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Comparative analysis of IL-2, IL-6, and IFN-γ responses in relation to human and animal Rotavirus VP7 Gene

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Abstract

Rotavirus infection is considered to be one of the zoonotic diseases, transmitted between humans and animals. The present study aimed to investigate the association between cytokine (IL-2, IL-6 and INF-γ) levels with Rotavirus VP7 gene distribution in humans and cattle by using Polymerase Chain Reaction (PCR) and Enzyme Linked Immunosorbent Assay (ELISA). Faecal samples (54) and 54 blood samples were collected from each diarrheic human and cattle. The PCR-VP7 results revealed that VP7-PCR positivity was observed in 12 human stool(22.2%) and 14 cattle faecal samples (25.9%). Non-significant effect (P>0.05) for human sex on Rota virusVP7 distribution, and (23.3%) of Rota virusVP7 detection was observed in males compared to females (20.8%). Significant effect (P<0.05) for cattle sex on Rota virus VP7 distribution, and a higher percentage (47.1%) of Rota virus VP7 infection in cattle was observed in females compared to males (16.2%). Non-significant effect (P>0.05) for human and cattle age was observed in this study. A higher percentage of Rota virus VP7 infection was observed in humans and cattle (23.5 and 34.6 % respectively) in the age group less than 5 years. ELISA-based IL-6, IL-2 and INF-γ results revealed that Positive cases with VP7 did not differ significantly by species, age, or sex. among the measured cytokines, only IL-6 in cattle showed a significant elevation in VP7-positive cases compared with VP7-negative controls (356.87±156.13 vs. 40.84±40.84 pg/mL, p = 0.020).

Keywords: Rotavirus A; Comparative Analysis; cattle; humans; VP7 gene, conventional RT- PCR, cytokines

Introduction

Rotaviruses were recognised as a significant etiological agent for acute gastroenteritis among neonates and young children under five years of age. Interspecies and intraspecies transmission have been reported for rotaviruses [1]. Both structural and non-structural proteins help mediate the intestinal dendritic cells during the infection course. Rotavirus-specific T cells could be stimulated by the activated intestinal dendritic cells to secrete the inflammatory cytokines [2]. The glycoprotein VP7 constitutes the viral outer layer, which elicits neutralising antibodies, enables viral entry through attachment [3], and is used to determine rotavirus groupings based on serotyping [4]. Rotaviral diarrheal diseases in calves and other domestic animals are referred to as white scours or milk scours. In this context, RVAs are referred to as important animal pathogens. An economic burden is normally associated with rotaviral infections in young cattle along with weight loss, high mortality rate, and unaffordable treatment expenditure [5].

A subset of antitransglutaminase IgA antibodies was recognised in active celiac disease (CD), where they recognise the viral protein VP7, improve the intestinal permeability, and elicit monocyte activation [6]. The antirotavirus VP7 antibodies may arise before the CD onset, whereas antitissue transglutaminase (tTG) and antiendomysium antibodies show a predictive role. The modulatory genes involved in biological processes in the human T84 intestinal cell line were reported to be induced by anti-tissue transglutaminase (tTG) and antiendomysium antibodies, referred to as a signature of CD [7]. These data suggested the cross-talk between rotavirus infection and CD occurrence.

Several studies addressed the essential role of cytokines, such as interleukin-2, interleukin-6 (IL-2, IL-6) and interferon-gamma (IFN-γ), in the pathogenesis of both inflammatory and immune disorders. The multifunctional cytokine IL-2, secreted by T-cells, plays a set of roles like T-cell activation, proliferation, and contraction [8]. IFN-γ, normally secreted by the immune cells, mostly natural killers and cells involved in both innate and acquired immunity, exerts antiviral capacities, contributes to T-cell activity, activates macrophages, and associates with T-helper responses [9:10].

Our study aim to Assess the cell mediated immune response associated with rotavirus infection and possible involvement of rotavirus infection in the development of autoimmune disease in cow and humans through ,Rotavirus detection by reverse transcriptase (RT-PCR) for extraction of high quality viral dsRNA from amplification of specific fragments from complex nucleic acid samples using PCR for stool and fecal samples and measurement of IL-6, IL-2 and IFN-γ concentration in the sera of samples by ELISA.

