RELATIONSHIP BETWEEN THE LEVEL OF SIGNALING LYMPHOCYTE ACTIVATION MOLECULE mRNA AND REPLICATION OF PESTE-DES-PETITS-RUMINANTS VIRUS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF HOST ANIMALS

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Summary. – Basal signaling lymphocyte activation molecule (SLAM) expression in the peripheral blood mononuclear cells (PBMCs) of cattle, buffalo, sheep, and goats was correlated with Peste-des-petits-ruminants virus (PPRV) replication assessed by real-time PCR and virus titers. PBMCs from goats had the highest level of SLAM mRNA followed by sheep, cattle, and buffalo. Different breeds of goats had different basal levels of SLAM mRNA. In the PBMCs of studied animals, basal SLAM expression had high correlation with their ability to replicate PPRV. After stimulation of PBMCs from different breeds of goats with concanavalin A (con A), the SLAM expression increased 4- to16-fold, what resulted in a 1.7- to 3.8-fold increase of viral mRNA and the virus titer increased by 0.4–1.3 log units. These findings revealed that SLAM expression and PPRV replication are highly correlated and different levels of SLAM mRNA could influence the virus replication in different animals.

Key words: Peste-des-petits-ruminants virus; signaling lymphocyte activation molecule; peripheral blood mononuclear cells; real-time PCR

Introduction

Peste des petits ruminants (PPR) is a contagious viral disease of goats and sheep that is widespread across sub-Saharan Africa, Arabian peninsula, and Indian subcontinent (Nanda *et al*., 1996). The causative agent PPRV belongs to the genus *Morbillivirus,* the family *Paramyxoviridae* and clinically affects goats and sheep. However, even though the virus multiplies in cattle and buffaloes, it does not cause any clinical symptoms in these animals. An exception is one clinical outbreak reported in buffaloes (Govindarajan *et al*., 1997).

Cellular receptors are one of the major determinants of the host range and tissue tropism of viruses. CD46, a complement regulatory molecule, was shown to act as a cellular receptor for Measles virus (MeV) (Dorig *et al*., 1993; Naniche *et al*., 1993). CD46 expression allows binding, entry, and replication of laboratory strains of MeV in normally non-susceptible rodent cells (Dorig *et al*., 1993; Naniche *et al*., 1993). Since MeV, Edmonston strain has been commonly used in laboratories, CD46 was generally accepted as the receptor for MeV. On the other hand, MeV strains (some clinical isolates and KA strain) isolated from B95a cells (Epstein-Barr virus-transformed marmoset B-cell line) were shown to grow only in a limited number of lymphoid cell lines (Tatsuo *et al*., 2000; Kobune *et al*., 1990). CD46 expressed on HeLa and Vero

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Abbreviations: CDV = Canine distemper virus; CHO = Chinese hamster ovary; con A = concanavalin A; H = hemagglutinin; MeV = Measles virus; PBMCs = peripheral blood mononuclear cells; PPR = peste des petits ruminants; PPRV = Peste-des-petits-ruminants virus; RPV = Rinderpest virus; SLAM = signaling lymphocyte activation molecule

cells is the receptor for vaccine or laboratory strains, but not for B-lymphotrophic isolates of MeV (Lecouturier *et al*., 1996). These observations led to the idea that B95a cell-isolated strains use as a receptor such a molecule that is different from CD46 (Tatsuo *et al*., 2000).

In order to identify a cellular receptor for B95a cell-isolated strains of MeV, Tatsuo *et al*. (2001) performed functional expression cloning and isolated cDNA clone that could render a resistant cell line to a cell line susceptible to a wild type strain isolated from B95a cells. Isolated cDNA was shown to encode SLAM also labeled CD150 (Cocks *et al*., 1995; Sidorenko *et al*., 1993). Chinese hamster ovary (CHO) cells were reported as non-susceptible to MeV, but transfected with human SLAM they became susceptible to the B95a cell-isolated MeV strains. Interestingly, MeV Edmonston strain infected both SLAM-expressing CHO cells as well as CD46-expressing CHO cells. Thus, the Edmonston strain was able to use both molecules, SLAM and CD46, as the receptors. When SLAM-expressing CHO cells were treated with anti-SLAM antibody, MeV infection was completely blocked (Tatsuo *et al*., 2001).

