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Seroepidemiology of Human Non-Species A Rotavirus Infections in Valencia, Spain

Noemi Navarro-Lleó[1] | Silvia Aznar-Córdoba[1] | Dunia Asensio-Cob[2] | Daniel Luque[2,3] | Javier M. Rodríguez[4] | Roberto Gozalbo-Rovira[1] | María Jesús Alcaraz-Soriano[5] | Roberto Cárcamo-Calvo[1] | Jesús Rodríguez-Díaz[1] | Javier Buesa[1,5]

[1]Faculty of Medicine, University of Valencia, Valencia, Spain | [2]Instituto de Salud Carlos III, Centro Nacional de Microbiología, Majadahonda, Spain | [3]School of Biomedical Sciences, University of New South Wales, Sydney, Australia | [4]Centro Nacional de Biotecnología, CSIC, Cantoblanco, Spain | [5]Hospital Clínico Universitario, INCLIVA, Valencia, Spain

Correspondence: Javier Buesa (javier.buesa@uv.es) | Noemi Navarro-Lleó (noemi.navarro@uv.es)

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ABSTRACT

Species A rotavirus (RVA) is a major cause of acute gastroenteritis in children. However, three other rotavirus species (RVB, RVC, and RVH) are also able to infect humans. It has been suggested that vaccination against RVA could facilitate an increase of non-A rotavirus infections. We investigated the antibody prevalence against RVA, RVB, RVC, and RVH in 420 human sera collected between 2020 and 2022 from different age groups in Valencia (Spain). Antibody prevalence rates to RVA, RVB, RVC, and RVH were 79.3%, 17.9%, 18.8%, and 14.5%, respectively. Antibody titers against RVA remained consistent across the different age groups, and RVB showed low titers except in younger individuals. RVC-specific antibodies peaked in children 5–10 years of age, whereas RVH exhibited the highest titers in the elderly. The detection of antibodies against non-A rotaviruses in humans in Spain, for the first time against RVB and RVH, highlights the need for their surveillance.

1 | Introduction

Species A rotavirus (RVA) is a major cause of acute gastroenteritis in infants and small children as well as in the young of many animal species worldwide [1]. It has been estimated that in 2021 RVA was the predominant cause of diarrheal deaths across all ages, with 176 000 (131 000–230 000) diarrheal deaths and 13.4 million (9.85–17.9) diarrheal disability-adjusted life-years [2]. The International Committee on Taxonomy of Viruses (ICTV) has established 9 different rotavirus species (A–D and F–J) according to the antigenicity of the VP6 protein, and four of them (RVA, RVB, RVC, and RVH) are able to infect humans [3, 4]. Rotavirus A (RVA), now named *Rotavirus alphagastroenteritidis* according to the binomial nomenclature for virus species, is responsible for most seasonal endemic diarrheal disease in young children. Members of RVA can also infect other mammals (e.g., pig, cattle, horse, rat, mouse, and bat) causing diarrhea, and birds (e.g., chicken, turkey, and pigeon) in which they cause enteric signs and runting and stunting syndrome.

Rotavirus B (RVB) (*Rotavirus betagastroenteritidis*) was first identified as the cause of severe gastroenteritis among adults in China from late 1982 to early 1983, and known as "adult diarrhea rotavirus" (ADRV) [5]. It was found to be responsible for diarrheal disease in humans in India, Bangladesh, Nepal, and Myanmar [6]). In addition to humans, RVB strains have been detected in different host species such as rats, cattle, sheep, goats, horses, and swine [7, 8]. RVB viruses have caused sporadic gastroenteritis epidemics in adults, but pigs are their main reservoir [9, 10].

Rotavirus C viruses (RVC) (*Rotavirus tritogastroenteritidis*) have been associated with sporadic diarrhea cases and self-limiting outbreaks of gastroenteritis in humans, primarily in the young, but they are also widely distributed in animals, mainly pigs, but also in cows, ferrets and dogs [9, 11–13]. RVC has been considered a potentially neglected cause of acute gastroenteritis [14]. Human RVCs have often been detected in sporadic cases and outbreaks of diarrhea in various age groups [15]. RVC infections in humans have been reported in many countries in North and South America, Asia, Africa, Europe, and Oceania [16–18]. They have also been reported in Spain, where 15% of 147 specimens negative for other enteric pathogens were found to contain RVC [19, 20].

Rotavirus H (RVH) (*Rotavirus aspergastroenteritidis*) was originally discovered in humans, leading to sporadic cases of acute gastroenteritis in China in 1987 and 1988 [21]. Initially designated as "new adult diarrhea rotavirus" (ADRV-N) [22], it was subsequently reclassified under the novel RV species H [3]. The same rotavirus strain was also involved in outbreaks of diarrhea among adults in Beijing and Shijiazhuang in 1994 and 1997, respectively [23]. Since then, RVH has been detected in pigs in Japan [24], USA [25], Brazil [26], South Africa [27], Vietnam [28], Italy [29], and Spain [30]. Recently, RVH has also been identified in bats in Cameroon [31]. To date, there have been only two reported human RVH sequences: J19, isolated from China, and B219, isolated from India [21, 32].

