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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

A SEROPREVALENCE STUDY OF ANTIBODIES AGAINST A NOVEL CANINE NOROVIRUS IN CANINE AND HUMAN POPULATIONS

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Este trabalho foi realizado no Laboratório de Microbiologia do Departamento de Ciências Biológicas da Faculdade de Farmácia da Universidade do Porto sob a orientação da Professora Doutora Maria de São José Garcia Alexandre.

Os resultados apresentados no presente trabalho fazem parte das seguintes comunicações em encontros científicos, apresentadas em anexos:

J.R. Mesquita, **I. Delgado**, M.S.J. Nascimento. “Seroprevalence of a Novel Canine Norovirus in pet dogs”. **IV Encontro de Jovens Investigadores da Universidade do Porto.** Porto, Portugal, 17-19 Fevereiro, 2011 (P-398).

J.R. Mesquita, **I. Delgado**, M.S.J. Nascimento. “Zoonose por Norovirus Canino: seroprevalência em veterinários e população geral”. **VII Congresso Hospital Veterinário Montenegro.** Santa Maria da Feira, Portugal, 12-13 Fevereiro, 2011

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ABSTRACT

Noroviruses are recognized as the leading cause of gastroenteritis worldwide among persons of all ages (Patel *et al.* 2009). They have a wide degree of genetic variability and are classified in 5 genogroups (GI-GV) three of which (GI, GII and GIV) contain human viruses (Mesquita *et al.* 2010). The most important modes of transmission are person-to-person contact and consumption of contaminated food, however, a zoonotic transmission has been suggested as a possibility due to the close genetic relatedness between human and some animal NoV (Mattison *et al.* 2007). A novel canine NoV (Viseu strain) was recently identified in Portugal and classified as genetically unrelated to any other animal or human norovirus known (Mesquita et al. 2010).

In this study, the seroprevalences for IgG antibodies against canine norovirus were determined for dogs from 14 European countries and from the USA. The Portuguese human population was also tested for IgG antibodies against canine norovirus and IgG antibodies against human norovirus and seroprevalences were determined for veterinarian and general populations.

A total of 780 canine serum samples and 493 human serum samples (373 veterinarians and 120 general population) were obtained and tested with an in-house VLP-based ELISA.

Overall, 69.2% of dogs tested positive for IgG anti-canine norovirus which supports the hypothesis that canine norovirus are circulating widespread throughout Europe and in the USA. The age of the dog was identified as a risk factor, with young dogs having a 16 fold increased odd of seropositivity to canine norovirus.

The veterinary professionals were found to have a higher risk for seropositivity to canine norovirus than the general population. Age, interaction with dogs while children and stick injury by a needle contaminated with dog blood were identified as risk factors for seropositivity to canine norovirus antibodies in the veterinarian population of Portugal.

AGRADECIMENTOS

Queria aproveitar este momento de conclusão de uma etapa para agradecer a todas as pessoas que se cruzaram comigo e me acompanharam neste percurso e em particular,

À Prof. ª Dr. ª Maria de São José Garcia Alexandre pela recepção, orientação e apoio dados.

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Aos meus amigos.

Às minhas famílias, a directa e a “amigável”.

Foram indispensáveis para que eu conseguisse chegar aqui.

Muito obrigada.

ABBREVIATIONS

aOD	adjusted odds ratio
aa	amino acid
χ^2	chi-square
CI	confidence interval
cOR	crude odds ratio
DEN	Denmark
ELISA	enzyme-linked immunosorbent assay
et al.	*et ally*
FIN	Finland
FR	France
G	genogroup
GER	Germany
HBGA	histo-blood group antigen
HUN	Hungary
Ig	immunoglobulin
ICTV	International Committee on Taxonomy of Viruses
IRE	Ireland
IT	Italy
LR	likelihood ratio
µL	microlitre
mL	mililitre
mM	milimolar
nm	nanometer
NE	Netherlands
NOR	Norway
ORF	open reading frame
OD	optical density
PBS	phosphate buffered saline
POL	Poland
PT	Portugal
P-domain	protrusion domain

Ref	reference level
S-domain	shell domain
SRSV	small round-structured viruses
Na^2CO^3	sodium carbonate
$NaHCO^3$	sodium bicarbonate
STAT	signal transducer and activator of transcription
SWE	Sweden
SWI	Switzerland
UK	United Kingdom
USA	United States of America
VP1	major viral protein
VP2	minor structural protein
VPg	genome-linked viral protein

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INTRODUCTION

Norovirus

The *Norovirus* genus belongs to the *Caliciviridae* family (Green *et al.* 2007). This family was initially proposed by Burroughs and Brown (1974) and described in the 3rd Report of the International Committee on Taxonomy of Viruses (ICTV) in 1979 (Barry *et al.* 2008, Thiel & König 1999). Until then the caliciviruses formed a *genus* belonging to the *Picornaviridae* family. The decision to classify the caliciviruses in a separate family was based on their different morphologic characteristics (size, viral particles density), capsid composition (caliciviruses have only one protein composing its capsid) and replication process from the picornaviruses. Based on sequence data and phylogenetic analysis the *Caliciviridae* family has recently been divided in 5 genera: *Vesivirus, Lagovirus, Norovirus*, *Sapovirus* and *Nebovirus*, a genus recently established by the ICTV including the Newbury Agent 1 strain as the type species (Figure 1; Bank-Wolf *et al.* 2010).

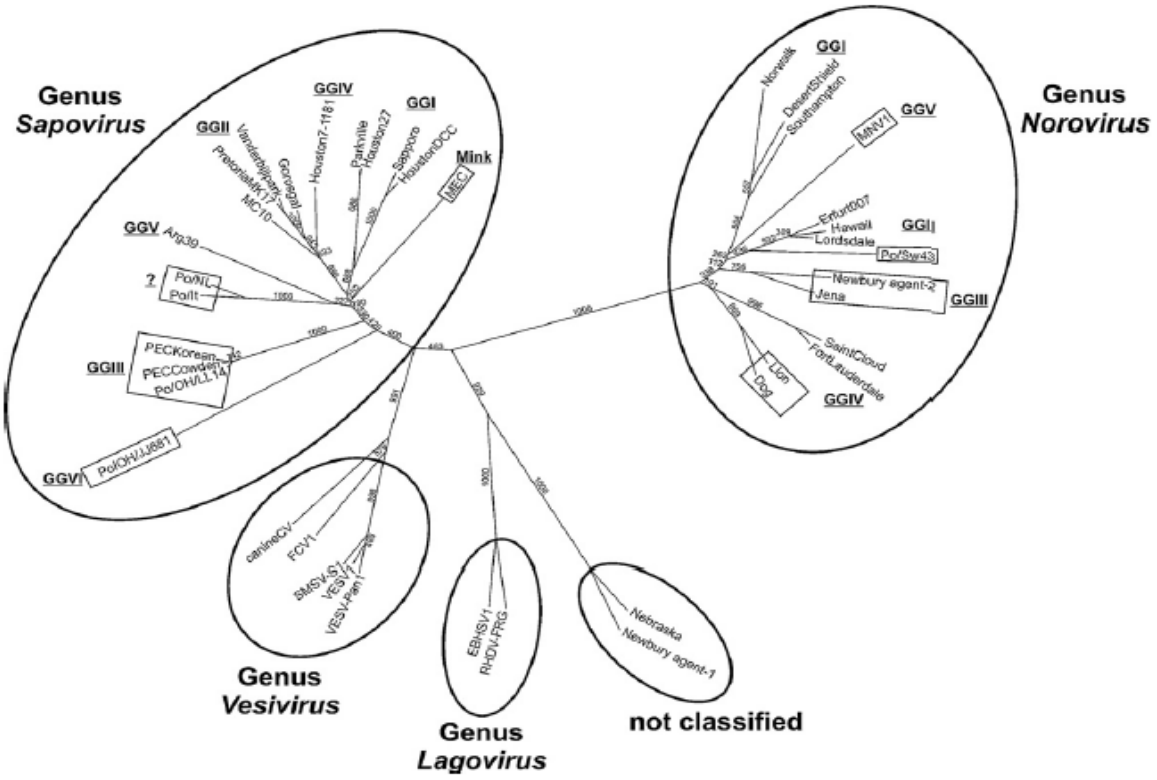

Figure 1. Phylogenetic tree of the *Caliciviridae* family. The analysis is based on nucleic acid sequences from the RNA-dependent RNA-polymerase encoding region . Strains of animal origin within the genera Norovirus and Sapovirus are highlighted by boxes. Branch lengths are proportional to genetic distances. Adapted from Bank-Wolf *et al*. 2010.

The previous 4 *genera* classification was established in 1998 by the ICTV (Scipioni *et al.* 2008, Yilmaz *et al.* 2011). Oliver *et al.* (2006) proposed the existence of a new genus in the *Caliciviridae* family named *Becovirus* or *Nabovirus* to distinguish two enteric bovine caliciviruses described (Newbury Agent 1 and Nebraska strain) that form a new clade now named *Nebovirus* (Yilmaz *et al.* 2011). Caliciviruses are small, nonenveloped viruses with icosahedral symmetry (Green *et al.* 2007). This family is characterized for having 32 cup-shaped depressions which led to the name *Caliciviridae*, derived from the Latin word *calix* for cup or goblet (Bank-Wolf *et al*. 2010). Caliciviruses have a wide range of hosts including human and animals and affect predominantly the enteric tract leading to infection and disease (Green *et al.* 2007). They have also been associated with vesicular lesions, reproductive failure, disease of the upper respiratory tract and systemic and hemorrhagic diseases (Scipioni *et al.* 2008).

Discovery of the Norwalk virus

The first Norovirus identified was associated with human disease (Kapikian *et al.* 1972). Trough the 20th century there were descriptions of several “acute infectious nonbacterial gastroenteritis” syndromes in humans (Blacklow NR 2004, Dolin *et al.* 1972, Kapikian *et al.* 1972, Dolin *et al.* 1971, Adler & Zickl 1969). These usually consisted of acute, self-limiting episodes of vomiting and/or diarrhea frequently presenting with an epidemic spread pattern and with no success in finding an etiological cause for the clinical manifestations. The first identification of a possible etiology occurred in 1972 when Kapikian and co-workers visualized a 27nm particle by immune electron microscopy in stool samples from volunteers fed with fecal filtrates from children who were affected four years before in an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio in 1968 (Kapikian *et al.* 1972). The virus-like particles identified were considered the etiological cause of the Norwalk gastroenteritis outbreak being named Norwalk virus and is considered the prototype strain of the genus *Norovirus* (Kapikian *et al.* 1972). The discovery of the Norwalk virus by immune electron microscopy was closely followed by the discovery of other similar enteric viruses classified as small round-structured viruses due to their appearance under the electron microscope and in the 1990´s this group of viruses was divided into the Norwalk-like viruses, now known as noroviruses, the Sapporo-like viruses, now called sapoviruses, and the astroviruses (Green *et al.* 2007).

Classification of noroviruses

The noroviruses are divided in 5 genogroups (GI-GV) according to the alignment of the amino acid sequence of the VP1, the major capsid protein (Mattison *et al.* 2007). Human norovirus strains are located in GI, GII and GIV. The porcine noroviruses are also in GII. The bovine noroviruses are in GIII and the murine noroviruses in GV (Bank-Wolf *et al.* 2010). The lion norovirus and the canine norovirus described by Martella and co-workers (Martella *et al.* 2008, Martella *et al.* 2007) were also located in GIV. Mesquita *et al.* (2010) reported a novel canine norovirus strain denominated Viseu which is closely related to the canine norovirus (Bari strain) and to a human norovirus (Chiba strain). The classification of a new genogroup (GVI) comprising these three strains was suggested (Figure 2).

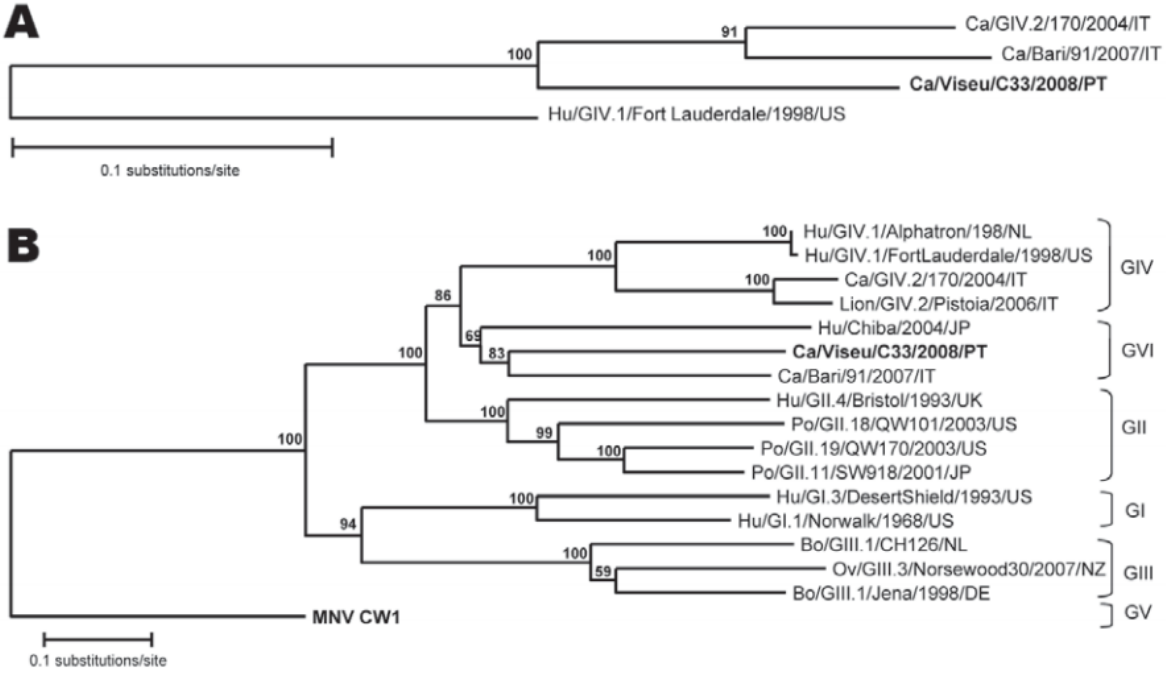

Figure 2. Genogroup division of the *Norovirus* genus. Phylogenetic trees of A) a 206-nucleotide region of the RNA-dependent polymerase gene of 1 human GIV strain (Hu/GIV.1/FortLauderdale/1998/US), 2 canine noroviruses (GIV.2/170/2004/IT, Bari/91/2007/IT) and the novel canine Viseu strain reported by Mesquita *et al*. 2010 (boldface); and B) full-length amino acid sequence of VP1 of norovirus strains of GI–GV. Ca, canine; Hu, human; Po, porcine; Bo, bovine; Ov, ovine; Mu, murine. Adapted from Mesquita *et al.* 2010.

Zheng *et al.* (2006) proposed a standardized method to genetically describe and classify norovirus strains that provides clear criteria for norovirus nomenclature below the genus level using the amino acid sequences of the VP1. Norovirus strains of the same genogroup share > 55% of amino acid identity while strains from the same genotype share > 85% of amino acid identity and as recombination can affect the correct classification of noroviruses, full capsid

sequencing should be used to classify new strains instead of partial sequences (Zheng *et al*. 2006). This method divides the 5 genogroups into 31 genotypes or genetic clusters with GI including 8 genotypes (GI.1-GI.8), GII with 19 (GII.1-GII.19), GIII with 2 (GIII.1 and GIII.2), GIV and GV each including 1 genotype (Karst 2010).