The vaccine strain Ondersteport of Canine distemper virus (CDV), another member of the genus *Morbillivirus,* and two B95a-isolated strains of CDV caused extensive CPE in normally resistant CHO and Vero cells after expression of canine SLAM (Seki *et al*., 2003). The vaccine strain AKO of Rinderpest virus (RPV) produced strong CPE in CHO cells expressing bovine SLAM. However, cell culture-adapted RPV efficiently infects CHO, Vero, and many other cell lines. But wild-type RPV, a virulent parent of the most common vaccine strains requires CD150 as a receptor (Baron, 2005). Dog SLAM has 65% amino acid identity to human SLAM, while bovine SLAM has 65 and 69% amino acid identity to human and dog SLAM, respectively. This fact suggested that SLAM might be a receptor for several, if not all morbilliviruses. Vero cells stably expressing canine SLAM are highly sensitive to CDV from clinical specimens and only a single amino acid substitution in the CDV hemagglutinin (H) can allow the virus to adapt to the SLAM of B95a cells (Seki *et al*., 2003). Vero cells expressing canine SLAM were not only useful for primary isolation but were also efficient for titration of the virus isolated from fresh tissues and for the study of growth profiles of CDV (Lan *et al*., 2005).

Our previous study has showed SLAM as a co-receptor for PPRV (Pawar *et al*., 2008). In this work we investigated basal SLAM mRNA levels in PBMCs of sheep, goats, cattle, and buffaloes in relation to their susceptibility to PPRV.

Materials and Methods

Cells and virus. Vero cells were used for growth and assay of the virus. PPRV, strain Arasur adapted to the Vero cells and available at 75th passage was used for infection of PBMCs of cattle, buffalo, sheep, and different breeds of goats. Virus titrations were performed in Vero cells following conventional techniques and virus titers were expressed as tissue culture infective doses 50/ml ($TCID_{50}$) (Dhinakar Raj *et al*., 2000).

Animals. Different breeds of apparently healthy and age-matched goats namely Barbari, Tellicherry, Jamunapari, and Kanni cross (University Research Farm and Embryo Biotechnology Laboratory, Madhavaram) were used for collection of blood followed by cultivation of PBMCs. Sheep, crossbred buffalo, and crossbred cattle were also used in the same way. Three animals were used from each breed for collection of blood.

PBMCs cultivation, stimulation, and infection. Blood was collected in heparinized vaccutte vials and thoroughly mixed with equal volume of RPMI-1640 medium, layered over 3 ml of Histopaque-1077 (Sigma) and centrifuged at 2,250 x g for 30 mins. The interphase rich in PBMCs was collected in a separate sterile tube and washed twice with RPMI-1640 and centrifuged at 1,650 x g for 10 mins. The washed PBMCs were resuspended in RPMI-1640 with 10% fetal calf serum (Gibco) and live cells were counted in hemocytometer using trypan blue staining. The PBMCs (10^6 cells/ml) from different species of animals were used after cultivation under a 5% CO_2 at 37°C for 4 hrs for determining SLAM expression using real-time PCR. The cells were infected with PPRV at $10^{4.0}$ $TCID_{50}$/ml and after 72 hrs the virus replication was assessed using real-time PCR for PPRV H gene with Sybr Green I chemistry. Another set of similarly infected cells was frozen and thawed and used for determination of the virus titers in Vero cells.

The PBMCs collected from different breeds of goats were seeded in 6-well plates at the concentration 10^6 cells/ml. One set of plates was cultured without mitogen stimulation and the other set was stimulated using con A with 10 µg/ml (Sigma) for 4 hrs. Then, SLAM expression was determined using real-time PCR. Stimulated and non-stimulated PBMCs were infected with PPRV and 72 hrs post infection the virus replication and titers were assessed.

