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Effect of peste des petits ruminants virus (PPRV) infection on the host immune response in naturally infected goats

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ABSTRACT

Toll like receptors (TLRs) expressed by various immune cells and tissues are known to play an important role in recognising the pathogens by the host. The study was carried out to envisage the expression of virus-recognising-TLRs like TLR-3, TLR-7 and TLR-8 as well as the Th1 and Th2 cytokines in the serum of naturally Peste des petits ruminants virus (PPRV) infected goats. Goat serum samples, collected from three districts of Asom (Kamrup, Nalbari, Darrang), were screened for Peste des petits ruminants virus (PPRV) antibody by Complementary-ELISA. Out of 227 samples screened, 72 samples showed presence of PPR viral antibody with a percentage prevalence of 31.72%. Out of the positive samples, 39 were selected randomly for testing the TLR and cytokine response after PPRV infection. The study indicated TLR-8 to have an enhanced expression in serum of PPRV infected goats along with IL-12 and IFN-γ of the Th1 pathway. Further, in infected group, a significant correlation was registered between IL-12 and IFN-γ. The present study showed the involvement of the Th-1 pathway in host immune response after PPRV natural infection which may help in proper disease management and control strategies.

Key words: Asom, Cytokines, cELISA, Goats, PPRV, TLRs

Peste des petits ruminants (PPR), also known as ovine rinderpest (or pseudo-rinderpest), is an important viral disease of goats, sheep and some small wild ruminants (Liu *et al.* 2013). PPR is an acute, highly contagious disease, and has been identified by the World Organization for Animal Health, as a notifiable and economically important transboundary viral disease of sheep and goats associated with high morbidity and mortality which can be as high as 100% (Al-Dubaib 2009, Balamurugan *et al.* 2012). The causative agent, PPR virus (PPRV), is a member of the Morbillivirus genus in the family Paramyxoviridae (Gibbs *et al.* 1979). PPRV is closely related to rinderpest virus (RPV), which infects cattle and other large ruminants and can cause disease in small ruminants (Couacy-Hymann *et al.* 1995). It is usually characterized by fever, respiratory distress, ocular, nasal and oral discharges, pneumonia, stomatitis and gastroenteritis leading to severe diarrhoea followed by death or recovery from the disease (Gibbs *et al.* 1979).

Although PPRV is a single serotype, it is genetically grouped into four lineages (I, II, III and IV) based on fusion (F) and Nucleoprotein (N) gene sequence analysis; of which, lineages I to III circulate in Africa and lineage IV circulates in Asia (Shaila *et al*. 1996, Dhar *et al*. 2002). In India, the first outbreak of PPR was described by Shaila *et al.* (1989), and since then, the disease has become endemic in India with outbreaks occurring regularly throughout the country, thus, causing severe economic losses and is presently considered as one of the major threats to about 200 million small ruminant population of the country (Balamurugan *et al.* 2012). As disease being the major reason for low productivity, it is necessary to understand immune response mechanism involved in disease resistance and to develop proper preventive measures.

In mammals, two different types of responses of the immune system are found namely innate and adaptive. Innate immunity is the first line of defence against pathogens and mainly reduces pathogen load and provides time for the development of highly specific and long-lasting adaptive immune response (Goyal 2012).The innate immune system can sense viruses, bacteria, parasites and fungi through the expression of pattern recognition receptors (PRRs) which recognize conserved structure in pathogens called pathogen associated molecular pattern (PAMPs),

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essential for the survival of the micro-organisms and are therefore difficult to alter (Griebel *et al.* 2005). The most common PRRs are toll like receptors (TLRs), retinoic acid-inducible gene I (RIG I) like receptors (RLRs) and Nod like receptors (NLRs) (Kumar *et al.* 2014a, Kawai and Akira 2010). Among these, TLRs constitute a multi-gene family of PRRs in vertebrates genome and are classified on the basis of recognition of distinctive PAMPs, playing a key role in protection against both viral and bacterial infections (Carpenter and O'Neill 2007, Goyal 2012). Identifying the TLRs, their ligands and the signal transduction events that they initiate, can provide an insight into our understanding of how the immune response to infection begins.