Studying this genus has been a challenge as the majority of noroviruses cannot be cultivated in cell or organ culture systems or in experimental animal models (Hutson *et al*. 2004, Green *et al.* 2007, Prasad *et al*. 1999). The only norovirus that can be cultivated in cell culture systems is the murine norovirus (Bank-Wolf *et al*. 2010). However, the pathogenesis of this virus is different from the rest of the genus making this an inadequate model (Wang *et al*. 2007). The acumulated knowledge of noroviruses is mostly due to outbreak and volunteer studies and to the development and application of molecular biology (Green *et al.* 2007, Hutson *et al*. 2004). Gnotobiotic animals are being studied as possible models for the study of noroviruses. Cheetham *et al.* (2006) tested gnotobiotic pigs as a model for the research of human noroviruses and replication of these viruses occurred in these animals. Gnotobiotic calves have also been investigated as possible model for the study of human norovirus (Souza *et al.* 2008). There has been a report of infection of a 3-dimensional model of human small intestine epithelium but viral replication was not possible to confirm by independent laboratories (Karst 2010).

Viral structure

Noroviruses have a spherical structure, are nonenveloped and when observed trough the electron microscope appear with an indistinct surface structure and a foamy or feathery outline. (Barry *et al.* 2008, Bank-Wolf *et al*. 2010). This is uncharacteristic as the rest of the calicivirus genera present a classic star-of-David-like appearance due to the 32 cup-shaped depressions the caliciviruses possess (Figure 3; Bank-Wolf *et al*. 2010, Barry *et al.* 2008, Scipioni *et al.* 2008, Sugieda *et al.* 1998). However the surface of noroviruses is also formed by 32 cup-shaped depressions (Scipioni *et al.* 2008). The norovirus capsid is composed by 180 copies of a single protein, the VP1 (Lochridge *et al.* 2005). Its architecture is based on a T = 3 icosahedral symmetry with 90 dimeric arch-like capsomers (Figure 4; Chen *et al.* 2004).

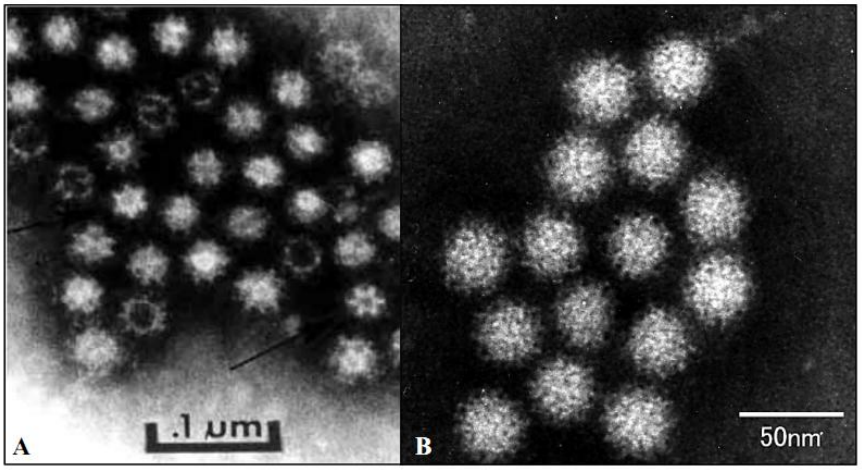

Figure 3. Two morphologic structures of caliciviruses by negative contrast electron microscopy. (A) Classic 32 cup-shaped depressions or star-of-David-like morphology presented by vesiviruses, lagoviruses and sapoviruses. (B) Small round-structured virus. Morphology presented by noroviruses and some feline caliciviruses. Adapted from Barry *et al*. 2008.

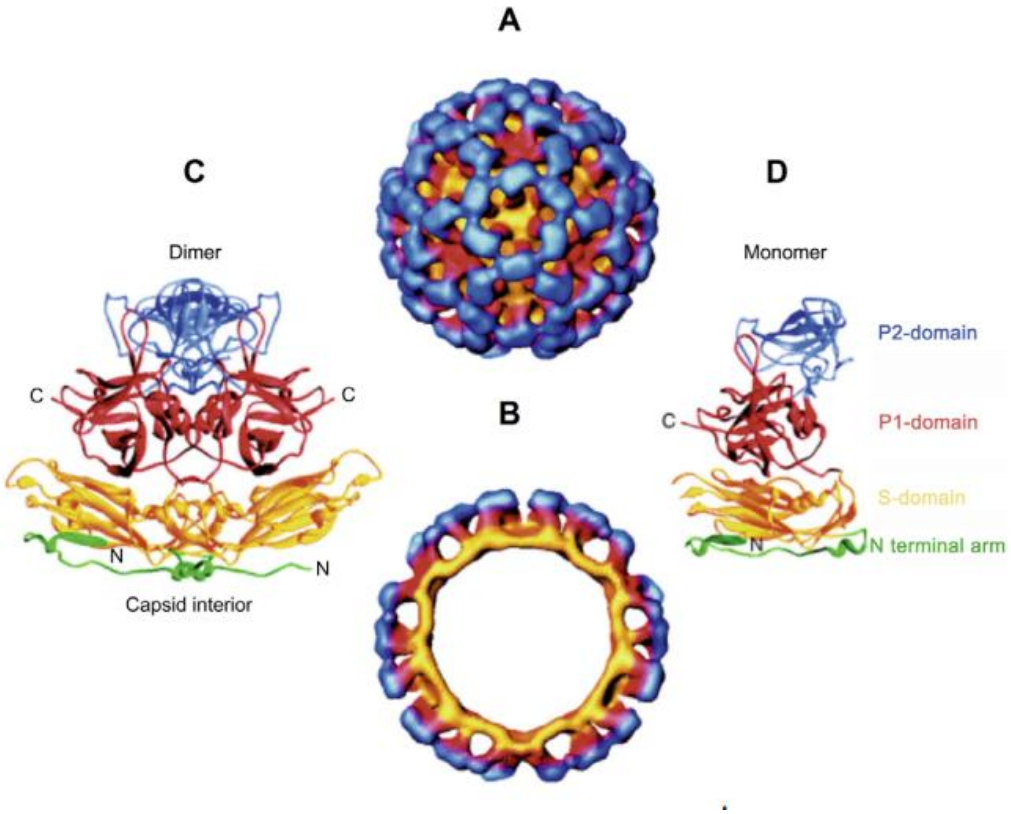

Figure 4. Capsid structure of the Norwalk virus-like particle (VLP). (A) Surface representation. (B) Capsid cross-section. (C) Dimer of the capsid protein. (D) The N-terminal arm region (green) is facing the interior of the VLP, a shell domain (S-domain, yellow) that forms the continuous surface of the VLP and the protruding domain (P-domain) that constitutes the arch at the surface of the VLP. The P-domain is further divided into subdomains P1 (red) and P2 (blue). Adapted from Scipioni *et al.* (2008).

Genome organization

The Norwalk virus genome was sequenced in 1990 by Jiang, X. *et al*. (1990). It consists of a positive-sense single-stranded RNA of around 7.5 kb organized in three open reading frames (ORF1-3) with a genome-linked viral protein (VPg) bound to the 5´-end of the genome and a polyA tail ate the 3´-end of the genome (Hardy 2005, Green *et al*. 2007). As noroviruses do not possess an internal ribossomal entry site or a cap structure (typical of eukaryotic mRNA) it is probable that a VPg may function in translation initiation (Daughenbaugh *et al*. 2003). A subgenomic mRNA is produced during norovirus replication (Karst 2010).

ORF1 located at the 5´-end of the genomic RNA encoding a polyprotein of approximately 195 kDa that is proteolytically cleaved into at least six non-structural proteins: protein p48, nucleoside triphosphatase, protein p22, VPg; 3C-like protease and RNA-dependent RNA-polymerase (Hardy 2005).

ORF2 and ORF3 encode the structural proteins, the major VP1 and the minor capsid protein (VP2), respectively (Green *et al*. 2007). VP1 is a 60 kDa protein divided into two domains: the S domain that contains the N-terminal region and the P domain that contains the C-terminal region (Phan *et al.* 2007, Lochridge *et al.* 2005). The S domain is a conserved region that forms the shell of the virion composed by 255 amino acids (N-terminal 225 aa) (Allen *et al.* 2008, Lochridge *et al.* 2005). The P domain forms a protrusion that extends from the capsid to the exterior of the virion. It contains 304 amino acids (aa 226–530) and is divided into two subdomains: P1 and P2. The interaction between these two subdomains increases the capsid stability and forms the protusion exhibited by this region (Lochridge *et al.* 2005). The P1 subdomain is also subdivided in two parts: P1-1 and P1-2, more conserved domains that flank the hypervariable P2 domain (Allen *et al.* 2008, Phan *et al.* 2007). P2 is the most variable segment of Noroviruses VP1 and occupies the most outer region of the capsid´s protrusions. These properties suggest that P2 bears a key role in receptor binding functions, antigenic properties and host immune response (Allen *et al.* 2008, Phan *et al.* 2007, Lochridge *et al.* 2005). VP1 is responsible for capsid formation and self-assembly also involved in host recognition and specificity, strain diversity and ability to elicit an immune response (Scipioni *et al.* 2008). VP2 is a smaller structural protein with around 20 kDa which participates in the expression, assembly and stability of VP1 (Bertolotti-Ciarlet *et al.* 2003).

The characteristics of the capsid proteins allowed the development of virus-like particles (VLPs) with a consequent evolution of diagnostic methods such as solid-fase antigen immunoassays as enzyme-linked immunosorbent assay (ELISA) to be genome specific (Hutson *et al*. 2004). VP1

and VP2 have been expressed in insect cells infected with a baculovirus and other protein expression systems suffering spontaneous self-assembly into VLPs that are similar to the virion capsid, presenting the same morphologic and antigenic properties than the norovirus, but lack the viral RNA genome (Green *et al.* 2007). These particles are immunogenic, eliciting the production of antibodies when given orally to humans or mice, which allows the production of hyperimmune sera (Hutson *et al.* 2004, Green *et al.* 2007).

Transmission and biological characteristics

Transmission of animal and human noroviruses occurs mainly through the fecal-oral route (Scipioni *et al.* 2008). Person-to-person contact and consumption of contaminated food are the most common routes in humans (Patel *et al.* 2009). The occurrence of infection from contaminated surfaces and infectious particles present in the vomitus, that can be aerosolized and cause infection through the respiratory tract, have also been suggested (Patel *et al.* 2009).

The Norwalk virus possess characteristics that increase the risk of infection by this virus as high stability, low infectious dose and resistance to inactivation, having been reported to retain infectivity after treatments with low pH (pH 2.7), high temperature (60 ºC), ether and chlorine, at a concentration similar to that of a drinking water distribution system (Green *et al.* 2007). Excretion of norovirus viral particles can last up to three weeks and has been reported in asymptomatic humans and animals (Bank-Wolf *et al.* 2010, Patel *et al.* 2009, Wang *et al.* 2005).

Virus–cell interactions

The site of entry and primary replication noroviruses is suspected to be the small intestine (Green *et al.* 2007). Murine noroviruses have been reported to have high tropism for hematopoietic cells, particularly macrophages and dendritic cells and a possible entry route for these viruses is their direct acquirement from the intestinal lumen by dendritic cells, a mechanism documented in humans (Scipioni *et al.* 2008).

Human noroviruses bind to carbohydrates linked to the human histo-blood group antigens (HBGAs) and the expression of these molecules is related to increased susceptibility to some norovirus strains (Tan & Jiang 2005). HBGAs are expressed by the majority of animals and other caliciviruses have also been reported to bind to HBGAs as rabbit hemorrhagic disease virus, a lagovirus and although the receptors of animal noroviruses are not yet known it´s logical to theorize that HBGAs may play this role (Scipioni *et al.* 2008). Noroviruses are a common cause of shellfishborne gastroenteritis outbreaks and mollusks have been found to concentrate

viruses in their tissues from the surrounding water through filter feeding (Constantini *et al*. 2006, Le Guyader *et al.* 2006). Oysters, a mollusk, express carbohydrates closely related to HBGAs in their digestive tissues which could be a mechanism of norovirus concentration (Le Guyader *et al.* 2006).

Pathogenesis

The majority of animal noroviruses are not associated to clinical disease occurring only with immunocompromised animals (Scipioni *et al.* 2008). In contrast, human noroviruses are the most common cause of acute gastroenteritis worldwide and norovirus outbreaks lead to large economic losses due to direct health care costs and indirect costs (Cannon *et al*. 2009).

Human noroviruses

Human noroviruses are today recognized as the leading cause of foodborne outbreaks of acute gastroenteritis worldwide as well as an important cause of sporadic acute gastroenteritis among persons of all ages (Patel *et al.* 2009). Infection by norovirus can cause vomiting, nausea, non-bloody diarrhea, fever, abdominal pain and cramps (Patel *et al.* 2009, Bank-Wolf *et al*. 2010). The onset of clinical signs that can present with relative severity is acute, with incubation periods lasting generally 24-48 hours and the signs are usually self-limiting, tending to resolve quickly (2-3 days). Elderly people, children and immunocompromised are most susceptible with more severe symptoms and the occurrence of fatalities having been reported in these groups (Cannon *et al.* 2009). Human volunteer studies showed broadening and blunting of the villi of the proximal small intestine with the mucosa remaining intact (Green *et al.* 2007). Outbreaks are common in locations with a high density of people such as hospitals, nursing homes, cruise ships, university dormitories and military barracks and GII.4 is the most common cause of acute gastroenteritis outbreaks in humans (Cannon *et al.* 2009).

Bovine noroviruses

Bovine norovirus are classified in GIII and strains Jena and Newbury Agent 2 genotypes are the prototypes of GIII.1 and GIII.2, respectively (Scipioni *et al.* 2008). The first bovine norovirus reported was the Newbury Agent 2, identified in calves from the United Kingdom (Wang *et al.* 2007). Since then, bovine noroviruses have been reported in Europe, North and South America and Asia (Turkey) (Bank-Wolf *et al*. 2010). Scipioni *et al.* (2008) state that bovine noroviruses are begnin pathogens, usually just facilitating or complicating gastroenteritis. These viruses are

mainly associated with diarrhea in calves (Bank-Wolf *et al.* 2010, Scipioni *et al.* 2008). The Jena agent was reported to be passaged 11 times in cattle fed with colostrums, leading to diarrhea at each passage (Scipioni *et al.* 2008). Gnotobiotic calves infected with the Newbury Agent 2 strain presented mainly with four clinical signs: non-hemorrhagic enteritis, mild diarrhea (more severe in 3-week-old calves than in neonates), transient anorexia and xylose mal-absorption (Bank-Wolf *et al*. 2010, Scipioni *et al.* 2008). The signs started after an incubation time of 12-24 hours and were present for up to 2 months of age with detection of viral shedding iniciating shortly before the appearance of the clinical signs (Scipioni *et al.* 2008). This norovirus appears to be less virulent then Newbury Agent 1, another bovine enteric calicivirus (Scipioni *et al.* 2008). Newbury Agent 2 and the Jena agent can cause villous atrophy, crypt hyperplasia and edema of the submucosa in the proximal small intestine (Scipioni *et al.* 2008).

Porcine noroviruses

Porcine noroviruses belong to three genotypes in the genogroup II: GII.11 and the newly described GII.18 and GII.19 by Wang *et al.* (2007). The first porcine norovirus was detected in cecal samples belonging to healthy adult pigs from Japan and since then, these viruses have also been detected in Europe and in the USA but detection of porcine noroviruses has only occurred in fecal samples of asymptomatic animals(Wang *et al.* 2007). Wang *et al*. (2005) reported mild diarrhea in gnotobiotic pigs coincident with viral shedding after inoculation with a porcine norovirus. The role of these viruses in porcine diarrhea is still unknown (Scipioni *et al.* 2008). Nevertheless pigs may serve as important reservoirs for noroviruses and have an important role in the evolution of caliciviruses (Wang *et al.* 2005). The close genetic and antigenical relatedness of human and porcine noroviruses, both classified in GII, causes concerns about the zoonotic potential of porcine noroviruses (Wang *et al.* 2007).