While RVA is well documented in Spain and Western Europe, there is limited epidemiological information available for RVB, RVC, and RVH. A study conducted in children in Barcelona (Spain) reported a low detection rate of RVC (1%) in fecal samples [20], whereas another study from Madrid (Spain) found a markedly higher detection rate of 15% [19]. Serological evidence of RVC has also been reported in Sweden (38%) [33] and the United Kingdom (43%) [34], with infections being more frequent among older children and adults, and less common in children under 5 years of age.

RVB and RVC have also been detected in porcine fecal samples from farms in Spain [35], and more recently, RVH has also been identified in swine populations [30]. Regarding RVB in humans, a single serological study conducted in the United Kingdom reported a seroprevalence of 10% [36]. In contrast, no data are currently available on the seroprevalence of RVH outside of Asia.

It has been suggested that vaccination against RVA could indirectly facilitate an increase in non-A rotavirus infections by reducing natural selective constraints [37]. However, little is known about the current epidemiology of these non-A rotavirus infections in humans. This study aims to address this gap by evaluating the seroprevalence of RVB, RVC, and RVH in a Spanish population. To this end, VP6 proteins from the intermediate capsid of RVA and RVC, and VP2 (core) and VP6 proteins of RVB and RVH, were produced by recombinant baculoviruses in insect cells and purified by molecular exclusion chromatography. Human serum IgG, IgA, and IgM antibodies to RVA, RVB, RVC, and RVH were analyzed by enzyme immunoassay in serum samples from individuals of different age groups.

2 | Materials and Methods

2.1 | Expression and Purification of Species A, B, C, and H VP6 Rotavirus Proteins in Insect Cells

Recombinant baculoviruses were used to express full-length VP6 proteins from RVA, RVB, RVC, and RVH of simian (SA11) and human (Bang373, Bristol, J19) strains, respectively. The following genes were used: VP2 (GenBank Acc. No. KJ450832) and VP6 (GenBank Acc. No. KJ450836) from RVA strain SA11; VP2 (GenBank NC_021545) and VP6 (GenBank NC_021544) from RVB strain Bang373; VP2 (GenBank NC_007546) and VP6 (GenBank NC_007570) from RVC strain Bristol, and VP2 (GenBank NC_007549) and VP6 (GenBank NC_007553) from RVH strain J19. The genes were synthesized by Synbio Technologies. Recombinant baculoviruses were constructed using the pFastBacDual plasmid for protein expression in the Bac-to-Bac system (Invitrogen). The VP2 and VP6 coding sequences from each rotavirus species were cloned under the control of the p10 and polh promoters, respectively. High Five (BTI-Tn-5B1-4) cells were infected at a multiplicity of infection of 0.1, adsorbed for 2 h at 27°C, and incubated in TC-100 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL). After 6 days, cells were harvested by centrifugation at 12 500 *g* for 40 min at 4°C. VP6 proteins from RVA/RVC were precipitated with 10% PEG 8000, 2.3% NaCl, and 0.02% sodium azide, incubated at 4°C overnight, and purified by ultracentrifugation on a 25% sucrose cushion (100 000*g*, 2 h). Further purification was achieved via ammonium sulfate precipitation (25%–30%) and gel filtration using a Superdex 200 column coupled to an Äkta avant chromatography system (Cytiva). VP2/VP6 proteins from RVB/RVH were extracted from lysed cells by sonication in TNE buffer with 0.8 mM PMSF and 0.2 U/mL DNase I, followed by centrifugation at 15 000*g* for 20 min at 4°C. Protein quantification was performed using a Qubit assay kit (Invitrogen) and samples were analyzed by SDS-PAGE and transmission electron microscopy (FEI Tecnai G2 Spirit Bio-Twin TEM) after negative staining with 2% uranyl acetate.

2.2 | Serum Samples

Serum samples were obtained from patients attended at the Hospital Clinico Universitario de Valencia from January 2020 to December 2022. A total of 420 serum samples were randomly selected from six different cohorts as follows: younger than 5 years ($n = 72$), 5 to 10 years ($n = 69$), 11 to 20 years ($n = 68$), 21 to 40 years ($n = 68$), 41 to 60 years ($n = 69$), and older than 60 years ($n = 74$). Samples were selected using a randomized approach, without prior knowledge of the donors clinical background or symptoms potentially associated with rotavirus infection. Information on RVA vaccination history was unavailable and was therefore not considered in the present analysis.

