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International Journal of Infectious Diseases 116 (2022) 68–73

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

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

Effective presence of antibodies against common human coronaviruses in immunoglobulin medicinal products

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ARTICLE INFO

Article history:
Received 30 September 2021
Revised 17 November 2021
Accepted 10 December 2021

Keywords:
Human coronavirus
Antibodies
Immunoglobulins

ABSTRACT

Background: Immunoglobulin products (for intravenous, intramuscular and subcutaneous administration) prepared from geographically diverse plasma pools were tested for activity against common human coronaviruses (HCoVs). Products from plasma obtained from Germany, Czech Republic, Slovak Republic, USA and Spain were tested for antibodies to common HCoVs: 229E, OC43, NL63 and HKU1. As these products are manufactured from pooled plasma from thousands of donors, the antibodies therein are representative of HCoV exposure in the population at large.

Methods: Immunoglobulin products were tested for antibodies to four common HCoVs by enzyme-linked immunosorbent assays (ELISAs). Neutralization assays were conducted using HCoV-229E cultured on to MRC5 cells.

Results: ELISAs showed that when expressed as specific activity (anti-HCoV activity/mg immunoglobulin), similar activity against the four common HCoVs was seen across the immunoglobulin products regardless of concentration or geographic origin. Highest anti-HCoV activity was seen against HCoV-229E, followed by HCoV-OC43, HCoV-NL63 and HCoV-HKU1. The neutralization assays showed similar potency for two immunoglobulin products prepared by different processes.

Conclusions: To the authors' knowledge, this is the first demonstration of antibodies to common HCoVs in immunoglobulin products. These results may explain the cross-reactivity seen with pre-pandemic immunoglobulin products and severe acute respiratory syndrome coronavirus-2, and contribute to differences in severity of illness between patients.

Introduction

Before the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic, relatively little attention was paid to the classical endemic human coronaviruses (HCoVs) (Li et al., 2021). Common HCoVs are globally distributed (Anthony et al., 2017), and are responsible for a large proportion of respiratory infections, most of which are mild for immunocompetent individuals. To date, four main subtypes of common HCoVs have been identified: HCoV-229E (Hamre and Procknow, 1966), HCoV-NL63 (Van Der Hoek et al., 2004), HCoV-OC43 (McIntosh et al., 1967) and HCoV-HKU1 (Woo et al., 2005). HCoV-229E and HCoV-OC43 were discovered in 1966 and 1967, respectively, and HCoV-NL63 and HCoV-HKU1 were identified in 2005. None of these viruses have been found to be maintained within an animal reservoir (Su et al., 2016). In addition, there are two known coronaviruses of animal origin that infect humans and have led to limited outbreaks: severe acute respiratory syndrome coronavirus (SARS-CoV) in China in 2002–2003; and Middle East respiratory syndrome coronavirus (MERS-CoV) which has been responsible for an ongoing outbreak of severe respiratory disease in the Middle East since 2012.

Due to the ubiquity of these viruses, antibodies against common HCoVs are expected to be widely distributed in the population. Nevertheless, as far as is known, few systematic epidemiological surveys have been performed at population level, and no global surveys have been undertaken (Killerby et al., 2018). Studies have investigated the proportion of infections in some specific groups of patients (Gaunt et al., 2010; Ruetalo et al., 2021). Since a large proportion of infections occur in childhood, it remains unknown whether the antibodies persist in the adult population and at what magnitude. Moreover, distinct antibody reservoirs against endemic

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https://doi.org/10.1016/j.ijid.2021.12.329

Table 1
Plasma collection periods of the products tested for antibodies to common human coronaviruses.

