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Interpretive summary

Human rotaviruses (HRoV) are a major cause of severe diarrheal disease in infants and young children. Whilst the vaccination of pregnant cows with HRoV boosts the release of HRoV-specific IgGs in bovine colostrum (BC), it raises regulatory and safety issues. In this study, we demonstrate that the conventional BRoV vaccine is sufficient to enhance the anti-HRoV protective efficacy of BC, thus providing a conservative approach to produce hyperimmune BC, making it exploitable as a functional food.

HYPERIMMUNE BOVINE COLOSTRUM: A NOVEL APPROACH

Colostrum from cows immunized with a veterinary vaccine against bovine rotavirus displays enhanced in vitro anti-human rotavirus activity

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ABSTRACT

Human rotaviruses represent a major cause of severe diarrheal disease in infants and young children. The limited impact of oral vaccines on global estimates of rotavirus mortality, and the suboptimal use of oral rehydration, justify the need for alternative prophylactic and therapeutic strategies, especially for immunocompromised hosts. The protective effects of colostrum - i.e. the first milk produced during the initial 24–48 hours post-parturition - is well documented in literature. In particular, the ingestion of hyperimmune bovine colostrum has been proposed as an alternative preventive approach against human rotavirus gastroenteritis. Whilst the immunization of pregnant cows with human rotavirus boosts the release of specific immunoglobulins G (IgGs) in bovine colostrum, it raises regulatory and safety issues. In this study, we demonstrate that the conventional bovine rotavirus vaccine is sufficient to enhance the anti-human rotavirus protective efficacy of bovine colostrum, thus providing a conservative approach to produce hyperimmune bovine colostrum, making it exploitable as a functional food.

Keywords: rotavirus, colostrum, cows, hyperimmune, immunoglobulins

INTRODUCTION

Viral gastroenteritis represents a relevant economic and public health burden, causing high morbidity and mortality rates, mainly in the poorest countries (Das, Salam, & Bhutta, 2014). Human rotaviruses (HRoVs) are a major cause of severe diarrheal disease in infants and young children, and the second cause of death in children less than 5 years old (Marcotte & Hammarström, 2016). Since no specific antiviral drug is available, the conventional treatment for HRoV acute gastroenteritis is largely symptomatic and involves fluid and electrolyte replacement and maintenance of nutrition. Despite the introduction of oral HRoV vaccines that significantly reduced the incidence of the disease in developed countries (Payne et al., 2013), the impact of this active prophylaxis on global estimates of HRoV mortality has been limited (Tate et al., 2016). The reason is mainly ascribable to the inadequate immunization coverage in lower income countries, where the burden of diarrheal disease is higher, and vaccines are mostly needed. As a matter of fact, oral vaccines are less immunogenic when given to infants in low-income compared with high-income countries, due to transplacental maternally-acquired antibodies, breastfeeding, histo blood group antigens, malnutrition, microbiota dysbiosis and environmental enteropathy. Moreover, the scarce availability of vaccines in these areas, along with their contraindications in immunodeficient patients (Babji & Kang, 2012; Glass et al., 2014; Binder et al., 2014; Gaspar et al.,2014) leave between a third to a half of children unprotected from severe HRoV disease (Babji & Kang, 2012). These hindrances, together with the suboptimal use of therapeutic oral rehydration solutions, justify the need for development of effective alternative prophylactic and therapeutic approaches to prevent and control HRoV gastroenteritis disease, especially for immunocompromised hosts.

Colostrum is the first milk produced by mammary glands during the initial 24–48 hours post-parturition (Tokuyama et al., 1990; Stelwagen et al., 2009), and represents a unique source of highly concentrated nutritional components (Macy et al., 1949), and growth factors (Pakkanen et al., 1997) for the gastrointestinal development of mammalian newborns.

More importantly, colostrum provides neonates with the essential passive immunity against infectious diseases (Ogra and Ogra, 1978; Cohen, 2006; Morris et al., 1980; Ebina et al., 1992; Majumdar and Ghose, 1982; Stephan et al., 1990; Tokuyama et al., 1990; Stelwagen et al., 2009). In particular, bovine colostrum (BC) has evolved into a highly effective host defense mechanism (Rainard & Riollet, 2006). In ruminants no transplacental exchange of immune factors occurs in utero, therefore colostrum and, to a lesser extent, mature milk provide protection through a high immunoglobulin (Ig) content, without which the newborn would not survive (Larson et al., 1980). The Igs present in BC are IgG1, IgG2, IgA, and IgM (Ogra and Ogra, 1978). The abundance of different Ig classes in colostrum and milk varies among species, with IgA being the predominant Ig in human mammary secretions. By contrast, in cow's colostrum IgG1 is the most represented (Barrington et al., 1997), while IgA and IgM are present at much reduced concentrations. The BC Igs, in conjunction with the ability of the ruminant neonatal gut to allow unrestricted passage of the large Ig molecules, provide the young animal with passive immunization (Bush & Staley, 1980; Moore et al., 2005).

Although the effect of colostrum is species-specific, a growing body of literature documented the protective effect of BC against several viral infections in humans (Benson et al., 2012; Ng et al., 2010; Inagaki et al., 2014; El-Fakharany et al., 2017; Bojsen et al., 2007).

In particular, vaccination of cows against specific human pathogens results in polyclonal pathogen-specific antibodies in BC. The antibodies purified from this hyperimmune BC (HBC) have successfully been exploited for the treatment of a variety of gastrointestinal infections caused by pathogenic bacteria (Hammarström and Weiner, 2008; Kelly, 2003; Playford et al., 2000; Struff and Sprotte, 2008) or virus (Korhonen et al., 2000; Mehra, 2006; Ng et al., 2010; Kramski et al., 2012a,b; Byakwaga et al., 2011; Inagaki et al., 2010; Ingaki et al., 2013) indicating HBC as an alternative source for low-cost virus-specific antibodies. These evidences, together with the high titer content of antimicrobial peptides and proteins, such as lactoferrin, lactoperoxidase and lysozyme, that can stimulate innate antiviral pathways and adaptive immune responses (Smolenski et al., 2007; Stelwagen et al., 2009; Tharpa, 2005) indicate BC as a functional food to provide protection against viral infections.

Consistently, ingestion of HBC has been proposed as an alternative prophylactic approach against HRoV gastroenteritis (Ebina et al., 1992; Sarker et al., 1998). HBC containing HRoV-specific, neutralizing IgGs has been produced so far by immunizing pregnant cows with HRoV and harvesting colostrum after delivery. However, the additional costs and regulatory and safety issues derived from the use of a HRoV vaccine, make impossible to generate large-scale amounts of HBC. The alternative use of BC from non-immunized cows (NHBC) may bypass these limits but, as expected, literature has clearly shown that NHBC is endowed with a significant lower ant-HRoV efficacy (Inagaki et al., 2010).

In this study, we present proof of concept data disclosing the protective effect against different HRoV genotypes of HBC from cows vaccinated with a conventional bovine rotavirus strain (BRoV). These results show that HBC generated by immunizing cows with the routinely-used bovine vaccine has a significantly higher anti-HRoV activity if compared to the one of NHBC, and contains crossreactive IgGs able to neutralize the infectivity of different HRoV strains, thus representing a functional food providing an alternative feasible and cost-effective strategy to manage HRoV infections.

MATERIALS AND METHODS

Chemicals

Laemmli buffer, molecular mass standards and electrophoresis apparatus for one-dimensional electrophoresis were supplied by Bio-Rad Laboratories, Inc., Hercules CA. β-mercaptoethanol, dithiothreitol (DTT), acetonitrile (ACN), sodium dodecyl sulphate (SDS), iodoacetamide (IAA), formic acid (FA), and all other chemicals used throughout the experimental work were current pure analytical grade products and purchased from Sigma-Aldrich Corporation, St Louis, MO. Water and acetonitrile (OPTIMA® LC/MS grade) for LC/MS analyses were purchased from Fisher Scientific, (Loughborough, UK).

