Virology 608 (2025) 110529

Contents lists available at ScienceDirect

Virology

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

In search of human protoparvovirus acute infections

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ARTICLE INFO

Keywords:
Bufavirus
Cutavirus
Tusavirus
Protoparvovirus
Acute infection
Epidemiology
Seroprevalence

ABSTRACT

Three protoparvoviruses (PPV)—bufavirus, cutavirus, and tusavirus—are the most recent members of the *Parvoviridae* virus family discovered in humans. All were initially found in stool but have since then been associated with gastroenteritis or cutaneous T-cell lymphoma, or found to be of zoonotic origin, respectively. In the current study, we developed novel PPV IgM enzyme immunoassays (EIA) and aimed to search for and characterize human protoparvovirus acute infections. We also provide a more comprehensive analysis of PPV seroprevalences. We screened, with in-house IgG, IgM, and PCR assays, a total of 1444 serum samples from ten different cohorts from six countries (Finland, Italy, Kenya, Latvia, Iran, and Iraq), with subjects varying in age and health status (e.g., unexplained fever, gastroenteritis, respiratory tract infections, chronic conditions, or constitutionally healthy). The geographic distributions of bufavirus seroprevalences were similar to previous findings, with a high (68 %) bufavirus seroprevalence found in *Iran adult* and low (<16 %) in *Finnish elderly* and *Italy adult* cohorts; the *Iran child* bufavirus seroprevalence was also significantly higher (16.5 %) than that of the *Italy child* cohort (4.5 %). Interestingly, we found surprisingly high (>10 %) cutavirus IgG seroprevalences among adults with chronic diseases and the elderly. We did not find any TuV IgG in any cohort. We also discovered some elevated human protoparvovirus IgM reactivity, but upon confirmatory competition EIA and PCR, none were true acute infections. These results suggest that acute human protoparvovirus infections are mild, local, rare, or not seen in respiratory tract infections or gastroenteritis.

This article is part of a special issue entitled: WSV 2025 published in Virology.

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https://doi.org/10.1016/j.virol.2025.110529
Received 22 December 2024; Received in revised form 14 March 2025; Accepted 8 April 2025
Available online 9 April 2025

1. Introduction

Parvoviridae is a family of small, nonenveloped, single-stranded DNA viruses that infect either vertebrates or invertebrates (Cotmore et al., 2014). The most recently discovered parvoviruses found in humans—bufavirus (BuV), tusavirus (TuV), and cutavirus (CuV) of the *Protoparvovirus* genus—were first detected via metagenomics in diarrhetic stools: from Burkina Faso in 2012, Tunisia in 2014, and Brazil and Botswana in 2016, respectively (Phan et al., 2012, 2014, 2016). CuV was also originally detected in skin of four patients with cutaneous T-cell lymphoma from France by in-silico analysis of metagenomic libraries or PCR (Phan et al., 2016). Primary seroepidemiological information is available for these viruses (Väisänen et al., 2018); however, their disease etiologies are largely unconfirmed or unknown, and there are currently no documented acute infections.

BuV DNA has been detected by PCR in a low worldwide prevalence (0–4 %)—almost entirely in stools (Phan et al., 2012; Yahiro et al., 2014; Altay et al., 2015; Altan et al., 2017; Okitsu et al., 2020; Simo-Fouda et al., 2021; Mohanraj et al., 2021; Daprà et al., 2021; Väisänen et al., 2014), but its etiological role in gastroenteritis (GE) remains unclear. These studies describe three genotypes of BuV (BuV1–3); interestingly, BuV2 DNA has been reported only in one stool sample from a single individual (Phan et al., 2012). Contrastingly, we earlier showed by serology, BuV2 to be globally the second-most common genotype—as well as serotype (Väisänen et al., 2018). In total, high (>60 %) BuV1–3 seroprevalences were found in Africa and the Middle East and lower (<10 %) in Europe and the Americas (Väisänen et al., 2018). CuV has further been shown to have a much lower, and globally more uniform, seroprevalence (≤5 %) than BuV (Väisänen et al., 2018), but reaching 9.5 % among patients with CTCL (Väisänen et al., 2019).

In contrast to BuV, CuV DNA has been primarily found in skin, and only occasionally in stools (Phan et al., 2016; Väisänen et al., 2019; Mollerup et al., 2017; Wieland et al., 2019; Hashida et al., 2023a, 2023b, 2024; Mohanraj et al., 2023, 2024). There is a significant association of persisting CuV DNA with cutaneous T-cell lymphoma (CTCL) and its precursor parapsoriasis (Väisänen et al., 2019; Hashida et al., 2023b, 2024; Mohanraj et al., 2023, 2024) while it has been found in much lower prevalences, if any, in skin from melanoma, transplant patients, and from healthy subjects (Väisänen et al., 2019; Mollerup et al., 2017; Wieland et al., 2019).

TuV DNA in humans has only been detected in the stools of five patients worldwide (Phan et al., 2014; Mohanraj et al., 2021; Väisänen et al., 2016; He et al., 2023), and only one child has been documented as TuV-IgG positive (Väisänen et al., 2016). Because there are much higher TuV genoprevalences in domestic goats and sheep (Reuter et al., 2022; Davies et al., 2024), and its close relatedness to other ruminant viruses (Boros et al., 2023), it is primarily considered an animal virus, with zoonotic potential.

Despite varying high geno- and seroprevalences globally, the signs and symptoms of serologically confirmed acute human protoparvovirus (PPV) infections are yet to be described, since viral DNA may be detected in stools from an earlier infection (Mohanraj et al., 2024). In this study, we utilized previously published in-house IgG enzyme immunoassay (EIA) (Väisänen et al., 2018) and multiplex qPCR (Mohanraj et al., 2021; Väisänen et al., 2014, 2016) as well as developed a novel multiplex EIA (with separate wells for each virus) to detect BuV1–3 and CuV IgM. We analyzed sera from 1444 patients comprising ten different cohorts with varying age groups, health conditions, and geographic distributions. Some of these cohorts have previously been studied for IgG (Väisänen et al., 2018; Mohanraj et al., 2021). However, this is the first study to measure IgM antibodies to investigate acute human protoparvovirus infections and the first study to screen sera from large cohorts of adults with underlying chronic conditions as well as elderly individuals.