2.3 | ELISA to Detect Antibodies Against Rotavirus A, B, C, and H

ELISA was used to assess IgG, IgA, and IgM antibodies in 420 serum samples against different VP2 and/or VP6 from RVA, RVB, RVC, and RVH strains, as described previously. The wells of 96-well microtiter plates (Costar) were coated with 100 μl of each RVA, RVB, RVC, and RVH VP2/VP6 proteins diluted in carbonate/bicarbonate buffer pH 9.6 at 1 μg/ml and incubated overnight at 4°C. Simultaneously, wells on the same plates were coated with bovine serum albumin (BSA) at a concentration of 1 μg/ml, to be used as negative controls. The plates were washed once with PBS and blocked with 3% BSA in PBS containing 0.05% Tween 20 (PBS-T) for 1 h at 37°C. After blocking, the plates were washed three times with PBS-T. Serum samples were serially diluted, starting at 1/100 in PBS-T containing BSA 1%, added in triplicate to the wells and incubated for 1 h at 37°C. After three washes with PBS-T, goat anti-human IgG + IgM + IgA antibody conjugated with horseradish peroxidase (Abcam) diluted to 1:7500 in PBS-T was added. The plates were incubated for 1 h at 37°C and washed four times. Colorimetric reactions were developed with SigmaFast OPD (Sigma-Aldrich). Reactions were stopped with 50 μL of H_2SO_4 3 M and the absorbance was read at 492 nm on a Multiskan FC spectrophotometer (ThermoFisher Scientific). A serum sample was taken to be antibody-positive when its OD_{492} value was higher than the mean optical density of the reagent blank plus three standard deviations. The titer of each sample was considered the inverse of the last dilution showing a positive reaction. Median titers of serum IgG antibodies were used to perform comparisons between groups.

2.4 | Mice Immunization With the Recombinant VP2/VP6 Proteins

To assess the potential cross-reactivity of serum antibodies against VP2/VP6 proteins from the different rotavirus species (RVA, RVB, RVC, and RVH), groups of 6 mice were immunized with the recombinant proteins from each single rotavirus species. Female BALB/c mice (Charles River Laboratories), aged 4–6 weeks, were given by the intraperitoneal route 4 doses of 5 ug of the recombinant proteins mixed with incomplete Freund's adjuvant at 2 week-intervals. Immunized mice were anesthetized with isoflurane and bled by cardiac puncture. Serum samples were collected and analyzed by ELISA for antibodies against the recombinant rotavirus VP2/VP6 proteins. The ELISA tests were performed as described above, except that the mouse sera were diluted to 1:5000 and a HRP-conjugated anti-mouse IgG (Abcam) diluted to 1:10 000 was used.

2.5 | Statistical Analysis

Data distribution of optical density values (OD_{492}) of mouse serum samples was assessed for normality using the Shapiro–Wilk test. Differences in OD_{492} values among groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test. Statistical analyzes were performed using R (version 4.1.2) with the rstatix package (version 0.7.2). Graphs were generated using GraphPad Prism version 10. A p-value < 0.05 was considered statistically significant.

The distribution of optical density values (OD_{492}) of all human serum samples was visualized using violin plots (Figure 3), which display both the data density and summary statistics (boxplots) for each age group. The plot was generated in R (version 4.1.2) using the ggplot2, ggprism, and ggpubr packages [38–40], allowing for a comprehensive visualization of distribution and group comparisons.

Differences in mean human serum antibody titers to the four rotavirus species across different age groups were assessed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison correction. All statistical analyzes were conducted using GraphPad Prism 10 software for Windows. A significance level of $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | Purification and Characterization of Recombinant VP2 and VP6 Proteins From RVA, RVB, RVC, and RVH

VP6 protein alone from simian RVA (SA11 strain) and RVC (Bristol strain), and VP2 plus VP6 proteins from human RVB (Bang373 strain) and RVH (J19 strain) species were purified from recombinant baculovirus-infected High Five cells as described in Material and Methods. Purified proteins were identified by liquid chromatography-mass spectrometry (LC-MS/MS) (results not shown). Assembled VP2/VP6 or VP6 proteins alone were characterized by SDS-PAGE (Supporting Information Figure 1) and electron microscopy (Figure 1). Molecular assembly of VP6 proteins from RVA and RVC generated 2D hexameric paracrystals with honeycomb appearance, whereas VP2 and VP6 coexpression produced tubular structures, but no typical virus-like particles (VLPs) were observed. This is the first time that these RVB proteins have been observed forming long and thin tubular structures. In all cases, these assemblies are likely artefactual and not representative of native virion architecture. Whether their formation results from non-physiological expression conditions or selective enrichment during purification remains unclear.

3.2 | Immune Response of Mouse Sera to Recombinant Proteins

Mice immunized with RVA VP6 exhibited a robust and specific response to the homologous RVA protein, with significantly higher OD_{492} values compared to RVB, RVC, RVH, and BSA ($p < 0.05$ for all comparisons; Figure 2A). Similarly, sera from RVB-immunized mice showed strong reactivity to the RVB VP6