Bovine colostrum collection

Fresh BC and HBC samples were supplied by the company Advances in Medicine (AIM, Bologna, IT). According to the supplier, colostrum was collected from both non-vaccinated and vaccinated pregnant dairy Holstein cows. Three cows were immunized by subcutaneous inoculation of the inactivated trivalent vaccine Trivacton 6 (MERIAL, Italia SpA) to maintain a maximum rate of antibodies in colostral secretions against *Escherichia coli*, Rotavirus and Coronavirus, which are implied in the establishment of neonatal diarrhea. Vaccination was performed with a two-injections schedule, administered two months and four weeks before parturition. Colostrum from the three vaccinated cows was collected until the fifth hour after birth, pooled and immediately frozen at -20°C. Concentrations of IgGs in whole colostra was 50 mg/ml (protein content: 12%), as assessed by previously described methods (Sacerdote et al., 2013).

After a suitable dilution with demineralized water, (1 volume), the suspension was introduced into a sterile beaker (controlled continuous stirring) and heated at ~38°C for about 1 hour. The suspension was then subjected to the skimming step, then caseins were removed by adjusting the pH to their isoelectric point (pH 4.6 with HCl 1M). After 1 hour, the product was centrifuged at 4000 rpm to definitively remove caseins. Low molecular weight components including salts and lactose were then removed by using hollow fiber cross flow filtration cartridges with 4000 NMWC (Nominal Molecular Weight Cutoff) coupled to a tangential flow filtration system equipped with a peristaltic pump essential to keep the flow recirculation continuous (Kross Flo®- Tangential flow Filtration System Research III). The pH of the retentate was then adjusted with NaOH 1M to pH 7.0 ± 0.2, and the neutralized sample centrifuged at 8500 rpm and the supernatant retained. The next steps consisted of clarification through 0:45 and 0:22 µm filters followed by lyophilisation.

Bovine IgG purification

Affinity Chromatography – IgG were purified by affinity chromatography, using protein G from Streptococci as stationary phase, immobilized in a preparative chromatographic column. In more detail, the affinity column was prepared by packing 400 mL of Protein G Sepharose 4 Fast Flow resin (GE Healthcare) in a column support HiScale ™ 50 (GE Healthcare) which was connected to an FPLC system (ÄKTAprime plus, GE Healthcare line-up). The chromatographic purification started by eluting the column with 5 volumes (5 x chromatographic bed volume) of buffer A (Binding Buffer: 20 mM sodium phosphate, pH 7), and then the sample was loaded at a flow rate of 20 ml min^{-1}. The eluate was monitored by a UV detector at 280nm, a conductivity meter (0.001-999.9 mS/cm), and a pH-meter; all the fractions characterized by a significant UV absorption were automatically collected (IgG depleted fractions). The subsequent step consisted of recovering the IgG fractions (IgG enriched fractions) by eluting the column with 100% of Elution Buffer (1 M glycine hydrochloride pH 2.5). The column regeneration was carried out by eluting 5 volumes (5 x chromatographic bed volume) of 20% ethanol.

Tangential Flow Filtration –IgG enriched fractions as well as IgG depleted fractions obtained as above reported were mixed and subjected to concentration and desalting using hollow fiber cross flow filtration cartridges with 3000 NMWC (Nominal Molecular Weight Cutoff) and a surface area of 650 cm^2 (GE Healthcare) coupled to a tangential flow filtration system equipped with a peristaltic pump essential to keep the flow recirculation continuous (Kross Flo®- Tangential flow Filtration System Research III). The IgG and IgG-depleted fractions were concentrated 20/30 times, dialyzed with 5 volumes of water, filtered through 0.22 micron membranes in sterile conditions, and finally lyophilized.

Separative methods of proteins on polyacrylamide gel

One-dimensional analysis (SDS-PAGE) - Protein separation was performed under reducing conditions; aliquots of 10µL of samples containing 20-25 µg of proteins were mixed with 10 µL of Laemmli sample buffer containing 50 mM DTT and heated at 95°C for 5 minutes. Samples and the standard proteins mixture (Precision Plus Protein Standards) were loaded on precast gels (Any KD ™ Mini Protean® TGX ™) and then placed in the electrophoresis cell (Mini-PROTEAN Tetra) and run at 200V (constant) for a variable time of about 30-40 min. Staining was carried out using Coomassie blue stain (Biosafe G250 Stain, Bio-Rad) and the images acquired by using the Bio-Rad GS800 densitometer and analyzed by using the software quantity One 1-D.

IgG and IgM analysis by SEC-UV

The IgG content of the IgG enriched and depleted fractions were then determined by a size exclusion chromatography (SEC) according to the method reported by Altomare and colleagues (Altomare et al., 2016a); SEC was performed on a Thermo Finnigan Surveyor HPLC system (ThermoFinnigan Italia, Milan, Italy) equipped with a variable wavelength detector and an auto-sampler, controlled by Xcalibur software (version 2.0.7 Thermo Fisher Scientific, Rodano, MI, Italy). The SEC separation was performed on a 4.6 × 300 mm Phenomenex Yarra™ 3u SEC-3000, with a 4 × 3 mm GFC4000 pre-column, by running an isocratic flow of mobile phase containing 0.1M sodium phosphate bibasic, 0.025% sodium azide pH 6.8, at a constant flow rate of 0.5 mL/min. The autosampler temperature was set at 8°C and UV detection was conducted at a wavelength of 280 nm, typical wavelength for protein detection. Due to its low molecular weight, the dipeptide Tyrosine-Histidine (TH) was chosen as internal standard (IS), since it marks the racing front, being the smallest analyte. All the samples to be analyzed were added by a fixed amount of the internal standard, the TH (0.3mM).

Cell lines and viruses

African green monkey kidney epithelial cells (MA104) and human epithelial adenocarcinoma HeLa cells (ATCC® CCL-2™) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco-BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany), at 37°C in an atmosphere of 5% of CO_2. African green monkey kidney cells (Vero) (ATCC CCL-81) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. HRoV strains Wa (ATCC® VR-2018), HRV 408 (ATCC® VR-2273), HRV 248 (ATCC® VR-2274), and BRoV strain NCDV (ATCC® VR-1290) were activated with 5µg/ml of porcine pancreatic trypsin type IX (Sigma) for 30 min at 37°C and propagated in MA104 cells using DMEM containing 0.5µg of trypsin per ml as described previously (Civra et al., 2015). Viral titers are expressed as focus-forming unit (FFU) per ml. A serologic characterization of RoVs exploited in this study is provided in Table 1. Human rhinovirus (HRhV) 1A (ATCC® VR-1559), was propagated in HeLa cells, at

34°C, in a humidified 5% CO_2 incubator. Clinical isolates of human herpes virus type 1 (HSV1) and type 2 (HSV2) were kindly provided by Prof. M. Pistello, University of Pisa, Italy. Human cytomegalovirus (HCMV) strain Towne was kindly provided by Prof. W. Brune, Heinrich Pette Institut, Hamburg, Germany. Vesicular stomatitis virus (VSV; ATCC® VR-1238), HCMV and HSV1 and HSV2 were propagated in Vero cells, at 37°C in a humidified 5% CO_2 incubator. When the full cytopathic effect (CPE) developed, cells and supernatants were harvested, pooled, frozen and thawed three times, clarified and aliquoted. Viruses were stored at −70°C. Viral titers were determined by the standard plaque method as described previously (Civra et al., 2014; Cagno et al., 2017), and expressed as plaque-forming units (PFU) per ml.