Lion norovirus

A novel norovirus was identified in the intestinal content of a captive lion cub that presented with severe hemorrhagic enteritis that was genetically related to human genogroup IV noroviruses and was classified as a new genotype, GIV.2 (Martella *et al.* 2007). However the potential pathogenicity of this new norovirus has not been established (Martella *et al.* 2007).

Canine noroviruses

Canine caliciviruses are rare pathogens not usually taken into account when diagnosing infectious diseases in dogs (Martella *et al*. 2008). The first canine norovirus reported was

identified in the feces of a 60-day-old pup that presented with vomiting and diarrhea and was classified as a canine norovirus, variant of the genotype GIV.2 as the highest sequence identity was found with the lion norovirus strain GIV.2 Pistoia (Martella *et al*. 2008). The virus was detected in the feces of the pup during 22 days which indicates that replication of the virus occurred in the dog however no conclusions about its pathogenicity could be taken as it was detected in coincidence with a canine parvovirus strain that is a likely cause of the gastroenteritis presented by the pup (Martella *et al*. 2008). Human noroviruses are classified in genotype GIV.1 which indicates that this canine norovirus is genetically very similar to human noroviruses (Martella *et al*. 2008).

Martella *et al.* (2009) studied the epidemiology of noroviruses in dogs by testing stool samples for the presence of these viruses. The prevalence of noroviruses found in the samples tested was low (4 positive samples in 183 tested samples [2,1%]). However this value cannot be assumed as a prevalence of canine norovirus as specific primers for animal GIV.2 weren´t used.

Recently Mesquita *et al.* (2010) reported a novel canine norovirus (Viseu strain) in 25 of 63 dogs presenting with diarrhea (40%) and in 4 of 42 dogs presenting without diarrhea (9%) that showed to be significantly associated with the disease and the highest genetic relatedness of the strain was found to two other known noroviruses strains, namely canine norovirus Bari strain and human norovirus Chiba strain with 63.2% and 55.1% amino acid identity, respectively. This led to the proposal of a sixth genotype (GVI) that would include these three norovirus strains (Mesquita *et al.* 2010).

Murine noroviruses

Murine noroviruses 1, 2, 3, and 4 were recently identified in immunocompetent and immunodeficient mice (Scipioni *et al.* 2008). Murine norovirus-1 is able replicate in immunocompetent mice causing histopathological changes, namely mild inflammation in the intestine and red pulp hypertrophy and white pulp activation the spleen, and disseminating to other tissues (spleen, liver, lung and lymph nodes) but intestinal replication and establishment of clinical disease are inhibited by STAT-1 (Mumphrey *et al.* 2007). In immunocompromised mice murine norovirus-1 can cause gastroenteritis, encephalitis, vasculitis, pneumonia, hepatitis and systemic infection (Karst 2010, Scipioni *et al.* 2008). Murine norovirus-1 has a different pathogenic pattern than murine noroviruses-2, 3 and 4 as these strains cause a longer time of fecal shedding and chronic tissue infection (Scipioni *et al.* 2008).

Immunity

The majority of existing data on immune response to norovirus infection results from the study of human norovirus outbreaks or volunteer studies (Green *et al.* 2007). Norovirus inoculation induces a specific serum IgG response, that can persists for months, serum IgA and IgM responses that have a shorter duration and mucosal IgA production (Karst 2010). However, data gathered has shown that, although a specific antibody response occurs, this does not correlate to resistance to illness (Green *et al.* 2007). Some hosts developed illness when re-exposed after short-term immunity has expired while others do not and resistant host present lower antibody titers than the susceptible hosts (Green *et al.* 2007). Studies have shown that HBGA expression in the intestinal mucosa is correlated to susceptibility to norovirus infection (Cannon *et al*. 2009).

Interspecies transmission of noroviruses and the risk of zoonosis

Noroviruses have a wide range of hosts, including humans, cattle, pigs, dogs and mice (Mattison *et al*. 2007). There have been reports of detection noroviruses and of anti-norovirus antibodies in different species than that of the natural host. Mattison *et al.* (2007) reported the presence of GII.4-like noroviral RNA in swine and bovine fecal samples as well as in a retail swine meat sample, which suggests another possible source of norovirus foodborne transmission to humans. Cheetham *et al.* (2006) and Souza *et al.* (2008) reported replication of human norovirus in gnotobiotic pigs and calves, respectively. Widdowson *et al*. (2005) studied the presence of IgG and IgA antibodies against bovine noroviruses in serum samples from veterinarians and matched population controls by enzyme immunoassay. Both IgG and IgA reactivity to bovine norovirus were present in veterinarian and control populations, with a higher IgG reactivity in veterinarians (Widdowson *et al*. 2005). Martino *et al.* (2010) tested sera from cats and dogs with a VLP-based ELISA using RNA from the prototype lion norovirus GIV Pistoia strain and found positive samples in both species which suggests that noroviruses may circulate among different carnivore populations. Farkas *et al.* (2010) detected the presence of human norovirus antibodies in serum samples of rhesus macaques using strains from genogroups GI and GII.

Cross-species transfer of viruses is a consequence of accumulation of genetic changes which may occur by different processes as mutation, recombination and reassortment (Louz *et al*. 2005). Mutations occur more quickly in RNA viruses than in DNA viruses because these are replicated by RNA polymerases that lack the proofreading function of DNA polymerases which

leads to a more rapid evolution of RNA viruses (Louz *et al*. 2005). Recombination of noroviruses occurs more commonly intragenogroups although intergenogroups recombination may also take place (Bank-Wolf *et al*. 2010). Recombinant strains of human genogroups GI and GII have been reported (Ambert-Balay *et al.* 2005). Phan *et al.* (2007) detected eight previously unreported recombinant noroviruses and three different types of recombination: intergenogroup recombination between GII and GIV strains and intergenotype recombination of GI and GII. Martella *et al.* (2009) reported a recombinant canine GIV strain (Bank-Wolf *et al*. 2010). Human and animal noroviruses can possess a close genetic and antigenic relationship of which porcine and human GII strains are an example (Farkas *et al.* 2005). Co-infection may result in the emergence of new recombinant norovirus between human and animal strains with unknown pathogenic potential and altered species tropism (Bank-Wolf *et al*. 2010).

Objectives

Following the detection of the novel canine norovirus by the team where this project was integrated (Mesquita *et al.* 2010) in Portuguese dogs and taking into account the concerns about the zoonotic potential of Noroviruses, this study aimed to:

i. Evaluate the prevalence of IgG antibodies against the novel canine norovirus Viseu strain in dogs from Portugal;
ii. Determine the presence of IgG antibodies against canine norovirus Viseu strain in the canine populations of other European countries (Denmark, France, Finland, Germany, Hungary, Ireland, Italy, Netherlands, Norway, Poland, Sweden, Switzerland, United Kingdom) and of the USA;
iii. Determine the presence of IgG antibodies against canine norovirus Viseu strain in the human population of Portugal, comparing veterinarian population *versus* general population;
iv. Evaluate if there are significant differences in the seroprevalences of veterinarian and general populations.

In order to fulfill these objectives, a VLP-based in house ELISA protocol was designed.

MATERIALS AND METHODS

Serum samples

A total of 780 serum samples were obtained from dogs of 15 countries (Table 1). Blood was collected by venepuncture from 309 Portuguese dogs. Sera were separated within 24 hours of collection and kept at -20ºC until use. Data regarding age, sex, breed and size were recorded. The 61 serum samples from dogs of the USA were kindly provided by Dr Jan Vinjé from the CDC, Atlanta, USA. Data regarding age, sex, breed and size were also obtained. The serum samples from dogs of the 13 European countries were kindly provided by Dr Thomas Vahlenkamp from the Friedrich Löffler Institute, Germany. No information about age, sex, breed and size was obtained for these samples. The number of samples obtained for each country is not uniform with samples numbers of 10 for six countries (Finland, Hungary, Ireland, Italy, Sweden and Switzerland), 50 for seven countries (Denmark, France, Germany, Netherlands, Norway, Poland and United Kingdom) 61 for the USA and 309 for Portugal.

Negative control sera consisted of sera from 6 specific pathogen free beagles that were kindly provided by Professor Anabela Silva from the IBMC, Porto, Portugal.

Table 1. Country of origin and number of canine serum samples.

Country	Nº of samples
Portugal	309
USA	61
Germany	50
France	50
United Kingdom	50
Poland	50
Denmark	50
Norway	50
Netherlands	50
Hungary	10
Italy	10
Ireland	10
Switzerland	10
Sweden	10
Finland	10
Total 15	**780**

A total of 373 samples from a veterinarian population were collected. The veterinary professionals attending the VII *Congresso Hospital Veterinário Montenegro* held at the

Europarque in *Santa Maria da Feira*, Portugal in February 2010 were asked to donate a blood sample to the present study. Blood was collected by venepuncture from all the participants who gave their informed consent and refrigerated at 4°C. The serum samples were separated within 24 hours of collection and kept at -20ºC until use. Each volunteer also answered a questionnaire which included information about demographics (age, sex, residence), professional details (years in practice, occurrence of accidental needle stick injury with animal/dog blood) and personal details (interaction with animals/dog while children).

A total of 120 samples from general population were obtained from anonymous donors during the same time period, matching the veterinarians´ sera by age group, sex and residence.

Sera samples obtained from the cord blood of 13 human newborns were used as negative controls and were kindly provided by Dr. Guilherme Gonçalves of the University of Porto, Portugal.

Virus-like particles

VLPs expressing the VP1 capsid gene of canine norovirus Viseu strain and of human norovirus GII.4 New Orleans strain were used as solid-fase antigen. Canine norovirus Viseu strain VLPs were produced by oral infection of synchronous *Trichoplusia ni* larvae and purified by cesium chloride ultracentrifugation. Human norovirus GII.4 New Orleans strain VLPs were produced in Sf9 insect cells and purified by cesium chloride ultracentrifugation. All the VLPs were gently provided by Dr Jan Vinjé from the CDC, Atlanta, USA.

Reagents, solutions and equipment

The following reagents were purchased:

- Difco ™ Skim Milk, Becton, Dickinson and Company, Le Pont de Claix, France;
- Goat anti-canine IgG conjugated with horseradish peroxidase, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA;
- Goat anti-human IgG conjugated with horseradish peroxidase, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA;
- Phosphate buffer saline (PBS) tablets, Gibco Invitrogen Co., Scotland, UK;
- Substrate, TMB Microwell Peroxidase System, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA;
- Stop Solution, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA;
- Tween® 20, Sigma-Aldrich GimbH, Steinheim, Germany;

- Sodium bicarbonate ($NaHCO^3$), Merck, Darmstadt, Germany;
- Sodium carbonate (Na^2CO^3), Merck, Darmstadt, Germany.

The following work solutions were prepared as needed:

- Coating buffer – Na^2CO^3 and $NaHCO^3$ were diluted in distilled water to a final concentration of 15 mM Na^2CO^3 and 35 mM $NaHCO^3$. The solution was adjusted for a pH 9,6 and stored at 4 ºC until use.
- Washing buffer – Tween ® 20 was diluted in PBS at a concentration of 0,1%.
- Blocking buffer – Difco ™ Skim Milk was diluted in washing buffer to a final concentration of 5%.

The following equipment was used:

- Incubator, EHRET GmbH & Co KG Labor, Austria;
- Multiwell spectrophotometer Bio-Tek Instruments Inc., PowerWave XS, USA;
- KCjunior™ Data Analysis Software.

Enzyme-linked immunosorbent assay

An indirect in house VLP-based micro ELISA was used to detect IgG antibodies against canine norovirus (Viseu strain) and human norovirus (New Orleans strain) in serum samples.

Microplates with 96 wells were coated with VLPs of canine norovirus used at the concentration of 5 µg/mL or of human norovirus used at the concentration of 1,25 µg/mL and incubated overnight at 4ºC.

After incubation the wells were washed 7 times for 3 minutes each with washing buffer and blocked with 200 µL of blocking buffer for 2 hours at 37ºC.

After blocking the wells were washed again in the same manner and 50 µL of diluted serum (canine sera at 1:100 and human sera at 1:1500) were added and incubated for 1 hour at 37ºC.

After washing 50 µL of conjugate diluted in blocking buffer were added and incubated. The conjugate consisted of goat anti-canine IgG and goat anti-human IgG both conjugated with horseradish peroxidase and were diluted at 1:6400 and 1:12800, respectively.

After 1 hour at 37 ºC wells were washed and 100 µL of substrate were added and incubated at room temperature for 10 minutes (without light). The enzymatic reaction was stopped with 100 µL of stop solution. The optical density (OD) values were measured at a wavelength of 450 nm. A serum was considered positive when the optical density (OD) value of the coated well was

higher than the cutoff (calculated as the mean of the negative control sera coated wells OD + 3 standard deviations).

Statistical analysis

The data collected was recorded in Excell (Microsoft Office 2007) and all the statistical analysis were performed using Epicalc package in the R software (R 2.1.2.0) (R Development Core Team, 2010).

RESULTS AND DISCUSSION

A total of 780 canine serum samples from 15 different countries, namely Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Netherlands, Norway, Poland, Portugal, Sweden, Switzerland, United Kingdom and USA, were tested with a VLP-based ELISA for canine norovirus IgG antibodies. Of the 780 samples, 540 (69.2%) were positive for IgG anti-canine norovirus, having been found throughout the 15 tested countries (Table 2; Figure 5).

Table 2. Seroprevalences for canine serum samples anti-canine norovirus IgG by a VLP-based ELISA.

Country	Number of tested serum samples tested	Number of positive samples	Percentage of positive samples (%)
Portugal	309	226	73.1
USA	61	51	83.6
Germany	50	26	52.0
France	50	26	52.0
Denmark	50	30	60.0
Poland	50	33	66.0
Netherlands	50	38	76.0
Norway	50	32	64.0
United Kingdom	50	42	84.0
Hungary	10	6	60.0
Italy	10	8	80.0
Ireland	10	1	10.0
Switzerland	10	3	30.0
Sweden	10	10	100.0
Finland	10	8	80.0
Total	**780**	**540**	**69.2**

Number of samples tested, number of positive samples detected and percentage of positive samples are presented for each country and in total.

The presence of positive sera throughout the European countries and USA is suggestive of widespread circulation of canine norovirus in the dog population. The calculated seroprevalences ranged from 10% (1/10) in Ireland to 100% (10/10) in Sweden, while in Portugal the value was of 73.1%. USA (83.6%), Netherlands (76%), United Kingdom (84%), Italy (80%) and Sweden (100%) were found to have higher seroprevalences than Portugal.