Product	Country of origin	Plasma collection start date	Plasma collection end date
Flebogamma DIF 5%	Germany	Jun2019	Aug 2019
Flebogamma DIF 5%	Czech Republic	Jan 2019	Jul 2019
Flebogamma DIF 5%	Slovak Republic	Jul 2017	Feb 2020
Flebogamma DIF 10%	Spain	Oct 2018	Jul 2019
Flebogamma DIF 10%	USA	Jul 2019	Sep 2019
Gamunex-C 10%	USA	Mar 2018	Oct 2019
Gamastan 15-18%	USA	Feb 2018	Apr 2019
Igamplia 16%	USA	May 2018	Nov 2019
Xembify 20%	USA	Sep 2019	May 2020

HCoVs in children and adults have been described (Khan et al., 2021). As purified medicinal immunoglobulin solutions are polyvalent and are prepared from donor plasma pools from thousands of individuals, they cover a broad spectrum of immunity in the general population, and would be expected to include anti-HCoV antibodies reflecting both the proportion of infections caused by each subtype and the specific antibody titer in the donor (general) population.

It is important to note that coronaviruses in the same subgroup, particularly betacoronaviruses such as HCoV-OC43, HCoV-HKU1, SARS-CoV, SARS-CoV-2 and MERS-CoV, show some interactivity in antigenic responses. Cross-reactivity between SARS-CoV and MERS-CoV with other human betacoronaviruses has become apparent (Che et al., 2005; Chan et al., 2013; Patrick et al., 2006). The fact that the new betacoronavirus SARS-CoV-2 is directly related to SARS-CoV (they share more than 90% sequence homology) (Guo et al., 2020) suggests that antigenic interactivity between them is possible, at least for some proteins. In addition, reactions to SARS-CoV-2 in pre-pandemic immunoglobulin solutions have been observed (Díez et al., 2020a). Furthermore, these solutions have some neutralizing capacity (Díez et al., 2020b). Neutralization activity is primarily mediated through the spike (S) glycoprotein, the primary protein involved in the binding of coronaviruses to host cells (Qian et al., 2015; Jiang et al., 2020).

In this study, immunoglobulin solutions for intravenous, intramuscular and subcutaneous administration were analysed for the presence of antibodies to common HCoVs. This study was designed to detect, for the first time, common HCoV antibodies in immunoglobulin solutions. The immunoglobulin solutions were obtained from plasma from different origins (Germany, Czech Republic, Slovak Republic, USA and Spain), allowing indirect comparison of the epidemiology of these viruses in these geographical areas.

Methods

Immunoglobulin products

The immunoglobulin solutions used in this study were all produced by Grifols (Barcelona, Spain, and Research Triangle Park, NC, USA). They included intravenous solutions (Flebogamma DIF 5% and 10% and Gamunex-C 10%), intramuscular solutions (Gamastan 15–18% and Igamplia 16%) and a subcutaneous solution (Xembify 20%). These products were obtained from plasma pools from different origins (Germany, Czech Republic, Slovak Republic, USA and Spain). The collection dates for the plasma units are shown in Table 1.

Immunoassays for immunoglobulins

Antibodies (immunoglobulins) to the common HCoVs were detected using enzyme-linked immunosorbent assay (ELISA) kits (Alpha Diagnostic Intl., San Antonio, TX, USA). For the alphacoronaviruses, the following kits were used: RV-406100 Recombivirus Human anti-HCoV 229E S1 IgG ELISA Kit and RV-406115 Recombivirus Human anti-HCoV NL63 S1 IgG ELISA Kit. For the betacoronaviruses, the following kits were used: RV-406130 Recombivirus Human anti-HCoV OC43 Spike IgG ELISA Kit and RV-406145 Recombivirus Human anti-HCoV HKU1 S1 IgG ELISA Kit. The ELISAs were performed according to the manufacturer's instructions. Data were analysed as suggested by the kit manufacturer. Antibody potency was calculated by multiplying the positivity ratio for the inverse of the most diluted positive sample relative to the low calibrator from the kit. Samples were tested in duplicate.