Among the group of TLRs, TLR-3, 7 and 8 are found on the surface of endosomes (not outer membrane bound), where they respond primarily to nucleic acid based PAMPs from viruses and bacteria (Hung *et al.* 2008, Heiden *et al.* 2014). Activation of these receptors leads to production of inflammatory cytokines as well as interferons, to tackle the viral infection. TLR-3 is activated by both double stranded/ single stranded viral RNA, whereas, both TLR-7 and 8, recognise single stranded viral RNA (Heiden *et al.* 2014, Hung *et al.* 2008)

The helper T cells (Th cells) are a type of T cell which plays an important role in adaptive immune system by amplifying immune responsiveness by releasing T cell cytokines. T-helper cell populations can be sub-classified based on the cytokines they secrete (Th1 and Th2 helper cells). Th1 cells are hypothesised to act as host immune effectors against intracellular pathogens like viruses and they rely on IFN-γ, IL-12 and IL-2. Th2 cells, on the other hand, are believed to act against extracellular pathogens and are influenced by IL-4, IL-5, IL-9, IL-10 and IL-13 (Kidd 2003). Thus, understanding exactly how the helper T cells respond to immune challenges may be helpful in the treatment of disease and in increasing effectiveness of vaccination.

The present study was designed to gain an insight on the expression of one of the important innate immune receptors like TLRs (TLR-3, TLR-7 and TLR-8) as well as adaptive immune response by understanding the expression of cytokines (IL4, IL10, IL12 and IFN-γ) in PPRV infected and apparently healthy goats.

MATERIALS AND METHODS

Collection of serum samples: Serum samples were collected to investigate the different parameters. Blood samples were collected by jugular vein-puncture from both clinically infected and apparently healthy adult animals from the same outbreak areas. The infected animals showed clinical signs of the disease like fever, ocular and nasal discharge, erosive stomatitis and diarrhoea. Serum was then separated and stored at –20°C without addition of any preservative for further use.

Screening by antibody detection by cELISA: Competitsive enzyme linked immunosorbent assay (cELISA) was used to detect the presence of antibodies against PPRV using the cELISA ID Screen® PPR Competitsion assay (ID VET, Montpellier, France). All procedures were carried out according to manufacturer's instructions. Results were expressed as percentage of negativity (PN) compared with the kit control and designated as positive, doubtful or negative according to the cut-off values recommended by the manufacturer (PN $\leq$35 is positive, 35 < PN $\leq$ 45 is doubtful, PN > 45 is negative).

Quantitation of Goat TLR and cytokine concentration in the serum: To evaluate the levels of TLRs (TLR-3, TLR-7 and TLR-8) and that of cytokines (IL-4, IL-10, IL-12, IFN-γ) present in the serum, of both the apparently healthy and clinically affected goats, sandwich ELISA was done using sandwich ELISA kit specific for each TLR and cytokine (Cusabio Biotech Co. Ltd, China) as per the procedure laid down by the manufacturer. The analysis was performed in duplicates. The total concentration of each was determined using standard curve, which was constructed by reducing the data using an online ElisaAnalysis software, version 3.2 (Leading Technology Group, Australia), capable of generating a four parameter logistic (4-PL) curve-fit. Total concentrations of TLRs were reported as picogram/ml (pg/ml).

Statistical analysis: Paired t-tests were used to compare the means between the apparently healthy and PPRV infected samples, and $P<0.05$ was considered significant. The correlation among the TLRs; between the cytokines and TLRs was evaluated using Pearson's correlation test. The r value from the correlation test was divided into three ranges of correlation: 0.0–0.3 (weak); 0.4–0.7 (intermediate); and 0.8–1.0 (strong). Also, the estimation of confidence interval (95%) and Mean ± SE were carried out as per standard statistical method using Statistical Analysis System (SAS) software version 9.3 (SAS India Ltd, Mumbai).

RESULTS AND DISCUSSION

Goats are known as the "poor man's cow" and its population in India is estimated to be around 1/6th of the world population (Tirumurugan *et al.* 2010). Till date, 13 TLRs have been discovered in mammals, that are differentially expressed in various cell types (Goyal 2012). In goats, 10 TLR genes have been reported and but not much work has been done on its further characterisation and expression (Goyal 2012, Raja *et al.* 2011). To check the host immune response, concentration levels of various TLRs (TLR 3, TLR 7 and TLR 8) as well as cytokines (IL-4, IL-10, IL-12, IFN-γ), in both apparently healthy and PPRV infected goats was determined by quantification of respective protein levels (TLRs/cytokine) in the serum of the animal.