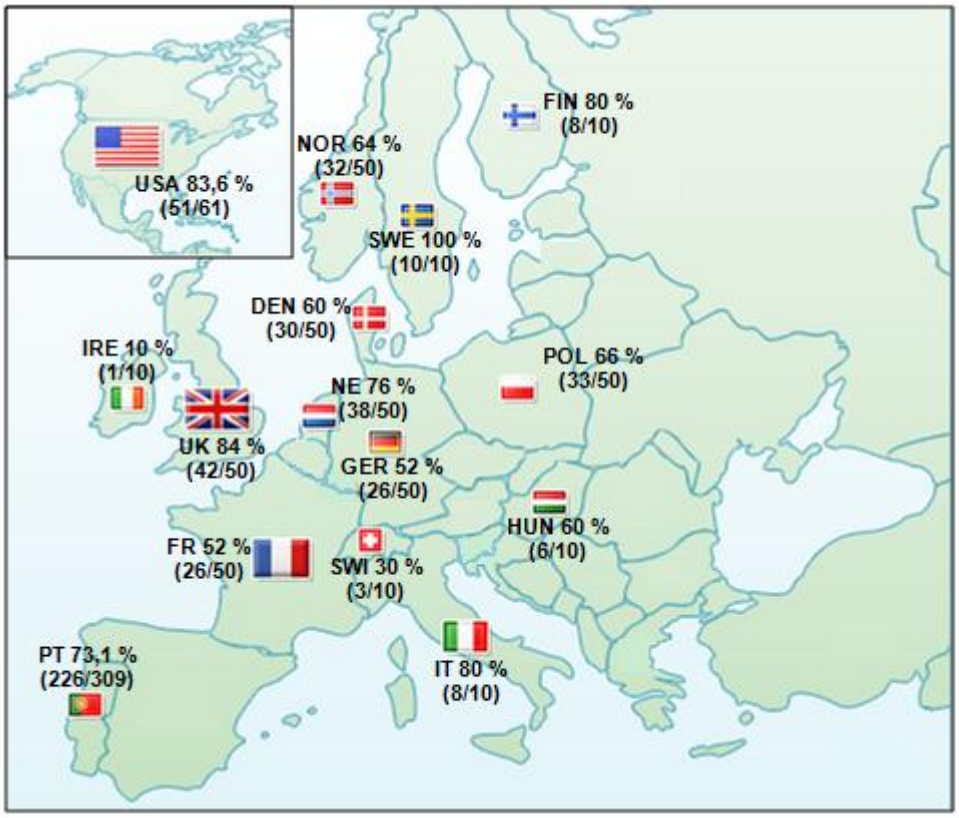

Figure 5. Seroprevalences for IgG anti-canine norovirus in the dog populations of European countries and USA. Denmark - DEN, Finland - FIN, France - FR, Germany - GER, Hungary - HUN, Ireland - IRE, Italy - IT, Netherlands - NE, Norway - NOR, Poland - POL, Portugal - PT, Sweden - SWE, Switzerland - SWI, United Kingdom - UK and United States of America – USA.

Previous studies reported the presence of canine norovirus genetically distinct and unrelated to any other norovirus in the feces of dogs of Italy and Portugal (Martella *et al*. 2009; Mesquita *et al.* 2010). The Portuguese and Italian reported canine norovirus strains possessed distinct nucleotide sequence identities in their VP1, while holding a high similarity among their RNA-dependent RNA-polymerase sequences. The ELISA screening of the current study used VLPs based on the Portuguese canine norovirus Viseu strain and probably detected antibodies for other canine norovirus strains across Europe and USA.

Seroprevalence studies of antibodies against other animal noroviruses have also reported high figures. Deng *et al*. (2003) found a 99.1% seroprevalence of antibodies against bovine norovirus (Jena virus) in bovines from Germany. Oliver *et al*. (2007) found seroprevalences of 87.5% and 76.5% to Jena virus and Newbury Agent 2 in the UK, respectively, and a 68.5% and 90.5% seroprevalence for Jena virus and Newbury Agent 2 in bovines from Germany, respectively. Farkas *et al.* (2005) detected high prevalences of antibodies against porcine norovirus in pigs from the USA (71%).

In order to assess possible cross-reactions between canine norovirus VLPs and antibodies to other noroviruses, a total of 23 canine serum samples were also tested with a VLP-based ELISA for human norovirus IgG antibodies. A Spearman´s correlation test was calculated to evaluate the correlation between the OD values of IgG reactivity for canine norovirus and for human norovirus obtained for the same canine sera (Figure 6).

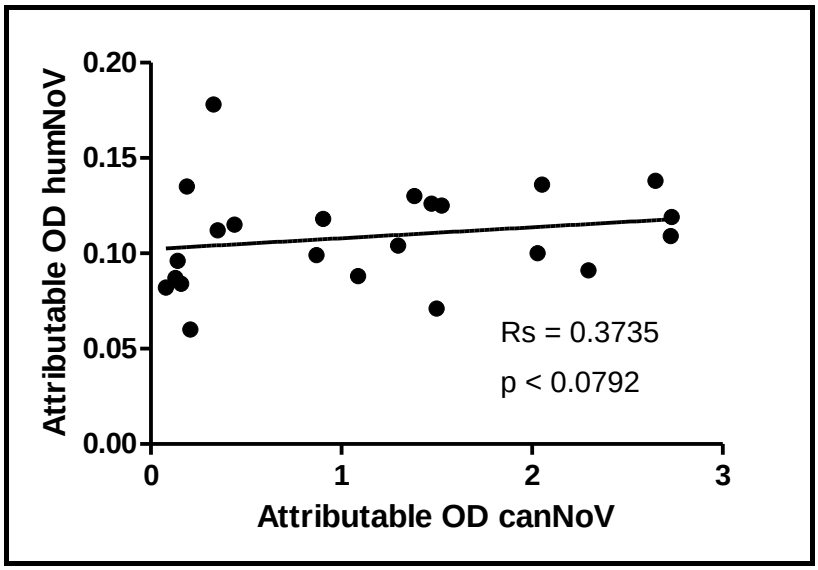

Figure 6. Study of cross-reactivity between canine norovirus VLPs and IgG anti-human norovirus. Net absorbances (ODs) attributable to serum IgG reactivity to human norovirus (humNoV) and canine norovirus (canNoV) VLPs. Rs - Spearman Rank Correlation.

The correlation coefficient observed was low (Rs = 0.3735, p < 0.0792) but the p-value was above the 0.05 level of significance. This indicates that the number of observations used to calculate this test was too low to state a final conclusion for this confidence level. Nevertheless, because the correlation coefficient value is low, there is confidence that a higher sample number would confirm a low correlation which would indicate a low cross-reactivity between canine norovirus VLPs and human norovirus antibodies. Although this would not rule out cross-reactions with different norovirus strains, it would suggest a low cross-reactivity between canine norovirus VLPs and antibodies against norovirus GII.

The country of origin was evaluated as a putative risk factor for IgG seropositivity against canine norovirus using multivariate analysis logistic regression models (Table 3). For statistical purposes, Portugal was considered the country of reference. Only four countries, namely France, Germany, Ireland and Switzerland, presented statistically significant results, and all posed lower risk of detection of a positive sample to IgG anti-canine norovirus than Portugal.

Table 3. Analysis of the country of origin as a risk factor for IgG seropositivity to canine norovirus.

	OR (95% CI)	P (Wald´s test)	P (LR-test)
Origin			< 0.001
Portugal	ref		
France	0.4 (0.22-0.73)	0.003	
Germany	0.4 (0.22-0.73)	0.003	
Ireland	0.04 (0.01-0.33)	0.003	
Switzerland	0.16 (0.04-0.62)	0.008	

Multivariate analysis logistic regression model. Only statistically significant countries are presented. OR - odds ratio, CI - confidence interval, LR - likelihood ratio test, ref - reference variable.

We further evaluated if age, sex, breed and size would be putative risk factors for IgG seropositivity against canine norovirus using univariate and multivariate analysis logistic regression models (Table 4). This analysis only included serum samples from Portugal and USA, as information regarding dogs´ age, sex, breed and size was only available from these countries.

Table 4. Risk factor analysis for detection of canine norovirus IgG antibodies in dogs.

	Univariate analysis		Multivariate analysis		
Variables	**cOR(95% CI)**	**p**	**aOR(95% CI)**	**p**	**p LR**
Age					<0.001
Adult (> 6 months)	Ref		Ref		
Young (≤ 6 months)	13.53 (3.73-49.12)	<0.001	15.82 (4.22-59.29)	<0.001	
Sex					0.638
Female	Ref		Ref		
Male	0.96 (0.6-1.53)	0.857	0.89 (0.54-1.46)	0.638	
Breed					0.024
Pure	Ref		Ref		
Mixed	1.59 (0.99-2.55)	0.054	1.85 (1.08-3.16)	0.026	
Size					0.126
Lap dog	Ref		Ref		
Larger breed	0.62 (0.28-1.38)	0.24	0.96 (0.39-2.34)	0.931	

Univariate and multivariate analysis of risk factors for IgG seropositivity to canine norovirus including dogs from Portugal and the USA (cOR: crude odds ratio; aOR: adjusted odds ratio; CI: confidence interval; LR: Likelihood ratio test; Ref: reference level).

The young age of dogs (≤ 6 months) was found to be strongly and independently associated as a risk factor having a 16 fold increased odd of seropositivity to canine norovirus infection

(aOR=15.82, P<0.001). Sex, breed and size of dogs showed not to be statistically significant when ascertaining putative risk factors for IgG seropositivity against canine norovirus.

The higher risk for seropositivity found in dogs under the age of 6 months can be explained by the increased susceptibility of younger dogs to infections caused by the loss of the acquired passive immunity received from their mothers (that generally occurs by the 8 to 12 weeks-of-age), and their still imature immune system (Day *et al.* 2010, Schultz *et al*. 2010).

Human sera were also tested for IgG reactivity against canine and human norovirus.

A total of 493 human sera were tested for IgG anti-canine norovirus with a VLP-based ELISA. Of the 373 veterinarians tested, 142 (38%) were found to be positive while in the 120 general population sera only 22 (18%) were positive. The OD values for the IgG anti-canine norovirus screening in human sera are presented in Figure 7.

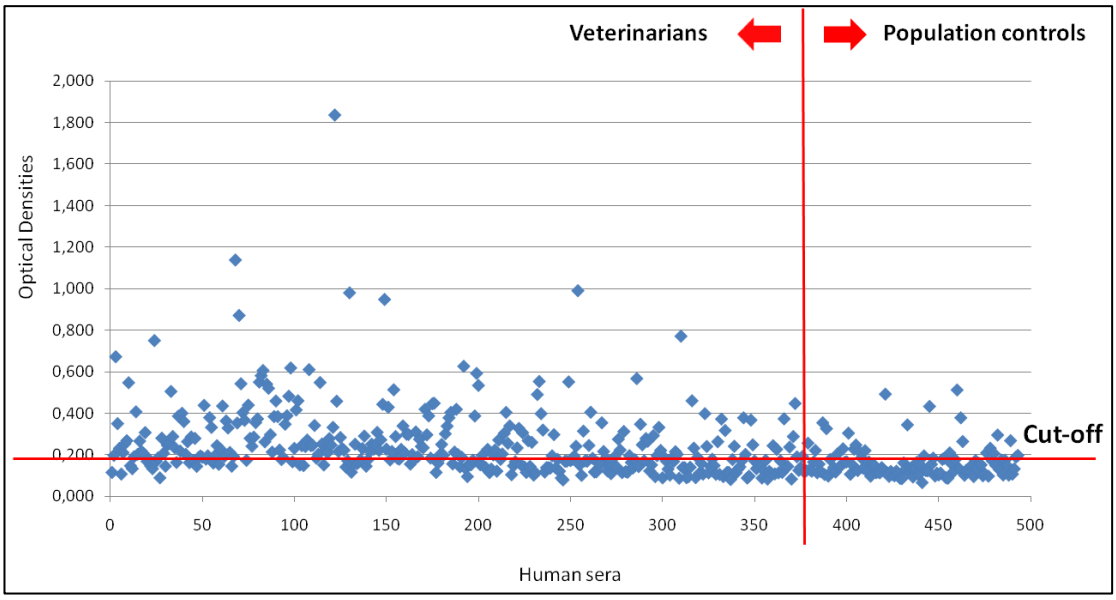

Figure 7. OD values for the IgG anti-canine norovirus screening in human sera.

All human sera were also tested for IgG anti-human norovirus with a VLP-based ELISA. Of the 373 veterinarians tested, 245 (93%) were found to be positive while in the 120 general

population sera 80 (85%) were positive. The OD values for the IgG anti-human norovirus screening in human sera are presented in Figure 8.

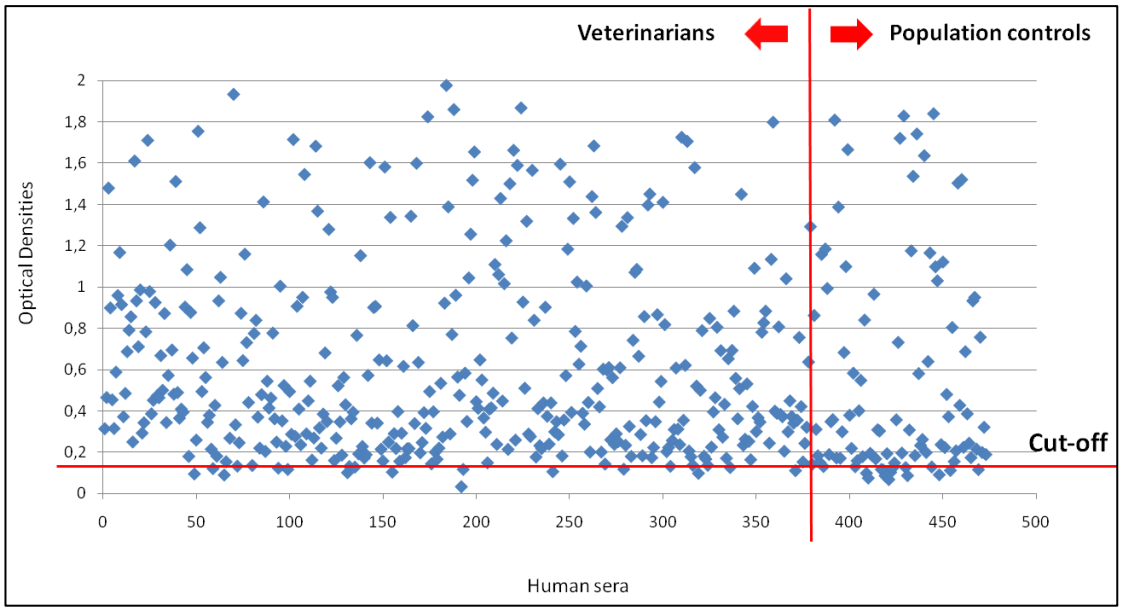

Figure 8. OD values for the IgG anti-human norovirus screening in human sera.

From the analysis of the ODs values presented in Figures 7 and 8, it can be observed that veterinarians have higher ODs against canine norovirus than the general population. Veterinarians also have a higher seroprevalence of IgG anti-canine norovirus than controls (38% vs 18%), while veterinarian and general populations have similar seroprevalences for human norovirus, 93% and 85%, respectively.

In order to compare the IgG anti-canine norovirus seroprevalences obtained for each group, a chi-square test was performed and the veterinarians' seroprevalence (a putative risk group because of increased contact with dogs) was found to be significantly higher when compared to the general population for IgG anti-canine norovirus (χ^2 test for unequal odds with Yates' continuity correction, p=0.0004).

Widdowson *et al.* (2005) screened for bovine norovirus antibodies in veterinarian and general population sera and reported significantly higher IgG seroprevalences to bovine norovirus in the veterinarian population (58/210: 28%) than in the general population (127/630: 20% [P=0.03]). A serological survey of infection by avian H5N2-subtype influenza virus in human populations from Japan used microneutralization to test two groups: poultry workers (a risk population, similar to the veterinarian population) and a general population and a significantly higher ($P < 0.05$) seroprevalence was found among poultry workers (15,3 %) than in a Japanese healthy

population (4 %) (Yamazaki *et al*. 2009).These studies indicate that a professional activity with increased contact with animals can pose as a risk factor for the occurrence of infection in humans by viral pathogens transmitted through animals.

