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# Presence of Antibodies against Genogroup VI Norovirus in Humans

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## Abstract

**Background:** Noroviruses are important enteric pathogens in humans and animals. Recently, we reported a novel canine norovirus (CaNoV) in dogs with diarrhea belonging to a new genogroup (GVI). No data are available on exposure of humans to this virus.

**Methods:** Sera from 373 small animal veterinarians and 120 age-matched population controls were tested for IgG antibodies to CaNoV by a recombinant virus like particle based enzyme-linked immunosorbent assay.

**Results:** Antibodies to CaNoV were found in 22.3% of the veterinarians and 5.8% of the control group ( $p < 0.001$ ). Mean corrected OD<sub>450</sub> values for CaNoV antibodies were significantly higher in small animal veterinarians compared to the control group.

**Conclusions:** These findings suggest that CaNoV may infect humans and small animal veterinarians are at an increased risk for exposure to this virus. Additional studies are needed to assess if this virus is able to cause disease in humans.

## Introduction

Noroviruses (NoVs) are the leading cause of epidemic and sporadic acute gastroenteritis in humans with worldwide an estimated 1 million hospitalizations and up to 200,000 deaths in children < 5 years of age each year [1,2]. Outbreaks occur in various settings including long-term care facilities, hospitals, schools, restaurants and cruise ships. The main modes of transmission of NoV are person-to-person and through the consumption of contaminated food or water. During outbreaks, however, multiple transmission routes may play a role [3]. In recent years NoVs have been detected in a number of mammalian species and several studies have suggested that zoonotic transmission from animal to humans may occur [4-6] and that an animal reservoir might be the source of the introduction of new strains in the human population. Although no zoonotic events have been reported, there are several indications that NoVs may be able to cross the species barrier. Gnotobiotic pigs have been experimentally infected with a human NoV strain [4], and

viruses closely related to human NoVs have been detected in swine [7]. Moreover, NoV sequences have been detected in livestock and in retail meat samples highlighting a possible route for indirect zoonotic transmission of NoVs through the food chain and the risk for emergence of animal/human recombinants [8].

Noroviruses are a group of non-enveloped, single-stranded, RNA viruses with an icosahedral capsid symmetry classified into the genus *Norovirus* of the family *Caliciviridae*. They can be grouped in at least 5 different genogroups (designated GI-GV) [1,9]. Strains infecting humans are found in GI, GII and GIV. Porcine NoVs are classified in distinct genotypes within GII, bovine and ovine viruses belong uniquely to GIII, and murine NoVs are grouped in GV. Recently, several research groups have reported NoVs in domestic carnivores with diarrhea [10,11]. Canine NoVs (CaNoVs) genetically related to GIV have been reported in Italy, Greece and Japan [10-13], whereas viruses belonging to a proposed new genogroup (GVI) were found in fecal samples from dogs with diarrhea in Portugal and Italy [9,14-16].

The zoonotic potential of an infectious disease agent has been inferred by comparing pathogen-specific antibody levels between individuals that are in close contact

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with a particular animal and a matched control population with no professional exposure to animals [17,18]. For example, a higher serum antibody level against bovine NoV was detected in large animal veterinarians compared to the general population, indicating that bovine NoV strains could infect the human population [18]. Additionally, antibodies to human NoVs have been detected in pigs highlighting the possibility of human-to-animal transmission of NoV [5].

In most industrialized countries, pets are an integral part of the household leading to well-documented health risks associated with owning a pet. Bites, scratches and allergies are more frequent; however, infections including parasitic, bacterial, fungal and viral diseases can be transmitted to humans [19]. In a recent report, human NoV sequences were detected in fecal samples from pet dogs which had been in direct contact with humans with NoV gastroenteritis, suggesting that human NoVs can at least survive in the gastrointestinal tract of dogs [20].

To investigate if CaNoV may infect humans, sera from pet veterinarians and age-matched population controls were tested for IgG antibodies to recombinant virus-like particles of CaNoV. This Study Protocol was published elsewhere [21].