2. Methods

2.1. Study cohorts

This study comprises sera from 1444 patients in ten cohorts across six countries. The study and all sampling were conducted in accordance with relevant guidelines and regulations. Samples and clinical information from the *Iran blood donor*, *Iraq adult*, *Kenya child* and *adult*, and *Latvia child* cohorts are described in previous studies (Väisänen et al., 2018; Mohanraj et al., 2021) and summarized here shortly (Table 1B). We now describe the clinical information for the cohorts that have previously not been analyzed for human protoparvoviruses: *Italy child* and *adult*, *Iran child and chronic adult*, and *Finland elderly* (>65 years) cohorts (Table 1A). Study cohorts were chosen to provide a more comprehensive analysis of age, health, and geographic distribution of PPVs; cohorts from areas with already high PPV IgG seroprevalence were also included to increase the likelihood of identifying acute infections.

Italy cohorts: The *Italy child* cohort consists of 22 sera from 22 children (1.3–17 years, mean age 12 years) and the *Italy adult* cohort of 178 sera from 178 adults (18.7–90 years, mean 48 years) sent for standard serological screening and were considered constitutionally healthy for this study. Several patients were born outside the EU (n = 24, 4 children and 20 adults), originating from the southern shore of the Mediterranean Sea, India or Pakistan. However, the length of residency in Italy or other locations is not known. According to Italian Privacy law and local ethical committee guidelines, informed consent was waived for analysis of these deidentified hospital samples.

Iran cohorts: The *Iran child* cohort is made up of 321 sera from 321 patients (1 month–15 years, mean 41 months). Notably, the predominant causes for hospitalization included fever with unknown etiology (n = 173), respiratory symptoms (n = 108), gastroenteritis (n = 25), skin rash (n = 13), and encephalitis (n = 2). In addition, some children exhibited underlying chronic diseases such as febrile seizures, cerebral palsy, X-linked (Burton) agammaglobulinemia, Crigler-Najjar syndrome, DiGeorge syndrome and primary immunodeficiency. The *Iran chronic adult* cohort comprises 78 sera from 78 adults (35–86 years, mean 52.5 years) with chronic conditions such as diabetes (n = 17), hyperlipidemia (n = 23), elevated liver enzymes (n = 3), thyroid disease (n = 6), iron deficiency anemia (n = 6), cancer (n = 2), renal disease (n = 2), low platelet count (n = 1) or multiple of the above conditions combined (n = 18), but with no acute illnesses. These studies were approved by the Ethics Committee of Hamadan University of Medical Sciences. Informed consent was waived for the Iran adult cohorts, and all parents have given informed consent for the Iran child cohort.

Finland elderly: The *Finland elderly* cohort consists of 374 sera from 374 elderly hospitalized patients (65–100 years, mean 83 years) from the VIREL study (Sadeghi et al., 2012; Aronen et al., 2016) with either respiratory symptoms, suspected pneumonia, cardiovascular, or other diseases in the University Hospital of Turku, Finland, between September 2007 and April 2009. Sera were previously serologically screened for Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa-associated polyomavirus (TSPyV) (Sadeghi et al., 2012), as well as acute human bocavirus (HBoV) 1–4 infections (Aronen et al., 2016). Ethical approval was obtained by the Ethics Committee of Turku University Hospital, and all individuals gave informed consent.

2.2. Serological analysis

PPV IgG EIA and BuV 1–3–CuV IgM EIA: All sera were analyzed for BuV1–3–CuV–TuV IgG in separate streptavidin-coated wells with a previously described enzyme immunoassay (EIA) (Väisänen et al., 2016, 2018) and a novel μ-capture BuV1–3–CuV IgM EIA that tests one virus antigen per well, detailed here. For the novel IgM EIA, microtiter plate types, buffers, incubation times, as well as biotinylated virus-like particle (VLP) and streptavidin-conjugate concentrations, were

systematically compared and optimized to reduce background. For the final IgM EIA, the wells (Costar, Corning Inc., Corning, NY, USA) were coated with goat anti-human IgM (MP Biomedicals, Solon, OH, USA) 1:1200 in 0.05 M carbonate buffer (pH 9.6) overnight. Wells were washed with phosphate-buffered saline with Tween 20 (PBST), blocked with 3 % bovine serum albumin (BSA), and washed again with PBST, after which they were left to dry and stored at −20 °C until use. Sera diluted 1:200 in PBST +0.5 % BSA were added to the wells. After incubation, biotinylated antigens consisting of BuV1–3 or CuV-VP2 VLPs (10 ng/well) were applied. Wells without applied antigens (blanks) were used as background controls for each serum. Finally, horseradish peroxidase-conjugated streptavidin (Dako, Agilent Technologies, Santa Clara, CA, USA) diluted 1:12,000 in PBST + 0.5 % BSA was added with 3,3′,5,5′-tetramethylbenzidine (TMB; BD OptEIA, BD BioSciences, New Jersey, USA) as the substrate. Optical densities (ODs) were measured at 450 nm (Multiskan EX; Thermo Fischer Scientific, Pittsburgh, PA, USA). As acute BuV1–3, CuV, and TuV infections have not yet been identified, we used VLP antigens and serum samples positive for IgM of the other human parvoviruses, HBoV1 and B19V (Kantola et al., 2011; Maple et al., 2014), as positive technical controls for the IgM assays.

