) that undergoes protease processing to produce several nonstructural proteins, including a RNA-dependent RNA polymerase (RdRp) and a capsid protein (VP1). ORF2 encodes a small protein (VP2) with an unknown function. A third ORF (ORF3), overlapping the 5′ end of the capsid gene, is present in some human SaVs (Hansman et al., 2007).

SaVs have been identified as causative agents of diarrhoea in humans and piglets (Flynn and Saif, 1988; Martella et al., 2008a; Pang et al., 2009). In humans they cause acute gastroenteritis in all ages in both outbreaks and sporadic cases (Oka et al., 2015).

Based on the full-length VP1 sequence, SaVs have been classified into five genogroups (GI–GV). More recently, fourteen additional genogroups (GVI–GXIX) have been proposed (Scheuer et al., 2013; Yinda et al., 2017), but only GI, GII, GIV and GV are known to infect humans. SaVs have been found in several mammalian species (Yinda et al., 2017), but strains genetically closely related to human SaVs have been identified only in chimpanzee (GI) (Mombo et al., 2014), rodents (GII) (Firth et al., 2014), California sea lions (GV) (Li et al., 2011a,b) and pigs (GV and GVIII) (Martella et al., 2008b; Scheuer et al., 2013).

SaVs have been occasionally found in stools collected from dogs (Li et al., 2011a,b; Soma et al., 2014; Bodnar et al., 2016). The first canine SaV has been detected by metagenomic approach in a dog with enteritis in USA (Li et al., 2011a,b). Sequence analysis of the full-length capsid protein revealed that these SaVs, that appeared more similar to GII of human and rodent strains (mean aa identity of 48.0–56.0%) than to other SaV genogroups, segregated into a monophyletic cluster, tentatively named as GXIII (≥60% pairwise aa inter-genogroup identity) (Scheuer et al., 2013; Bodnar et al., 2016). To date, the circulation of these novel SaVs in dog populations has been documented only in two molecular investigations based on collection of rectal swabs from diarrhoeic and clinically healthy puppies. In both studies SaV infections have been found only in dogs with enteritis, with rates ranging from 2.0% to 2.2% (Soma et al., 2014; Bodnar et al., 2016). These rather low prevalence rates were of difficult interpretation and they could be accounted for host-restricted patterns of susceptibility to SaV infections or

* Corresponding author.
E-mail address: bdimartino@unite.it (B. Di Martino).

https://doi.org/10.1016/j.virusres.2018.04.014
Received 17 February 2018; Received in revised form 17 April 2018; Accepted 20 April 2018
Available online 24 April 2018
0168-1702/

could be subsequent to the genetic variability of the virus. Indeed, genetic distance calculations using the 0.8 kb portion of the polymerase region, commonly employed as target of molecular diagnostic tools for caliciviruses, revealed that these group of SaVs are genetically highly heterogeneous (77.6% to 99.5% aa identity) (Bodnar et al., 2016). Whether these canine SaVs are common in dogs or whether they were detected serendipitously, is still unclear. In order to start filling this gap and to understand more in depth the epidemiology of these viruses, we screened an age-stratified collection of canine sera by using an enzyme-linked immunosorbent assay (ELISA) based on virus-like particles (VLPs) of the canine SaV strain Bari/4076/2007/ITA (Bodnar et al., 2016).

2. Materials and methods

2.1. Serum sample collection

A total of 516 serum samples were collected between March 2013 and July 2015 by a convenience

sampling of household dogs admitted to veterinary clinics from different Italian regions. The samples were divided in six age groups: < 1 year, 1–3 years, 4–6 years, 7–9 years, 10–12 and > 12 years of age.

2.2. Construction of the recombinant baculovirus

The full-length VP1 capsid gene of the canine SaV strain Bari/4076/2007/ITA (Genbank accession no. KT207828) (Bodnar et al., 2016) was amplified by reverse transcription-PCR (RT-PCR) using forward primer VP1_Start (5′-AGA GGA TCC ACC ATG GAG GGC CCC AAG CCC A-3′) and reverse primer VP1_Stop (5′-AGA GGA TCC CTA GAC ATT ACG ACG GAC A-3′), both including recognition sites for *Bam*H1. The amplified gene was cloned into the *Bam*HI site of the transfer vector pAcYM1 (Matsuura et al., 1987) under the control of the polyhedrin promoter. The correct orientation of the insert was evaluated by PCR and sequence analysis. Recombinant vector pAcYM1 with VP1 gene was purified and co-transfected into *Spodoptera frugiperda* (*Sf21*) cells with linearized baculovirus DNA (BacPAK6, Bsu36 I digest, Clontech), using Cellfectin II Reagent (Invitrogen Ltd, Milan, Italy). The recombinant baculovirus was plaque purified on *Sf21* cells and selected using X-Gal blue/white screening.