Material and Methods

Cohorts study and their demographic characteristics

This study ran from January to September 2024. This study was conducted in Al Basra province. Fifty-four human and bovine samples (for each faeces and blood) were used in the investigation. There were thirty men and twenty-four females in the human group and thirty-seven males and seventeen females in the bovine group.. During the three weeks before to collection, diarrhoea was experienced by every study participant. Frequent passing of unusually loose or watery faeces, happening three or more times in 24 hours and lasting no more than 14 days, was defined as acute diarrhoea. [11].

Detection of IL-2, IL-6, IFN-γ concentration by ELISA

Commercial enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, China) were used for *in vitro* quantitative determination of IL-2, IL-6 and INF- γ concentration in human and cattle sera. The micro ELISA plate provided in these kits has been pre-coated with an antibody specific to each of IL-2, IL-6, INF- γ. Samples (or Standards) were added to the micro ELISA plate wells and combined with the specific antibody. Depending on the manufacturer's instructions for each of the three ELISA kits.

Genomic RNA extraction from human and cattle faecal samples

Using the QIAamp Viral RNA micro kit (Qiagen, USA), rotavirus RNA was isolated from faecal samples used in this study in accordance with the manufacturer's instructions. All of the extracted RNA was kept at -80°C until it was examined.

Quality and quantity assessment of rotavirus RNA

To evaluate the quality and quantity of the viral RNA extracted from each sample included in this investigation, spectroscopic examination was performed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Co., USA). The 260/280 and 230/280 ratios of 2.0 and >2.0, respectively, were interpreted as indicating the RNA's purity and the absence of protein and humic acid/carbohydrate contamination.

Reverse transcription of rotavirus dsRNA

Using the Omniscript RT Kit (Qiagen, Valencia, CA, USA), the dsRNA was reverse transcribed into cDNA in accordance with the manufacturer's instructions.

Primers design and synthesis

The primer sets (Table 1) used for rotavirus genotyping using traditional RT-PCR were obtained from the literature [12]. The internet application Primer-Blast, which is available on the following server: https://www.ncbi.nlm.nih.gov/tools/primer-blast/, was used to test the primer's specificity. Integrated DNA Technologies generated the primer set (IDT CO, USA).

VP7 amplification using conventional RT-PCR

A specific primer set (Table 1) specific for the VP7 region of the gene in rotaviruses was used to amplify the full coding sequence (cds) of VP7 using the cDNA, which was generated from all dsRNA viral genomes isolated from all samples included in this investigation. cDNA(2 µL), 1.5 µL (15 pmole) of each of the forward primer (VP7-UF) and reverse primer (VP7-RV), 25 µL of the all Taq Master Mix kit (Qiagen Co., USA), and 30 µL of nuclease-free water were all included in the 50 µL RT-PCR analysis. Initial denaturation at 95 °C for one minute, 30 cycles of 94 °C denaturation for 30 seconds, 55 °C annealing for 30 seconds, 72 °C extension for 45 seconds, and a final extension step at 72 °C for five minutes were the programming parameters for the thermocycler. This procedure amplified a PCR fragment of the VP7 region with a length of 897 bp. Following the completion of RT-PCR, all PCR products were visualised using A UV-Transilluminator (Cleaver Co., UK) after 1% agarose gel electrophoresis. This method was used

to confirm the presence of PCR products with their expected 897 bp.

Table 1: The *VP7-* specific Primer used in conventional RT-PCR

Genes		Sequence 5'-3'	bp	Reference
VP7- **universal**	F	5-ATGTATGGTATTGAATATACCAC-3	897	(12)
	R	5-AACTTGCCACCATTTTTTCC-3		

Statistical analysis

The collected data were analysed statistically using PRISM software 5.0 to assess the potential association between demographic factors and rotavirus infectivity rates. The significance was determined by calculating the expected frequency and chi-square value at a specific degree of freedom, with a threshold of $P<0.05$.

Results

Demographic Characteristics of Human vs. Cattle

Table 1 compares the distributions of age and sex between humans and cattle. The mean age in humans was 8.33 ± 11.08 years compared to 4.27 ± 2.95 years in the cattle. A greater proportion (42.59%) of humans fell into the age group <1, and 22.22% were 1-5 years. In contrast, calves aged less than one year constituted 27.78% cattle, and 31.48% were observed in the age group 1-5 years. A higher proportion of cattle was detected in the age group 5–10 years. Regarding sex distribution, 55.56% of human patients were male and 44.44% were female, compared to 68.52% male (31.48% female) in the cattle group

Table 1: Age and sex distribution according to the study groups.