Neutralization assays

Neutralization assays was performed using HCoV-229E. Briefly, different immunoglobulin solutions (Flebogamma DIF and Gamunex-C) were incubated with 100 infectious units of HCoV-229E for 1.5 h at 37 ± 2 °C. MRC5 cells (ATCC CCL-171, Manassas, VA, USA) in confluent culture in 96-well microtiter plates were infected with 200 µL per well of virus/antibody mixture. The microtiter plates were incubated at 35 ± 2 °C for 4 days, and cytopathic effects were observed using an inverted microscope (Axiovert 40, ACHROPLAN 10X/0·25 Ph1 objective, Karl Zeiss, Göttingen, Germany). Concentration–effect curves were generated, and half-maximal inhibitory concentration values were calculated using GraphPad Prism Version 9.1.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

The immunoglobulin titers (anti-HCoV activity/mL) for the immunoglobulin products are shown in Figure 1. When expressed in this manner, the lower concentration of immunoglobulin (5%) showed less activity than the higher concentrations (10–20%). For products of similar concentration, immunoglobulin activity was similar regardless of the geographic origin of the plasma pool. Overall, the highest activity was seen against HCoV-229E and HCoV-OC43.

The similarity is clearer when the data are expressed as specific activity (anti-HCoV activity/mg immunoglobulin: Figure 2). These data show that anti-HCoV activity was consistent across the products regardless of the total immunoglobulin concentration and the origin of the plasma pool. Activity was highest against HCoV-229E followed by HCoV-OC43. A similar lower level of activity was seen against HCoV-NL63 and HCoV-HKU1.

When the data from all the products were combined, the mean specific activity against the individual virus strains (Figure 3) followed the same profile as that noted for the individual products (Figure 2). Greatest activity was seen against HCoV-229E (885 ± 267units anti-HCoV activity/mg immunoglobulin), followed by HCoV-OC43 (633 ± 76 units anti-HCoV activity/mg immunoglobulin), with similar lower levels of activity observed against HCoV-NL63 (306 ± 53 units anti-HCoV activity/mg immunoglobulin) and

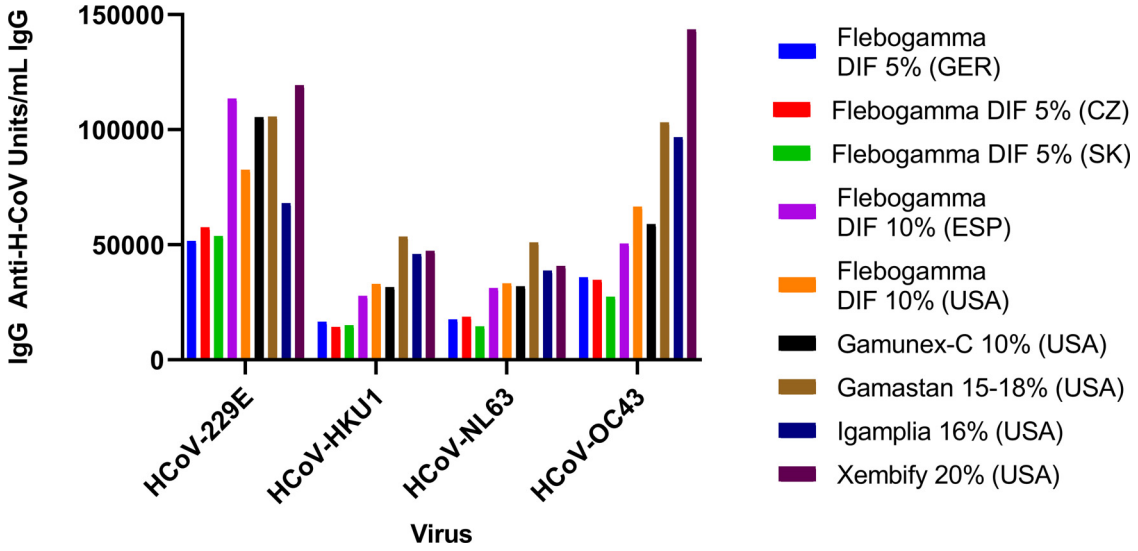

Figure 1. Immunoglobulins against common human coronaviruses (HCoV) per product mL. Anti-HCoV activity (measured by enzyme-linked immunosorbent assay as immunoglobulin anti-HCoV units per /mL of immunoglobulin product) to common HCoVs in different immunoglobulin solutions manufactured using plasma from different countries. The levels of antibodies (immunoglobulins) against the same virus were similar in all products with a similar immunoglobulin concentration. However, differences were seen between the viruses. GER, Germany; ESP, Spain; Cz, Czech Republic; SK, Slovak Republic.