In order to assess possible cross-reactions between canine norovirus VLPs and antibodies to other noroviruses in humans, 360 veterinarian sera were also tested for IgG anti-human norovirus with a VLP-based ELISA. A Spearmans´ correlation test was calculated to evaluate the correlation between the OD values of IgG reactivity for canine norovirus and for human norovirus obtained for the same human sera (Figure 9).

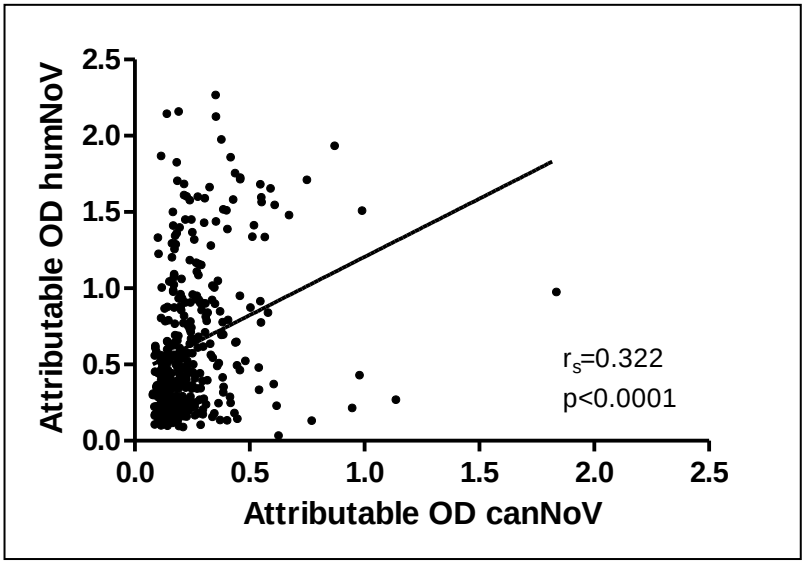

Figure 9. Study of cross-reactivity between canine norovirus VLPs and IgG anti-human norovirus. Net absorbances (ODs) attributable to serum IgG reactivity to human norovirus (humNoV) and canine norovirus VLPs (canNoV). Rs - Spearman Rank Correlation.

The correlation coefficient observed was low (Rs=0.3222, p<0.0001) which indicates a low cross-reactivity between canine norovirus VLPs and IgG anti-human norovirus.

Putative risk factors for IgG seropositivity to canine norovirus within veterinarians were evaluated using univariate and multivariate logistic regression models (Table 5).

Table 5. Risk factor analysis for IgG anti-canine norovirus positivity in veterinarians.

Variables	Univariate analysis		Multivariate analysis		
	cOR(95% CI)	p	aOR(95% CI)	p	p LR
Age					0.004
19-29	Ref		Ref		
30-39	2 (1.28-3.13)	0.002	3.15 (1.58-6.3)	0.001	
40-49	2.17 (0.99-4.79)	0.054	8.75 (1.84-41.66)	0.006	
≥50	2.33 (0.46-11.88)	0.31	6.15 (0.55-69.22)	0.142	
Sex					0.607
Male	Ref		Ref		
Female	1.11 (0.7-1.79)	0.661	1.19 (0.61-2.32)	0.607	
Residence					0.525
Portugal	Ref		Ref		
Foreign	0.59 (0.26-1.36)	0.218	0.65 (0.18-2.44)	0.528	
Years in practice					0.099
1-10	Ref		Ref		
11-20	1.32 (0.75-2.32)	0.332	0.37 (0.14-0.95)	0.039	
>20	1.48 (0.49-4.52)	0.491	0.26 (0.04-1.87)	0.18	
Work with dogs					0.649
No	Ref		Ref		
Yes	0.92 (0.15-5.58)	0.929	0.53 (0.04-7.82)	0.645	
Interaction with animals while children (<18 years-old)					0.024
No	Ref		Ref		
Yes	2.6 (0.95-7.08)	0.062	0.1 (0.02-0.71)	0.021	
Interaction with dogs while children (<18 years-old)					<0.001
No	Ref		Ref		
Yes	11.48 (5.57-23.67)	<0.001	20.8 (6.46-66.91)	<0.001	
Blood-contaminated needle stick injuries					0.026
No	Ref		Ref		
Yes	1.17 (0.7-1.93)	0.549	0.31 (0.11-0.89)	0.03	
Needle stick injury with dog blood					<0.001
No	Ref		Ref		
Yes	24.92 (12.06-51.49)	<0.001	28.7 (11.94-69.19)	<0.001	

Univariate and multivariate analysis of risk factors for canine norovirus infection in veterinarians. cOR - crude odds ratio, aOR - adjusted odds ratio, CI - confidence interval, LR - Likelihood ratio test, Ref - reference level.

Age was identified as a risk factor for IgG seropositivity to canine norovirus in this population. This is reflected significantly among the age groups of 30-39 (aOR=3.15, p<0.001) and 40-49 years of age (aOR=8.75, p<0.006) but not in the ≥50 years of age group (aOR=6.15, p<0.142). This could be due to an immune burst (repeated stimuli) due to repeated exposures to canine norovirus over the continuous years of veterinary practice. However, this assumption could not be supported because no statistical significance was observed with the variable “years in practice”.

On the other hand, the interaction with dogs while children (< 18 years-old) was strongly and independently associated (aOR=20.8, p<0.001) with seropositivity, with a 20 fold increased odds when comparing with the group without dog interaction while children. Stick injury with dog

blood contaminated needles was also identified as a risk factor showing a strong and independent association (cOR=24.92, p<0.001; aOR=28.7, p<0.001) with seropositivity to canine norovirus.

It was interesting to find that stick injuries with dog blood contaminated needles was an important risk factor for seropositivity. Norovirus RNA has been detected in sera of children with gastroenteritis, suggesting that viremia could be a common event during norovirus gastroenteritis (Takanashi *et al.* 2009; Medici *et al.* 2010). Therefore, it is possible that the encounter with canine norovirus can occur through dog blood contaminated needle injuries, a common event within veterinarians, providing a large burst of antibodies and explaining the strong association found in this study.

CONCLUSIONS

This is the first study detecting antibodies against canine norovirus in the dog and human populations.

The completion of this research supports the hypothesis that canine norovirus are circulating widespread throughout Europe, namely in Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Netherlands, Norway, Poland, Portugal, Sweden, Switzerland, United Kingdom, and USA, with an average seroprevalence of 69.2%. Moreover, young dogs (less than 6 months of age) were identified as having a higher susceptibility to seropositivity by canine norovirus.

The data from this study also confirms that the veterinary professionals are at higher risk for seropositivity to canine norovirus than the general population. Age (between 30-49 years), interaction with dogs while children (< 18 years) and stick injury by a needle contaminated with dog blood were identified as risk factors for seropositivity to canine norovirus antibodies in the veterinarian population of Portugal.

Zoonotic transmission between dogs and humans is not new, and the close and often intimate interactions between these species have been suggested as a major disease risk for humans. Given the recent detection of canine noroviruses, the elucidation of the role of this virus in dogs and its importance as a zoonotic pathogen is keen to be searched for in the upcoming years.

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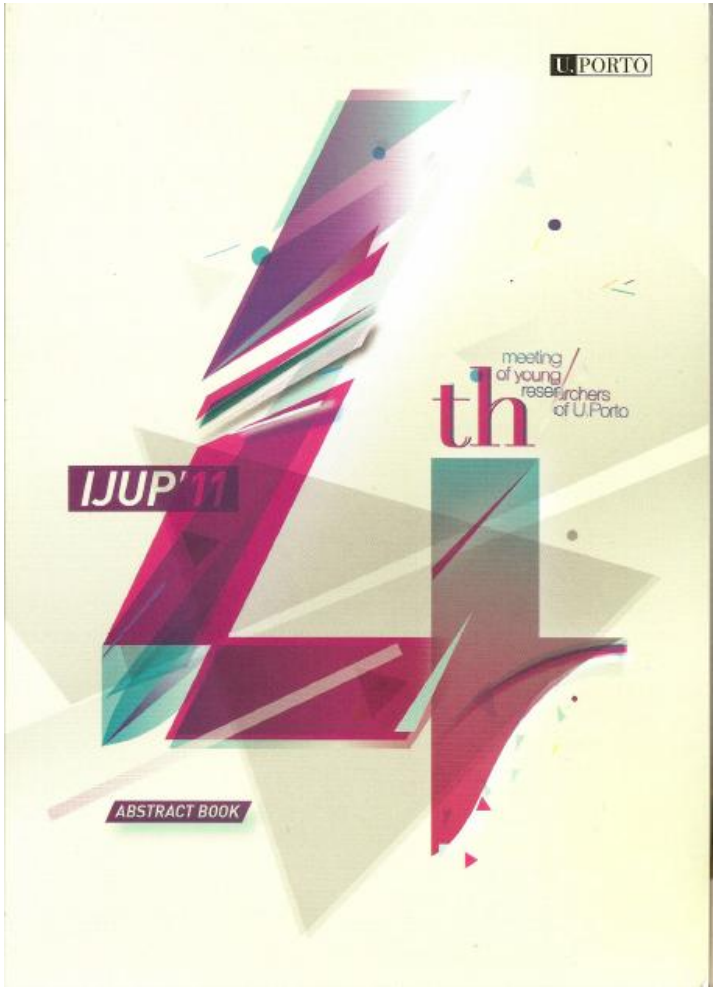
ATTACHMENT I

Abstract in the book of congress/ Poster presentation

J.R. Mesquita, **I. Delgado**, M.S.J. Nascimento. "Seroprevalence of a Novel Canine Norovirus in pet dogs". **IV Encontro de Jovens Investigadores da Universidade do Porto.** Porto, Portugal, 17-19 Fevereiro, 2011 (P-398).



Seroprevalence of a novel Canine Norovirus in pet dogs

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Noroviruses (NoV) are today recognized as the leading cause of gastroenteritis worldwide among persons of all ages [1]. They have a wide degree of genetic variability and are classified in 5 genogroups (GI-GV) three of which (GI, GII and GIV) contain human viruses [2]. The most important modes of transmission are person-to-person contact and consumption of contaminated food , however, a zoonotic transmission has been suggested as a possibility due to the close genetic relatedness between human and some animal NoV [3]. Recently, our group identified in Portugal a novel canine NoV (Viseu strain) that was genetically unrelated to any other animal or human norovirus known [2].

In order to evaluate the prevalence of antibodies to the novel canine NoV in dogs we developed and validated an enzyme immune assay (EIA) based on recombinant virus-like particles (VLPs). The VLPs were produced in Sf9 insect cells infected with a recombinant baculovirus containing the capsid protein gene (VP1) of the Viseu strain.

Serum samples were obtained from 309 dogs from Portugal and 61 dogs from the USA. Sera from 8 specific pathogen free (SPF) beagle dogs were used as negative control. Each serum sample was tested for the presence of IgG antibodies against canine NoV (Viseu strain) using a direct VLP based EIA. A serum was considered positive when the optical density (OD) value was higher than the cutoff (mean of negative control serum OD + 3 standard deviations).

Overall, 226 (73%) of dogs from Portugal and 51 (83%) of dogs from the USA tested positive for IgG against the Viseu canine NoV. The age of the animal was identified as a risk factor, with young dogs (< 6 months) having a 16 fold increased odd of seropositivity to canine NoV (aOR=15.82, P<0,001).

The high canine seroprevalence found in the present study indicates that infection with this novel canine NoV strain is common among dogs of Portugal and of the USA. Given the intimate contact of dogs with humans, this virus may represent a potential zoonotic risk.

Acknowledgments: To Fundação para a Ciência e a Tecnologia for the grant SFRH/BD/45407/2008 to J.R.M. To Dr. Jan Vinjé, CDC, Atlanta, USA for the generous provision of the VLPs.

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Seroprevalence of a novel Canine Norovirus in pet dogs

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INTRODUCTION

Human noroviruses (NoV) are now recognized as the leading cause of foodborne outbreaks of acute gastroenteritis worldwide as well as an important cause of sporadic acute gastroenteritis among persons of all ages [1]. NoV have a wide degree of genetic variability and are classified in 5 genogroups (GI-GV) three of which (GI, GII and GIV) contain the human noroviruses. The most important modes of transmission are person-to-person contact and consumption of contaminated food however a zoonotic transmission has been suggested as a possibility due to the close genetic relatedness between human and some animal NoV [2]. Recently, our group identified in Portugal a novel canine NoV (Viseu strain) in 40% and 9% of dogs with and without diarrhea, respectively [3]. Sequence analysis of the 3-kb fragment including ORF2 (major capsid protein gene) indicated that the Viseu strain was genetically unrelated with any other animal or human NoVs and comprises a tentative new genogroup (GVI). The detection of this novel canine NoV strain raises the question on whether this potential enteric pathogen is widely circulating in the dog population, with the ability to establish infection.

OBJECTIVE

The aim of the present work was to evaluate the prevalence of antibodies (IgG) to canine NoV (Viseu strain) in pet dogs from Portugal and from the USA using an enzyme immune assay (EIA) based on recombinant virus-like particles (VLPs).

MATERIAL & METHODS

Samples: Blood was collected by venepuncture from 370 pet dogs (309 dogs from Portugal and 61 dogs from the USA). Sera were separated and kept at -20ºC until use. Data regarding age, sex, country, breed and size was recorded.

Recombinant Canine NoV-VLPs: Virus-like particles (VLPs) produced in Sf9 insect cells infected with a recombinant baculovirus containing the capsid gene (VP1) of canine NoV Viseu strain were gently provided by Dr Jan Vinjé from the CDC, Atlanta, USA. VLPs were stored at 4º C until use.

VLP-Based EIA: For the development of the EIA, 96-well microplates (Immulon 2HB, Thermo Electron Corporation, Milford, USA) were coated with 50 µL VLP suspension (5 µg/ml) in Coating Buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) or Coating Buffer without VLPs. After incubation for 1 h at 37ºC, wells were washed three times with PBS (0.01 M, pH 7.4)/Tween 0.005% and blocked with 200 µL of Blocking Buffer (PBS/Tween 0.005% - 10% Non-Fat Dry Milk) for 2 h at 37ºC. After washing 50 µL of serum samples (diluted 1:100 in Blocking Buffer) were added in duplicate and incubated for 1 h at 37ºC. After washing 50 µL of Goat-anti-Dog IgG-horseradish peroxidase (1:6400) were added and incubated for 1 h at 37ºC. The wells were washed before development with 100 µL substrate (TMB Microwell Peroxidase System, Kirkegaard & Perry Laboratories Inc., USA) at room temperature for 10 minutes in the dark. Enzymatic reaction was stopped by adding 100 µL of Stop Solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA) and optical density (OD) was measured at 450 nm (MRX revelation spectrophotometer, Dynex, Magellan Biosciences). A serum was considered positive when the optical density (OD) value was higher than the cutoff (mean of negative control serum OD + 3 standard deviations).

Statistical Analysis: All statistical analyses were performed using Epicalc package in the R software (R 2.1.2.0) (R Development Core Team, 2010). Age, breed and size were divided into 2 groups (≤ 6 months = young and > 6months = adult, pure-breeds and mixed-breeds, lap dog and larger breed respectively).

RESULTS

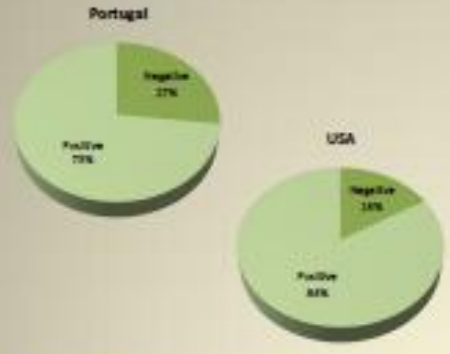

Figure- Seroprevalence of IgG antibodies against canine NoV (Viseu strain) in pet dogs from Portugal and USA.