Competitive IgG EIA: Competitive IgG EIAs were performed as described (Väisänen et al., 2016, 2018). Because of complications with cross-reactivity of the human PPV capsids, samples with absorbances >0.2 for more than one of the viruses were characterized according to given rules (Supplementary Material) and subjected to competitive IgG accordingly, whereas ODs >0.2 for a single virus were considered a true positive result.

Competitive IgM EIA: To differentiate possible cross-reactions between the VLPs, all samples with absorbance values above a determined cutoff were verified in a competitive EIA as described for IgG assays (Väisänen et al., 2016, 2018), with modifications described here for IgM: In the CuV-IgM competition assay, self (homotypic) or BuV (heterotypic) non-biotinylated VLP antigens (at 30 ng/μL) block the antibodies in the sera against itself or against BuV, respectively, in separate wells at 37 °C for 45 min before the addition of the biotinylated CuV-VLP antigen, and vice versa for the BuV-IgM EIAs.

Cutoffs to determine which samples proceed to competitive EIA were calculated with two different methods (Supplementary Material). Samples above or equal to the cutoff were then subjected to competitive IgM-EIA assays. Samples that after competitive EIA showed no cross reactivities, and with absorbance values greater than or equal to the calculated cutoffs, were considered IgM positive.

2.3. Protoparvovirus (PPV) multiplex qPCR

All sera exhibiting elevated IgM absorbances (55 samples) and subjected to competitive IgM EIA were also screened for virus DNA by a multiplex PPV real-time qPCR, as previously described (Väisänen et al., 2019; Mohanraj et al., 2021). Due to insufficient volumes of these samples, five μl of serum were used directly as template for qPCR without undergoing DNA extraction, but with the initial heating in the PCR program. A second round of qPCR was performed on 5 μL of the reaction products as template to confirm the negative results, and the possible positives were sequenced. BuV, CuV, and TuV qPCR plasmids served as positive and water as negative controls.

2.4. Statistical analysis

We performed statistical analysis by Chi-square tests using OriginPro 6 (OriginLab Corp.). In the case of small sample sizes, a Fisher's exact test was used. P-values <0.05 were considered statistically significant.

3. Results

3.1. Human protoparvovirus IgG seroprevalences in adults

We analyzed the IgG seroprevalences for BuV1–3, CuV, and TuV in adults from Italy, Iran, and Finland (Table 2A). IgG seroprevalences for

Table 1
Description of cohorts used in the current study.

	Cohort	Persons (N)	Health status	Age range[b], (mean)	N (%) male: N (%) female	Other publications of clinical samples	Human PPV IgG published
A)	**Finland elderly**	374	Hospitalized for respiratory symptoms or suspected pneumonia, or cardiovascular and other diseases	65–100 y (83 y)	170 (45.5): 204 (54.5)	(Sadeghi et al., 2012), (Aronen et al., 2016)	Current study
	Italy child	22	Constitutionally healthy	1–18 y (12 y)	10 (45.4): 12 (54.5)	Current study	Current study
	Italy adult	178	Constitutionally healthy	19–90 y (48 y)	68 (38.2): 110 (61.8)	Current study	Current study
	Iran child	321	Hospitalized for respiratory symptoms, gastroenteritis, skin rash, encephalitis, or fever with unknown etiology	1 m–15 y (41 m)	184 (57.3): 137(42.7)	Current study	Current study
	Iran chronic adult	78	Chronic conditions[a]	35–86 y (53 y)	39 (50): 39 (50)	Current study	Current study
B)	**Iran blood donor**	89	Constitutionally healthy	18–77 y (45 y)	44 (49.4): 45 (50.6)	Väisänen et al. (2018)	Väisänen et al. (2018)
	Iraq adult	79	Constitutionally healthy	18–60 (39 y)	58 (73.4): 21 (26.6)	Väisänen et al. (2018)	Väisänen et al. (2018)
	Kenya child[c]	102	Febrile at time of sampling (mean temperature 38.6, range 36.4–40.4)	0.5–18 y (7 y)	58 (56.9): 40 (39.2), 4 unknown (3.9)	Väisänen et al. (2018)	Väisänen et al. (2018)
	Kenya adult[c]	117	Febrile at time of sampling (mean temperature 38.9, range 37.5–39.8)	18–88 y (43 y)	44 (37.6): 72 (61.5), 1 unknown (0.9)	Väisänen et al. (2018)	Väisänen et al. (2018)
	Latvia child	84	GE and/or RTI	1–59 m (23 m)	54 (64.3): 30 (35.7)	(Nora-Krukle et al., 2018), (Mohanraj et al., 2021)	Mohanraj et al. (2021)
	Total no. of persons	1444					

A) Cohorts newly screened for protoparvovirus IgG. B) Cohorts previously screened for protoparvovirus IgG in other publications.
[a] Chronic underlying diseases were e.g., diabetes, hyperlipidemia, thyroid disease, cancer, and iron deficiency anemia.
[b] Age is reported either in months (m) or years (y).
[c] Gender information was not provided for one adult and four children. GE, gastroenteritis; RTI, respiratory tract infection.

Table 2
IgG seroprevalence of BuV1–3, CuV, and TuV in the adult study cohorts.