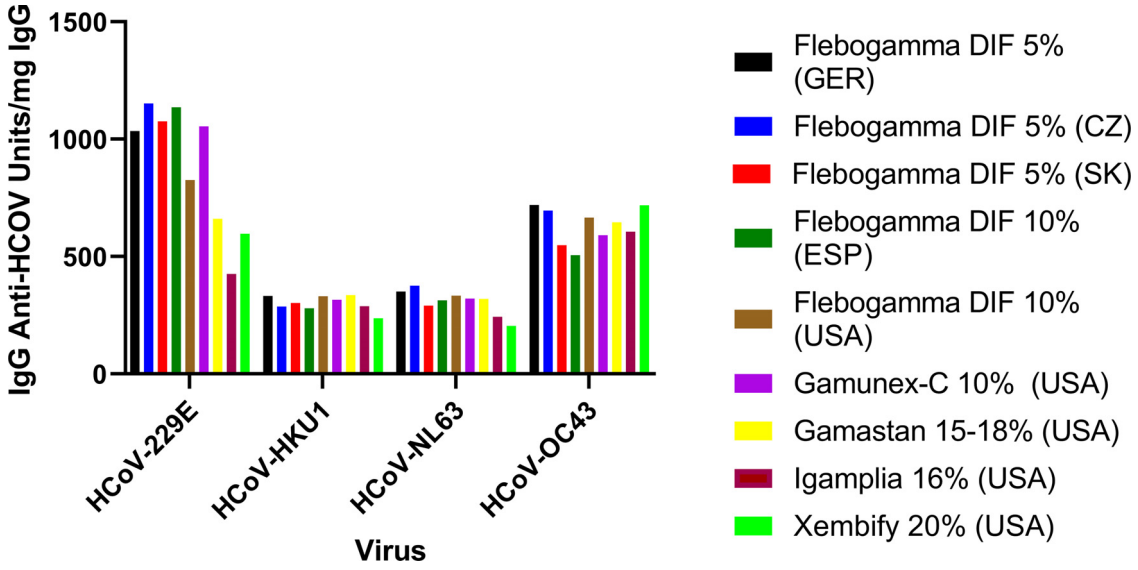

Figure 2. Immunoglobulin activity against common human coronaviruses (HCoV) per mg whole immunoglobulin. Anti-HCoV activity measured by immunoglobulin enzyme-linked immunosorbent assay (expressed as immunoglobulin anti-HCoV units/mg total immunoglobulin) against common HCoVs. Specific activity of the anti-HCoV antibodies was similar regardless of the geographic origin of the plasma pool. GER, Germany; ESP, Spain; Cz, Czech Republic; SK, Slovak Republic.

HCoV-HKU1 (301 ± 32 units anti-HCoV activity/mg immunoglobulin).

Immunoglobulin activity results were also analysed after segregating the results by the geographic origin of the plasma into three groups: Central Europe (Czech Republic and Slovak Republic), Spain and USA (Figure 4). Immunoglobulin products had similar activity against all four HCoVs regardless of the geographic origin of the plasma.

Functional characterization of the antibodies was performed by infectivity neutralization assays using HCoV-229E (Figure 5). When neutralization assays were performed using HCoV-229E in MRC5 cells, the concentration–effect curves for two types of intravenous immunoglobulin (IVIG) 10% produced by different manufacturing processes (Flebogamma-DIF 10%, origin USA; Gamunex-C 10%, origin USA) were nearly superimposable. This shows that the neutralization activity of the antibodies present in these products is essentially the same regardless of the manufacturing process. This demonstrates that immunoglobulin medicinal products contain functional antibodies against common HCoVs.