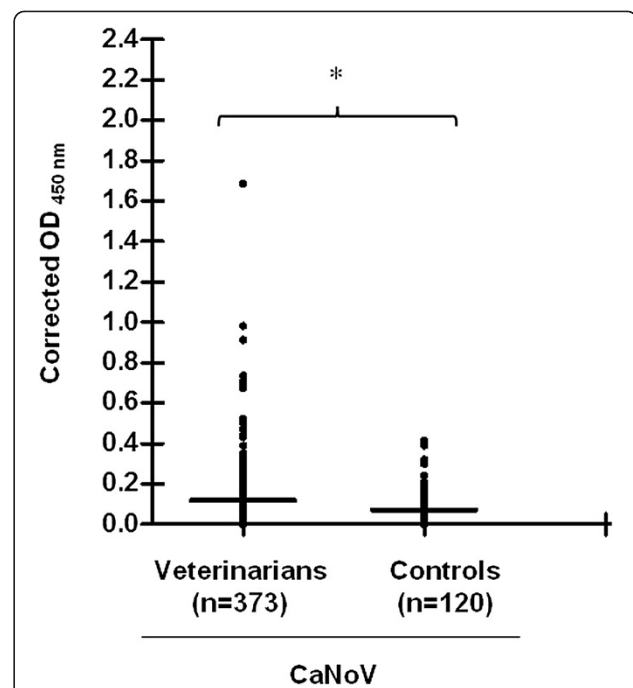
## Results

Of the 373 veterinarians, 83 (22.3%) had IgG antibodies against CaNoV compared to 7 (5.8%) of the 120 matched population controls ( $p < 0.001$ ). Moreover, the mean corrected OD<sub>450</sub> values for CaNoV antibodies was significantly higher in veterinarians than in controls ( $p < 0.001$ ) (Figure 1). CaNoV antibodies were detected in veterinarians from all four countries.

To evaluate possible cross-reactivity between these antibodies and human NoV, two serum samples from veterinarians with high (HAT) and low antibody titers (LAT) to CaNoV were pre-incubated with 2-fold serial dilutions of CaNoV VLPs. The corrected OD<sub>450</sub> values for CaNoV antibodies in both serum samples decreased significantly with increasing concentration of CaNoV VLPs ( $\beta = -0.150 \pm 0.043$ , 95%CI  $-0.287$  to  $-0.013$ ,  $p < 0.05$  and  $\beta = -0.073 \pm 0.018$ , 95%CI  $-0.132$  to  $-0.014$ ,  $p < 0.05$  for serum HAT and LAT, respectively) (Figure 2A). By contrast, no significant change in the corrected OD<sub>450</sub> values was observed when the pre-incubated sera were tested for the presence of GII.4 New Orleans antibodies ( $\beta = -0.011 \pm 0.031$ ; 95%CI  $-0.11$  to  $0.089$  and  $\beta = 0.0219 \pm 0.056$ ; 95%CI  $0.158$  to  $0.202$  for samples 68 and 25, respectively) (Figure 2B).

## Discussion

We detected IgG antibodies against CaNoV in 22.3% of the small animal veterinarians and in 5.8% of the age-matched population controls. These findings suggest

