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Kinetics of Interferon gamma and Interleukin-21 response following foot and mouth disease virus infection

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ABSTRACT

Foot and mouth disease (FMD) is one of the most contagious diseases of cloven footed animals causing significant economic impediment in livestock production system. The immune response to FMD virus (FMDV) infection is regulated by a complex interplay between various cells, cytokines and other immune components. Based on the well established role of Interferon-gamma (IFN-γ) and Interleukin-21 (IL-21) in viral infections, this study aimed to determine expression level of these cytokines in clinically infected adults and calves; and the results were compared with those in the subclinically infected animals up to 120 days post outbreak (DPO) in a vaccinated cattle herd. The expression level of IFN-γ and IL-21 was assayed on 0, 7, 14, 28, 60, 90, and 120 DPO by enzyme linked immunosorbent assay (ELISA) with simultaneous assessment of FMDV structural protein-antibody titer against serotype 'O' by liquid phase blocking ELISA (LPBE) and nonstructural protein-antibody, a differential marker of infection, using r3AB3 indirect ELISA (r3AB3 I-ELISA). Although, the peak expression of IFN-γ was observed on 14 DPO across all categories of animals, the clinically infected animals registered a significant increase in IFN-γ level as compared to the subclinically infected population possibly due to the difference in the extent of virus replication and inflammation. The IL-21 level increased significantly during 14-28 DPO and highest expression was noticed on 28 DPO. The increase in the expression level of IFN-γ and IL-21 at 28 DPO correlated with the increase in antibody titer as determined by LPBE suggesting the role of these cytokines in augmenting immune response to FMDV infection.

1. Introduction

Foot and mouth disease (FMD) is one of the highly contagious diseases of cloven hoofed animals. This OIE listed disease poses threat to the economy of intensive and extensive food-producing animal systems. The etiological agent, FMD virus (FMDV) is a single stranded positive-sense RNA virus which is classified under the genus *Aphthovirus* within the family *Picornaviridae* [1]. The primary route of transmission in cattle is aerosol and virus establishes primarily in naso-pharyngeal and associated epithelial tissues [2]. Although it is well documented that immunity against FMDV depends primarily on the neutralizing antibody response [3], cell mediated immune (CMI) response may also play a vital role to confer protection [4–6]. The production of various cytokines upon antigen stimulation *in vitro* or *in vivo* (infection) provides insights into the mechanism of protection, pathogenesis, host-pathogen interaction and immune evasion mechanism of any pathogen. The cytokine response is an integral part of the immune response and it can be used to monitor immune status of animals during disease progression [4,6]. A spurt of inflammatory cytokines including tumor necrosis factor–α (TNF-α), interferon-α (IFNα), IFN-β, IFN-γ, IL-6, IL-8 and IL-10 is released subsequent to FMDV infection [4–6].

IFN-γ and IL-21 are T-cell associated cytokines and play a significant role in the regulation of the immune system. The importance of IFN- γ in FMD infection has been reported earlier in antigen stimulated whole blood of vaccinated, infected and vaccinated-then-infected cattle [4]. Also IFN-γ inhibited FMDV replication *in vitro* and *in vivo* when used along with type I IFNs [7]. IL-21 has an overwhelming role in the proliferation and differentiation of B cells into plasma cells, Ig (immunoglobulin) class switching and antibody production [8,9]. In an earlier study, the probable role of IL-21 in antibody production upon FMDV vaccination was suggested [10]. Both IFN-γ and IL-21 are critical cytokines for conferring immunity against viral infections [11,12].

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Reports on use of these cytokines as adjuvants to improve antiviral [13,14] and antibacterial immunity [15] do exist. However, studies pertaining to the expression kinetics of IL-21 and IFN-γ in case of natural FMDV infection are scanty. Considering the role of INF-γ and IL21 in stimulating the adaptive immune response, the primary objective of this study was to determine the differential expression pattern of IFN-γ and IL-21 subsequent to FMD outbreak in animal population categorized according to their clinical manifestations and age. The cytokine profile was also correlated with the anti-FMDV antibody titre.