Discussion

To the authors' knowledge, this is the first study to measure the presence of antibodies to common HCoVs in therapeutic immunoglobulin solutions (intravenous, intramuscular and subcutaneous administration). Anti-HCoV immunoglobulin levels were similar across products for each virus regardless of the product concentration or the geographic origin of the plasma. However, there were differences in antibody levels between viruses, with the highest levels for HCoV-229E, lower levels for HCoV-OC43, and yet lower levels for HCoV-HKU1 and HCoV-NL63.

Studies on the incidence of HCoV infections treated by a healthcare provider have shown that the most common strain and

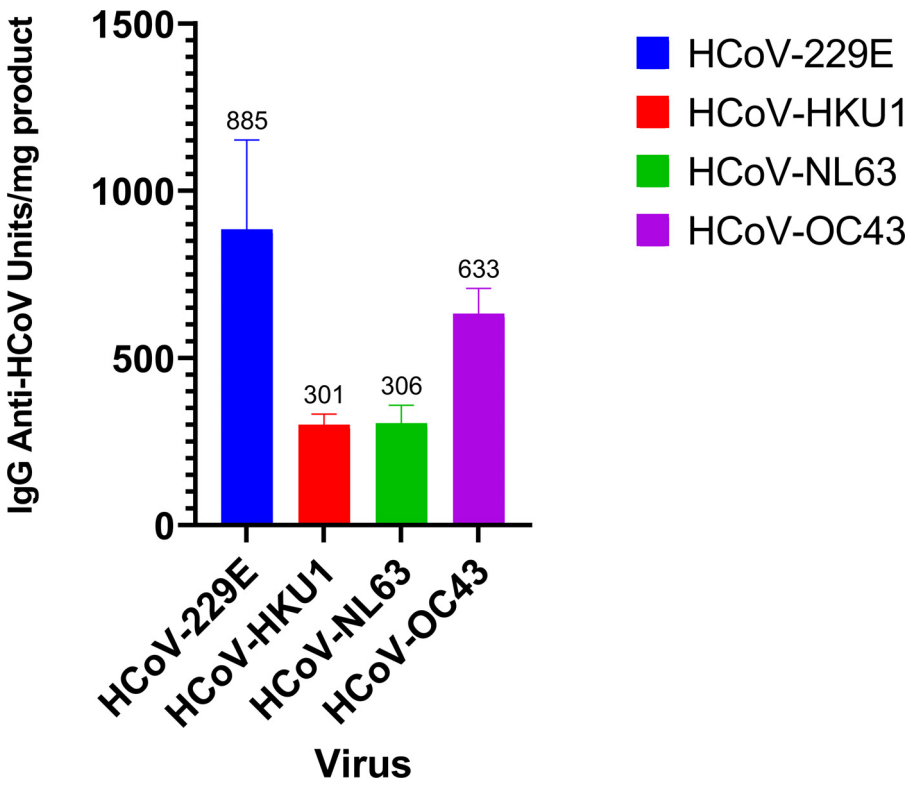

Figure 3. Levels of anti-human coronavirus (HCoV) activity against common HCoVs in immunoglobulin products. Mean anti-HCoV antibody levels (measured by immunoglobulin enzyme-linked immunosorbent assay and expressed as immunoglobulin anti-HCoV units/mg product) across all products were different for each virus (analysis of variance, $P<0.0001$), except for HCoV-HKU1 and HCoV-NL63 which showed similar antibody levels.