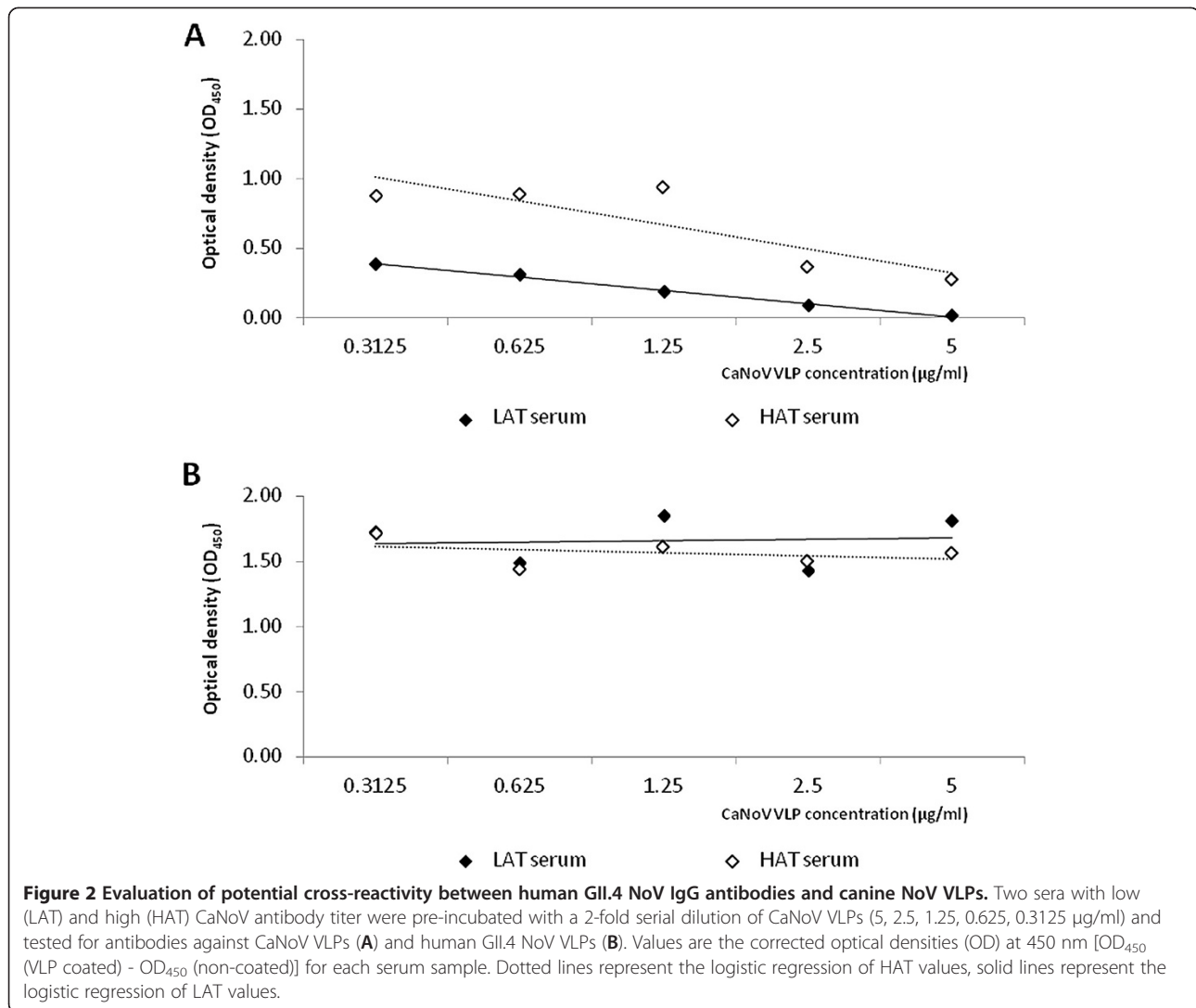


**Figure 1** Corrected optical densities [OD] at 450 nm for canine norovirus antibodies in sera from veterinarians and controls.

Sera were tested for the presence of CaNoV antibodies in a VLP-based ELISA at 1:1,500 dilution. Values are the corrected optical densities (OD) at 450 nm [OD<sub>450</sub> (VLP coated) - OD<sub>450</sub> (non-coated)] for each serum sample and the mean corrected OD<sub>450</sub> value of each group (horizontal bars). Groups were compared and analyzed by Mann-Whitney U-test. Differences were considered significant (\*) at  $p < 0.05$ .

that CaNoV may infect humans and that small animal veterinarians are at an increased risk for exposure and possibly infection with this virus. The presence of antibodies in the population control samples may be explained by household contact as dogs are popular pet animals.