Table - Risk factor analysis for canine NoV (Viseu strain) infection in pet dogs.

	Univariate analysis		Multivariate analysis		
	cOR (95% CI)	p	aOR (95% CI)	p	pLR
Age					< 0.001
Adult (> 6 months)	Ref.		Ref.		
Young (≤ 6 months)	15,83 (8,73-28,72)	< 0,001	15,82 (8,22-30,29)	< 0,001	
Sex					0,658
Female	Ref.		Ref.		
Male	0,94 (0,6-1,45)	0,807	0,89 (0,54-1,46)	0,658	
Country					0,103
USA	Ref.		Ref.		
Portugal	1,87 (0,91-3,86)	0,069	1,99 (0,79-5,03)	0,259	
Breed					0,024
Pure	Ref.		Ref.		
Mixed	1,69 (1,09-2,63)	0,084	1,83 (1,08-3,10)	0,039	
Size					0,136
Lap dog	Ref.		Ref.		
Larger breed	0,63 (0,39-1,04)	0,26	0,64 (0,39-1,04)	0,691	

cOR: crude odds ratio; aOR: adjusted odds ratio; CI: confidence interval; LR: Likelihood ratio test; Ref: reference level

CONCLUSIONS

- IgG antibodies against canine NoV (Viseu strain) were detected in 226 (73 %) dogs from Portugal and 51 (84%) dogs from the USA (Figure) which indicates that infection with this recently identified strain is common among dogs of Portugal and of the USA.
- The age of dogs was identified as a risk factor with young pets (≤ 6 months) having a 16 fold increased odd of seropositivity to canine NoV (aOR=15.82, P<0.001).
- No other statistically significant risk factors were found as demonstrated by the multivariate analysis presented in Table.
- Given the intimate contact of dogs with the generalized human population the circulation of this new canine NoV may represent a potential zoonotic risk.
- Further studies aiming the evaluation of the prevalence of antibodies against canine NoV (Viseu strain) in human population are necessary to clarify the zoonotic potential of this novel canine NoV.

ACKNOWLEDGEMENTS

To Fundação para a Ciência e a Tecnologia for the grant SFRH/BD/45407/2008 to J.R.M. To Dr. Jan Vinjé, CDC, Atlanta, USA for the generous provision of the VLPs.

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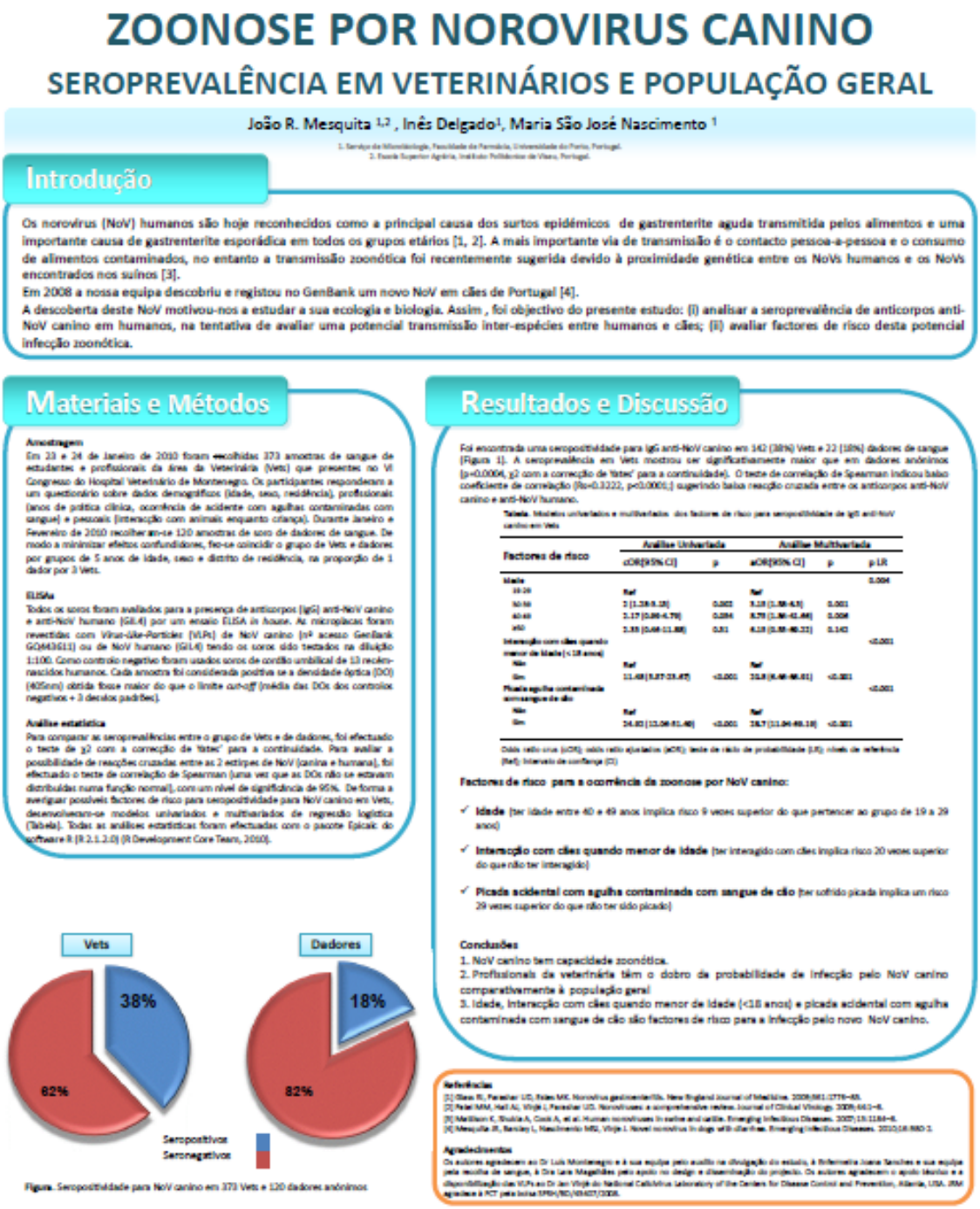
ATTACHMENT II

Poster presentation

J.R. Mesquita, **I. Delgado**, M.S.J. Nascimento. “Zoonose por Norovirus Canino: seroprevalência em veterinários e população geral”. **VII Congresso Hospital Veterinário Montenegro.** Santa Maria da Feira, Portugal, 12-13 Fevereiro, 2011.



ZOONOSE POR NOROVIRUS CANINO

SEROPREVALÊNCIA EM VETERINÁRIOS E POPULAÇÃO GERAL

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Introdução

Os norovirus (NoV) humanos são hoje reconhecidos como a principal causa dos surtos epidémicos de gastrenterite aguda transmitida pelos alimentos e uma importante causa de gastrenterite esporádica em todos os grupos etários [1, 2]. A mais importante via de transmissão é o contacto pessoa-a-pessoa e o consumo de alimentos contaminados, no entanto a transmissão zoonótica foi recentemente sugerida devido à proximidade genética entre os NoVs humanos e os NoVs encontrados nos suínos [3].
Em 2008 a nossa equipa descobriu e registou no GenBank um novo NoV em cães de Portugal [4].
A descoberta deste NoV motivou-nos a estudar a sua ecologia e biologia. Assim , foi objectivo do presente estudo: (i) analisar a seroprevalência de anticorpos anti-NoV canino em humanos, na tentativa de avaliar uma potencial transmissão inter-espécies entre humanos e cães; (ii) avaliar factores de risco desta potencial infecção zoonótica.

Materiais e Métodos

Amostragem
Em 23 e 24 de Janeiro de 2010 foram recolhidas 373 amostras de sangue de estudantes e profissionais da área da Veterinária (Vets) que presentes no VI Congresso do Hospital Veterinário de Montenegro. Os participantes responderam a um questionário sobre dados demográficos (idade, sexo, residência), profissionais (anos de prática clínica, ocorrência de acidente com agulhas contaminadas com sangue) e pessoais (interacção com animais enquanto criança). Durante Janeiro e Fevereiro de 2010 recolheram-se 120 amostras de soro de dadores de sangue. De modo a minimizar efeitos confundidores, fez-se coincidir o grupo de Vets e dadores por grupos de 5 anos de idade, sexo e distrito de residência, na proporção de 1 dador por 3 Vets.

ELISAs
Todos os soros foram avaliados para a presença de anticorpos [IgG] anti-NoV canino e anti-NoV humano (GII.4) por um ensaio ELISA *in house*. As microplacas foram revestidas com *Virus-like-Particles* (VLPs) de NoV canino (nº acesso GenBank GQ463611) ou de NoV humano (GII.4) tendo os soros sido testados na diluição 1:100. Como controlo negativo foram usados soros de cordão umbilical de 13 recém-nascidos humanos. Cada amostra foi considerada positiva se a densidade óptica (DO) (405nm) obtida fosse maior do que o limite *cut-off* (média das DOs dos controlos negativos + 3 desvios padrões).

Análise estatística
Para comparar as seroprevalências entre o grupo de Vets e de dadores, foi efectuado o teste de $\chi 2$ com a correcção de 'Yates' para a continuidade. Para avaliar a possibilidade de reacções cruzadas entre as 2 estirpes de NoV (canina e humana), foi efectuado o teste de correlação de Spearman (uma vez que as DOs não se estavam distribuídas numa função normal), com um nível de significância de 95%. De forma a averiguar possíveis factores de risco para seropositividade para NoV canino em Vets, desenvolveram-se modelos univariados e multivariados de regressão logística (Tabela). Todas as análises estatísticas foram efectuadas com o pacote Epicalc do software R (R 2.1.2.0) (R Development Core Team, 2010).

Resultados e Discussão

Foi encontrada uma seropositividade para IgG anti-NoV canino em 142 (38%) Vets e 22 (18%) dadores de sangue (Figura 1). A seroprevalência em Vets mostrou ser significativamente maior que em dadores anónimos ($p<0.0004$, $\chi 2$ com a correcção de 'Yates' para a continuidade). O teste de correlação de Spearman indicou baixo coeficiente de correlação ($Rs=0.3222$, $p<0.0001$,) sugerindo baixa reacção cruzada entre os anticorpos anti-NoV canino e anti-NoV humano.

Tabela. Modelos univariados e multivariados dos factores de risco para seropositividade de IgG anti-NoV canino em Vets

Factores de risco	Análise Univariada		Análise Multivariada		
	cOR[95% CI]	p	aOR[95% CI]	p	p LR
Idade					0.004
19-29	Ref		Ref		
30-39	3 [1.28-8.18]	0.002	3.18 [1.38-8.5]	0.001	
40-49	3.17 [0.88-8.79]	0.054	8.79 [1.96-42.44]	0.004	
≥50	1.33 [0.68-11.88]	0.51	4.18 [0.88-40.22]	0.143	
Interacção com cães quando menor de idade (<18 anos)					<0.001
Não	Ref		Ref		
Sim	11.48 [5.27-28.47]	<0.001	23.8 [8.48-88.01]	<0.001	
Picada agulha contaminada com sangue de cão					<0.001
Não	Ref		Ref		
Sim	24.83 [13.08-51.48]	<0.001	28.7 [11.04-88.18]	<0.001	

Odds ratio cruz (cOR); odds ratio ajustados (aOR); teste de rácio de probabilidade (LR); nível de referência (Ref); Intervalo de confiança (CI)

Factores de risco para a ocorrência de zoonose por NoV canino:

- ✓ **Idade** (ter idade entre 40 e 49 anos implica risco 9 vezes superior do que pertencer ao grupo de 19 a 29 anos)
- ✓ **Interacção com cães quando menor de idade** (ter interagido com cães implica risco 20 vezes superior do que não ter interagido)
- ✓ **Picada acidental com agulha contaminada com sangue de cão** (ter sofrido picada implica um risco 29 vezes superior do que não ter sido picado)

Conclusões
1. NoV canino tem capacidade zoonótica.
2. Profissionais da veterinária têm o dobro da probabilidade de infecção pelo NoV canino comparativamente à população geral
3. Idade, Interacção com cães quando menor de idade (<18 anos) e picada acidental com agulha contaminada com sangue de cão são factores de risco para a infecção pelo novo NoV canino.

Figura. Seropositividade para NoV canino em 373 Vets e 120 dadores anónimos

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ATTACHMENT III

The OD values obtained for IgG reactivity detected by a VLP-based ELISA for the tested serum samples are presented in Tables 1-5.

Table 1. ODs for IgG anti-canine norovirus of canine sera. Denmark - DEN, Finland - FIN, France - FR, Germany - GER, Hungary - HUN, Ireland - IRE, Italy - IT, Netherlands - NE, Norway - NOR, Poland - POL, Portugal - PT, Sweden - SWE, Switzerland - SWI, United Kingdom - UK and United States of America – USA.