Focus reduction assays

Antiviral activity of NHBC, HBC, or IgGs against RoV Wa, HRV408, HRV248, and NCDV was determined by focus reduction assay or plaque reduction assays. Assays of inhibition of rotavirus infectivity were carried out with confluent MA104 cell monolayers plated in 96-well trays, as described elsewhere (Civra et al., 2014). Cells were treated for 2 hours at 37°C with serial dilutions of colostrum, at protein concentrations ranging from 0.02 to 3340µg protein/ml in serum-free medium prior to virus addition. HRoV infection was performed at a multiplicity of infection (MOI) of 0.02 FFU/ml for 1 hour at 37°C, in presence of the colostra. Infected cells were washed with serum-free medium, fresh methanol extract was added, and cells were incubated in this medium at 37°C in a humidified incubator in 5% (vol/vol) CO_2–95% (vol/vol) air. After 16 hours of incubation, infected cells were fixed with cold acetone-methanol (50:50), and viral titers determined by indirect immunostaining by using a mouse monoclonal antibody directed to human rotavirus VP6 (0036; Villeurbanne, France), and the secondary antibody peroxidase-conjugated AffiniPure $F(ab')_2$ Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., 872 W. Baltimore Pike, West Grove, PA 19390).

Plaque reduction assay

HeLa or Vero cells were first seeded (at 8×10^4 cells/well) in 24 well plates. 24 hours later the HBC, NHBC, or IgGs were serially diluted in medium (from 0.02 to 3340µg protein/ml) and added to cell monolayers. After 2 hours of incubation (37°C, 5% CO_2), medium was removed and infection was performed with 200 µL/well with HRhV 1A, VSV, HSV-1, HSV-2, or HCMV at a MOI of 0.0002 PFU/ml in presence of colostra. The infected cells were incubated at 34°C for HRhV infections or

37°C for the other viruses for 1 hour, then washed with medium, and overlaid with a 1:1 combination of 1.6% SeaPlaque Agarose and 2X DMEM containing the colostra. Control wells (100% of infection) were prepared by treating cells with equal volumes of culture medium. The plates were incubated at 34°C or 37°C for 3 days. After incubation, the plates were fixed with 7.5% formaldehyde (Fluka) and stained with crystal violet (Sigma, St. Louis, Mo.). The number of plaques formed was counted.

Rotavirus (RoV) neutralization assay

IgG precipitated with ammonium sulphate at respective 90% effective concentration (EC_{90}) or equal volume of culture medium or ammonium sulphate supernatant were added to $2x10^5$ FFU/ml of trypsin-activated RoV suspension and mixed in a total volume of 200 µl. The virus-compound mixtures were incubated for 1 hour at 37 °C then serially diluted to the non-inhibitory concentration of test IgG, and the residual viral infectivity was determined as previously described (Civra et al., 2014).

RoV-cell binding assay

RoV-cell binding assays were performed as described previously (Civra et al., 2015). Trypsin-activated RoVs Wa, HRV248, HRV408, and NCDV were treated as described for neutralization assays. After 1 hour, cells were washed with fresh medium and cooled on ice. RoVs were then cooled to 4°C, and allowed to attach to cells for 1 h (MOI=3 FFU/cell) at 4°C. After a wash with cold DMEM, cells were subjected to two rounds of freeze-thawing and then incubated at 37°C for 30 minutes with 10 µg/ml porcine trypsin to release bound virus. The lysates were then clarified by low speed centrifugation for 10 min, and cell-bound virus titers were determined by indirect immunostaining as above.

Cell viability assay

Cells were seeded at a density of 5 x 10^3/well in 96-well plates and treated the next day with HBC, NHBC, IgGs or ammonium sulfate supernatant at concentrations ranging from 0.02 to 7140µg/ml to generate dose-response curves. Control samples (100% of viability) were prepared by treating cells with culture medium. After 24 or 72 hours of incubation, cell viability was determined using a CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) and following the manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) at 490nm. Viability of treated cells is expressed as a percentage relative to cells incubated with culture medium.

Statistical assessment

Blockades of viral infectivity are expressed as mean % ± standard deviation (SD). Where possible, anti-viral effective concentration (EC_{50}) values were calculated by regression analysis using the dose-response curves generated from the experimental data, using PRISM 4 (GraphPad Software, San Diego, CA, U.S.A.). The 50% cytotoxic concentration (CC_{50}) was determined using logarithmic viability curves. Where possible, a selectivity index (SI) was calculated dividing the CC_{50} by the EC_{50} value. One-way ANOVA, followed by Bonferroni test, was used to assess the statistical significance of the differences between treated and untreated samples. EC_{50} values were compared using the sum-of-squares F test. Significance was set at the 95% level.

RESULTS

1D gel electrophoresis

Figure 1 displays the SDS-PAGE profile of colostrum proteins (reducing conditions) relative to the following samples: colostrum from non-immunized (lane 2) and immunized (lane 3) cows and the corresponding defatted / casein-depleted / dialyzed / filtered samples (lanes 4 and 5). As expected, all the protein profiles show two intense bands at ca. 50 and 25 kDa, representing the heavy and light IgG chains, respectively. The other bands can be ascribable to the classical set of high-abundance species normally found in just about any type of milk of animal origin (although in colostrum IgGs alone represent ca. 80% of the total protein mass), including: α-lactalbumin (14.1 kDa), ß-lactoglobulin (19.9 kDa), serum transferrin (77.7 kDa) and α2-macroglobulin (167.5 kDa). As expected, the protein patterns relative to colostrum samples differ in respect to the corresponding defatted / casein-depleted / dialyzed / filtered ones, by a significant reduction of the bands attributable to the caseins (lane 2 /3, figure 1).

Bovine IgG purification by affinity chromatography

Purification of IgG from colostrum collected from non-immunized and immunized cows was achieved by using a preparative affinity chromatography system based on protein G from Streptococci which is highly selective against the bovine IgG.

The affinity chromatogram trace, recorded at 280nm, displayed two peaks: the first, not retained, corresponding to the non-immunoglobulin protein fraction, and the second to the IgG fraction (Figure 2 – panel a) whose peak accounts for the 80%. The electrophoretic patterns obtained for the two collected fractions (Figure 2 – panel b), indicate a good depletion of immunoglobulins, whose characteristic bands at 160-50-25 kDa, are very weak in the aliquot eluted within the first peak, and clearly much more intense in the fraction of the second peak corresponding to the immunoglobulins.

SEC-UV for IgG analysis

A SEC-UV method was then optimized to achieve a reproducible separation of the most abundant analytes in bovine colostrum; the method was then applied to verify the purity of the fractions obtained: the non-immunoglobulin protein fraction and the IgG eluted fraction.

Figure 3 represents typical chromatograms recorded by setting the UV detector at 280nm and relative to the IgG-depleted (panel a) and IgG-enriched (panel b) fractions, each spiked with the internal standard TH (0.3mM). The typical retention times for IgG and the dipeptide TH are 9.15min and 11.57min, respectively.