An increased exposure risk to bovine NoV has been reported for large animal veterinarians in the Netherlands who had a higher seroprevalence of bovine NoV antibodies than the general population [18]. Conversely, a high prevalence of antibodies against human NoV (Norwalk strain) was detected in pigs and captive juvenile macaques [5,22]. These data suggest that NoV may be able to cross the species barrier. Exposure to zoonotic agents is a widely recognized risk in veterinary medicine. In an Australian survey, 4% of veterinarians reported having acquired at least one zoonotic disease from animal-related exposure [23]. In another study, IgG antibodies against *Brucella* spp and *Coxiella burnetii* were higher among veterinarians working in an endemic region [24]. The zoonotic risk of veterinarians and pig handlers for hepatitis E virus (HEV) infections has been



demonstrated in studies in Taiwan and the US [25,26]. In the Taiwan study, 27% of pig handlers tested positive for anti-HEV antibodies compared to 8% of the control subjects [25], while in the US study 26% of veterinarians working with swine and 17% of blood donors were seropositive for HEV, suggesting that veterinarians may be at a higher risk of HEV infection through animal contact, compared to normal blood donors [26].

A limitation of our study was that the antibodies detected by the CaNoV VLPs may be cross-reactive against human NoVs. However, the blocking assay data showed that binding of CaNoV antibodies but not human NoV antibodies, could be blocked by CaNoV VLPs, demonstrating the VLP-based ELISA used in this study measured CaNoV-specific antibodies.

In conclusion, our data suggest that CaNoV may infect humans and that small animal veterinarians are at an increased risk. Studies that test human stool samples in

households with dogs with CaNoV diarrhea are needed to confirm that this virus is able to cause diarrheal disease in humans.

## Materials and methods

### Serum samples

A total of 373 pet veterinarians from four different countries (Portugal, Spain, Brazil, and United Kingdom) who attended the Annual Veterinary Meeting in January 2012 in Santa Maria da Feira, Portugal, were enrolled in the study after giving informed consent (Table 1). Blood was obtained by venipuncture from all enrolled veterinarians. In addition, 120 sera matched by age (in 5-year age groups) and sex were collected from anonymous volunteers from the University of Porto. This study was approved by the institutional review board at the University of Porto (Parecer n°18/CEUP/2011).

**Table 1 Descriptive epidemiology of 373 veterinarians participating in the study**

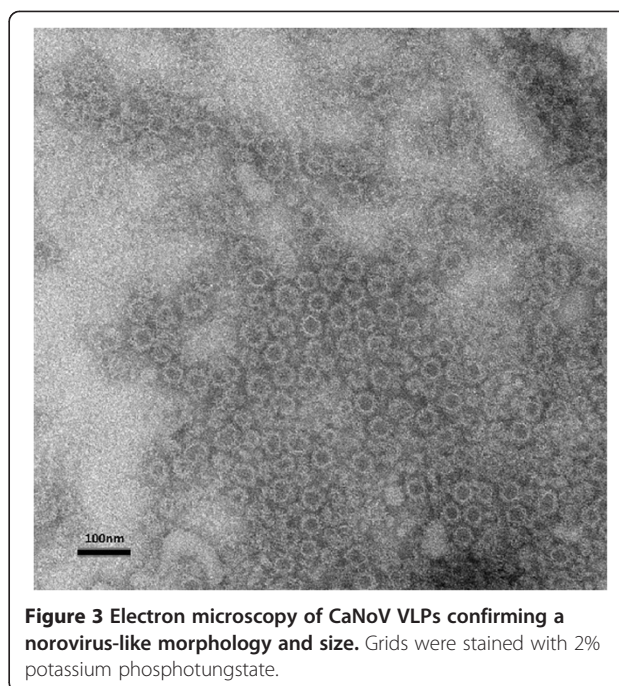
	% (N <sup>1</sup> )	95%CI <sup>2</sup>
Age		
19-29	51.7 (193)	46.7-56.8
30-39	38.8 (145)	33.9-43.8
40-49	7.8 (29)	5.1-10.5
≥50	1.7 (6)	0.3-2.9
Sex		
Female	70.5 (263)	65.9-75.1
Male	29.5 (110)	24.9-34.1
Residence		
Foreign <sup>3</sup>	6.4 (24)	3.9-8.9
Portugal	93.6 (349)	91.1-96.1
Years in practice		
1-10	80.2 (299)	76.1-84.2
11-20	16.1 (60)	12.4-19.8
>20	3.7 (14)	1.8-5.7