Variables	Human (N=54)	Cattle (N=54)
Age (years)		
Mean±SD	8.33±11.08	4.27±2.95
Range	1 month to 40 years	1 month to 9 years
Age groups		
<1 year (n. %)	23(42.59)	15(27.78)
1 to 5 years (n. %)	12(22.22)	17(31.48)
5 to 10 years (n. %)	3(5.56)	22(40.74)
10 to 20 years (n. %)	7(12.96)	0(0)
>20 years (n. %)	9(16.67)	0(0)
Sex		
Male (n. %)	30(55.56)	37(68.52)
Female (n. %)	24(44.44)	17(31.48)

Distribution of VP7according to faecal sample sources

Table (2) and Figures (1,2) displayed the results of rotavirus *VP7 gene*-based PCR in 54 human stool and 54 cattle faecal samples. The VP7-PCR positive results were observed in 12 human stool(22.2%) and 14 cattle faecal samples (25.9%).. Whereas the VP7-negative results were detected in 42 human stool (77.8%) and 40 cattle faecal samples (74.1%). The proportional distribution of rotavirus infection in human and cattle cases did not differ significantly (P>0.05).

Table 2. Distribution of rotavirus infection in relation to the Source of clinical samples

Source of clinical samples	VP7-Positive n.(%)	VP7-negative n.(%)	Chi-squared	95% Confidence Interval	P-Value
Human (n. %)	12(22.2)	42(77.8)	0.200	- 12.354% to 19.527%	0.654
Bovine (n. %)	14(25.9)	40(74.1)			

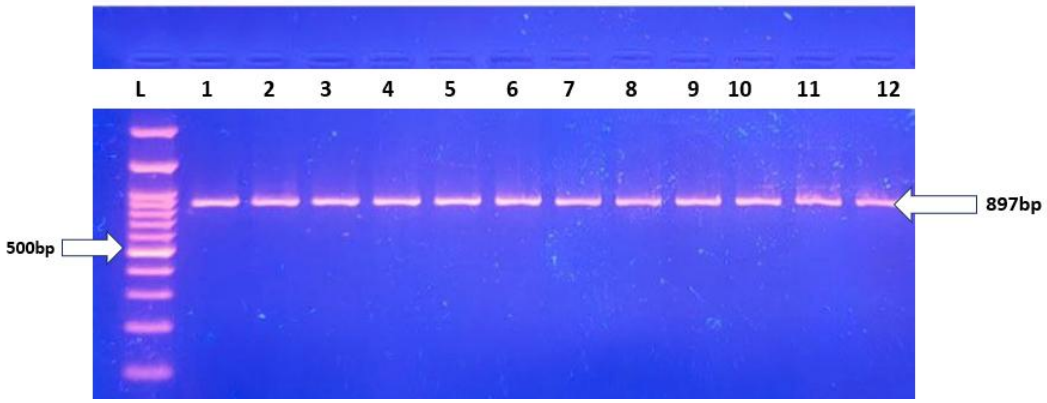

Figure 1: 1% agarose gel electrophoresis showing PCR product for the amplified rotavirus VP7 genes. Lane L: DNA ladder. Lanes 1-6: PCR product for amplified VP7 gene of human stool; Lanes 7-10: PCR product for amplified VP7 gene of cattle