The peak relative to IgG is well evident only in the IgG enriched fraction and absent in the depleted IgG fraction which is characterized by other peaks such as those relative to IgM and beta-lactoglobulin

Antiviral activity assessment

After their biochemical characterization, colostra and IgG samples were tested for antiviral activity. The results summarized in table 2 clearly show that nor NHBC neither HBC are endowed with antiviral activity against human pathogens such as HSV-1, HSV-2, HCMV, and HRhV. Not surprisingly, NHBC is effective against bovine pathogens such as BRoV NCDV (EC_{50}=61.5μg protein/ml) and VSV (EC_{50}=64μg protein/ml) (table 2). Of note, NHBC shows non-strain restricted antiviral efficacy against the human RoV strains Wa (EC_{50}=2.3μg protein/ml), HRV248 (EC_{50}=4.2μg protein/ml), and HRV408 (EC_{50}=12.6μg protein/ml) with percentages of inhibition to maxima of 100% (figure 4). To test the hypothesis that colostrum from cows immunized with a veterinary anti

BRoV vaccine may exert a higher anti-HRoV activity than the one from non immunized cows, a second set of antiviral assays was performed. As expected, HBC exerts a significantly (p_{Ftest}<0.0001) higher antiviral activity against BRoV NCDV (EC_{50}=5.5µg protein/ml), if compared with the one of non-immunized cows (table 2). This result indicates that vaccination elicited an anti-BRoV immune response. Notably, HBC is significantly (0.0001<p_{Ftest}<0.0005) more effective than NHBC also against Wa (EC_{50}=0.3µg protein/ml), HRV248 (EC_{50}=1.6µg protein/ml), and HRV408 (EC_{50}=2.1µg protein/ml) human strains (table 2), suggesting a high titer of cross-reactive IgG in HBC. Therefore, a third set of experiments was performed to test the presence of anti-HRoV IgG in HBC-derived IgGs. The results shown in table 3 and figure 5 demonstrate that these IgGs inhibit the infectivity of BRoV strain NCDV (EC_{50}=6.2µg protein/ml), but more interestingly are endowed with a strong antiviral activity also against HRoV strains Wa (EC_{50}=1.9µg protein/ml), HRV248 (EC_{50}=0.7µg protein/ml), and HRV408 (EC_{50}=1.8µg protein/ml) with percentages of inhibition to maxima of 100%. As a negative control to IgG antiviral assays, we treated cells with equal volumes of IgG-depleted ammonium sulfate supernatant; results shown in table 3 and figure 5 clearly show that the IgG-depleted ammonium sulfate supernatant is not endowed with a significant antiviral activity. Notably, treatment of the different cell lines with NHBC, HBC and IgG do not affect cell viability even at high concentrations, showing that the antiviral activity we observed is not ascribable to non-specific cytotoxic effects.

Mechanism of action experiments show that these IgGs neutralize virus infectivity by significantly (0.0103<p_{ANOVA}<0.0155) inhibit RoV-cell binding (figure 6, panels A, B, and C). More importantly, the significant (0.0005<p_{ANOVA}<0.0183) neutralization of viral infectivity observed in virus inactivation assays (figure 6, panel D, E, and F) is consistent with a neutralizing activity of virus-specific antibodies targeting the RoV surface antigens, rather than cellular receptors.

DISCUSSION

The supportive properties of BC when consumed by other mammalian species, including pigs and humans, are well documented in the medical literature (Boudry et al., 2007; Bridger & Brown, 1981; Gopal & Gill, 2000; He et al., 2001; Pakkanen & Aalto, 1997; Solomons, 2002; Struff and Sprotte, 2007; Uruakpa et al., 2002). Emerging evidences indicate BC as a promising nutraceutical which can prevent or mitigate various diseases in newborns and adults (Bagwe et al., 2015), in particular gastrointestinal infections. Consistently with these findings, in this study we confirmed the protective activity of NHBC against HRoV, which is well documented in literature (Inagaki et al., 2010; Inagaki et al., 2013). Of note, NHBC shows antiviral activity also against several HRoV strains and, as expected, against BRoV strain NCDV, at concentrations comparable to the ones previously showed (Inagaki et al., 2013).

In our experimental setting NHBC showed no significant antiviral activity against three different viral pathogens, namely HRhV, HCMV, HSV-1, and HSV-2. These results show that BC is not a "broad spectrum antiviral", but it rather exerts specific antiviral activities. It is likely that this antiviral specificity and potency most probably reflects the immunological status of the animal.

Boosting the natural concentrations of immune components in colostrum and milk through vaccination of cows offers great potential for their use as prophylactic or therapeutic products in humans. HBC from cows vaccinated with HRoV showed to be an effective therapeutic in reducing the duration and severity of RoV-caused diarrhea in a double-blind controlled clinical study with infants of 6 to 24 months of age (Mitra et al., 1995). In a second study, Davidson and colleagues produced a HBC by introducing a vaccine containing four HRoV into pregnant Freisian cows. They demonstrated that this HBC administered orally mediated protection by preventing infection of HRoV infection (Davidson et al., 1989). Efficacy of passive immunization with HBC-derived IgG is documented in literature. Hilpert and colleagues (Hilpert et al., 1987) treated infants hospitalized with acute diarrhea with anti-rotavirus immunoglobulin concentrate without a significant decrease in duration of diarrhea or excretion of virus, while Turner and colleagues demonstrated a reduction in incidence and duration of diarrhea in treated infants (Turner and Kelsey, 1987). In 1998, Sarker and colleagues produced a HBC by immunizing pregnant cows with HRoV strains, i.e. Wa, RV3, RV5 and ST3, representing serotypes 1 to 4, respectively. HBC-purified IgGs were then administered in a double blind placebo-controlled trial to children with diagnosed HRoV diarrhea (Sarker et al., 1998). Children who received HBC-IgGs had significantly less daily and total stool output and stool frequency and required a smaller amount of oral rehydration solution than did children who received placebo. However, it is difficult to generate large-scale amounts of HBC by immunizing cows with a non-scheduled and thus non-conventional HRoV vaccine; indeed, the yield is not enough to successfully cover the global requirement since over 500,000 deaths occur across the world every year due to rotavirus-induced diarrhoea (Bagwe et al., 2015). To overcome these limitations we put forward and tested the hypothesis that HBC from cows immunized with a conventional veterinary vaccine against BRoV would exert a higher anti HRoV activity compared to NHBC due to a high titer of cross-reactive anti HRoV IgG. Indeed this study confirms this hypothesis and propose an easier and cheaper approach for the production anti-HRoV HBC or IgG in cows. Our results show an in vitro HRoV inhibition efficacy (i.e. EC50) of HBC comparable with the one that Inagaki and colleagues obtained by treating MA104 cells with skimmed and concentrated bovine late colostrum (SCBLC) from HRoV-immunized cows (Inagaki et al., 2010; Inagaki et al., 2013). Notably, we demonstrated that HBC and its IgGs can inhibit the infectivity of four RoVs having four different GP genotypes (Table 1), thus suggesting that vaccination with BRoV stimulates the production of cross-reactive neutralizing antibodies.

With a view to exploit HBC as source of anti-HRoV IgG, quality assessment procedures would be necessary in order to monitor the IgG content; nevertheless, these techniques are already available and well documented in literature (Altomare et al., 2016b). Moreover, cows produce BC in large excess respective to the amount needed to feed their calves (cows produce about 33 liters of colostrum each day in the first days after parturition, while just 4-6 liters per day are administered to the calf during the first two days) (Devery-Pocius and Larson, 1983).

Overall, this study demonstrate that the conventional BRoV vaccine is sufficient to boost the anti-HRoV protective efficacy of BC. This is by itself a conservative, feasible, and not yield-limiting approach to produce HBC exploitable as a functional food to prevent and treat HRoV infections.

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Table 1. Rotavirus strains used in this study.

Strain	Origin	Serotype
Wa	Human	G1(P8)
HRV 408	Human	Natural reassortant G3(P?)
HRV 248	Human	Natural reassortant G4(P4)
NCDV	Bovine	G6(P1)

(P?) means P genotype is unknown

The characteristics of the rotavirus strains are from Parrón et al., 2017 and Rahman et al., 2012.

Table 2. Antiviral activities of BCs.