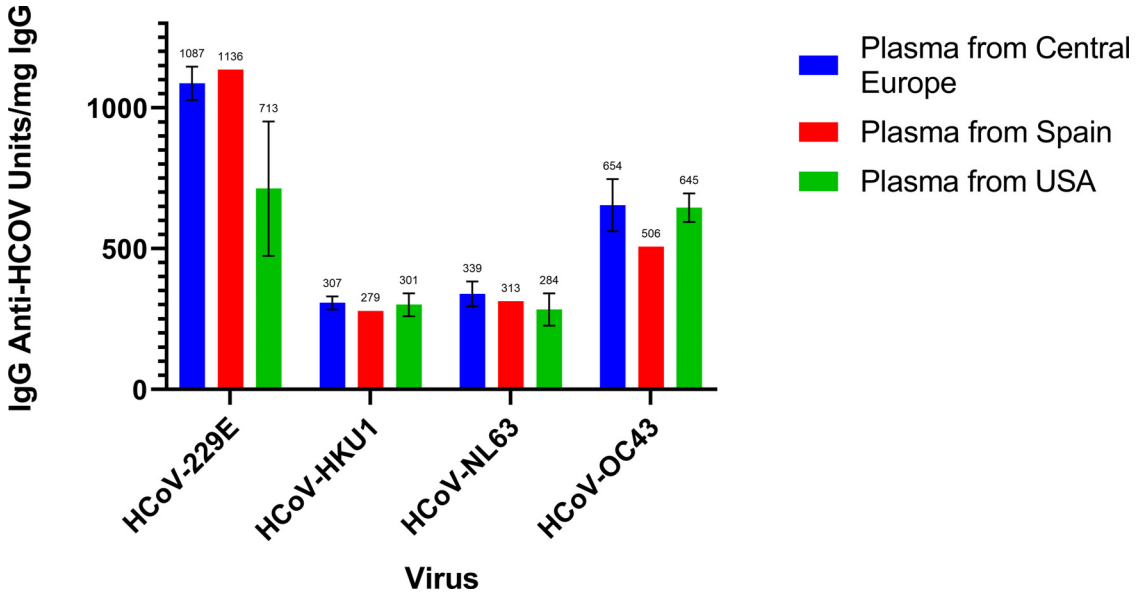

Figure 4. Antibodies to common human coronaviruses (HCoVs) by plasma origin. Antibody levels (measured by immunoglobulin enzyme-linked immunosorbent assay and expressed as immunoglobulin anti-HCoV units/mg immunoglobulin) to common HCoVs grouped by geographic origin of the plasma pool. Differences were seen between the common HCoV strains studied, but there were no significant differences between products derived from plasma of different geographic origin (analysis of variance, $P<0.90$).

prevalence depend on the geographic region and the time of year. Gaunt et al. (2010) found that the most prevalent strain of common HCoV in Edinburgh, Scotland varied from year to year, and that respiratory infections due to common HCoVs showed marked seasonality. However, over the 3-year period of data collection, HCoV-OC43 and HCoV-NL63 were the most frequently detected common HCoVs (Gaunt et al., 2010). Similar seasonality and variation in the predominant viral strain from year to year were found in a study conducted in the USA (Killerby et al., 2018).

A study in France found that HCoV-229E and HCoV-HKU1 were the most common HCoVs causing respiratory infections (Lepiller et al., 2013). In Japan, HCoV infections were most commonly caused by HCoV-NL63 and HCoV-HKU1, with peak prevalence in the winter months and annual variation in the relative prevalence of the different common HCoV strains (Matoba et al.,