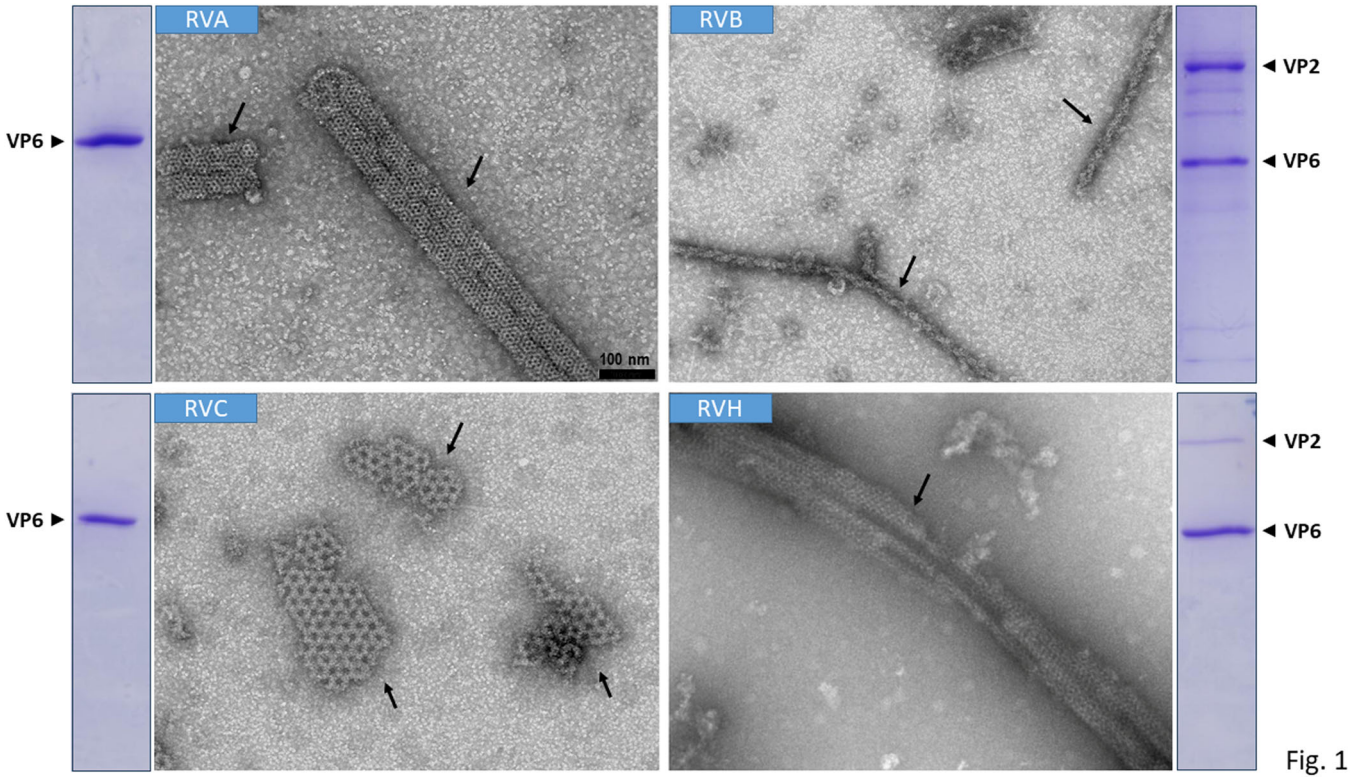

FIGURE 1 | Electron microscopy analysis of negative staining of the purified assemblies from the expression of VP6 from rotaviruses A and C, and coexpression of VP2/VP6 from rotaviruses B and H. Coomassie blue-stained SDS-PAGE gels of purified rotavirus proteins are shown on the sides, with the positions of VP2 and VP6 marked. Purified assemblies of VP6 proteins from RVA and RVC generated hexameric 2D paracrystals with a honeycomb appearance, whereas VP2 and VP6 coexpression produced tubular structures. Additionally, panels A, B, and C show a background of smaller structures compatible with unassembled VP6 trimers. Black arrows indicate characteristic VP6 oligomers. All four electron microscopy panels are shown at the same magnification; the scale bar in panel A (100 nm) applies to all panels.