	Cohort	Persons >18 years N (M/F)	Any BuV IgG	Two or more BuV IgG	CuV and any BuV IgG	BuV1 IgG	BuV2 IgG	BuV3 IgG	CuV IgG
A)	**Finland elderly**	374 (170/204)	46 (12.3)	4 (1.1)	9 (2.4)	32 (8.6)	5 (1.3)	10 (2.7)	67 (17.9)
	Italy adult[b]	178 (68/110)	28 (15.7)	6 (3.4)	4 (2.2)	21 (11.8)	6 (3.4)	7 (3.9)	11 (6.2)
	Iran chronic adult	78 (39/39)	53 (67.9)	13 (16.7)	10 (12.8)	46 (59.0)	14 (17.9)	7 (9.0)	10 (12.8)
B)	**Iran blood donor**[b]	89 (44/45)	53 (59.6)	15 (16.9)	5 (5.6)	49 (55.1)	16 (18.0)	5 (5.6)	6 (6.7)
	Iraq adult	79 (58/21)	66 (83.5)	28 (35.4)	0	63 (79.7)	26 (32.9)	12 (15.2)	1 (1.3)
	Kenya adult[a, b]	117 (44/72/1 unknown)	81 (69.2)	37 (31.6)	4 (3.4)	30 (25.6)	41 (35.0)	54 (46.2)	5 (4.3),
	Total from all cohorts	915 423/491/1 unknown	327 (35.7)	103 (11.3)	34 (3.7)	241 (26.3)	108 (11.8)	95 (10.4)	100 (10.9)

A) Cohorts that are novel to this study. B) Cohorts that have been previously analyzed for PPV IgG but shown here for comparison (Väisänen et al., 2018). Values and percentages here reflect only the samples that could be screened for IgM in this study, and thus differ from those of the original publication. Values are no. sera (% of no. sera per cohort) unless otherwise noted. All samples were TuV-IgG negative.

[a] Gender information was not provided for one adult.

[b] Unclear BuV2 and CuV blocking results were observed for some samples: 12 (*Finland elderly*), 5 (*Italy adult*), and 3 (*Kenya adult*). These values are not included in the cohorts' BuV2 or CuV seroprevalence calculations. BuV, bufavirus; CuV, cutavirus; TuV, tusavirus.

these PPVs in the *Iran blood donor*, *Iraq adult*, and *Kenya adult* cohorts have been previously published (Väisänen et al., 2018), but the IgM results are described in this current study. Some of the previously screened samples had insufficient remaining serum volumes, therefore, the IgG seroprevalences summarized here in Table 2B reflect only samples for which also IgM assays were completed.

We found seroprevalences of any BuV-serotype IgG of 12.3 %, 15.7 %, and 67.9 % in the *Finland elderly*, *Italy adult*, and *Iran chronic adult* cohorts, respectively. BuV1 was the predominant IgG serotype among these cohorts. BuV3 and BuV2 were the next predominant serotypes in both the *Iran adult* and the European adult cohorts. IgG positivity to multiple BuV serotypes was much higher in the *Iran chronic adult* (16.7 %) than in the *Italy adult* and *Finland elderly* cohorts (3.4 % and 1.1 %, respectively). Of the 28 individuals in the *Italy adult* cohort, who were IgG positive for at least one BuV type, nine (32.1 %) originated from outside Italy, compared to 7.6 % (11/145) of the BuV IgG-negative adults. For these nine individuals, BuV1 was the predominant serotype, followed by BuV3.

The *Finland elderly* cohort was the only cohort with higher CuV seroprevalences (17.9 %) than the total BuV1–3 seroprevalences (12.3 %), and it had the highest CuV seroprevalences of any cohort hitherto studied. Also, the *Iran chronic adult* cohort had a higher CuV seroprevalence (12.8 %) than previously reported for healthy and febrile adults from the Middle East (less than 6 %) (Väisänen et al., 2018), but the difference was not statistically significant. Of the patients with CuV IgG in the *Iran chronic adult* cohort, three had diabetes, one had elevated liver enzymes, four had hyperlipidemia, one had renal disease, and one had thyroid disease. TuV IgG was not detected in any cohort.

In the *Italy adult* and *Iran chronic adult* cohorts (Table 2A), multiple BuV-IgG positivity was more common than dual BuV- and CuV-IgG

Table 3
IgG seroprevalence of BuV1–3, CuV, and TuV in the adult study cohorts below and above 65 years of age.

	Cohort	Age, years (range, mean)	Persons N M/F	Any BuV IgG	Two or more BuV IgG	CuV and any BuV IgG	BuV1 IgG	BuV2 IgG	BuV3 IgG	CuV IgG
A)	**Finland elderly**	≥65 (65–100, 83)	374 170/204	46 (12.3)	4 (1.1)	9 (2.4)	32 (8.6)	5 (1.3)	10 (2.7)	67 (17.9)
	Italy adult[b]	<65 (18–64, 40)	139 46/93	20 (14.4)	4 (2.9)	4 (2.9)	15 (10.8)	4 (2.9)	5 (3.6)	9 (6.5)
		≥65 (66–90, 75)	39 22/17	8 (20.5)	2 (5.1)	0	6 (15.4)	2 (5.1)	2 (5.1)	2 (5.1)
	Iran chronic adult	<65 (35–64, 53)	67 33/34	45 (67.2)	12 (17.9)	10 (14.9)	40 (59.7)	12 (17.9)	6 (9.0)	10 (14.9)
		≥65 (65–86, 73)	11 6/5	8 (72.7)	1 (9.1)	0	6 (54.5)	2 (18.2)	1 (9.1)	0
B)	**Iran blood donor**[b]	<65 (18–64, 42)	81 37/44	47 (58.0)	14 (17.3)	2 (2.5)	43 (53.1)	15 (18.5)	4 (4.9)	3 (3.7)
		≥65 (67–77, 72)	8 7/1	6 (75)	1 (12.5)	3 (37.5)	6 (75)	1 (12.5)	1 (12.5)	3 (37.5)
	Kenya, adult[a, b]	<65 (18–64, 40)	107 43/63/1 unknown	77 (72.0)	33 (30.6)	4 (3.7)	28 (26.2)	37 (34.6)	51 (47.7)	5 (4.7)
		≥65 (66–88, 73)	10 9/1	4 (40)	4 (40)	0	2 (20)	4 (40)	3 (30)	0
	Total from all cohorts		836 373/462/1 unknown	261 (31.2)	75 (9.0)	32 (3.8)	178 (21.3)	82 (9.8)	83 (9.9)	99 (11.8)

A) Cohorts that are novel to this study. B) Cohorts that have been previously analyzed for BuV1–3, CuV, and TuV IgG, but shown here for comparison (Väisänen et al., 2018). Values and percentages here reflect only the samples that could be screened for IgM in this study, and thus differ from the original publication. Values are no. (%) unless otherwise noted. NB: In some cases the N is too small for the prevalences to be reliable. All samples were TuV-IgG negative.