The sera samples (227), from naturally PPRV infected goats (Fig. 1), collected from 3 districts of Asom (Kamrup, Nalbari and Darrang), were tested for the presence of PPR viral antibody by c-ELISA. Sera samples (30) from apparently healthy goats were also tested by cELISA for

Table 1. Details of the samples screened for peste des petits ruminants virus antibodies in goats during outbreaks (March 2013-December 2013) in a few districts of Asom

Name of district	Total no. of samples screened	Positive in Competitive ELISA	Prevalence (%)
Kamrup	118	38	32.20
Nalbari	51	28	54.90
Darrang	58	6	10.34
Total	227	72	31.72

presence of viral antibody and were diagnosed negative.

Out of 227 samples tested, 72 samples showed presence of PPR viral antibody. The percentage prevalence (Table 1) of PPRV antibodies in goats was found to be 31.72% (0.31±0.02; Confidence Interval (CI) = 0.27–0.35). Similar findings were also observed by Balamurugan *et al.* (2012) who also observed 38.2% goats to be positive for PPRV.

Out of the positive samples (72), samples which had sufficient quantity were selected (39) for analysis of the TLRs and cytokine profile to study the immune response of the host. To check the immune status in healthy goats, sera samples (16) from young, apparently healthy goats were randomly selected and tested.

Studies on the expression profile of TLRs and cytokines (proteins) in the serum of goats of Asom have not been documented from this part of the country until now. The sandwich ELISA data, fitted to the four parameter logistic (4-PL) curve, gave the total concentrations of the different variables employed in the study. Among the TLRs, all of them including TLR 3, TLR 7 and TLR 8 showed a higher expression in PPRV infected goats (Fig. 2). However, only TLR 8 was found to have significant ($P<0.05$) up-regulation in PPRV infected goats in comparison with the apparently healthy goats (Fig. 2). In contrary to our findings, Kumar *et al.* (2014b) had observed a dose dependent enhanced expression of TLR 3 and TLR 7 in PPR virus infected Vero cells.

As reported by many, PPRV infection in goats leads to a classic inflammatory immune response, which is characterised by enhanced expression of cytokines such as IFN-β, IFN-γ, IL-4, IL-1β, IL-8, IL-10, IL-6 and IL-12 (Kumar *et al.* 2014b, Baron *et al.* 2014). In this study, an elevated response of both IL 12 and IFN-γ ($P<0.05$) was observed (Fig. 2). Our results are in accordance with previous studies where IFN-γ showed a significant ($P<0.05$) increase upon PPRV infection in the serum of goats (Truong *et al.* 2014). However, no significant increase ($P>0.05$) was observed in the concentrations of IL-4 and IL-10 in PPRV infected goats.

According to the Th1-Th2 balance hypothesis, Th1 cells drive the type-1 pathway (cellular immunity) to fight viruses and other intracellular pathogens; and Th2 cells drive the type-2 pathway (humoral immunity) and up-regulate antibody production to fight extracellular organisms; and both mutually inhibit each other (Kidd 2003). Further, there

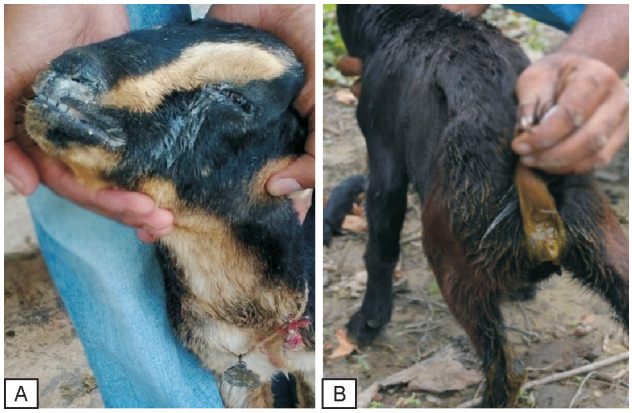

Fig. 1. Infected goats showing typical symptoms of PPR. (A) Ocular and Nasal Discharge (B) Diarrhoea

are reports stating that IFN-γ and IL-12 can exert inhibitory activity on the production of other Th2 cytokines like IL-10 (Gessani and Belardelli 1998, Trinchieri 1997). This goes consistent with our finding that the up-regulation of IFN-γ and IL-12 (Th1 Cytokines) is accompanied by significant down-regulation of Th2 cytokine (IL-10) in PPRV infected goats (Fig. 2). Also, our result is in accordance with Erb *et al.* (1998), where Th1 pathway is up-regulated during intracellular infection by *Mycobacterium bovis,* whereas Th2 pathway seems to be unaffected.