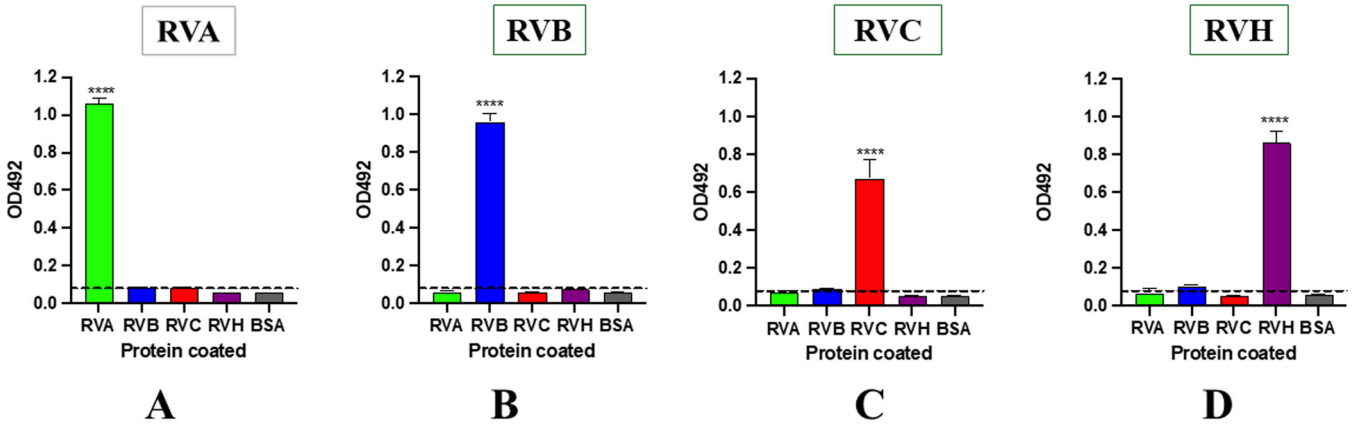

FIGURE 2 | Mouse sera reactivity against VP2/VP6 proteins from RVA (A), RVB (B), RVC (C), RVH (D), and against bovine serum albumin (BSA) as a negative control. Cut-off value was considered the BSA value plus 3 standard deviation (SD). Significance levels $p < 0.05$ are indicated by asterisks (****).

protein, with minimal cross-reactivity to other VP6 proteins or BSA ($p < 0.05$; Figure 2B).

Sera from mice immunized with RVC VP6 displayed significantly elevated OD_{492} values only in response to RVC protein ($p < 0.05$), indicating antigen-specific recognition with no substantial cross-reactivity (Figure 2C). Finally, anti-RVH sera reacted selectively with RVH VP6 ($p < 0.05$ for all heterologous comparisons), demonstrating clear species-specific antibody responses (Figure 2D).

These results collectively indicate that the VP6 proteins from different rotavirus species elicit highly specific humoral immune responses, with no detectable cross-reactivity among the tested species under the conditions used.

TABLE 1 | Seroprevalence of total antibodies against VP6 proteins of rotavirus A (RVA), B (RVB), C (RVC), and H (RVH) species in different age groups in Valencia (Spain).

	Positive serum samples			
Age group	**VP6 RVA**	**VP2 + VP6 RVB**	**VP6 RVC**	**VP2 + VP6 RVH**
< 5	46/72 (63.9%)	21/72 (29.2%)	3/72 (4.2%)	5/72 (6.9%)
5–10	48/69 (69.6%)	11/69 (15.9%)	8/69 (11.6%)	16/69 (23.2%)
11–20	55/68 (80.9%)	11/68 (16.2%)	10/68 (14.7%)	1/68 (1.5%)
21–40	59/68 (86.8%)	8/68 (11.8%)	15/68 (22.1%)	2/68 (2.9%)
41–60	58/69 (84.1%)	17/69 (24.6%)	19/69 (27.5%)	4/69 (5.8%)
> 60	67/74 (90.5%)	7/74 (9.5%)	24/74 (32.4%)	33/74 (44.6%)
Total	333/420 (79.3%)	75/420 (17.9%)	79/420 (18.8%)	61/420 (14.5%)

3.3 | Analysis of the Prevalence of Serum Antibodies Targeting Rotavirus A, B, C, and H

A total of 333 (79.3%; 95% confidence intervals (CI): 68.4%–90.2%) human sera were positive for RVA-specific antibody, 75 (17.9%; 95% CI: 9.9%–25.8%) were positive for RVB VP2 and VP6-specific antibody, 79 (18.8%; 95% CI: 7.7%–29.8%) were positive for RVC VP6-specific antibody and 61 (14.5%; 95% CI: 3.5%–31.8%) were positive for RVH VP2 and VP6-specific antibody (Table 1 and Figure 3). As expected, the highest seroprevalence was found against RVA VP6. The average prevalence of antibodies against RVB, RVC and RVH VP6 proteins in the studied population overall was below 20%, slightly higher against RVC (18.8%) than against RVB and RVH.

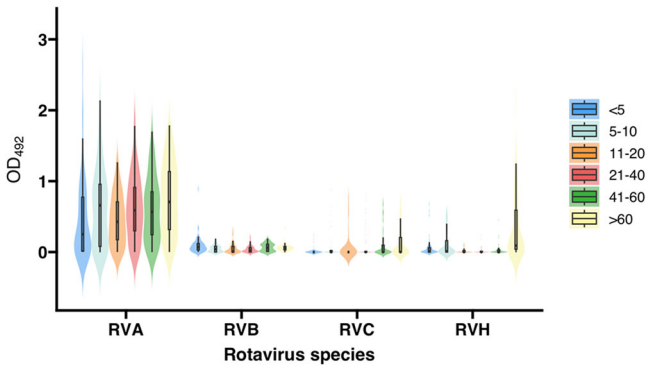

FIGURE 3 | Optical density (OD_{492}) violin plots for all human serum samples tested against VP6 RVA and RVC and against VP2/VP6 proteins of RVB and RVH in different age groups. The violin plot depicts distributions of numeric data for the age groups using density curves. The width of each curve corresponds with the approximate frequency of data points in each region. Densities are frequently accompanied by an overlaid chart type, such as box plot, to provide additional information.

3.4 | Comparison of the Serum Antibody Titers Against VP6 or VP2/VP6 Proteins in Different Age Groups

Regarding RVA VP6-specific antibody, the prevalence rose progressively from 63.9% in children < 5 years old to 90.5% in adults > 60 years of age. Similar progression in antibody prevalence was only found in RVC, rising from 4.2% in children < 5 years old to 32.4% in adults > 60 years of age (Table 1 and Figure 4).