2. Methods

2.1. Animals and sampling

The present study involved a vaccinated organized herd of crossbred Holstein Friesians (HF) cattle located in the Kumaon ranges of Himalaya (29°28′N and 79°39′E, 7500 feet above mean sea level). The animals had been vaccinated regularly at six months interval with oil adjuvanted trivalent (consisting of serotype O, A and Asia 1 strains) inactivated whole virus FMD vaccine. The last round of vaccination before outbreak was done on 19th October 2013 and the index FMD case was noticed on 22nd October 2013 with typical clinical signs *i.e.* rise of body temperature to 106–107 °F and eruption of vesicles on the tongue, hard and soft palate and erosion of tongue epithelium. The outbreak was further confirmed to be due to serotype O FMDV by virus isolation and sequencing of capsid coding region.

Animals were classified into two broad groups such as "clinically infected" and "subclinically infected" based on reverse transcription-multiplex polymerase chain reaction (RT-mPCR) assay results and presence or absence of clinical signs [16]. Based on age and number of vaccinations received, they were further sub-grouped into vaccinated clinically infected adults (n = 13), vaccinated subclinically infected adults (n = 9), primo-vaccinated clinically infected calves (n = 7) and primo-vaccinated subclinically infected calves (n = 5). Age classification was done taking 6 months as the cutoff and primo-vaccinated suggests that the calves received the first ever dose of vaccine just before the outbreak. Blood samples were collected for separation of serum on the day of vaccination (0 days post vaccination (DPV) *i.e.,* 19th October 2013) and the day of first appearance of symptoms (0 days post outbreak (DPO) i.e., 22nd October 2013) followed by on 7, 14, 28, 60, 90 and 120 DPO.

2.2. FMD diagnosis and assessment of FMDV-antibody status

The presence of FMDV genome and serotype was diagnosed by RT-mPCR assay [17] on RNA extracted from saliva of calves and saliva and milk of adult animals. The serum samples were tested using recombinant 3AB3 nonstructural protein (NSP) indirect ELISA (r3AB3 I-ELISA) [18] to detect infection-associated antibodies against 3AB NSP of FMDV. Serum samples producing corrected optical density (OD) values > 40% of that of the positive control was scored positive for FMDV infection. The kit reportedly has a diagnostic sensitivity of 96%, while the diagnostic specificity varied between the naïve and vaccinates as 99.1% and 96.4%, respectively [18]. Two-fold dilution (from 1:32 to 1:256) of serum samples were tested in LPB ELISA as per the procedure described earlier [19] for determining the serotype O-specific structural protein-antibody titre to assess the protective antibody level. The results were expressed as percentage reactivity (Percentage reactivity = $OD_{\text{mean of each test serum dilution}}/OD_{\text{mean of antigen control}}*100$) for each serum dilution and antibody titres were expressed as logarithm of reciprocal of serum dilutions giving 50% of the absorbance recorded in the antigen control wells. The samples showing $\log_{10}$ titre of > 1.8 were considered to have protective antibody titre [20].

2.3. Measurement of serum IFN-γ and IL-21 levels

The IFN-γ and IL-21 concentrations in the serum were determined by commercially available sandwich ELISA kits; bovine IFN-γ ELISA kit (Bio-Rad, USA) and bovine IL-21 ELISA kit (Cusabio, Wuhan Huamei Biotech Co. Ltd, China), respectively. The plates were developed and OD was read at 450 nm in microplate reader (Bio-Rad, USA). All samples were run in duplicate and standard curve generated from OD of standards was used to interpolate cytokine levels, expressed as protein concentration (pg/ml), in the samples.

2.4. Statistical analysis

Analysis of Variance (ANOVA) procedure of Statistical Package for the Social Sciences (SPSS) (15.0) was used for analyzing titre of IL-21 and IFN-γ at different time points from 0 till 120 DPO. The results with $P \leq 0.01$ were considered statistically significant.