[a] Gender information was not provided for one adult.

[b] As in Table 2, unclear BuV2 and CuV blocking results were observed for 2.2 % of the adult cohorts: 12 (*Finland elderly*), 5 (*Italy adult*), and 3 (*Kenya adult*), and were thus not included in this table. BuV, bufavirus; CuV, cutavirus; TuV, tusavirus.

positivity ($p<0.05$ for all cohorts). We observed initial IgG cross-reactivities between any BuV and CuV in 2.4 %, 2.2 %, and 12.8 % of the sera in the *Finland elderly*, *Italy adult*, and *Iran chronic* cohorts, respectively, which were differentiated by competitive EIAs. We could not, however, discern between BuV2 and CuV IgG positivity in 2.2 % of the total samples (12 in *Finland elderly*, 5 in *Italy adult*, and 3 in *Kenya adult* cohorts), hence we considered them inconclusive and did thus not include them in the seroprevalence of BuV2 or CuV.

3.2. Age distribution of BuV1–3 and CuV IgG in adults versus the elderly

We also compared PPV IgG seroprevalences among elderly individuals, ≥65 years of age, from Finland, Italy, Iran, and Kenya (Table 3). The *Finland elderly* cohort did not have individuals less than 65 years of age for within-cohort comparison, but there was an increase with age in seroprevalences for any BuV type in all other populations, except the *Kenya adult* cohort, but for CuV, we saw a slight decrease in seroprevalences for all groups except for *Iran blood donor*. However, the differences in CuV seroprevalence from those younger and older than 65 years, was only significant in the *Iran blood donor* cohort ($p < 0.01$). Nevertheless, the sample sizes were small ($n<12$) of elderly individuals in the Middle East cohorts, and thus the generalizability of findings presented here should be interpreted with caution.

3.3. Age distribution of BuV1–3 and CuV IgG seroprevalences in children

The seroprevalences for BuV1–3, CuV, and TuV were also determined for two newly screened pediatric cohorts from Italy and Iran (Table 4A). Previously published seroprevalences for the *Latvia* and *Kenya child* cohorts are provided for comparison (Table 4B), but their IgM-antibody findings are newly described in this study.

In the *Iran child* cohort ($n = 321$), we found relatively high (16.5 %) BuV and low (2.2 %) CuV seroprevalences (Table 4). Like the *Iran adult* cohort, the *Iran child* cohort had BuV1 seroprevalence predominance followed by BuV2. Only 1 sample in the *Italy child* cohort was seropositive for any protoparvovirus. This patient, a 17-year-old male, was IgG positive for BuV2. There were no statistically significant differences between male and female BuV or CuV seropositives in any child cohort. However, there was a difference in total BuV-IgG positivity between Kenyan children younger and older than 5 years of age ($p < 0.02$). A summary of the seroprevalences of all newly screened cohorts is provided in Fig. 1.

3.3.1. Elevated BuV- and CuV-IgM absorbances and qPCR

Sera from all cohorts were screened for acute infections with the novel BuV1–3- and CuV-IgM assays. TuV IgM was not analyzed due to its rare IgG seropositivity in this and previous studies (Väisänen et al., 2016, 2018; Mohanraj et al., 2021). Cutoff values based on initial absorbances were calculated with two different methods and averaged to set a more balanced threshold and minimize cohort-specific variation (Supplementary Material). Because of validity concerns of calculated IgM-EIA cutoff values, all samples with elevated IgM absorbances equal to or above the cutoff value (i.e., 0.25 in all cohorts) were verified through competitive EIA and multiplex PPV qPCR. Prevalences of elevated IgM absorbances for BuV1–3 and CuV in the cohorts are described in Supplementary Material, Table S2.

We found in total 55 (3.8 %) sera from six cohorts, all child cohorts and the two European adult cohorts (*Finland elderly* and *Italy adult*), with elevated (>0.25) initial IgM absorbances (range 0.25–0.56, mean 0.32) before competitive EIA, but none in the adult cohorts from the Middle East or Kenya. We found the greatest number of samples with elevated IgM absorbances in the European cohorts, *Latvia child* (35 samples) and *Italy adult* (14 samples). BuV1 IgM reactivity was predominant among the *Italy adult* cohort (10 samples), and CuV IgM in the *Latvia child* cohort (24 samples). However, only 0.2 % of the total individuals (all within three cohorts) had both IgG and elevated levels of IgM for the same virus: one from the *Kenya child* cohort for BuV3, two from the *Iraq adult* cohort for BuV2 and one each from the *Finland elderly* and *Italy adult* cohorts for BuV1.

All samples with elevated IgM absorbances ($n = 55$; OD >0.25) (Supplementary Material) were subjected to both competitive IgM EIA and a multiplex PPV qPCR. We did not observe self-blocking in any of the samples with elevated IgM, even after lowering the cutoff for the *Finland elderly* cohort because of possible immunosenescence (Wang et al., 2022), suggesting that all were IgM negative for the tested human PPVs. Likewise, by qPCR we did not detect any human PPV DNA in the sera. Taken together, these results suggest that all patients with elevated IgM absorbances in sera did not have acute infections at the time of sampling.

Table 4
Seroprevalence by age of PPV IgG in the pediatric cohorts.