Serum	OD	GER45	0,319	FR40	0,284	DEN35	0,445	POL30	0,158	NE25	0,255	NOR20	0,469	UK15	0,217	HUN10	0,271
GER1	0,488	GER46	0,316	FR41	0,208	DEN36	0,326	POL31	0,422	NE26	0,542	NOR21	0,306	UK16	0,292	TI1	0,166
GER2	0,148	GER47	0,176	FR42	0,337	DEN37	0,308	POL32	0,420	NE27	0,256	NOR22	0,396	UK17	0,606	TI2	0,220
GER3	0,486	GER48	0,284	FR43	0,348	DEN38	0,341	POL33	0,320	NE28	0,252	NOR23	0,307	UK18	0,377	TI3	0,205
GER4	0,268	GER49	0,246	FR44	0,349	DEN39	0,362	POL34	0,404	NE29	0,437	NOR24	0,364	UK19	0,384	TI4	0,268
GER5	0,589	GER50	0,187	FR45	0,363	DEN40	0,272	POL35	0,418	NE30	0,394	NOR25	0,465	UK20	0,194	TI5	0,352
GER6	0,511	FR1	0,193	FR46	0,592	DEN41	0,221	POL36	0,464	NE31	0,220	NOR26	0,238	UK21	0,269	TI6	0,315
GER7	0,363	FR2	0,686	FR47	0,412	DEN42	0,348	POL37	0,260	NE32	0,373	NOR27	0,406	UK22	0,250	TI7	0,192
GER8	0,449	FR3	0,178	FR48	0,367	DEN43	0,313	POL38	0,502	NE33	0,494	NOR28	0,263	UK23	0,372	TI8	0,302
GER9	0,324	FR4	0,289	FR49	0,155	DEN44	0,326	POL39	0,261	NE34	0,138	NOR29	0,205	UK24	0,476	TI9	0,232
GER10	0,141	FR5	0,214	FR50	0,372	DEN45	0,053	POL40	0,268	NE35	0,620	NOR30	0,489	UK25	0,417	TI10	0,417
GER11	0,703	FR6	0,233	DEN1	0,216	DEN46	0,354	POL41	0,175	NE36	0,502	NOR31	0,324	UK26	0,235	IRE1	0,121
GER12	0,350	FR7	0,213	DEN2	0,292	DEN47	0,660	POL42	0,851	NE37	0,410	NOR32	0,362	UK27	0,187	IRE2	0,200
GER13	0,259	FR8	0,152	DEN3	0,227	DEN48	0,560	POL43	0,864	NE38	0,427	NOR33	0,463	UK28	0,333	IRE3	0,241
GER14	0,287	FR9	0,220	DEN4	0,223	DEN49	0,352	POL44	0,618	NE39	0,313	NOR34	0,455	UK29	0,476	IRE4	0,268
GER15	0,298	FR10	0,314	DEN5	0,257	DEN50	0,279	POL45	0,219	NE40	0,482	NOR35	0,365	UK30	0,547	IRE5	0,190
GER16	0,416	FR11	0,340	DEN6	0,491	POL1	0,418	POL46	0,299	NE41	0,390	NOR36	0,783	UK31	0,393	IRE6	0,143
GER17	0,189	FR12	0,267	DEN7	0,442	POL2	0,325	POL47	0,382	NE42	0,418	NOR37	0,679	UK32	0,340	IRE7	0,224
GER18	0,322	FR13	0,560	DEN8	0,279	POL3	0,289	POL48	0,273	NE43	0,487	NOR38	0,258	UK33	0,532	IRE8	0,183
GER19	0,248	FR14	0,367	DEN9	0,516	POL4	0,234	POL49	0,322	NE44	0,520	NOR39	0,474	UK34	0,544	IRE9	0,130
GER20	0,469	FR15	0,244	DEN10	0,204	POL5	0,259	POL50	0,284	NE45	0,352	NOR40	0,264	UK35	0,306	IRE10	0,182
GER21	0,241	FR16	0,362	DEN11	0,340	POL6	0,734	NE1	0,325	NE46	0,243	NOR41	0,638	UK36	0,290	SWI1	0,161
GER22	0,181	FR17	0,512	DEN12	0,332	POL7	0,631	NE2	0,846	NE47	0,335	NOR42	0,699	UK37	0,629	SWI2	0,131
GER23	0,267	FR18	0,448	DEN13	0,221	POL8	0,325	NE3	0,441	NE48	0,379	NOR43	0,303	UK38	0,389	SWI3	0,212
GER24	0,847	FR19	0,284	DEN14	0,258	POL9	0,801	NE4	0,245	NE49	0,317	NOR44	0,436	UK39	0,523	SWI4	0,181
GER25	0,139	FR20	0,430	DEN15	0,423	POL10	0,439	NE5	0,562	NE50	0,214	NOR45	0,376	UK40	0,494	SWI5	0,125
GER26	0,245	FR21	0,297	DEN16	0,282	POL11	0,243	NE6	0,570	NOR1	0,213	NOR46	0,229	UK41	0,320	SWI6	0,242
GER27	0,194	FR22	0,405	DEN17	0,442	POL12	0,604	NE7	0,330	NOR2	0,216	NOR47	0,321	UK42	0,495	SWI7	0,504
GER28	0,282	FR23	0,340	DEN18	0,508	POL13	0,309	NE8	0,417	NOR3	0,291	NOR48	0,450	UK43	0,434	SWI8	0,353
GER29	0,171	FR24	0,219	DEN19	0,355	POL14	0,242	NE9	0,266	NOR4	0,234	NOR49	0,408	UK44	0,326	SWI9	0,167
GER30	0,297	FR25	0,678	DEN20	0,375	POL15	0,130	NE10	0,403	NOR5	0,648	NOR50	0,249	UK45	0,403	SWI10	0,485
GER31	0,186	FR26	0,216	DEN21	0,607	POL16	0,412	NE11	0,398	NOR6	0,425	UK1	0,613	UK46	0,342	SWE1	0,461
GER32	0,176	FR27	0,110	DEN22	0,274	POL17	0,506	NE12	0,477	NOR7	0,294	UK2	0,341	UK47	0,426	SWE2	0,425
GER33	0,234	FR28	0,288	DEN23	0,571	POL18	0,399	NE13	0,420	NOR8	0,270	UK3	0,425	UK48	0,259	SWE3	0,321
GER34	0,247	FR29	0,180	DEN24	0,270	POL19	0,237	NE14	0,371	NOR9	0,332	UK4	0,535	UK49	0,300	SWE4	0,422
GER35	0,414	FR30	0,343	DEN25	0,225	POL20	0,436	NE15	0,244	NOR10	0,361	UK5	0,478	UK50	0,505	SWE5	0,362
GER36	0,145	FR31	0,486	DEN26	0,328	POL21	0,464	NE16	0,399	NOR11	0,677	UK6	0,441	HUN1	0,263	SWE6	0,323
GER37	0,415	FR32	0,915	DEN27	0,282	POL22	0,776	NE17	0,676	NOR12	0,219	UK7	0,413	HUN2	0,381	SWE7	0,645
GER38	0,334	FR33	0,139	DEN28	0,272	POL23	0,331	NE18	0,572	NOR13	0,361	UK8	0,708	HUN3	0,177	SWE8	0,561
GER39	0,346	FR34	0,200	DEN29	0,257	POL24	0,246	NE19	0,293	NOR14	0,536	UK9	0,638	HUN4	0,149	SWE9	0,359
GER40	0,247	FR35	0,134	DEN30	0,261	POL25	0,373	NE20	0,248	NOR15	0,154	UK10	0,530	HUN5	0,185	SWE10	0,449
GER41	0,345	FR36	0,511	DEN31	0,421	POL26	0,363	NE21	0,437	NOR16	0,257	UK11	0,646	HUN6	0,216	FIN1	0,231
GER42	0,326	FR37	0,206	DEN32	0,417	POL27	0,312	NE22	0,400	NOR17	0,236	UK12	0,454	HUN7	0,194	FIN2	0,562
GER43	0,215	FR38	0,273	DEN33	0,291	POL28	0,666	NE23	0,396	NOR18	0,375	UK13	0,589	HUN8	0,171	FIN3	0,751
GER44	0,265	FR39	0,288	DEN34	0,324	POL29	0,515	NE24	0,403	NOR19	0,538	UK14	0,827	HUN9	0,345	FIN4	0,318

FIN5	0,203	PT46	0,745	PT97	1,346	PT148	2,733	PT199	2,658	PT250	1,002	PT301	1,081	USA43	1,451
FIN6	0,433	PT47	0,897	PT98	0,836	PT149	2,271	PT200	0,715	PT251	0,094	PT302	0,980	USA44	0,594
FIN7	0,439	PT48	1,939	PT99	0,649	PT150	1,313	PT201	0,150	PT252	0,567	PT303	0,167	USA45	0,374
FIN8	0,527	PT49	0,293	PT100	1,527	PT151	0,398	PT202	0,603	PT253	0,159	PT304	0,598	USA46	0,496
FIN9	0,533	PT50	0,251	PT101	0,264	PT152	1,061	PT203	1,440	PT254	1,052	PT305	0,603	USA47	0,494
FIN10	0,441	PT51	0,747	PT102	0,828	PT153	2,752	PT204	0,328	PT255	0,395	PT306	0,438	USA48	0,300
PT1	1,484	PT52	1,344	PT103	0,377	PT154	1,383	PT205	0,931	PT256	0,983	PT307	0,203	USA49	1,213
PT2	0,111	PT53	1,088	PT104	2,045	PT155	1,971	PT206	0,168	PT257	1,419	PT308	0,759	USA50	0,865
PT3	0,797	PT54	1,271	PT105	1,289	PT156	1,290	PT207	0,255	PT258	1,314	PT309	0,636	USA51	0,959
PT4	0,092	PT55	0,121	PT106	0,847	PT157	1,543	PT208	0,786	PT259	0,198	USA1	1,211	USA52	0,670
PT5	0,833	PT56	0,280	PT107	0,520	PT158	2,453	PT209	0,387	PT260	0,329	USA2	0,394	USA53	0,861
PT6	0,419	PT57	0,713	PT108	1,232	PT159	0,374	PT210	1,070	PT261	0,695	USA3	0,297	USA54	0,871
PT7	0,223	PT58	0,830	PT109	1,307	PT160	0,568	PT211	0,189	PT262	0,640	USA4	0,822	USA55	0,797
PT8	1,298	PT59	2,406	PT110	0,545	PT161	0,393	PT212	0,218	PT263	0,335	USA5	0,528	USA56	0,592
PT9	0,337	PT60	1,953	PT111	1,379	PT162	1,482	PT213	0,477	PT264	0,488	USA6	0,767	USA57	1,581
PT10	0,843	PT61	2,062	PT112	0,703	PT163	0,646	PT214	0,258	PT265	0,220	USA7	0,403	USA58	0,771
PT11	0,141	PT62	1,455	PT113	2,053	PT164	1,799	PT215	0,572	PT266	0,463	USA8	0,553	USA59	0,433
PT12	1,294	PT63	0,806	PT114	0,480	PT165	0,895	PT216	0,453	PT267	0,212	USA9	0,177	USA60	0,439
PT13	0,080	PT64	0,557	PT115	0,668	PT166	0,584	PT217	0,579	PT268	0,194	USA10	1,596	USA61	0,415
PT14	1,738	PT65	0,215	PT116	0,219	PT167	0,550	PT218	0,387	PT269	0,111	USA11	0,468	B1	0,122
PT15	0,830	PT66	0,372	PT117	0,436	PT168	0,136	PT219	0,125	PT270	0,163	USA12	0,742	B2	0,211
PT16	0,223	PT67	0,572	PT118	0,879	PT169	0,107	PT220	0,654	PT271	1,000	USA13	0,401	B3	0,184
PT17	0,127	PT68	0,567	PT119	0,876	PT170	0,353	PT221	0,458	PT272	1,183	USA14	0,635	B4	0,164
PT18	0,409	PT69	0,705	PT120	0,938	PT171	1,086	PT222	0,385	PT273	0,211	USA15	0,812	B5	0,170
PT19	0,820	PT70	0,630	PT121	0,440	PT172	0,312	PT223	0,276	PT274	0,809	USA16	0,371	B6	0,150
PT20	2,031	PT71	0,534	PT122	0,743	PT173	1,019	PT224	0,538	PT275	1,505	USA17	0,662		
PT21	0,904	PT72	1,024	PT123	1,122	PT174	0,710	PT225	0,420	PT276	1,690	USA18	1,018		
PT22	1,215	PT73	0,761	PT124	0,303	PT175	1,346	PT226	1,517	PT277	0,358	USA19	0,433		
PT23	1,746	PT74	1,582	PT125	1,384	PT176	0,102	PT227	0,183	PT278	0,505	USA20	0,351		
PT24	0,068	PT75	0,272	PT126	1,658	PT177	0,212	PT228	0,219	PT279	0,165	USA21	0,247		
PT25	1,115	PT76	1,763	PT127	1,803	PT178	0,123	PT229	0,327	PT280	0,918	USA22	0,354		
PT26	0,582	PT77	0,870	PT128	2,205	PT179	1,048	PT230	2,008	PT281	0,353	USA23	0,426		
PT27	0,138	PT78	0,119	PT129	0,456	PT180	0,150	PT231	0,472	PT282	0,391	USA24	1,138		
PT28	1,289	PT79	0,129	PT130	0,431	PT181	0,523	PT232	0,438	PT283	0,759	USA25	0,829		
PT29	0,352	PT80	1,206	PT131	0,686	PT182	0,290	PT233	1,914	PT284	0,278	USA26	1,621		
PT30	1,901	PT81	0,446	PT132	0,387	PT183	0,490	PT234	0,374	PT285	0,260	USA27	0,818		
PT31	0,208	PT82	0,759	PT133	0,413	PT184	1,783	PT235	0,351	PT286	0,502	USA28	0,990		
PT32	0,140	PT83	0,307	PT134	2,295	PT185	0,221	PT236	0,994	PT287	0,076	USA29	1,239		
PT33	0,180	PT84	0,079	PT135	0,572	PT186	0,716	PT237	0,539	PT288	0,485	USA30	0,216		
PT34	0,351	PT85	0,159	PT136	1,519	PT187	0,108	PT238	1,032	PT289	0,470	USA31	0,913		
PT35	0,189	PT86	0,329	PT137	1,778	PT188	0,158	PT239	0,422	PT290	0,465	USA32	0,347		
PT36	0,355	PT87	0,317	PT138	1,114	PT189	0,487	PT240	1,372	PT291	0,089	USA33	0,825		
PT37	0,291	PT88	0,761	PT139	1,167	PT190	0,431	PT241	1,142	PT292	0,389	USA34	0,223		
PT38	0,457	PT89	1,373	PT140	1,500	PT191	0,275	PT242	0,225	PT293	2,017	USA35	1,338		
PT39	0,712	PT90	0,301	PT141	1,020	PT192	0,503	PT243	0,683	PT294	0,462	USA36	0,027		
PT40	0,833	PT91	0,514	PT142	2,728	PT193	1,773	PT244	0,940	PT295	0,479	USA37	0,786		
PT41	0,818	PT92	0,565	PT143	0,400	PT194	0,911	PT245	0,381	PT296	0,523	USA38	0,643		
PT42	0,712	PT93	0,493	PT144	2,648	PT195	1,311	PT246	0,609	PT297	0,141	USA39	1,277		
PT43	0,441	PT94	0,839	PT145	0,410	PT196	1,025	PT247	0,753	PT298	0,131	USA40	0,457		
PT44	1,396	PT95	1,024	PT146	1,472	PT197	0,273	PT248	0,159	PT299	0,160	USA41	0,726		
PT45	1,283	PT96	0,959	PT147	1,560	PT198	0,377	PT249	0,198	PT300	0,454	USA42	1,019		

Table 2. ODs for IgG anti-human norovirus of canine sera. Portugal - PT

Sera	ODs
PT8	0,104
PT20	0,100
PT21	0,118
PT31	0,060
PT32	0,096
PT34	0,112
PT35	0,135
PT53	0,088
PT77	0,099
PT79	0,087
PT84	0,082
PT85	0,084
PT86	0,178
PT100	0,125
PT113	0,136
PT121	0,115
PT125	0,130
PT134	0,091
PT140	0,071
PT142	0,109
PT144	0,138
PT146	0,126
PT148	0,119

Table 3. ODs for IgG anti-canine norovirus of human sera. Veterinarian – V, General population – G.