	Virus	EC50 (µg/ml) (95% C.I.)	EC90 (µg/ml) (95% C.I.)	CC50 (µg/ml)	SI
Bovine not immune colostrum					
	HSV-1	n.a.	n.a.	> 6680	-
	HSV-2	n.a	n.a	> 6680	-
	HCMV	n.a.	n.a.	> 6680	-
	HRhV	n.a.	n.a.	> 6680	-
	HRoV (#Wa)	2.3 (1.6-3.5)	16.3 (6.8-39.1)	> 6680	>2855
	HRoV (#HRV 408)	12.6 (8.6-18.7)	90 (38-215)	> 6680	>529
	HRoV (#HRV 248)	4.2 (2.4-7.3)	134 (39-454)	> 6680	>1591
	BRoV (#NCDV)	61 (44-86)	143 (97-211)	> 6680	>109
	VSV	64 (55-75)	343 (252-467)	> 6680	> 104
Bovine hypermmune colostrum					
	HSV-1	n.a	n.a.	> 7140	-
	HSV-2	6018 (2560-14110)	n.a.	> 7140	> 1.18
	HCMV	n.a.	n.a.	> 7140	-
	HRhV	n.a.	n.a.	> 7140	-
	HRoV (#Wa)	0.3 (0.3-0.5)	4.5 (2.4-8.5)	> 7140	>21000
	HRoV (#HRV 408)	2.1 (1.7-2.6)	7.6 (4.7-12.5)	> 7140	>3449
	HRoV (#HRV 248)	1.6 (1.2-2.8)	8.1 (5.6-11.6)	> 7140	>4519
	BRoV (#NCDV)	5.5 (3.7-8.2)	51 (21-122)	> 7140	>1301
	VSV	89 (60-132)	489 (200-1198)	> 7140	>80

Herpes simplex type 1 (HSV-1); herpes simplex type 2 (HSV-2); human cytomegalovirus (HCMV); human rhinovirus (HRhV); human rotavirus (HRoV); bovine rotavirus (BRoV); vesicular stomatitis virus (VSV). EC_{50} half-maximal effective concentration; CI confidence interval; EC_{90} 90% effective concentration; CC_{50} half maximal cytotoxic concentration; SI selectivity index; n.a. not assessable

Table 3. Antiviral activities of IgGs from HBC.

	Virus	EC50 (µg/ml) (95% C.I.)	EC90 (µg/ml) (95% C.I.)	CC50 (µg/ml)	SI
IgG	HRoV (#Wa)	1.9 (1.4-2.6)	19.7 (10.4-37.1)	> 5910	> 3110
	HRoV (#HRV 408)	1.8 (0.8-3.9)	12.6 (2.5-63.2)	> 5910	> 3283
	HRoV (#HRV 248)	0.7 (0.6-0.9)	8.2 (4.9-13.9)	> 5910	> 8443
	BRoV (#NCDV)	6.2 (5.2-7.4)	30.2 (19.7-49.0)	> 5910	> 953
AS supernatant	HRoV (#Wa)	n.a.	n.a.	> 5910	n.a.
	HRoV (#HRV 408)	n.a.	n.a.	> 5910	n.a.
	HRoV (#HRV 248)	n.a.	n.a.	> 5910	n.a.
	BRoV (#NCDV)	n.a.	n.a.	> 5910	n.a.

Herpes simplex type 1 (HSV-1); herpes simplex type 2 (HSV-2); human cytomegalovirus (HCMV); human rhinovirus (HRhV); human rotavirus (HRoV); bovine rotavirus (BRoV); vesicular stomatitis virus (VSV). EC_{50} half-maximal effective concentration; CI confidence interval; EC_{90} 90% effective concentration; CC_{50} half maximal cytotoxic concentration; SI selectivity index; n.a. not assessable

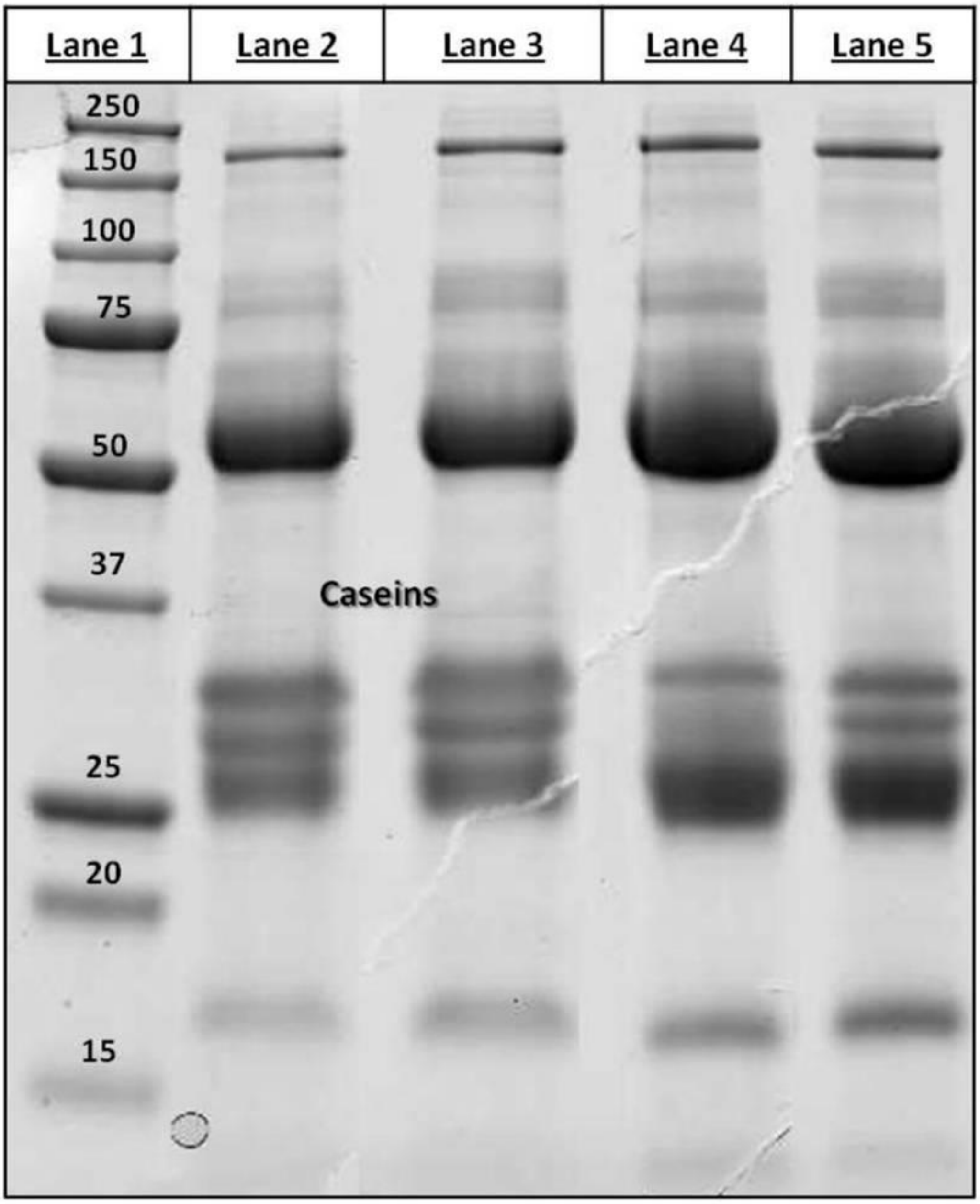

Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
250
150
100
75
50
37
Caseins
25
20
15

Figure 1.