	Cohort	Age[a] (range, mean)	Persons N (M/F)	Any BuV IgG	Two or more BuV IgG	CuV and any BuV IgG	BuV1 IgG	BuV2 IgG[c]	BuV3 IgG	CuV IgG[c]
A	**Italy child**	<5 y (1–4.0 y, 2 y)	3 (1/2)	0	0	0	0	0	0	0
		5 ≤ 18 y (5.2–17 y, 13 y)	19 (9/10)	1 (5.3)	0	0	0	1 (5.3)	0	0
	Iran child	<5 y (1 m–5 y, 2 y)	236 (132/104)	42 (17.8)	10 (4.2)	2 (0.8)	37 (15.7)	14 (5.9)	3 (1.3)	3 (1.3)
		5 ≤ 18 y (5–15 y, 8 y)	85 (52/33)	11 (12.9)	4 (4.7)	4 (4.7)	9 (10.6)	5 (5.9)	3 (3.5)	4 (4.7)
B	**Latvia child**	<5 y (1–51 m, 24 m)	84 (54/30)	0	0	0	0	0	0	3 (3.6)
		5 ≤ 18 y	0	0	0	0	0	0	0	0
	Kenya child[b]	<5 y (5 m–4 y, 3 y)	47 (26/19, 2 unknown)	6 (12.8)	0	0	0	0	6 (12.8)	2 (4.3)
		5 ≤ 18 y (5–17 y, 11 y)	55 (32/21, 2 unknown)	18 (32.7)	2 (3.6)	0	2 (3.6)	3 (5.5)	15 (27.3)	0
	All children	1 m–18 y	529	78 (14.7)	16 (3.0)	6 (1.1)	48 (9.1)	23 (4.3)	27 (5.1)	12 (2.3)

A) Cohorts that are novel to this study. B) Cohorts that have been previously analyzed for BuV1–3, CuV, and TuV IgG, but shown here for comparison (Väisänen et al., 2018). Values and percentages here reflect only the samples that could be screened for IgM in this study, and thus differ from the original publication. Values are no. (%) unless otherwise noted. All samples were TuV-IgG negative.

[a] Age is reported either in months (m) or years (y).

[b] Gender information was not provided for four children.

[c] Unclear BuV2 and CuV blocking results were observed for two samples: one from Italy and one from Iran and were thus not included in the overall seroprevalence calculations. BuV, bufavirus; CuV, cutavirus; TuV, tusavirus.

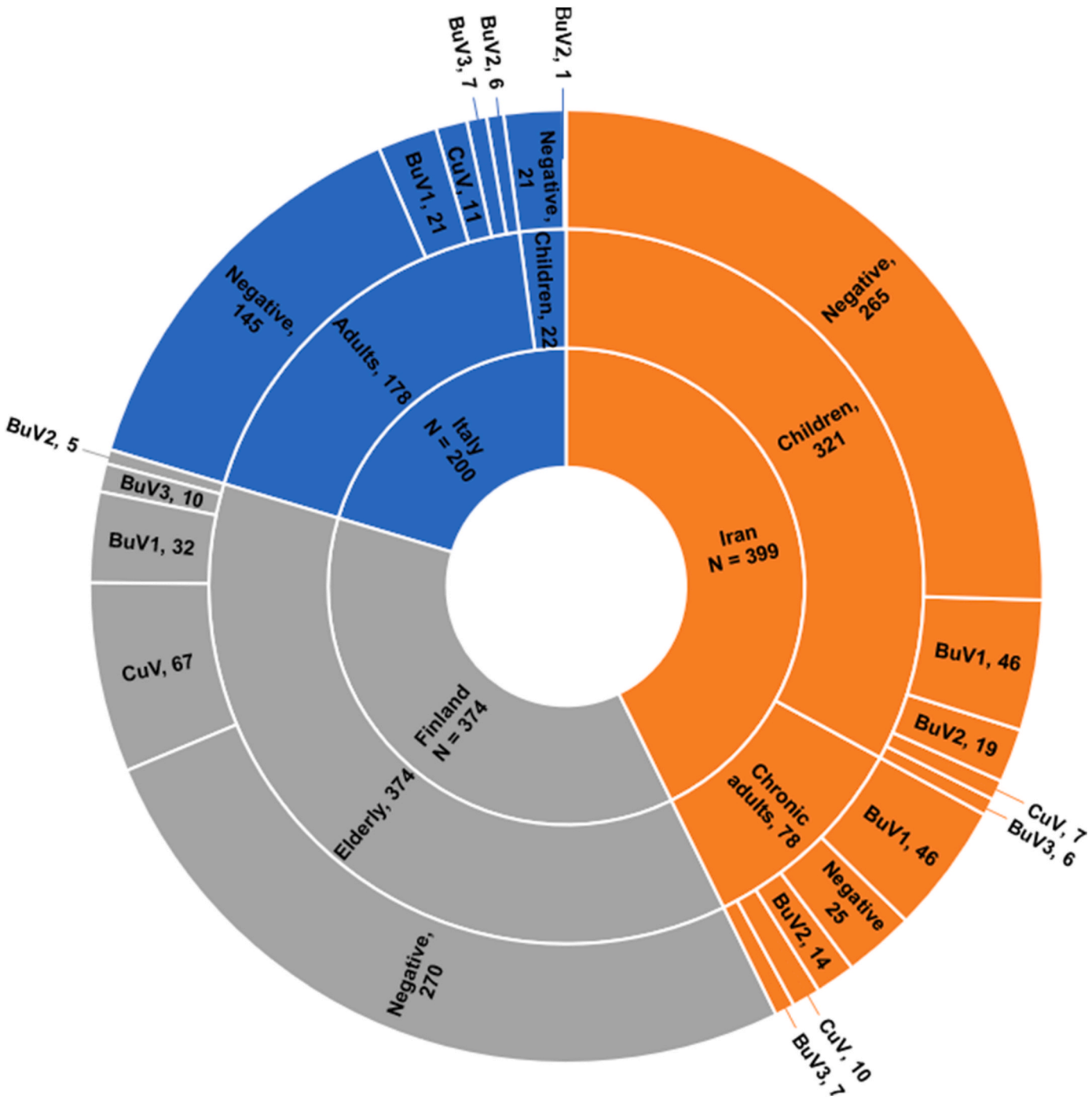

Fig. 1. Summary of the distribution of IgG seroprevalences in the newly screened cohorts. Inner circle represents country of cohort and number of individuals (N). Subsequent rings depict age (child vs adult, n) and virus positivity (negative, BuV1–3, CuV). Note that samples can be positive for more than one virus. No TuV-IgG positives were found. BuV, bufavirus; CuV, cutavirus; TuV, tusavirus.