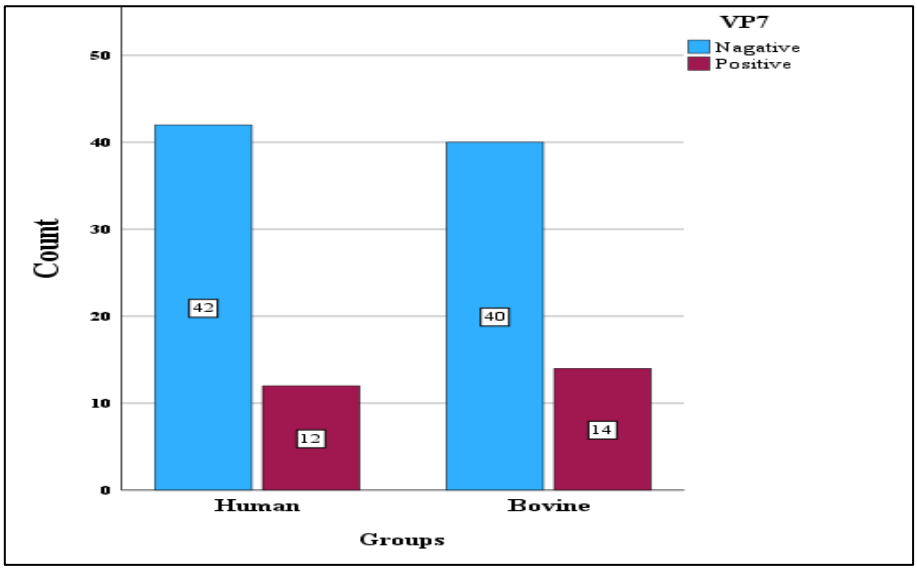

Figure 2: VP7 rotavirus-based PCR positive results according to sample sources

Rota Virus distribution according to sex and age

The effect of sex and age on Rota virus VP7 distribution in humans and cattle was statistically analysed by using the Chi-squared test. Non-significant effect (P>0.05) for human sex on Rota virus VP7 distribution in males and females. However higher percentage (23.3%) of Rota virus VP7 infection was observed in males compared to females (20.8%). In contrast significant effect (P<0.05) for cattle sex on Rota virus VP7 distribution in males and females. A higher percentage (47.1%) of Rota virus VP7 infection was observed in females compared to males (16.2%). Concerning the effect of age on Rota virus VP7 distribution in humans and cattle, a non-significant effect (P>0.05) for human and cattle age was observed in this study. A higher percentage of Rota virus VP7 infection was observed in humans and cattle (23.5 and 34.6 % respectively) in the age group less than 5 years (Table 3,4).

Table 3: Rotavirus infection in humans in relation to sex and age

Variables	VP7-PCR Positive(n.%)	VP7-PCR Negative(n.%)	Chi-squared	95% Confidence Interval	P-Value
Sex					
Males	7 (23.3%)	23 (76.7%)	0.047	- 20.269% to 23.559%	0.827
Females	5 (20.8%)	19 (79.2%)			
Age groups					
<5 years	8 (23.5)	26 (76.5)	0.088	- 20.779% to 23.835%	0.767
> 5-year	4(20)	16 (80)			

Table 4: Rota Virus infection in cattle according to sex and age

Variables	VP7-PCR Positive(n.%)	VP7-PCR Negative(n.%)	Chi-squared	95% Confidence Intervale	P-Value
Sex					
Males	6 (16.2)	31(83.8)	5.684	5.220% to 54.479%	0.0171*
Females	8(47.1)	9(52.9)			
Age groups					
<5 year	9(34.6)	17(65.4)	1.921	- 6.660% to 38.314%	0.166
>5 year	5(17.9)	23(82.1)			

*Significant difference

Cytokine concentrations according to serum samples sources

The results of ELISA testing of human sera for cytokine concentrations are listed in Table 5. These results revealed that IL-6 concentrations were nearly identical between the VP7-positive and negative human (354.76 ± 69.6 vs. 359.71 ± 176.5 pg/mL; p=0.925). IL-2 was higher on average in VP7-positive samples (679 ± 227.81 pg/mL) compared to VP7-negative ones (555.39 ± 265.71 pg/mL), but this difference was not significant (P>0.05). IFN-γ concentration values were likewise close between the VP7-positive and negative human (117.84 ± 172.35 vs. 137.47 ± 204.52 pg/mL) with a non-significant difference (P>0.05).

As was displayed in table (6) the IL-6 levels were significantly(P<0.05) differed between VP7-positive cattle (356.87 ± 156.13 pg/mL) and VP7-negative ones (40.84 ± 40.84 pg/mL), IL-2 mean concentrations showed non-significant difference (P>0.05) between the VP7-positive and negative cattle (869.98 ± 347.08 vs. 853.14 ± 570.85 pg/mL). IFN-γ levels were also non significantly(P>0.05). differed between VP7-positive and negative cattle (104.57 ± 102.43 vs. 150.52 ± 163.93 pg/mL) .