2.3. Production of virus-like particles (VLPs) of the canine SaV

For baculovirus amplification, each plaque was resuspended in 500 µl TC-100 medium (Invitrogen Ltd, Milan, Italy) and subjected to 3 rounds of amplification in fresh Sf9 insect cells. The expression of VP1 capsid protein was analyzed by SDS-12% polyacrylamide gel electrophoresis (PAGE), followed by Coomassie brilliant blue staining. A time course (from day 1 to 8) was performed to optimize the expression of the recombinant protein. For large-scale production of VLPs, 100 ml *Sf9* cells (1×10^6 cell/ml) suspension culture, were infected with the recombinant baculovirus at a multiplicity of infection of 3 plaque forming units/cell. The culture medium after separation from the cell debris at 96 h post-infection (PI), 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 to 60% (wt/vol) sucrose gradient, as previously described (Di Martino et al., 2010). The gradients were fractionated by bottom puncture, and aliquots of each fraction were analysed by SDS-12% PAGE. Gradient fractions containing a band of approximately 60 kDa, corresponding in size to the predicted molecular weight of the capsid protein of the canine SaV, were assessed by negative staining transmission electron microscopy (TEM) (Zeiss, EM 900) observation, to evaluate the VLPs morphology. The concentration of VLPs (~ 100 µg/ml) was determined by measuring the optical density at 280 nm (OD_{280}) and by running aliquots of purified VLPs on SDS-12% PAGE containing bovine serum albumin (BSA) standards.

2.4. Antibody-detection ELISA

For development of the ELISA, mock infected *Sf9* insect cells and SaV VLPs (final concentration of 1 µg/ml) were coated in 96 well EIA plates (Costar, Italy) at 100 µl per well in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plates were incubated at 4 °C overnight. The wells were washed five times with 0.1% Tween-Phosphate-Buffered Saline (PBS-T) and then blocked with 200 µl of PBS containing 1% BSA at room temperature for two hours. After washing five times with PBS-T, each dog serum sample (100 µl), diluted to 1:100 in PBS, was added to the antigen-coated wells, and the plates were incubated at 37 °C for 1 h. Plates were washed five times with 0.1% PBS-T and then incubated with horseradish peroxidase-conjugated goat anti-dog IgG (Sigma-Aldrich, Italy) at 1:5000 dilution for 30 min at 37 °C. The plates were washed five times in PBS-T prior to the addition of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate (Invitrogen Ltd, Milan, Italy). Each reaction was completed by incubation at room temperature for 20 min, and the absorbance was measured at 405 nm. The cut-off point of the test ($OD_{405} \geq 0.5$) was established as the mean of the OD_{405} readings of 50 dog serum samples negative in western blotting (WB) for the presence of SaV specific antibodies, plus 2 standard deviations, using antigen concentration of 1 µg/ml. Mock infected *Sf9* insect cells were used to obtain a positive/negative ratio (OD_{405} of SaV VLPs/ OD_{405} of mock infected *Sf9* insect cells ≥2) to evaluate the background binding.

2.5. Evaluation of cross-reactivity between the canine SaV and other caliciviruses

In order to investigate the antigenic relationships between the canine SaV VLPs and other caliciviruses, we assessed by ELISA the reactivity of rabbit hyperimmune sera raised against the feline calicivirus (FCV) vaccine strain F9 (Di Martino et al., 2007) and against carnivore noroviruses (NoVs) GIV.2, strain lion/Pistoia/387/06/ITA and GVI.2, strain dog/FD53/2007/ITA (Di Martino et al., 2010, 2017) with the SaV antigen. Each hyperimmune serum was 2-fold diluted starting at initial dilution of 1:50 until 1:3,200, using an antigen concentration of 1 µg/ml.

2.6. Statistical analysis

The data were analysed using Prism Graphpad Software. Fisher's exact test was used to determine the differences in prevalence among the age groups. A P value of < 0.05 was considered statistically significant.

3. Results and discussion

The full-length VP1 capsid protein gene of the canine SaV strain Bari/4076/2007/ITA, (Bodnar et al., 2016) was cloned into a baculovirus transfer vector to generate a recombinant baculovirus. By SDS-12% PAGE, a band with a molecular mass of ~60 kDa corresponding in size to the SaV VP1 protein, was observed in the supernatant of *Sf9* insect cells from 48 h PI (Fig. 1A). The antigenicity of the recombinant VP1 was confirmed by WB (Fig. 1B), using a field serum collected from one dog resulted positive in RT-PCR for the presence of canine SaV RNA (Bodnar et al., 2016). Electron microscopy (EM) analysis revealed that the SaV VP1 protein was able to assembly in VLPs with a diameter of approximately 35 nm (Fig. 2).

The canine SaV VLPs were employed to develop an antibody detection ELISA in order to investigate the prevalence of IgG antibodies in an age-stratified canine population. Out of 516 sera, a total of 208 (40.3%) reacted with the SaV VLPs at a dilution of 1:100 with an OD_{405}