4. Discussion

The protoparvoviruses BuV, CuV, and TuV are among the newest parvoviruses discovered in humans, thus serologic studies of them are rare and the signs and symptoms of acute infections are unknown (Väisänen et al., 2016, 2018). With our novel PPV IgM EIAs, despite some false elevated IgM absorbances, none of the individuals of our ten cohorts exhibited true PPV IgM or viremia, and were thus all considered non-acute. Our current in-house BuV1–3, CuV, and TuV IgG EIA results disclosed surprisingly high seroprevalences for CuV among the elderly from Finland (18 %), and in adults with chronic diseases from Iran (12 %), patient groups that have not been studied before. This is particularly intriguing when considering the association of CuV with CTCL (Phan et al., 2016; Väisänen et al., 2019; Mollerup et al., 2017; Hashida et al., 2023b; Mohanraj et al., 2024). While the IgG EIA was published before, here we developed novel in-house competitive IgM EIAs to screen for also IgM for BuV1–3 and CuV in 1444 samples from both healthy and diseased (i.e., respiratory tract infection [RTI], gastroenteritis [GE], fever with unknown cause, and chronic conditions) individuals from ten different cohorts, spanning all age groups and different geographic locations within Europe, Africa, and the Middle East.

Given that this is the first study to screen sera for BuV1–3 and CuV

IgM, there are no positive or negative controls or established absorbance cutoff values to discern IgM-positive and -negative samples. We therefore utilized two different methods for cutoff calculations (Supplemental Material). When initially screened, 3.8 % of patient samples (0.2 % of those exhibiting IgG for the same virus) across our ten cohorts appeared to have elevated IgM levels above these determined cutoffs; yet they were all negative in our confirmatory tests; competitive IgM EIA and qPCR, i.e., the IgM reactivity could not be self-blocked, and they were all non-viremic. Our data thus suggests symptoms in acute infections are mild, rare, or occur as other disease manifestations than here included.

We previously determined that insect-cell lysates used to isolate VLPs may cause background in the EIA assays (Väisänen et al., 2016). It is possible that our elevated IgM absorbances found here were affected by these components, as some samples had elevated IgM for multiple viruses. However, the no-VLP controls remained negative and our IgM EIA is validated for both human bocaviruses (HBoV) and B19V (Kantola et al., 2011; Maple et al., 2014). Thus, the absence of PPV IgM-positive samples in our study is likely due to the lack of detectable acute infections in the sample population, rather than an issue with assay performance. It may further be that infections are subclinical, local, or very acute so the antibody response is not yet detected, or the target populations may not be optimal for these protoparvoviruses. We did, however, include different age groups, countries, and disease manifestations, but the target populations may still have been too small or the incubation time (from infection to symptom onset) too short for production of a detectable level of IgM. At least Iran, Iraq and Kenya show very high BuV IgG seroprevalences, so we did expect some BuV acute infections particularly in these cohorts, among patients with fever of unknown etiology, RTI or GE, but interestingly, no samples from these endemic regions exhibited IgM, or even elevated IgM responses, while Latvian children (for BuV1 and CuV) and Italian adults (for BuV1), with lower seroprevalences, had the most elevated IgM findings. It is possible that this false IgM reactivity could be due to cross-reactivity with yet unidentified related PPVs, since we do not see such elevated IgM for parvovirus B19, comprising only one serotype (Maple et al., 2014; Ekman et al.).

We found congruent BuV1–3 IgG seroprevalences in the newly screened cohorts to those previously determined for geographically close regions (Väisänen et al., 2018). In the newly screened *Finland elderly*, *Italy adult*, and *Iran chronic adult* cohorts, BuV1 was the predominant BuV serotype. We found higher BuV3 than BuV2 seroprevalences in the European adult cohorts; the opposite was observed for adult cohorts from the Middle East, with comparable ages. The global BuV distribution and serotype frequency are consistent with trends in our previously published data (Väisänen et al., 2018). However, we found surprisingly high CuV IgG seroprevalences in the newly screened cohorts compared to previous results. While previous studies have found mostly low CuV IgG seroprevalences (<5 %) (Väisänen et al., 2018), we found CuV IgG seroprevalences of 17.9 %, 6.5 %, and 12.8 % in our Finnish and Italian adults, and the *Iran chronic adult* cohort. The latter patients did not have any acute illnesses, but had underlying chronic conditions (like diabetes, hyperlipidemia, elevated liver enzymes, thyroid disease, and cancer). Previous studies have linked CuV genoprevalence to another chronic condition, cutaneous T-cell lymphoma and a chronic inflammatory condition often preceding this lymphoma, called parapsoriasis (Väisänen et al., 2019; Hashida et al., 2023a, 2023b; Mohanraj et al., 2023, 2024) The CuV-IgG seroprevalence was 9.5 % among 42 Finnish patients with CTCL (Väisänen et al., 2019). It is possible that there is a link between CuV infection and chronic inflammation in general. Further studies of the sero- and genoprevalences of CuV in patients with underlying conditions is further necessary to confirm any association or susceptibility to infection.