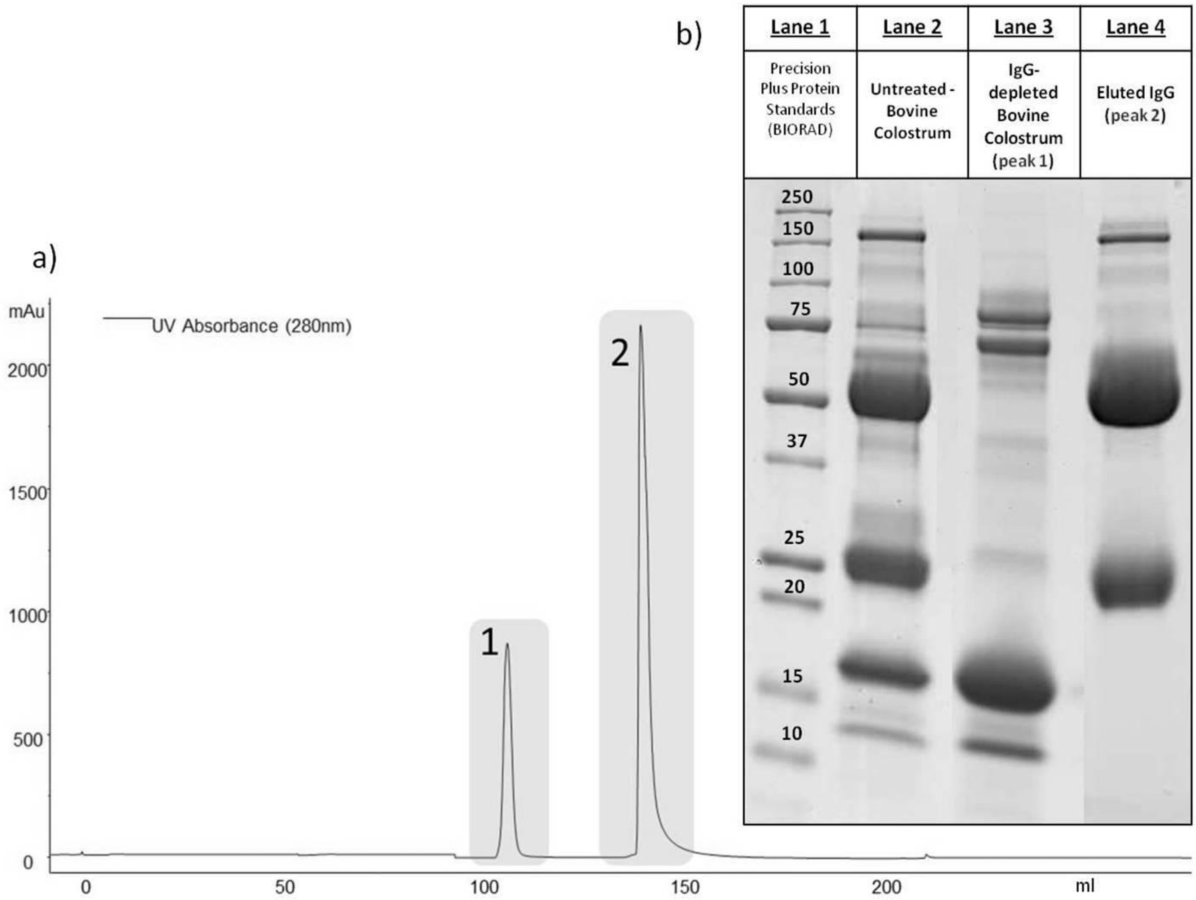
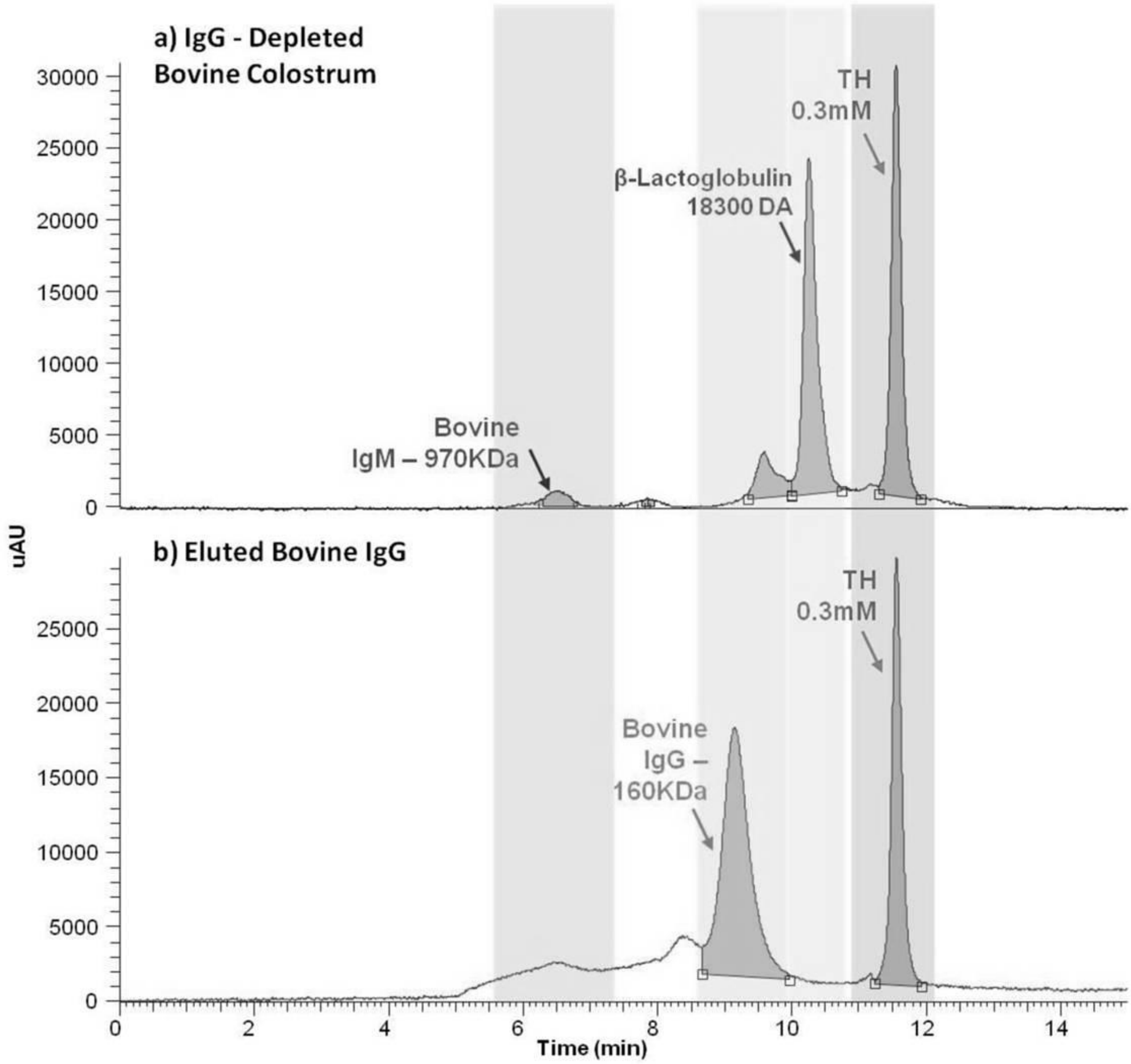

a)
mAu
UV Absorbance (280nm)
2000
1500
1000
500
0
1
2
0
50
100
150
200
ml
b)
Lane 1
Lane 2
Lane 3
Lane 4
Precision Plus Protein Standards (BIORAD)
Untreated - Bovine Colostrum
IgG-depleted Bovine Colostrum (peak 1)
Eluted IgG (peak 2)
250
150
100
75
50
37
25
20
15
10

Figure 2.