Further, correlation was checked among TLR-3, TLR-7 and TLR-8 using the Pearson's correlation test. In PPRV

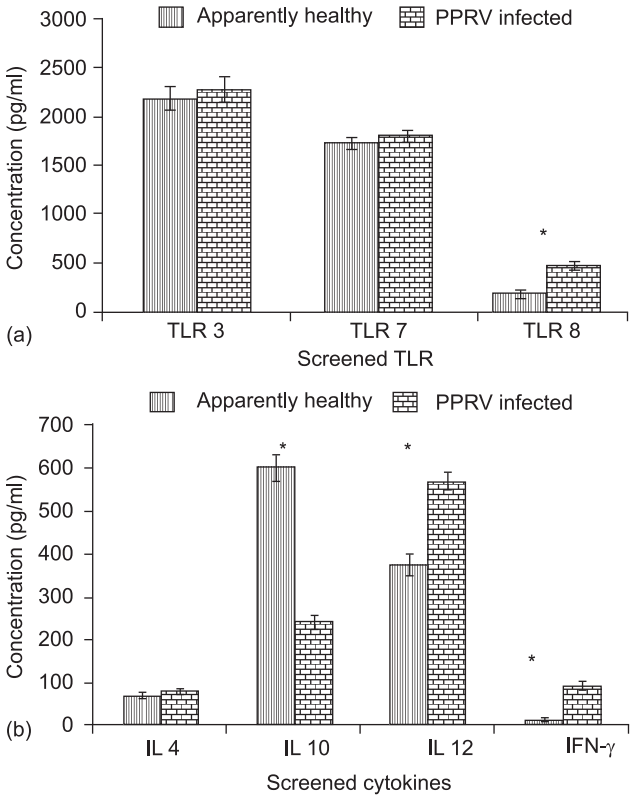

Fig. 2. Concentrations of a) TLR-3, TLR-7, TLR-8 and b) IL-4, IL-10, IL-12, IFN-γ in apparently healthy and PPRV infected goats. *Significant ($P<0.05$)

Table 2. Overview of Pearson correlation coefficients (r) between the immunogenic markers of apparently healthy animals (16) and PPRV infected animals (39)

Comparison	Apparently healthy Pearson correlation coefficient (r)	Clinically infected Pearson correlation coefficient (r)
TLR 7 and TLR 8	0.23	0.51^{**+}
TLR 3 and TLR 8	0.22	0.50^{**+}
TLR 3 and TLR 7	0.18	0.69^{**+}
IL 12 and IFN-γ	0.03	0.41^{**+}
IL 4 and IL 10	0.03	0.02

**Represents significance (P<0.01) level, +Represents intermediate correlation.

infected group, a moderate statistically significant (P<0.05) correlation was found between TLR-3 and TLR-7; TLR-7 and TLR-8; TLR-3 and TLR-8 (Table 2). This finding showed that there is a significant relation among all the virus-recognising-TLRs. This may be explained from the fact that TLR 3 recognises both double/single stranded viral RNA whereas TLR-7/8 recognises single stranded RNA only (Hung *et al.* 2008). PPR virus being a single stranded RNA, is thus, recognised by all of them. Moreover, cytokines of Th1 pathway, IL 12 and IFN-γ showed a significant (P<0.05) intermediate correlation in PPRV infected goats (Table 2) whereas they showed a poor correlation in healthy goats with no significance (P>0.05).

On the other hand, cytokines of Th2 pathway, IL 4 and IL 10 showed a poor correlation in both PPRV infected goats (P>0.05) and apparently healthy goats (Table 2).

Previous reports suggest that, for induction of Th1 immune response, activation of innate immune response is a prerequisite. Zhao *et al.* (2006) observed a relation between TLR signalling and IFN-γ. Therefore, to check the correlation between the innate response and the cytokines, all the TLRs were checked with the cytokines. However, statistically significant correlation was not observed in any of them.

The present study provides preliminary information on host immune response after PPRV infection. The information may be useful in getting a picture about the immune response in goats, which may in turn help to formulate disease control strategies as well as implementation of PPR vaccination programme in Asom. The study revealed that TLR-8 shows an elevated response upon PPRV infection. Th1 pathway also seems to be triggered by up-regulation of IFN-γ and IL-12 cytokines. The findings thus reflect the immune response after natural infection of goats by PPR virus. However, detailed studies on TLRs needs to be undertaken to understand its role in generation of persistent antibody and cell mediated immune response against PPRV.

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