Differences in PPV seroprevalences in adults versus elderly individuals were not observed in the *Italy*, *Iran chronic*, or *Kenya* cohorts, likely due to the small sample sizes of elderly individuals (n<12). We observed higher BuV (12.3 %) and CuV (17.9 %) seroprevalences in the *Finland elderly* cohort than were previously described for younger Finnish adults (1.9 % and 4.9 %, respectively) (Väisänen et al., 2018), which is logical. Higher BuV and CuV IgG seroprevalences (25 % and 33 %, respectively) in adults younger than 65 years have been described, but these patients had parapsoriasis or CTCL, and the sample size was only 12 (Mohanraj et al., 2024). We did also find significantly decreased CuV IgG seroprevalences in the *Iran blood donor* cohort, in adults over 65 compared to those under 65 years of age, but also here, the small sample size in the elderly group warrants caution in the interpretation of these findings. Further studies with larger sample sizes are needed to validate these differences.

We were not able to distinguish BuV2 and CuV IgG cross-reactivity in 1.5 % of the total samples screened, a rate similar to that in our previous study (Väisänen et al., 2018). BuV2 and CuV share high (82 %) amino acid sequence identities within the VP2 gene, which may explain the antibody cross reactivities and cross-blocking results observed here. Interestingly, this BuV2-CuV cross-reaction was, however, not seen in our IgM assay, which otherwise exhibited some unexplained nonspecific elevated IgM responses, supporting the possible existence of unknown related cross-reactive PPVs.

We previously published somewhat high BuV and low CuV seroprevalences (23.6 %, 1.9 % respectively) in the *Kenya child* cohort (Väisänen et al., 2018), and no BuV and low (3.6 %) CuV seroprevalences in the *Latvia child* cohort (Mohanraj et al., 2021). We now found a high (12 %) BuV1 seroprevalence in the *Iran child* cohort and only one BuV2 IgG-positive serum in the *Italy child* cohort. The number of Iranian children positive for any BuV IgG was similar between those younger and older than 5 years of age. Among the Kenyan children older than 5 years, there was a significant, but expected, increase in IgG positivity for BuV, with a BuV3 predominance, compared to those younger than 5 years of age. These data suggest that children in Kenya and Iran perhaps have greater susceptibility to BuV infections due to geographic or sociocultural factors than those from Europe, but children in Iran may be more exposed to these viruses at a younger age than Kenyan children, perhaps depending on differences in epidemic years. Despite this, we could not document any acute infections among the children in these regions.

TuV DNA has been detected only rarely in humans—in diarrhetic stool samples from a child in Tunisia (Phan et al., 2014), in two Finnish adults with gastroenteritis (Mohanraj et al., 2021), and in two Chinese adults with chronic illnesses (He et al., 2023). TuV-specific antibodies have been detected only once, in a child from Finland (Väisänen et al., 2016). We did not find any TuV IgG-positive samples in our screening, and thus eliminated TuV from our IgM EIAs. Higher TuV genoprevalences have been noted among goats and sheep (Reuter et al., 2022), suggesting a possible zoonotic origin for human TuV infection, possibly by passive transfer when ingesting TuV in food.

While human protoparvoviruses are occasionally associated with certain chronic health conditions, they are, based on our results, not likely the primary cause of any acute illnesses, however, it is impossible to cover all disease possibilities (Söderlund-Venermo, 2019). Whether these viruses are a natural part of the human virome is also currently debated. In one study, a considerable CuV-DNA prevalence was found in skin swabs of healthy elderly (>60 years of age) and also in HIV-positive subjects (Wieland et al., 2019; Hashida et al., 2023a). Contrastingly, other studies have detected only occasional, if any, CuV DNA in healthy tissues or skin swabs (Mohanraj et al., 2023; Pyöriä et al., 2023).

5. Conclusion

Our data support that acute human protoparvovirus infections appear globally and with accumulating seroprevalences from adolescence to adulthood. The symptoms are likely mild, brief, or unusual. Infections appear to affect all ages but are more common during adolescence and adulthood than early childhood, as we found surprisingly high seroprevalences particularly among adults in Kenya and the

Middle East, and of CuV among the Finnish elderly. Although there is growing evidence that CuV infections are associated with cutaneous T-cell lymphoma, and that BuV1–3 are enteric, their full clinical significances remain undefined. However, our data do not support their role as respiratory pathogens.

CRediT authorship contribution statement

Sally K. Chesnut: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Ushanandini Mohanraj:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Rajita Rayamajhi Thapa:** Investigation. **Farid A. Jalilian:** Writing – review & editing, Resources. **Razieh Amini:** Resources. **Iraj Sedighi:** Resources. **Parinaz Sedighi:** Resources. **Haider Al-Hello:** Resources. **Ali M. Barakat:** Resources. **Moses Masika:** Resources. **Dufton Mwaengo:** Resources. **Omu Anzala:** Resources. **Zaiga Nora-Krukle:** Writing – review & editing, Resources. **Anda Vilmane:** Resources. **Inga Ziemele:** Resources. **Elisabetta Manaresi:** Resources. **Giorgio Gallinella:** Writing – review & editing, Resources. **Laura Viikari:** Resources. **Tuomas Jartti:** Writing – review & editing, Resources. **Maria Söderlund-Venermo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Funding SOURCES

This work was supported by the Sigrid Jusélius Foundation (MSV), the Life and Health Medical Grant Association (MSV), the Finnish-Norwegian Medical Foundation (UM), Ida Montin Foundation (UM), Kirsti and Tor Johansson Foundation (MSV), the Viral Disease Research Foundation (UM, SKC), the Finnish Cultural Foundation (SKC), the Instrumentarium Foundation (UM), and the Nordic Society of Clinical Microbiology and Infectious Diseases (SKC).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank MD Heli Ylä-Outinen and MD Henriikka Langen for active patient recruit, sample collection and geriatric patient monitoring.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2025.110529.

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