3. Results

3.1. Clinical picture of FMD and diagnosis

Following the outbreak, the affected cattle suffered from pyrexia (106–107 °F). High temperature was observed on first day and was maintained up to 24–48 h. The animals started drooling saliva second day onwards and severe vesicular lesions appeared on the feet, teats and oral cavity on 2nd -3rd day (Fig. 1a and b). Two out of twelve clinically infected adult animals showed mild FMD lesions; however, the symptoms were severe in all the clinically infected calves. The RT-mPCR revealed presence of serotype O FMDV specific genomes in all the animals (n = 34) included in this study.

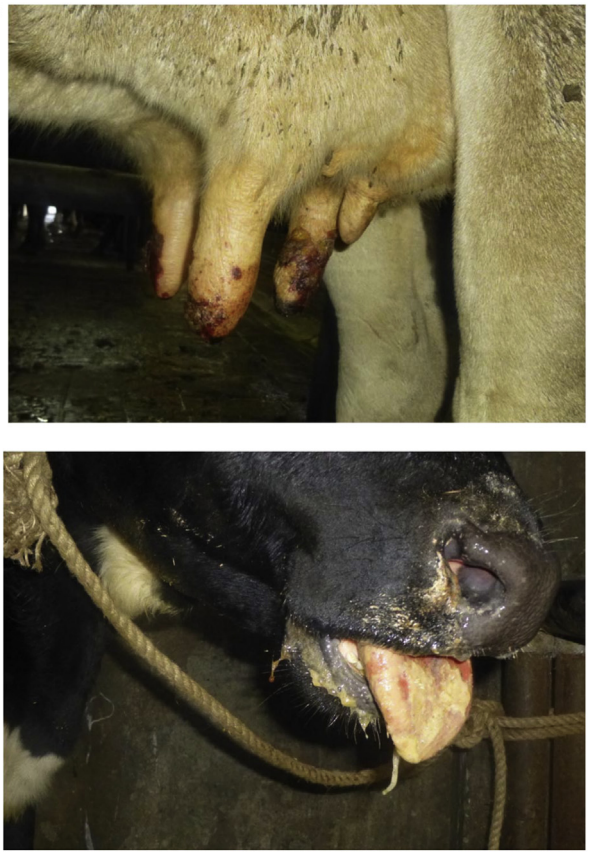
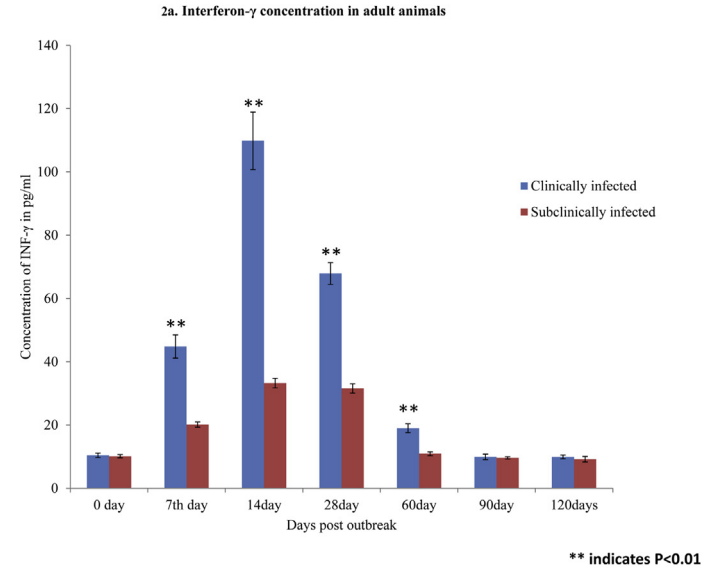
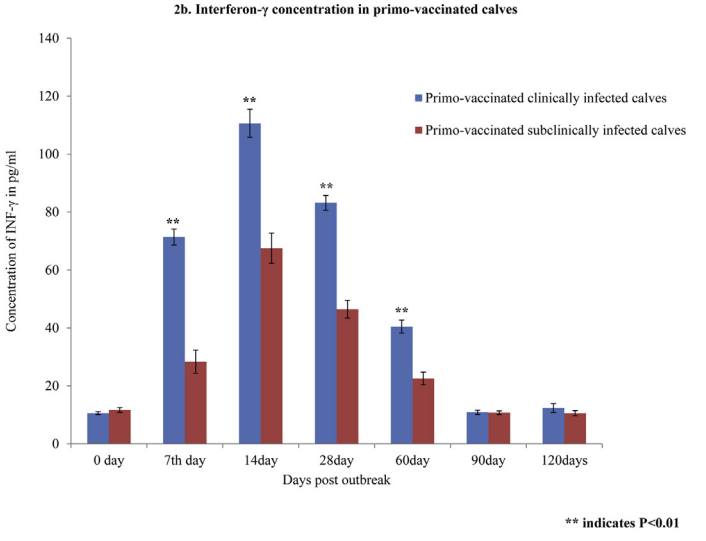