624

625 **Figure 3.**

626

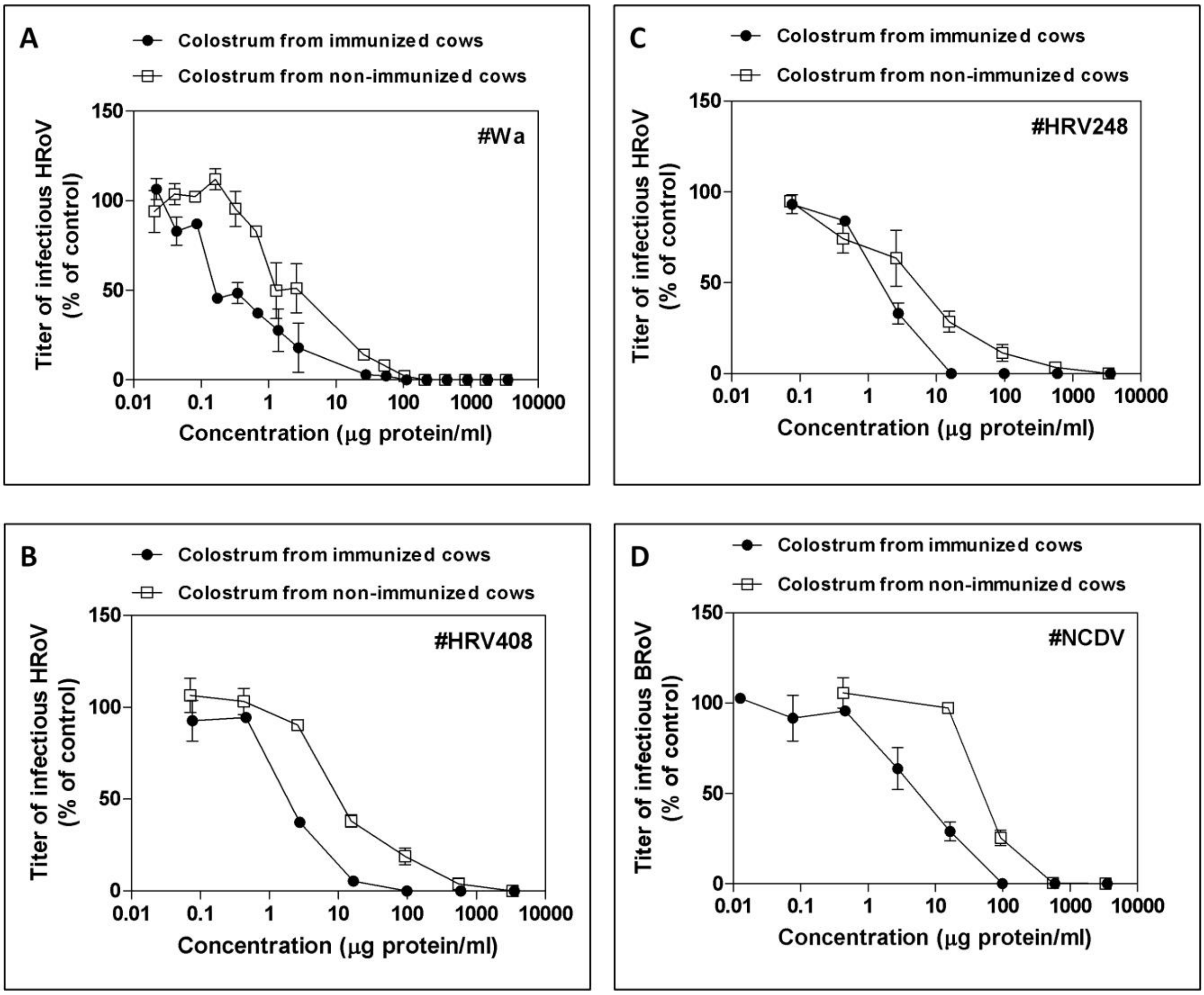
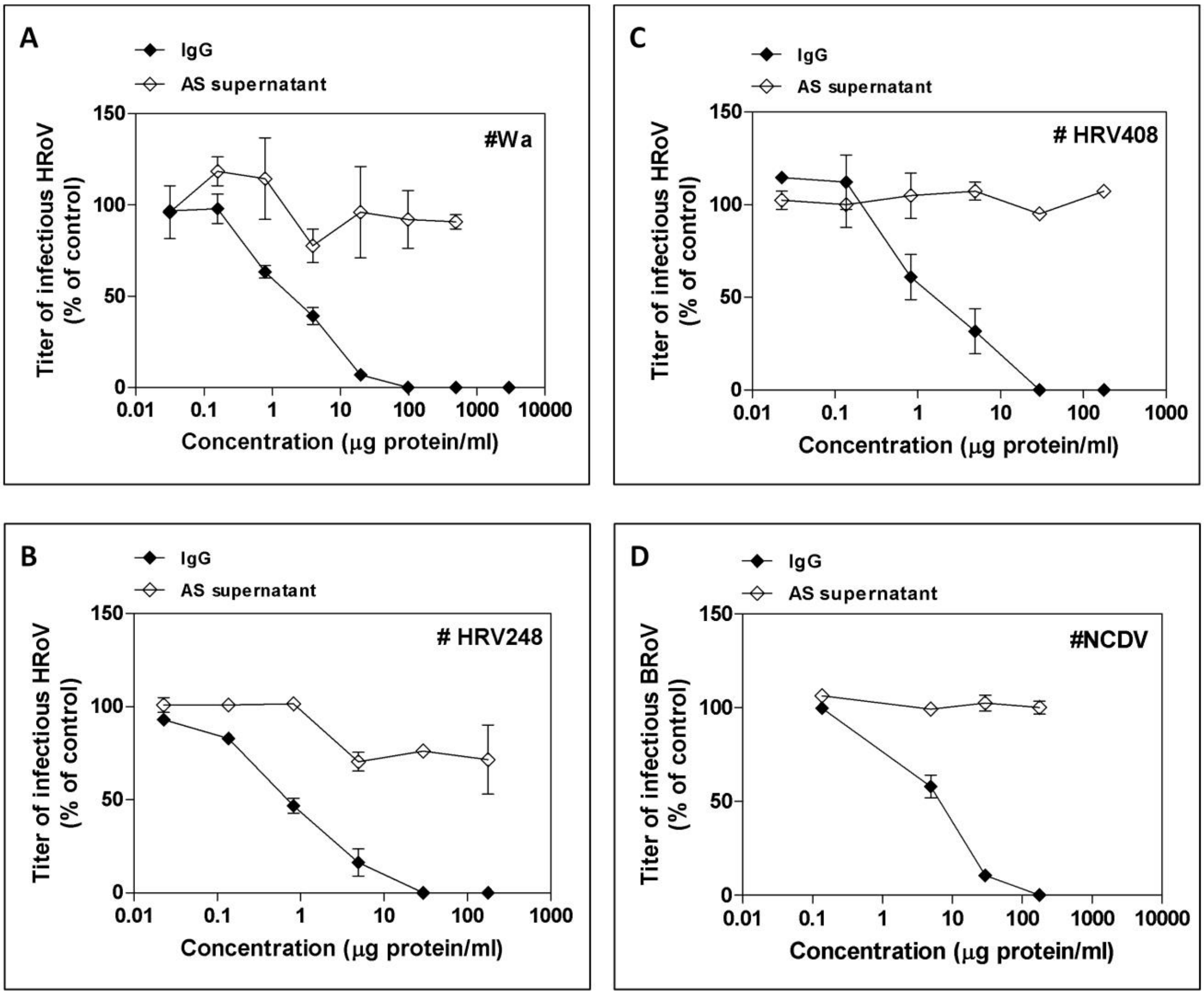

A
Colostrum from immunized cows
Colostrum from non-immunized cows
#Wa
Titer of infectious HRoV (% of control)
150
100
50
0
0.01
0.1
1
10
100
1000
10000
Concentration (μg protein/ml)
C
Colostrum from immunized cows
Colostrum from non-immunized cows
#HRV248
Titer of infectious HRoV (% of control)
Concentration (μg protein/ml)
B
Colostrum from immunized cows
Colostrum from non-immunized cows
#HRV408
Titer of infectious HRoV (% of control)
Concentration (μg protein/ml)
D
Colostrum from immunized cows
Colostrum from non-immunized cows
#NCDV
Titer of infectious BRoV (% of control)
Concentration (μg protein/ml)

Figure 4.



A
IgG
AS supernatant
#Wa
Titer of infectious HRoV (% of control)
Concentration (μg protein/ml)
C
IgG
AS supernatant
HRV408
Titer of infectious HRoV (% of control)
Concentration (μg protein/ml)
B
IgG
AS supernatant
HRV248
Titer of infectious HRoV (% of control)
Concentration (μg protein/ml)
D
IgG
AS supernatant
#NCDV
Titer of infectious BRoV (% of control)
Concentration (μg protein/ml)

Figure 5.