The prevalence of RVB VP2/VP6-specific antibody was higher in children < 5 years old and in adults of 41–60 years. Interestingly, the prevalence of RVH VP6-specific antibody was highest in people aged > 60 years (44.6%), followed by the 5–10 year age group (23.2%). Antibody-positive sera to RVC showed a very neat progression across age groups, from 4.2% in children < 5 years to 32.4% in the > 60 years group.

The highest prevalence of anti-RVH VP2/VP6 was found in the oldest age group (> 60 years), with 44.6% of positive serum samples (Table 1). Interestingly, the group of children 5 to 10 years old showed a prevalence of 23.2%, with a significant dip in the following age groups (Figure 4).

The analysis of serum samples in this study revealed varying antibody titers against the VP6 and VP2 proteins of different viral species (Figure 5). A comparison of mean IgA, IgG, and

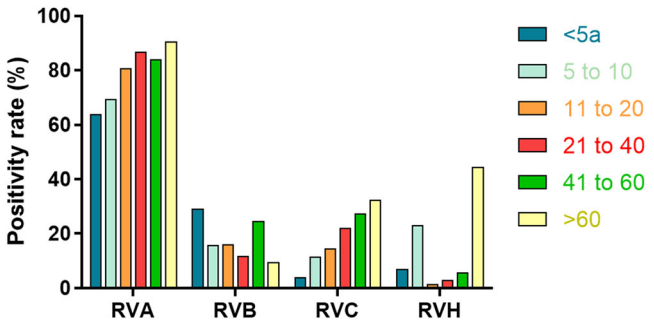

FIGURE 4 | Seroprevalence of total antibodies against VP6 proteins of rotavirus species A, B, C, and H in different age groups in Valencia, Spain.

IgM antibody titers across age groups showed significant variations depending on the virus. For RVA, antibody levels peaked in individuals aged 5–10 years and those over 60, with mean titers reaching up to 26 600 (Figure 5A). In contrast, RVB titers were lower than those observed for RVA, though significantly higher in the youngest age group, averaging around 2000