Fig. 1. a.FMD lesions in teats of milch animals. b. FMD lesions in mouth of milch animals.





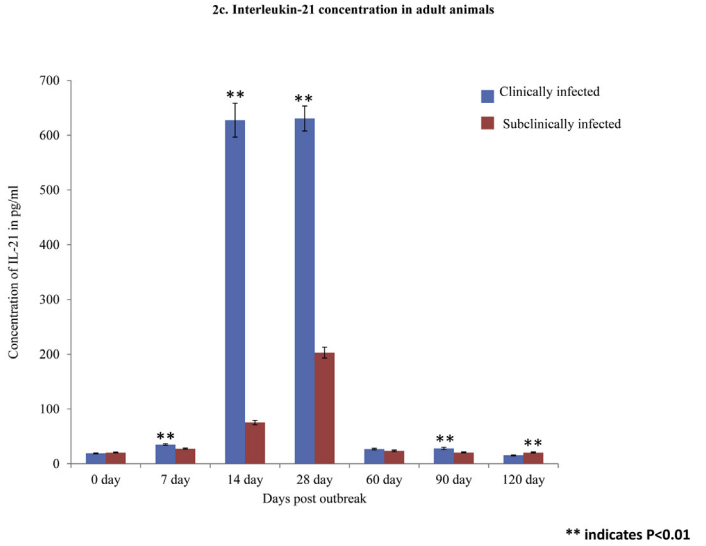


Fig. 2. a. Interferon-γ concentration in adult animals. b. Interferon-γ concentration in primo-vaccinated calves. c. Interleukin-21 concentration in adult animals. d. Interleukin-21 concentration in primo-vaccinated calves

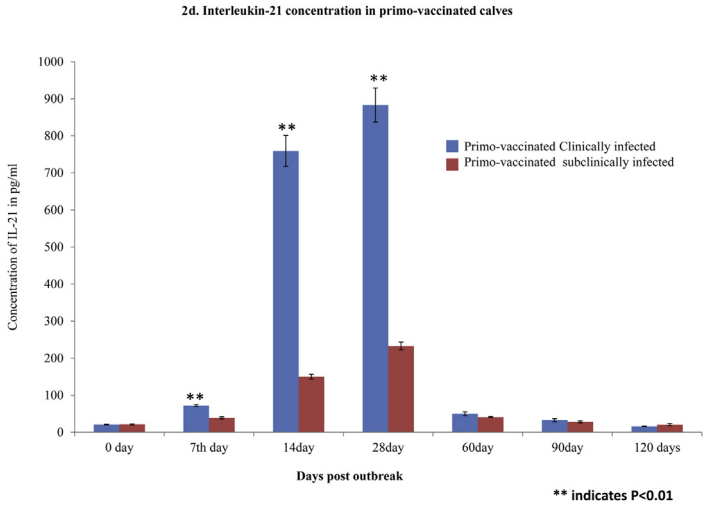


Fig. 2. (*continued*)