Sera	ODs	V45	0,385	V90	0,580	V135	0,140	V180	0,386	V225	0,232	V270	0,183	V315	0,139	V360	0,107	G32	0,246	G77	0,102
V1	0,106	V46	0,217	V91	0,605	V136	0,170	V181	0,183	V226	0,326	V271	0,122	V316	0,459	V361	0,225	G33	0,123	G78	0,111
V2	0,287	V47	0,399	V92	0,261	V137	0,979	V182	0,445	V227	0,152	V272	0,150	V317	0,231	V362	0,112	G34	0,213	G79	0,177
V3	0,335	V48	0,359	V93	0,539	V138	0,115	V183	0,447	V228	0,305	V273	0,108	V318	0,089	V363	0,170	G35	0,184	G80	0,182
V4	0,324	V49	0,201	V94	0,519	V139	0,153	V184	0,114	V229	0,140	V274	0,146	V319	0,186	V364	0,114	G36	0,134	G81	0,088
V5	0,243	V50	0,262	V95	0,296	V140	0,250	V185	0,159	V230	0,114	V275	0,190	V320	0,110	V365	0,137	G37	0,219	G82	0,086
V6	0,114	V51	0,161	V96	0,212	V141	0,156	V186	0,199	V231	0,267	V276	0,141	V321	0,158	V366	0,143	G38	0,122	G83	0,208
V7	0,196	V52	0,285	V97	0,383	V142	0,164	V187	0,249	V232	0,143	V277	0,274	V322	0,106	V367	0,236	G39	0,165	G84	0,155
V8	0,671	V53	0,172	V98	0,458	V143	0,175	V188	0,197	V233	0,259	V278	0,222	V323	0,239	V368	0,082	G40	0,096	G85	0,118
V9	0,349	V54	0,278	V99	0,383	V144	0,229	V189	0,300	V234	0,101	V279	0,113	V324	0,112	V369	0,287	G41	0,155	G86	0,175
V10	0,230	V55	0,143	V100	0,222	V145	0,289	V190	0,337	V235	0,112	V280	0,311	V325	0,142	V370	0,201	G42	0,148	G87	0,511
V11	0,106	V56	0,186	V101	0,176	V146	0,144	V191	0,377	V236	0,552	V281	0,118	V326	0,178	V371	0,197	G43	0,113	G88	0,133
V12	0,209	V57	0,193	V102	0,185	V147	0,175	V192	0,403	V237	0,318	V282	0,115	V327	0,147	V372	0,221	G44	0,116	G89	0,377
V13	0,252	V58	0,187	V103	0,346	V148	0,216	V193	0,155	V238	0,116	V283	0,195	V328	0,105	V373	0,173	G45	0,112	G90	0,263
V14	0,267	V59	0,437	V104	0,387	V149	0,250	V194	0,212	V239	0,137	V284	0,162	V329	0,262	G1	0,124	G46	0,137	G91	0,100
V15	0,546	V60	0,178	V105	0,481	V150	0,226	V195	0,418	V240	0,137	V285	0,136	V330	0,104	G2	0,137	G47	0,128	G92	0,125
V16	0,146	V61	0,194	V106	0,617	V151	0,248	V196	0,199	V241	0,135	V286	0,175	V331	0,371	G3	0,191	G48	0,491	G93	0,129
V17	0,132	V62	0,378	V107	0,165	V152	0,307	V197	0,141	V242	0,194	V287	0,566	V332	0,094	G4	0,116	G49	0,112	G94	0,118
V18	0,190	V63	0,331	V108	0,230	V153	0,220	V198	0,187	V243	0,296	V288	0,142	V333	0,316	G5	0,122	G50	0,095	G95	0,149
V19	0,406	V64	0,159	V109	0,415	V154	0,271	V199	0,132	V244	0,135	V289	0,346	V334	0,114	G6	0,255	G51	0,119	G96	0,094
V20	0,202	V65	0,196	V110	0,459	V155	0,442	V200	0,094	V245	0,177	V290	0,247	V335	0,114	G7	0,128	G52	0,103	G97	0,171
V21	0,189	V66	0,243	V111	0,150	V156	0,947	V201	0,155	V246	0,120	V291	0,170	V336	0,080	G8	0,120	G53	0,098	G98	0,128
V22	0,446	V67	0,153	V112	0,147	V157	0,226	V202	0,151	V247	0,087	V292	0,277	V337	0,176	G9	0,164	G54	0,120	G99	0,157
V23	0,196	V68	0,225	V113	0,239	V158	0,428	V203	0,173	V248	0,078	V293	0,264	V338	0,240	G10	0,220	G55	0,107	G100	0,150
V24	0,265	V69	0,434	V114	0,270	V159	0,174	V204	0,386	V249	0,168	V294	0,121	V339	0,112	G11	0,112	G56	0,086	G101	0,098
V25	0,215	V70	0,191	V115	0,609	V160	0,215	V205	0,591	V250	0,168	V295	0,150	V340	0,130	G12	0,154	G57	0,084	G102	0,107
V26	0,206	V71	0,362	V116	0,243	V161	0,512	V206	0,151	V251	0,550	V296	0,291	V341	0,141	G13	0,118	G58	0,160	G103	0,208
V27	0,306	V72	0,328	V117	0,245	V162	0,287	V207	0,196	V252	0,171	V297	0,094	V342	0,170	G14	0,353	G59	0,129	G104	0,096
V28	0,167	V73	0,210	V118	0,340	V163	0,182	V208	0,142	V253	0,177	V298	0,195	V343	0,377	G15	0,104	G60	0,343	G105	0,231
V29	0,178	V74	0,144	V119	0,182	V164	0,178	V209	0,137	V254	0,192	V299	0,220	V344	0,201	G16	0,325	G61	0,113	G106	0,170
V30	0,145	V75	0,191	V120	0,201	V165	0,225	V210	0,225	V255	0,240	V300	0,088	V345	0,087	G17	0,096	G62	0,133	G107	0,205
V31	0,132	V76	1,137	V121	0,547	V166	0,338	V211	0,113	V256	0,989	V301	0,186	V346	0,096	G18	0,176	G63	0,092	G108	0,147
V32	0,749	V77	0,352	V122	0,251	V167	0,208	V212	0,130	V257	0,150	V302	0,169	V347	0,366	G19	0,121	G64	0,136	G109	0,294
V33	0,167	V78	0,870	V123	0,150	V168	0,298	V213	0,198	V258	0,100	V303	0,130	V348	0,247	G20	0,138	G65	0,162	G110	0,127
V34	0,184	V79	0,541	V124	0,224	V169	0,297	V214	0,222	V259	0,313	V304	0,101	V349	0,169	G21	0,116	G66	0,103	G111	0,174
V35	0,088	V80	0,402	V125	0,209	V170	0,154	V215	0,120	V260	0,170	V305	0,184	V350	0,132	G22	0,203	G67	0,150	G112	0,102
V36	0,279	V81	0,365	V126	0,277	V171	0,200	V216	0,270	V261	0,168	V306	0,167	V351	0,153	G23	0,141	G68	0,065	G113	0,173
V37	0,206	V82	0,172	V127	0,246	V172	0,176	V217	0,177	V262	0,244	V307	0,214	V352	0,145	G24	0,148	G69	0,194	G114	0,148
V38	0,144	V83	0,438	V128	0,331	V173	0,309	V218	0,203	V263	0,182	V308	0,201	V353	0,087	G25	0,228	G70	0,123	G115	0,101
V39	0,245	V84	0,278	V129	1,835	V174	0,190	V219	0,302	V264	0,116	V309	0,086	V354	0,123	G26	0,163	G71	0,122	G116	0,267
V40	0,255	V85	0,240	V130	0,457	V175	0,274	V220	0,254	V265	0,117	V310	0,770	V355	0,172	G27	0,165	G72	0,432	G117	0,106
V41	0,504	V86	0,284	V131	0,215	V176	0,239	V221	0,338	V266	0,160	V311	0,137	V356	0,082	G28	0,303	G73	0,153	G118	0,131
V42	0,287	V87	0,354	V132	0,282	V177	0,233	V222	0,103	V267	0,151	V312	0,137	V357	0,119	G29	0,105	G74	0,183	G119	0,188
V43	0,233	V88	0,372	V133	0,229	V178	0,418	V223	0,146	V268	0,353	V313	0,089	V358	0,110	G30	0,151	G75	0,097	G120	0,145
V44	0,162	V89	0,549	V134	0,221	V179	0,295	V224	0,166	V269	0,215	V314	0,093	V359	0,243	G31	0,226	G76	0,110		

Table 4. ODs for IgG anti-canine norovirus of sera of human newborns - NB.

Sera	Ods	NB3	0,134	NB6	0,084	NB9	0,096	NB12	0,115
NB1	0,073	NB4	0,068	NB7	0,086	NB10	0,088	NB13	0,093
NB2	0,136	NB5	0,131	NB8	0,124	NB11	0,084		

Table 5. ODs for IgG anti-human norovirus of human sera. Veterinarian – V, General population – G.

Sera	ODs	V45	0,128	V90	0,545	V135	0,363	V180	0,202	V225	1,868	V270	0,599	V315	0,179	V360	1,364	G32	1,159	G77	0,127
V1	0,315	V46	0,615	V91	0,415	V136	0,395	V181	0,167	V226	0,928	V271	0,611	V316	0,138	V361	0,517	G33	0,132	G78	0,088
V2	0,466	V47	0,684	V92	0,463	V137	0,129	V182	0,221	V227	0,511	V272	0,261	V317	1,579	V362	0,634	G34	1,185	G79	0,309
V3	1,480	V48	0,181	V93	0,778	V138	0,767	V183	0,535	V228	1,319	V273	0,562	V318	0,521	V363	1,032	G35	0,994	G80	1,176
V4	0,900	V49	0,878	V94	0,363	V139	0,193	V184	0,275	V229	0,289	V274	0,239	V319	0,099	V364	0,301	G36	0,190	G81	1,537
V5	0,455	V50	0,657	V95	0,250	V140	1,153	V185	0,923	V230	0,277	V275	0,290	V320	0,501	V365	0,754	G37	0,349	G82	0,185
V6	0,589	V51	0,096	V96	0,124	V141	0,229	V186	1,977	V231	1,566	V276	0,253	V321	0,790	V366	0,876	G38	0,359	G83	1,742
V7	0,960	V52	0,260	V97	1,005	V142	0,173	V187	1,389	V232	0,840	V277	0,610	V322	0,182	V367	0,498	G39	1,809	G84	0,582
V8	1,168	V53	1,755	V98	0,353	V143	0,189	V188	0,290	V233	0,178	V278	1,295	V323	0,168	V368	0,304	G40	0,173	G85	0,234
V9	0,916	V54	1,288	V99	0,524	V144	0,573	V189	0,771	V234	0,412	V279	0,119	V324	0,138	V369	1,512	G41	1,388	G86	0,263
V10	0,373	V55	0,495	V100	0,230	V145	1,603	V190	1,860	V235	0,233	V280	0,235	V325	0,849	V370	0,607	G42	0,172	G87	1,637
V11	0,486	V56	0,707	V101	0,118	V146	0,342	V191	0,961	V236	0,219	V281	1,337	V326	0,226	V371	0,543	G43	0,300	G88	0,200
V12	0,688	V57	0,563	V102	0,495	V147	0,902	V192	0,565	V237	0,440	V282	0,326	V327	0,396	V372	0,278	G44	0,684	G89	0,640
V13	0,792	V58	0,346	V103	0,288	V148	0,907	V193	0,477	V238	0,903	V283	0,181	V328	0,465	V373	0,980	G45	1,100	G90	1,166
V14	0,857	V59	0,380	V104	1,715	V149	0,342	V194	0,118	V239	0,375	V284	0,743	V329	0,806	G1	0,828	G46	1,666	G91	0,130
V15	0,251	V60	0,216	V105	0,278	V150	0,648	V195	0,584	V240	0,240	V285	1,072	V330	0,310	G2	0,884	G47	0,380	G92	1,840
V16	1,611	V61	0,122	V106	0,908	V151	0,216	V196	0,350	V241	0,440	V286	1,086	V331	0,693	G3	2,223	G48	0,220	G93	1,099
V17	0,934	V62	0,428	V107	0,410	V152	0,159	V197	1,045	V242	0,107	V287	0,666	V332	0,274	G4	0,245	G49	0,584	G94	1,031
V18	0,712	V63	0,182	V108	0,237	V153	1,582	V198	1,257	V243	0,300	V288	0,287	V333	0,433	G5	1,135	G50	0,131	G95	0,092
V19	0,987	V64	0,934	V109	0,951	V154	0,644	V199	1,518	V244	0,349	V289	0,184	V334	0,172	G6	1,799	G51	0,160	G96	0,238
V20	0,294	V65	1,048	V110	1,546	V155	0,250	V200	1,655	V245	0,286	V290	0,858	V335	0,654	G7	0,400	G52	0,402	G97	1,122
V21	0,344	V66	0,636	V111	0,290	V156	1,338	V201	0,446	V246	1,596	V291	0,354	V336	0,127	G8	0,390	G53	0,549	G98	0,224
V22	0,784	V67	0,091	V112	0,450	V157	0,105	V202	0,414	V247	0,183	V292	1,398	V337	0,694	G9	0,808	G54	0,180	G99	0,481
V23	1,711	V68	0,155	V113	0,545	V158	0,291	V203	0,648	V248	0,357	V293	1,451	V338	0,884	G10	0,384	G55	0,841	G100	0,373
V24	0,979	V69	2,159	V114	0,163	V159	0,219	V204	0,551	V249	0,572	V294	0,175	V339	0,559	G11	0,373	G56	0,100	G101	0,113
V25	0,387	V70	0,269	V115	0,270	V160	0,398	V205	0,367	V250	1,184	V295	0,224	V340	0,363	G12	0,206	G57	0,076	G102	0,805
V26	0,453	V71	2,268	V116	1,683	V161	0,157	V206	0,298	V251	0,394	V296	0,349	V341	0,509	G13	1,041	G58	0,196	G103	0,156
V27	0,926	V72	1,934	V117	1,368	V162	0,293	V207	0,150	V252	1,333	V297	0,867	V342	1,450	G14	0,300	G59	0,186	G104	0,208
V28	0,474	V73	0,334	V118	0,319	V163	0,617	V208	0,410	V253	0,786	V298	0,444	V343	0,235	G15	0,450	G60	0,966	G105	1,504
V29	0,465	V74	0,134	V119	0,221	V164	0,175	V209	0,416	V254	1,025	V299	0,544	V344	0,264	G16	0,374	G61	0,169	G106	0,428
V30	0,669	V75	0,247	V120	0,386	V165	0,218	V210	0,487	V255	0,627	V300	1,411	V345	0,532	G17	0,342	G62	0,310	G107	1,521
V31	0,501	V76	0,874	V121	0,682	V166	0,219	V211	1,110	V256	0,714	V301	0,819	V346	0,254	G18	0,112	G63	0,304	G108	0,225
V32	0,873	V77	0,645	V122	0,350	V167	1,344	V212	0,239	V257	0,390	V302	0,204	V347	0,165	G19	0,356	G64	0,118	G109	0,688
V33	0,345	V78	1,160	V123	1,280	V168	0,814	V213	1,061	V258	0,337	V303	0,224	V348	0,423	G20	0,757	G65	0,115	G110	0,387
V34	0,573	V79	0,732	V124	0,977	V169	0,340	V214	1,430	V259	1,006	V304	0,133	V349	1,092	G21	0,422	G66	0,087	G111	0,245
V35	1,204	V80	0,442	V125	0,951	V170	1,600	V215	0,449	V260	0,442	V305	0,258	V350	0,301	G22	0,154	G67	0,194	G112	0,175
V36	0,696	V81	2,127	V126	0,161	V171	0,636	V216	1,017	V261	0,202	V306	0,311	V351	0,368	G23	0,244	G68	0,069	G113	0,934
V37	0,482	V82	0,137	V127	0,269	V172	0,198	V217	1,225	V262	1,439	V307	0,609	V352	0,347	G24	0,322	G69	0,104	G114	0,951
V38	1,512	V83	0,776	V128	0,523	V173	0,248	V218	0,215	V263	1,684	V308	0,313	V353	0,782	G25	0,638	G70	0,124	G115	0,216
V39	0,490	V84	0,840	V129	0,348	V174	0,393	V219	1,501	V264	1,362	V309	0,238	V354	0,177	G26	1,293	G71	0,149	G116	0,117
V40	0,366	V85	0,371	V130	0,187	V175	0,317	V220	0,754	V265	0,510	V310	1,726	V355	0,499	G27	0,143	G72	0,358	G117	0,758
V41	0,410	V86	0,221	V131	0,563	V176	1,825	V221	1,663	V266	0,421	V311	0,356	V356	0,278	G28	0,863	G73	0,733	G118	0,200
V42	0,395	V87	0,480	V132	0,431	V177	0,495	V222	0,260	V267	0,202	V312	0,622	V357	1,715	G29	0,312	G74	1,721	G119	0,322
V43	0,904	V88	1,413	V133	0,102	V178	0,145	V223	1,590	V268	0,603	V313	1,706	V358	1,352	G30	0,184	G75	0,197	G120	0,190
V44	1,085	V89	0,205	V134	0,132	V179	0,397	V224	2,144	V269	0,144	V314	0,207	V359	0,484	G31	0,152	G76	1,829		