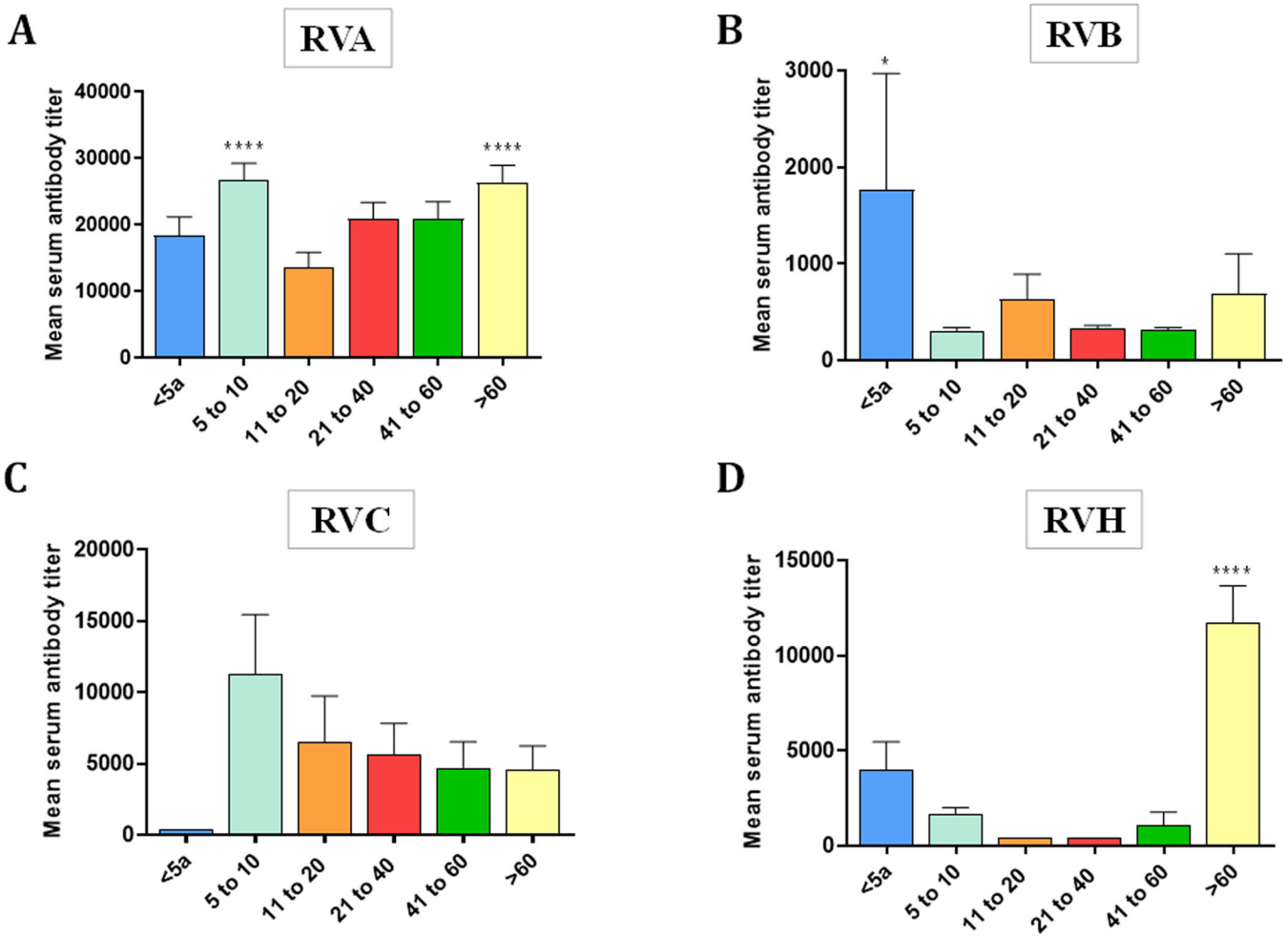

FIGURE 5 | Mean serum antibody titers across six different age cohorts against VP6 RVA (A) and RVC (C) and against VP2/VP6 proteins of RVB (B) and RVH (D). Error bars represent the standard error of the mean (SEM). Statistical analyzes using one-way ANOVA revealed the following significant findings: antibody levels against RVA were significantly higher in the 5–10 years group and the > 60 years group compared to other age groups (A); for RVB, the < 5 years group exhibited significantly higher antibody titers than the other cohorts (B); for RVH, the > 60 years group showed significantly greater mean serum antibody levels compared to all other groups (D). Significance levels are indicated as follows: $p < 0.05$ (*) and $p < 0.0001$ (****).

(Figure 5B). Meanwhile, RVC titers also peaked in the 5–10-year-old group, although these differences were not statistically significant (Figure 5C). As for RVH, the highest mean titers, reaching up to 12 000, were detected in individuals over 60 years old, with values significantly surpassing those of other age groups (Figure 5D). These findings suggest that each virus may display age-specific tropism.

3.5 | Serum Samples With Antibodies to One or Multiple Rotavirus Species

One hundred and eighty-seven sera (51.4% of the positive samples) were positive only for RVA, 12 (3.3%) only for RVB, 3 (0.8%) only for RVC and 13 (3.6%) only for RVH. The numbers and percentages of serum samples showing reactivity to more than one rotavirus species are shown in Figure 6. A total of 149 (40.9%) sera were considered positive to more than one rotavirus species: 116 (31.9%) to two species, 31 (8.5%) to three species and 2 (0.5%) to four species. The most common combination of antibodies was against RVA and RVC (13.5%), followed by RVA and RVB (10.7%). Serum samples showing reactivity against three or four rotavirus species were predominantly from individuals over the age of 60 (results not shown).

4 | Discussion

Seroepidemiological studies are relevant to elucidate the circulating viruses in a particular area. The main aim of this study was to determine the serum antibody prevalence and titers to a panel of VP2 and/or VP6 proteins from four rotavirus species (RVA, RVB, RVC, and RVH) in different age groups in Valencia, Spain. RVA infections are common in our geographical area, even after the introduction of rotavirus vaccines, which showed over 85% effectiveness against rotavirus hospitalization [41–43]. Vaccines against rotavirus have been available in Spain since 2006, but it was not until 2024 that they began to be funded by the Public Health System [44].

Immunization of mice with the recombinant VP6 or VP2/VP6 proteins from the four rotavirus species used as coating antigens

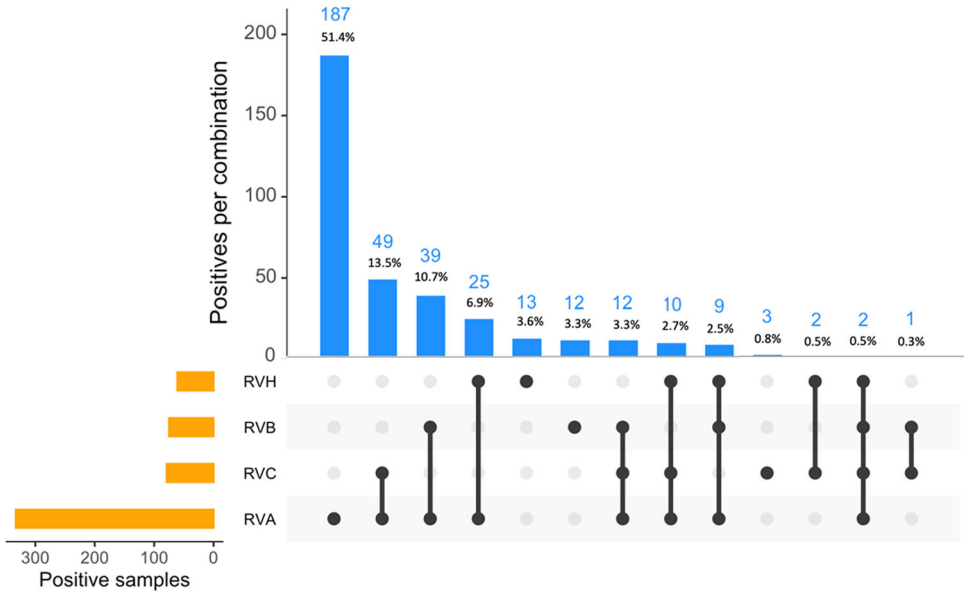

FIGURE 6 | Serum samples with antibodies against one or several rotavirus species. Dots represent serum samples with antibodies against rotavirus species indicated on the left side. Bars connect samples considered positive to 2, 3, or 4 rotavirus species. Bar chart shows numbers of positive serum samples for each combination.