3.2. Kinetics of Interferon gamma and IL-21 response

The level of cytokines, IFN-γ and IL-21 in the serum of all the animals was determined by ELISA and the results obtained are presented in Fig. 2a–d. The titre of IFN-γ was at basal level in all the animals initially; however, the levels showed an increase at 7 DPO. Furthermore, the difference in the level between the clinically and subclinically infected categories was significant on day 7 ($P < 0.01$) with relatively higher titre in the clinically infected groups (Fig. 2a and b). The differences further widened between the groups and were highly significant at 7, 14, 28 and 60 DPO ($P < 0.01$). An increase of 2.22, 3.3 and 2.15 fold was observed on 7, 14 and 28 DPO, respectively in clinically infected adult animals when compared with subclinically infected ones. In subclinically infected adult animals, the increased level of IFN- γ (although significantly lower than the clinically infected group) dropped back to basal level by 60th day, while it declined to basal level by 90 DPO in the clinically infected group. In primo-vaccinated clinically infected calves the increase was 2.51, 1.64, 1.79 and 1.79 fold on 7, 14, 28 and 60 DPO, respectively when compared with primo-vaccinated subclinically infected calves. The peak level was observed at 14 DPO in both groups and dropped to basal level by 90 DPO. No difference could be detected on 90 DPO and thereafter till end of the sampling in the expression level between the clinically and subclinically infected calves possibly due to the infected animals recovered completely from infection.

The differences between clinically and subclinically infected adult groups with respect to the expression of IL-21 were statistically highly significant on day 7, 14 and 28 ($P < 0.01$) (Fig. 2c and d). The increase was 8.34, 3.12 folds at 14 and 28 DPO, respectively in clinically infected group as compared to the subclinically infected group. Likewise, primo-vaccinated clinically infected calves revealed 5 fold increases at 14 DPO and 3.78 fold increases on 28 DPO when compared to the primo-vaccinated subclinically infected calves. By 60 DPO, the IL-21 level in clinically and subclinically infected calves showed a marked decline and almost reached the basal level and remained comparable thereafter.

Table 1
Results of LPB ELISA and r3AB3 NSP-ELISA.

Group	Animal identity number	Log_{10} titre against serotype O in LPB ELISA			r3AB3 NSP ELISA		
		0 day	28th day	90th day	0 day	28th day	90th day
Calves clinically infected	631	< 1.5	1.98	1.9	N	P	P
	632	< 1.5	1.98	> 2.4	N	P	N
	633	< 1.5	2.28	> 2.4	N	P	P
	635	< 1.5	1.68	> 2.4	N	P	P
	637	< 1.5	2.4	1.11	N	P	P
	638	< 1.5	2.28	1	N	P	P
	639	< 1.5	2.4	0.15	N	P	P
Calves subclinically infected	616	< 1.5	1.68	1.67	N	P	P
	622	< 1.5	2.28	1.91	N	P	N
	625	< 1.5	2.28	2.1	N	P	P
	629	< 1.5	> 2.4	1.87	N	N	N
	636	< 1.5	> 2.4	1.9	N	P	P
Adults clinically infected	372	< 1.5	> 2.4	> 2.4	N	P	P
	284	< 1.5	> 2.4	2.27	N	P	N
	337	1.68	> 2.4	> 2.4	N	P	P
	567	1.68	> 2.4	> 2.4	N	P	N
	450	1.98	> 2.4	> 2.4	N	P	P
	354	1.68	> 2.4	2.23	N	P	P
	456	1.68	> 2.4	2.28	N	P	P
	359	1.98	> 2.4	1.94	N	N	P
	307	1.98	> 2.4	> 2.4	N	P	P
	562	1.5	> 2.4	> 2.4	N	P	P
	564	1.68	> 2.4	1.39	N	P	P
	454	1.68	> 2.4	> 2.4	N	P	P
	107	< 1.5	> 2.4	1.84	N	P	P
Adults subclinically infected	363	2.4	> 2.4	> 2.4	N	P	P
	218	2.4	> 2.4	> 2.4	N	P	N
	483	2.28	> 2.4	> 2.4	N	P	P
	464	1.98	> 2.4	> 2.4	N	P	P
	239	1.98	> 2.4	> 2.4	N	P	N
	271	2.28	> 2.4	> 2.4	N	P	P
	119	2.28	> 2.4	> 2.4	N	P	P
	131	2.28	> 2.4	2.29	N	P	N
	161	2.28	2.28	> 2.4	N	P	N
						94.11%	73.52%