Table 5: IL-6, IL-2, IFN-γ concentrations in human serum samples

Variables	VP7-Positive (n=12)	VP7-negative (n=42)	P-Value
IL-6 (pg/mL)			
Mean±SD	354.76± 69.6	359.71± 176.5	0.925
Range	267.16-487.72	14.2-1065.56	
IL-2 (pg/mL)			
Mean±SD	679± 227.81	555.39± 265.71	0.150
Range	415.13-1233.03	2.09-1704.3	
IFN-γ (pg/mL)			
Mean±SD	117.84± 172.35	137.47± 204.52	0.763
Range	5.57-5.57	0.64-715.03	

Table 4.6: IL-6, IL-2, IFN-γ concentrations in cattle serum samples

Variables	VP7-Positive (n=14)	VP7-negative (n=40)	P-Value
IL-6 (pg/mL)			
Mean±SD	356.87±156.13	40.84±40.84	0.020*
Range	459.8-57.47	350.76-350.76	
IL-2 (pg/mL)			
Mean±SD	869.98±347.08	853.14± 570.85	0.918
Range	524.18-1606.93	1.6-2720.85	
IFN-γ (pg/mL)			
Mean±SD	104.57±102.43	150.52±163.93	0.331
Range	1.6-309.2	8.87-591.6	

*Significant difference

Discussion

Young children mostly suffer from severe disease outcomes associated with rotavirus infection; meanwhile, adults have experienced less severe disease outcomes (presenting as either mild or asymptomatic cases) because of the acquired immune response elicited by the previous rotavirus infections. Despite the efficacy of the rotavirus vaccine to reduce the mortality rate by 60%, a high mortality rate is still being recorded in low-income countries [13]. From the standpoint of epidemiological point of view, RVA is still the most important etiological agent of diarrheal diseases in humans and animals [14;15;16;17]. Our study aimed to evaluate whether there was any correlation between the cytokine level(IL-2, IL-6, INF-γ) with the expression of the VP7 gene in humans and cattle, to elucidate the similarities and differences in immune response across species and enhance the understanding of the role of VP7 in immune interaction. Present finding reveals that (22.2%) human samples were positive, the same prevalence was shown in Pakistan in 2017, disclosed (23%) samples were positive for Rotavirus [18], also a study conducted by Bizuneh et al [19] showed that 26.6% of faecal specimens. Other studies exhibit a higher RVA prevalence (87%) in Shanghai [20] and (41.37%) [21] compared to our study. The positivity of rotavirus was observed to

be 39.65% in Goa and 36% in Meghalaya [22]. Relating to rotavirus A prevalence, our findings revealed the prevalence of Rotavirus among bovine samples was 25.93%. In India, the prevalence of BRV ranges from 11.8% to 26.8% [23]. Many studies detected positive samples for bovine Rotavirus (23.15%, 10.52%) by polyacrylamide gel [24], and 28.89% of diarrheal cases were positive for BRV virus in Kuwait [25]. A study by [26] detected samples from dairy and beef calves were RVA was detected higher rate in dairy calves (59.5%) than in beef (28.4%) calves.

Young children are the main victims of rotavirus infection, with children under five years old having the highest incidence and severity of the illness. While older children and adults typically have milder or asymptomatic infections, infants under three months old typically exhibit reduced illness rates because of maternal antibodies and breastfeeding protection. As a result, our findings' tendency toward a younger mean age among VP7-positive individuals is consistent with known epidemiology, even if it was not statistically significant[27:28].

At the age group less than 5 years in the current study, we revealed that a higher percentage of RotavirusVP7 infection was observed in humans and cattle (23.5 and 34.6 % respectively). A study by [29], according to the RT PCR results, 56.3% of samples in Karbala province were positive in children under the age of five, while 58.5% were positive in Basrah province. A prior study in Ramadi City, Iraq, reported a 56% RV infection prevalence rate in infants aged 1-24 months. However, another Iraqi city, Kurdistan, reported a 37% frequency of RV infection among youngsters [30:31]. In Turkey, a 19% RV infection prevalence rate was found in children under the age of five suffering from gastroenteritis [32]. Our findings reveal no significant relationship between sex and VP7 positivity in humans. The literature yields mixed results: some pooled analyses reveal a somewhat greater prevalence in males, particularly in young infants, but females have a higher rate of rotaviral enteritis in adults. However, many observational studies have revealed no consistent or significant sex difference, which agrees with our findings [33]

One of the main causes of diarrhoea in newborn calves, particularly those under 5–8 weeks old, is group A rotavirus. The risk of rotavirus infection is highest in calves younger than one

month, especially in the first two weeks of life, according to several epidemiological studies. Our study found no significant age differences between VP7-positive and VP7-negative calves, which could be attributed to the large age range and sample size limitations. Nonetheless, the biological expectation is that younger calves are more vulnerable, reflecting underdeveloped immunity and reliance on colostrum protection [34:35:36].