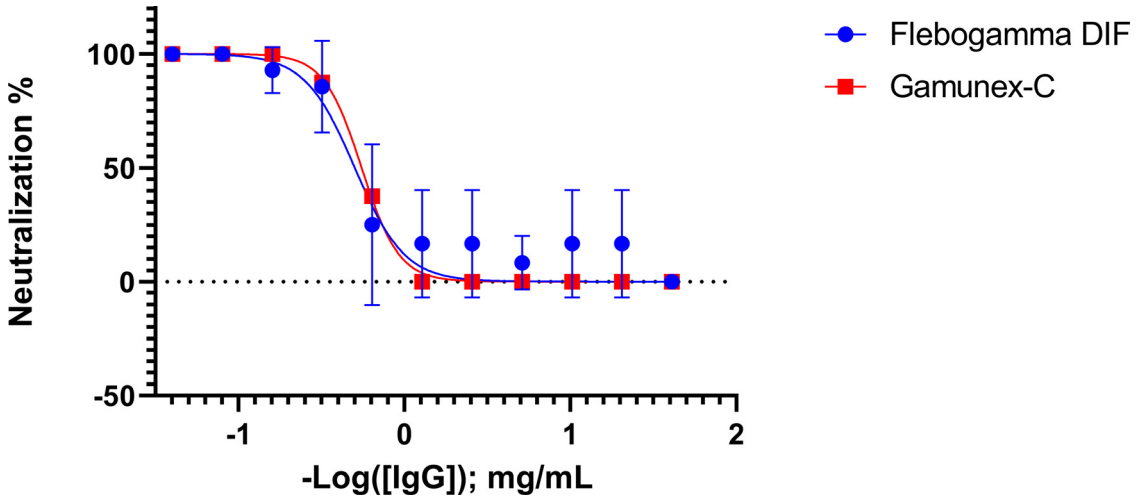

Figure 5. HCoV-229E virus neutralization. Neutralization of HCoV-229E was measured in a cytopathic assay in MRC5 cells. Concentration–effect curves (mg immunoglobulin/mL-neutralization %) were generated for virus neutralization and half-maximal inhibitory concentration (IC_{50}) values were calculated. The IC_{50} for Flebogamma-DIF was 0.503 mg immunoglobulin/mL which was very similar to the IC_{50} for Gamunex-C (0.553 mg immunoglobulin/mL).

2015). One paediatric study in China found that HCoV-229E and HCoV-OC43 had the highest prevalence among the common strains causing respiratory infections (Lin et al., 2020), while another study found HCoV-NL63 to be the most prevalent (Zhang et al., 2021). Co-infection with other respiratory viruses was also a common finding (Gaunt et al., 2010; Lepiller et al., 2013; Lin et al., 2020).

A global systematic review and meta-analysis of data from 1995 to 2020 in paediatric and adult patients showed that HCoV-OC43 was the most prevalent common HCoV (estimated prevalence 2.40%), followed by HCoV-NL63 (1.60%), HCoV-HKU1 (1.04%) and HCoV-229E (0.97%). These data were collected almost exclusively in developed countries (97%) (Li et al., 2021).

Given the above studies showing differences in the prevalence of common HCoV strains in different parts of the world, it is somewhat surprising that all the immunoglobulin samples in this study showed a similar pattern of anti-HCoV activity. This could be explained by the seasonal variability of the prevalence of common HCoVs (i.e. the predominance of one strain in a given winter season followed by the predominance of a different strain in the following winter season), and that the plasma pool likely reflects HCoV exposure over time in the donors. In addition, three of the epidemiological studies cited previously were conducted in Asia (Matoba et al., 2015; Lin et al., 2020; Zhang et al., 2021), while the immunoglobulin products tested in this study were from Central Europe, Spain and the USA. The predominance of different HCoV strains varies in different geographical areas over time.

It was also surprising that the antibody profile in the immunoglobulin products (highest levels in HCoV-229E and HCoV-OC43) did not match the prevalence of HCoVs in the longitudinal meta-analysis (HCoV-OC43 most prevalent, HCoV-229E least prevalent; Li et al., 2021). This may be because the geographic source of the plasma used to produce these products is reflective of these specific regions and not representative of worldwide prevalence. Another factor that could contribute to the apparent disparity may be that the published studies represent clinical samples from patients that sought medical attention, while the immunoglobulin products represent a population that included individuals who had milder infections and did not seek medical attention. In other words, the epidemiology reflects patients with more symptomatic infections, while the immunoglobulin products include asymptomatic individuals, as well as patients with mild infections and symptomatic infections.

In addition, these studies demonstrated that these antibodies had neutralizing activity against HCoV-229E in MRC5 cells. Neutralization activity is an important factor in the use of plasma-derived products employed in the treatment and/or prevention of viral diseases. The neutralizing capacity in this study was demonstrated with two different products with different manufacturing methods. This finding suggests that the ubiquity of anti-HCoV binding activity is accompanied by neutralization activity. HCoV-229E was employed to demonstrate neutralization activity of the antibodies detected by ELISAs. Direct extrapolation cannot be made to the other common HCoVs, but it is logical that the antibodies would also have neutralization activity against them.