3.3. Detection of FMDV structural and nonstructural protein-antibodies

In r3AB3 I-ELISA (DIVA test) none of the sera samples showed NSP-antibodies at '0' DPO suggesting no past exposure of the herd to FMDV. However, 32 out of 34 (94.11%) animals turned seropositive for 3AB-antibodies by 28 DPO indicating recent circulation of virus (Table 1). The proportion of 3AB-antibody positive animals declined to 73.52% by 90 DPO. Although it was difficult to distinguish between vaccination and infection induced structural protein-antibody titres obtained in LPBE since the wave of infection followed the routine vaccination drive, the proportion of subclinically infected adults showing log_{10} antibody titre of more than 1.8, the cutoff adapted for inferring protective antibody level [20], was considerably more than the clinically infected adult animals at 0 DPV (Table 1). Therefore the titre of protective antibodies existing in the host body at the face of an outbreak appears to

have a direct bearing on the clinical severity of the disease. Antibody titre determined at that point of time just before manifestation of clinical symptoms is expected to be a result of previous vaccinations rather than infection associated antibodies. This could be verified from the observation that all of the primo-vaccinated infected calves revealed a $\log_{10}$ titre of less than 1.5 at 0 DPV. By 28 DPO, animals across categories demonstrated a spike in the antibody titre against serotype O which could be related to infection associated seroconversion and also vaccination-infection boosting effect. The titre at 90 DPO although registered a marginal decline in some of the animals compared to those at 28 DPO, it persisted at a high level in a majority of the population.

4. Discussion

The cytokines are inflammatory mediators in viral infections and their levels differ dramatically from steady state conditions in the infected animals exhibiting acute disease as compared to the uninfected ones. During FMDV infection or vaccination, there is a highly heterogeneous host immune response augmented by various cytokines depending on the severity of the disease [4,5]. In the present study, we aimed at determining the kinetics of relevant cytokines such as IFN-γ and IL-21in case of FMDV clinical infection in cattle as compared to that in the subclinically infected animals in the herd.

IFN-γ is an important cytokine that regulates the immune response in FMDV infection [4]. This cytokine is produced predominantly by NK and natural killer T (NKT) cells as part of the innate immune response, and by antigen induced $CD4^+$ and $CD8^+$ T cells [21]. Present study showed significant variation in IFN-γ level among the categories of animals post-outbreak which peaked on 14 DPO (10.42 ± 0.68 to 109.82 ± 9.0 pg/ml in clinically infected adults, 10.13 ± 0.52 to 33.25 ± 1.51 pg/ml in subclinically infected adults, 10.59 ± 0.48 to 110.61 ± 4.84 pg/ml in primo-vaccinated clinically infected calves, 11.67 ± 0.83 to 67.51 ± 5.21 pg/ml in primo-vaccinated subclinically infected calves) and thereafter started declining and plummeted to basal level by 90 DPO. The difference in the level of IFN-γ between the clinically and the subclinically infected groups was statistically significant in both adult animals and calves. Consistent with the present study, specific induction of IFN-γ was detected in the antigen stimulated whole blood samples from FMD vaccinated, infected and vaccinated-then-infected cattle [4] *in vitro*. The increased level of IFN-γ in the current study reinforces its importance in eliciting immune response against FMD infection as this cytokine is known to play a critical role in regulating immune response against viral infections [4,22]. The increased level of IFN-γ is critical not only in FMDV vaccinated and infected animals; it has also been shown to inhibit FMDV replication *in vitro* and *in vivo* when used along with type I IFNs [7].