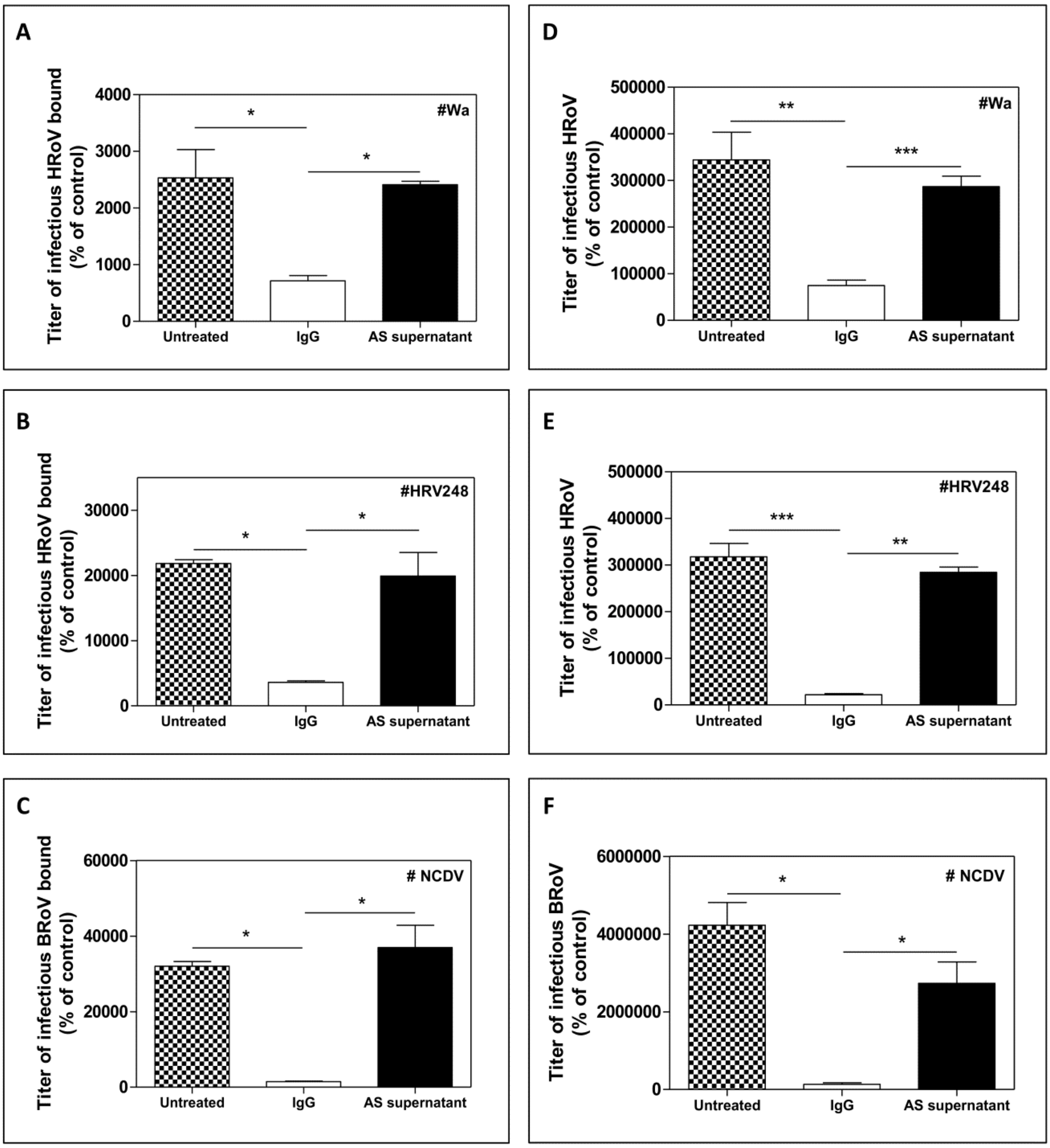

A
#Wa
Titer of infectious HRoV bound
(% of control)
4000
3000
2000
1000
0
Untreated
IgG
AS supernatant
*
*
B
#HRV248
Titer of infectious HRoV bound
(% of control)
30000
20000
10000
0
Untreated
IgG
AS supernatant
*
*
C
NCDV
Titer of infectious BRoV bound
(% of control)
60000
40000
20000
0
Untreated
IgG
AS supernatant
*
*
D
#Wa
Titer of infectious HRoV
(% of control)
500000
400000
300000
200000
100000
0
Untreated
IgG
AS supernatant
**
***
E
#HRV248
Titer of infectious HRoV
(% of control)
500000
400000
300000
200000
100000
0
Untreated
IgG
AS supernatant
***
**
F
NCDV
Titer of infectious BRoV
(% of control)
6000000
4000000
2000000
0
Untreated
IgG
AS supernatant
*
*

Figure 6.

Legends

Figure 1. SDS-PAGE profiling of colostrum proteins. Lane 1. Precision Plus Protein Standards (BIORAD); lane 2. Untreated - Bovine Colostrum; lane 3. Untreated Hyperimmune Bovine Colostrum; lane 4. Bovine Colostrum Defatted/Casein-depleted/Dialyzed/Filtered; lane 5. Hyperimmune Bovine Colostrum Defatted/Casein-depleted/Dialyzed/Filtered.

Figure 2. IgG purification by affinity chromatography. Panel a) FPLC-UV chromatogram of the untreated-Bovine Colostrum (casein-depleted and defatted bovine colostrum) obtained by using protein G as affinity stationary phase; peaks 1 and 2 are attributed to the IgG-depleted and IgG fractions as demonstrated by the SDS-gel electrophoresis patterns. Panels b) reports the gel electrophoretic pattern of peak 1 (lane 3) and 2 (lane 4) compared to the untreated bovine colostrum pattern, run in reducing conditions. The characteristic bands at 25 and 50 Da are well evident in peak 2 and only in negligible amounts in peak 1.

Figure 3. IgG analysis by SEC-UV; panels a) shows the SEC-UV chromatogram of IgG - Depleted Bovine Colostrum sample, using TH as internal standard; panel b) shows the SEC-UV chromatogram of IgG purified fraction from bovine colostrum.

Figure 4. Antiviral activity of colostrum from immunized cows (HBC, solid circles) and colostrum from non-immunized cows (NHBC, open squares) against HRoV strains Wa (A), HRV408 (B), HRV248 (C), and NCDV (D) on MA104 cells. Cells were treated for 2h with increasing concentrations of colostra and then infected in presence of colostrum. Viral infections were detected as described in the Material and Methods section. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SEM for duplicates.

Figure 5. Antiviral activity of hyperimmune colostrum (HBC)-derived immunoglobulin G (IgG, solid diamonds) or ammonium sulfate (AS, open diamonds) supernatant against HRoV strains Wa (A), HRV408 (B), HRV248 (C), and NCDV (D) on MA104 cells. Viral infections were detected as described in the Material and Methods section. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SEM for duplicates.

Figure 6. Mechanism of action of HBC-derived IgGs. Panels A, B, and C show the effect of HBC-derived IgGs respectively on Wa, HRV248, and NCDV binding to the MA104 cell surface. Panels D, E, and F show the results of virus inactivation assays on infectious particles respectively of Wa, HRV248, and NCDV. On the *y* axis, the Wa infectious titers are expressed as focus-forming units per

ml (FFU/ml). On the *y* axis, the infectious titer of Wa bound to cells is expressed as a % of the titer bound to control untreated MA104 cells. Error bars represent the SEM of independent experiments. *$p_{ANOVA}<0.05$ **$p_{ANOVA}<0.001$ ***$p_{ANOVA}<0.0001$