<sup>1</sup>Number of individuals in each category; <sup>2</sup>Confidence Interval; <sup>3</sup>Foreign: residents of Brazil, Spain or United Kingdom.

#### Canine norovirus VLP-based antibody ELISA

Recombinant virus-like particles (VLPs) of CaNoV [dog/C33/Visseu/2007/PRT, GenBank accession number: GQ443611.1] were produced by cloning full-length VP1/VP2 (ORF2 and ORF3 of the genome) in a baculovirus-insect cell expression system. Recombinant VLPs were recovered from the culture media and purified through sucrose and CsCl gradients [27]. Norovirus morphology and size of the purified VLPs was confirmed by electron microscopy (Figure 3).

Canine NoV VLPs (0.25 µg per well) were coated into 96-well microtiter plates (NUNC, Milford, USA) in carbonate-bicarbonate buffer (0.01 M, pH 9.6), and incubated overnight at 4°C. Coated plates were washed with PBS/0.5% Tween-20 and blocked with PBS/0.5% Tween 20/ 5% non-fat dry milk (blocking buffer) for 2 h at 37°C. Serum samples were diluted 1:1,500 in blocking buffer and tested in duplicate in VLP-coated and non-coated wells, to correct for sample background. After 1 hour incubation at 37°C, bound IgG was detected by peroxidase-labeled goat anti-dog IgG (H+L) (1:12,800) and TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, USA). Background signal [OD<sub>450</sub> (non-coated wells)] was subtracted from each sample to obtain a corrected OD<sub>450</sub> [OD<sub>450</sub> (VLP coated) - OD<sub>450</sub> (non-coated)]. Cut off value of the test was established as the mean of the OD<sub>450</sub> (non-coated wells) plus 3 standard deviations (3SD). A serum sample was considered positive when the corrected OD<sub>450</sub> was higher than the cut off.



**Figure 3 Electron microscopy of CaNoV VLPs confirming a norovirus-like morphology and size.** Grids were stained with 2% potassium phosphotungstate.

#### Blocking assay

Two serum samples with high (HAT) and low antibodies levels (LAT) against CaNoV and high levels of anti GII.4 New Orleans antibodies were pre-incubated with 2-fold serial dilutions of CaNoV VLPs (5, 2.5, 1.25, 0.625, 0.3125 µg/ml) for 1 h at 37°C. After incubation, 50 µL of pre-incubated sera were tested in duplicate for the presence of CaNoV antibodies as described above, and for the presence of human NoV antibodies using GII.4 New Orleans VLPs [Hu/GII.4/New Orleans1805/2009/USA, GenBank accession number: GU445325.2] with slight modifications (coating with 0.0625 µg per well and detection of bound IgG by peroxidase-labeled goat anti-human IgG (H+L) (1:12,800)).

#### Statistical analysis

A  $\chi^2$  test for unequal odds with Yates' continuity correction was used to determine significant differences in CaNoV prevalence between study groups. Mann-Whitney U-test was used to assess differences in CaNoV antibody magnitude between study groups. P values less than 0.05 were considered statistically significant. Statistical analyses were performed with R software [28].

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

JM conceived the study, collected the sera, carried out the immunoassays and drafted the manuscript. VC helped design the immunoassays, and helped draft the manuscript. JC cloned and sequenced the canine norovirus strain. SL produced the virus-like particles. MSJN conceived the study and participated in the design of the study. JV participated in the design and



coordination of the study and drafted the manuscript. All authors read and approved the final manuscript.

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The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. This article did receive clearance through the appropriate channels at the CDC prior to submission.

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