A study by [37] revealed no significant relationship with the animals' age or sex, only with the farms' locations. The current findings contradicted prior data that showed a considerable incidence of rotavirus in both age and sex [37:38]. There were no discernible sex differences, which is in line with several epidemiological studies that show environmental factors and calf management to be more important in determining infection risk than sex. The bulk of data indicates that sex is not a reliable indicator of rotavirus infection, despite some reports indicating variation within herds [25].

One helpful indicator of pro-inflammatory cytokine activity is IL-6. The notable rise in IL-6 among calves that tested positive for VP7 is in line with its function as an acute-phase cytokine and indicator of the severity of diarrhoea in newborn calves. [39]. Reviews that highlight the crucial role inflammation plays in clinical outcomes and zoonotic risk further corroborate this [36]. An essential component of rotavirus pathogenesis, the nonstructural protein NSP4 functions as a pathogen-associated molecular pattern (PAMP), directly triggering pro-inflammatory cytokines such as IL-6 [40:41]. Therefore, our finding of increased IL-6 in VP7-positive people most likely indicates strong innate immune activation brought on by viral elements like NSP4. During convalescence, IL-6 levels significantly decreased [42]. These results confirm that in cases of active rotavirus infection, IL-6 is a valid indicator of systemic inflammation. The notion that measuring this interleukin could be a useful tool in treating acute gastroenteritis is supported by expert studies that show higher levels of IL-6 and IL-8 in rotavirus-induced gastroenteritis. [43:44:45]. Furthermore, Mangiarotti et al. came to the conclusion that rotavirus and bacterial gastroenteritis in a pediatric population cannot be distinguished by IL-6 levels [46].

One important mediator of T-cell-driven adaptive immunity is IL-2. Clinical evidence showed that during the first four days of an acute rotavirus infection, IL-2 levels are low and only start to rise considerably, indicating a delayed adaptive immune response [47]. There was no discernible change in serum levels, indicating that T-cell activation (induced by IL-2) might stay confined in gut-associated lymphoid tissue instead of emerging in the bloodstream.

Although IFN-γ is a crucial antiviral cytokine, there is no significant systemic change (P=0.331), suggesting that its production in cattle is predominantly limited to the intestinal mucosa [48; 49]. An immunobiotics review verified that intestinal epithelial cells play a key role in generating cytokines that protect against rotavirus[50].

Previous research has shown that rotavirus largely induces mucosal immune responses in the gut rather than systemic responses that can be detected in blood, and the absence of serum differences confirms this finding [51:52]. Another study found that while cytokine levels like IL-6 and IFN-γ may temporarily increase during the acute phase of infection, measurement at a single time point may miss these peaks [53:54]. IFN-γ levels in peripheral blood are frequently fluctuating and temporary, even though they can increase during rotavirus infection [55]. Children and adults also seem to have different IFN-γ responses to rotavirus, especially to NSP4. According to Malik et al., by days 4–30 post-onset, IFN-γ-secreting cells specific to the rotavirus were found in roughly 68% of infected children, suggesting a varied and delayed cell-mediated response [41]. A study that used salivary samples showed that NSP4 by itself can produce a significant IFN-γ response and that, in vaccination situations, IFN-γ was associated with viral shedding [56]. This explains why there were no appreciable variations in serum IFN-γ levels between VP7-positive and negative subjects in our investigation, suggesting that IFN-γ production may be dependent on antigen presentation and the mucosal compartment rather than systemic circulation. Nutritional therapies such as immunobiotics and lactoferrin may improve mucosal immunity while reducing inflammation [57].

The potential of dietary therapies to influence immune responses was highlighted by an experimental investigation conducted in weanling pigs that showed spray-dried bovine plasma reduced excessive production of inflammatory cytokines (IL-6, IFN-γ) after rotavirus infection [58].

Conclusion

Pathogenic enteric viruses implicated in gastroenteritis are the etiological agents of infantile diarrhea, a major public health ailment. The PCR-VP7 results revealed that a higher percentage of Rota virus VP7 infection was observed in humans and cattle (23.5 and 34.6 % respectively). Among the measured cytokines, only IL-6 in cattle showed a significant elevation in VP7-positive cases compared with VP7-negative controls. Increased IL-6 in VP7-positive people most likely indicates strong innate immune activation brought on by viral elements like NSP4. Although IFN-γ is a crucial antiviral cytokine, there is no significant systemic change (P=0.331), suggesting that its production in cattle is predominantly limited to the intestinal mucosa.

Conflict of Interest: Authors declare there is no conflict of interest.

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