It is also important to note that coronaviruses in the same subgroup, particularly betacoronaviruses such as HCoV-OC43, HCoV-HKU1, SARS-CoV, SARS-CoV-2 and MERS-CoV, show some interactivity in antigenicity. Cross-reactivity between SARS-CoV and MERS-CoV and other human betacoronaviruses has been reported (Che et al., 2005; Chan et al., 2013; Patrick et al., 2006). The fact that SARS-CoV-2 is closely related to SARS-CoV (>90% sequence homology) (Guo et al., 2020) suggests that antigenic interactivity between them is possible, at least for some proteins.

In addition, reactivity to SARS-CoV-2 in pre-pandemic immunoglobulin solutions has been observed recently (Díez et al., 2020a). As demonstrated in this study, these solutions have the capacity to neutralize common HCoVs such as HCoV-229E. Furthermore, these solutions have demonstrated some neutralizing capacity towards SARS-CoV-2 (Díez et al., 2020b). The worldwide presence of these common HCoVs may affect the current SARS-CoV-2 pandemic. Pre-existing immunity to common HCoVs may have a role in both humoral and cellular responses to SARS-CoV-2 (Díez et al., 2020b; Anderson et al., 2021; Meyerholz and Perlman 2021), and could explain, in part, the differences in severity of illness between patients.

This observed reactivity between pre-pandemic IVIG and SARS-CoV-2 occurs despite the low protein sequence homology between the SARS-CoV-2 S protein and common HCoVs (HCoV-OC43: 30% identity, 41% similarity; HCoV-HKU1: 29% identity, 40% similarity). However, despite low overall homology, higher homology was observed in the C-terminal regions of the S proteins. This region is instrumental in the insertion of the fusion protein into the cell membrane of the host cell (Hicks et al., 2021). The C-terminus homology could underly potential cross-reactivity of antibodies of the common HCoVs with SARS-CoV-2.

As shown in Table 1, the majority of the pooled plasma used in the manufacture of the products tested in this study was collected prior to the COVID-19 pandemic. Two products contained plasma collected in the early stages of the pandemic (until May 2020). A study examining the presence of anti-SARS-CoV-2 antibodies in immunoglobulin products demonstrated that these antibodies were not detected until late 2020 (products produced in September and October 2020) (Romero et al., 2021). This suggests that the observed activity against common HCoVs was not due to cross-reactivity with anti-SARS-CoV-2 antibodies.

In conclusion, this study demonstrated the presence of antibodies to common HCoVs in parenteral immunoglobulin products. The level of anti-HCoV activity for each virus was similar regardless of the geographic origin of the plasma. Neutralization activity was demonstrated against a representative strain of HCoV (HCoV-229E) in MRC5 cells. These findings may help to explain the previously evidenced cross-reactivity and neutralization activity for SARS-CoV-2 observed with pre-pandemic immunoglobulin products (Díez et al., 2020a,b), and differences in severity of illness between patients.

Acknowledgements

The authors wish to thank Michael K. James, PhD (Grifols) for medical writing and editorial support in the preparation of this manuscript. In addition, the authors wish to thank D. Casals, E. Sala and J. Luque for expert technical assistance.

Conflict of interest statement

The authors (JMD, CR and RG) are employees of Grifols, which manufactures the immunoglobulin products studied in this paper.

Funding

This study was funded by Grifols (Barcelona, Spain). The funding source played no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the paper for publication.

Ethical approval

These studies did not directly involve human subjects or animals; therefore, approval by an institutional review board or animal care committee was not required. These studies complied with all applicable regulations.

Author contributions

JMD, CR and RG contributed to the conceptualization, investigation, visualization and writing of this manuscript (original draft and subsequent review and editing). JMD, CR and RG have verified the underlying data described in this paper. All the authors confirm that they had full access to all the data and accept responsibility for its submission for publication.

Data sharing

All the relevant data that support the findings of this study are available within the article and its supplementary material. Complementary data are available from the corresponding author upon reasonable request (josemaria.diez@grifols.com).

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