IL-21 is secreted by $CD4^+$ T cells upon antigen stimulation and it acts on B cells to promote antibody production [9,23]. IL-21 could be very important cytokine to support the humoral immune response through the proliferation and differentiation of B cells into plasma cells, enhancing immunoglobulin production and isotype class switching [8,9]. Clinical and basic research has established the protective role of IL-21 in other viral diseases, including HIV [24], hepatitis B virus [25] and Dengue virus [26]. IL-21 acting on B cells and $CD4^+$ T cells is critical for generating long-lived plasma cells after infection with an acute strain of LCMV, vesicular stomatitis virus, and influenza virus, highlighting the importance of IL-21 in humoral immunity during viral infection [12]. The potent antiviral properties of IL-21 [9,27] make this cytokine a suitable adjuvant in vaccine formulations. In the present study, for the first time, we present the evidence of higher levels of IL-21 production in case of FMDV infection. It is apparent from this study that the level of IL-21 increases significantly during FMDV infection in clinically infected compared to the subclinically infected animals. High IL-21 level was observed from 7 DPO, that increased considerably on 14 DPO and was maintained till 28 DPO. This trend of IL-21 response could be correlated with the increase in the specific antibody titre against FMDV as well as recovery of animals. An enhanced expression of IL-21 at mRNA level at 28 DPV in the crossbred calves has been reported earlier that supports the role of IL-21 in mediating *in vivo* humoral response post FMD vaccination [10].

The level of both cytokines, IL-21 and IFN-γ, increased significantly during recovery phase and was associated positively with the increase in structural protein-antibody titre against FMDV as detected by LPBE which suggest a protective role of IL-21 and IFN-γ in FMDV infection. Elevated levels of IFN-γ and IL-21 from 14 to 28 DPO were also found in subclinically infected animals; however, the magnitude of response was significantly lower than that in the clinically infected animals which could be due to the inability of virus to establish clinical disease and absence of inflammatory changes in subclinically infected animals. On the other hand, the elevated level in clinically infected animals might be due to relatively more viral load and associated severe inflammation in acute infection. The disease outbreak in the herd despite recent vaccination might be because of failure to develop protective immune response over a short period of time post recent vaccination (the last round of vaccination was conducted just 4 days prior to the index case) and waning antibody level elicited from past vaccination (about 6 months prior to the outbreak) thereby creating a window of susceptibility. The cause of the outbreak was possibly a spill over from surrounding outbreaks and not by vaccination. This has been discussed in detail elsewhere [16]. In that study the farm outbreak causing strain has already been shown to be of O/ME-SA/Ind-2001d lineage by capsid sequence analysis, which is quite distinct from O/R2/75, the type O vaccine strain. Therefore, it could be established that the outbreak was not due to any residual virulence in the vaccine, although outbreak occurred a couple of days post-vaccination.

Although, the entire group of animals under study was found to be positive for FMDV genome by RT-mPCR and nonstructural protein-antibodies by r3AB3 I-ELISA, a proportion of infected animals remained asymptomatic in the herd which is a salient feature in a regularly vaccinated herd and might be due to the variables such as individual susceptibility, antibody level at the face of the outbreak etc. [16]. Previous studies have also shown that major histocompatibility BoLA-DRB3 (bovine leukocyte antigen-DRB3) plays a key role in immune response to FMDV infection [28] and polymorphism in BoLA-DRB3 determines the susceptibility to FMD infection [29].

5. Conclusion

In conclusion, significantly higher levels of IFN-γ and IL-21 post FMD outbreak in the crossbred cows, particularly in the clinically infected animals as compared to the subclinically infected ones, between 7 and 28 DPO suggests an immune-protective role of these cytokines in FMDV infection in synergy with other mediators of immune response. This was supported by the observation that the timing of increase in the level of these cytokines overlapped with both increase in the protective antibody titre in the animals and their recovery from clinical disease. Further study is warranted to understand the kinetics and mechanism of action of these cytokines in detail. It is also important to elucidate immune cell populations responsible for secreting these cytokines during the course of FMDV infection. The observations made in this study could be relevant to trials on these cytokines administered as adjuvants for enhancing the levels of protective IgG expression.

Conflicts of interest

None of the authors has any conflict with other people or organizations that could unprofessionally influence the content of the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.micpath.2018.08.049.

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