in the in-house ELISA demonstrated the lack of cross-reactivity of the serum antibodies against them, in accordance with their taxonomic differentiation [3].

Our results confirm the high prevalence of RVA infections in the studied population, as 63.9% of children younger than 5 years of age has RVA VP6-specific antibodies. However, it should be considered that some children had been vaccinated, although the percentage does not exceed 40% according to our data. The seroprevalence increased progressively in each age group, reaching a ratio of 90.5% in individuals over 60 years of age. It must be considered that both asymptomatic and symptomatic rotavirus infections occur in adults, and they may be important in the transmission of rotavirus in the community.

In this survey, an average seroprevalence of 18.8% for anti-RVC antibodies was found. This level of prevalence is lower than those reported in other studies. A seroepidemiological study performed in 2004 in England and Wales demonstrated a prevalence of IgG antibodies against RVC of 39% (95% CI 37.0%–40.4%). Seroprevalence was highest (46.0%) in the oldest age group (61–70 years of age) [45]. The overall seroprevalence in India for RVC antibodies was 25.32% (95% CI 22.64–28.21) [46], whereas in Japan was 30% [47]. Nevertheless, the seroprevalence found in the present study in individuals aged > 60 years (32.4%) is similar to those previously reported in other countries [18, 45, 46].

Serological evidence suggests that RVC infections are more common than indicated by the low detection rates (< 7%) reported in antigen-based studies [34, 48, 49]. This discrepancy may be due to the focus of these studies on an unsuitable population group (children under 5 years old), despite findings showing a higher prevalence in individuals over this age threshold. This interpretation is reinforced by the titer data from the present study, where the 5–10 year old group displayed the highest average titer (12,000) compared to other age groups.

Studies on the seroepidemiology of RVB in humans are scarce. Nakata et al. (1987) tested 219 human sera and 18 immunoglobulin pools collected from eight countries for antibodies to RVB. Overall, a low proportion (4.2%) of sera contained RVB-specific antibodies [50]. Penaranda et al. (1989) found that serum samples from patients from a large epidemic of RVB had elevated titers of specific antibodies 3 and 16 months after the onset of symptoms. Also, antibodies were present in gamma-globulin pools prepared years before the RVB epidemic, indicating that this rotavirus species is a common enteric pathogen in China [51]. However, a low seroprevalence of RVB antibodies was found in other countries outside China [36]. In this study, 10.7% of the serum samples showed antibodies against both RVA and RVB simultaneously. This suggests that infections involving multiple rotavirus species may be more common than previously considered. This is supported by studies in swine, where co-infections with up to three rotavirus species (RVA, RVB, and RVC) have been documented [9, 52]. The finding of sera with reactivity to more than one rotavirus species may reflect lifetime infections with different rotavirus species. It may also mean cross-reactivity of some serum samples to VP6 and/or VP2 proteins of different rotavirus species, which is unlikely, as if this were the case, many more samples would be cross-reactive.

The present study has some limitations, such as the low number of serum samples analyzed, all of them belonging to individuals from urban and suburban areas of the city of Valencia, but not from rural areas. Our ELISA test detects IgA, IgG, and IgM antibodies simultaneously, so it does not distinguish between recent and old infections.

To our knowledge, this is the first time that antibodies to human RVH have been detected. The detection of sera with antibodies to rotavirus species B, C and H in this study reinforces the interest in these viral pathogens, which are not usually investigated in patients with diarrhea using

commercial immunological or molecular methods designed exclusively for the diagnosis of RVA infections. Searching for antibodies to rare pathogens is more likely to yield positive results than studying the pathogen itself. Surveillance of sporadic cases and of outbreaks of undiagnosed diarrheal disease should clarify the prevalence of human non-species A rotaviruses.

Acknowledgments

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Ethics Statement

This study was conducted with the approval of the Ethics Committee of the University of Valencia (code H1544010468380). The ethics committee waived the need for informed consent since human blood samples from Hospital Clinico Universitario de Valencia were anonymized previously for their inclusion in the present study. The study was performed following the declaration of Helsinki on ethical principles for medical research involving human subjects. The animal experiments were approved by the Ethics Committee for Animal Welfare of the Universitat de València and the Directorate General of Agriculture, Livestock, and Fisheries (code 2024/VSC/PAA/0113) of the Generalitat Valenciana.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supplementary Figure 1.