Total RNA extraction and cDNA synthesis. Total RNA was extracted using TriZol (Invitrogen) from con A stimulated or non-stimulated PBMCs and cDNA was synthesized using the High capacity cDNA archive kit (Applied Biosystems) following manufacturer's instructions.

Real-time PCR for SLAM mRNA. The primers and labeled probe synthesized using goat SLAM sequences (Table 1) were cross-reacting with sheep, cattle, and buffalo SLAM, so the same primers and probes were used for SLAM expression study in PBMCs from different species. PBMCs isolated from different breeds of goats were also used for studying SLAM expression with and without stimulation with con A. ß-actin was used as an endogenous control for the real-time PCR. Separate master-mixes were prepared for endogenous control and target gene (SLAM) using TaqMan Universal PCR master mix 2x, 10 µl, TaqMan primers and probe (1 µl, 20x) and 100 ng cDNA according manufacturer's procedure (Applied Biosystems). Each reaction was prepared in triplicate. A no-template control (NTC) was prepared using only water (instead of cDNA). The plate was centrifuged in the cooling plate centrifuge (4ºC) at 560 rpm for 3–5 mins to get rid the sample of air bubbles. Then the plate was kept inside the real-time PCR machine, Model 7500 (Applied Biosystems). The program was run using the following universal cycling condition consisting of

Table 1. Primers and probes used in real-time PCR

mRNA	Primer/probe	Sequence*	Position (nt)
SLAM	Forward	CACTTTAGCCTGCAAATGAAGCT	410–432
	Probe	FAM ACTCCGCAAATTAAG MGB	449–463
	Reverse	TTCCTGGGTGGAGTTCAACAC	464–484
ß-actin	Forward	GGCTGTGCTGTCCCTGTAC	156–174
	Probe	FAM CTGGCCGCACCACTG MGB	179–193
	Reverse	CCGGAGTCCATCACGATGC	194–214
PPRV H	Forward	GCCGACGATGGACCTGAG	1568–1585
	Reverse	GTAGCACCACACCTTATGA	1757–1737

*Acc. Nos. of SLAM, ß-actin, and PPRV H genes: DQ 228869, AF481159.1, AJ 512718.2, respectively.

one cycle at 50ºC for 2 mins, one cycle at 95ºC for 10 mins, and 40 cycles at 95ºC for 15 secs and 60ºC for 1 min. The C_t values were recorded for both the target and endogenous controls. The data was considered only in the case, when NTC showed no amplification. The basal SLAM expression and PPRV H expression in buffalo PBMCs was used as a calibrator and the fold increase in SLAM expression in cattle, sheep, and goat were determined and correlated with the respective virus titers. Similar experiments were performed with both stimulated and non-stimulated PBMCs collected from different breeds of goats. Using the Kanni cross breed as a calibrator, the increase in SLAM expression in non-stimulated PBMCs from other breeds of goats was calculated. However, when comparisons was made between SLAM expression between non-stimulated and stimulated PBMCs of different breeds of goats, the levels of SLAM expression in non-stimulated PBMCs were used as calibrator for each respective breed of goat.

Real-time PCR for PPRV H mRNA. The replication of PPRV propagated in PBMCs from different species and breeds of goats were assayed by real-time PCR for PPRV H gene with Sybr Green I chemistry. RNA was extracted and cDNA synthesized from PPRV infected PBMCs as described earlier. The PCR mix for Sybr Green I assay was prepared using 10 µl of the Sybr Green I PCR master mix (Applied Biosystems), 1 µl (1 pmol) of PPRV H/actin, forward primers, 1 µl (1 pmol) of PPRV H/actin reverse primers, and 100 ng cDNA adjusted in water in 8 µl to give a total reaction volume of 20 µl. The real-time PCR was run under the universal cycling condition. In addition to C_t values the melting curve was recorded to assess the specificity of amplification. When con A was used to stimulate the PBMCs, the increase in PPRV H expression and the increase in virus titers were determined and their correlations established.

Real-time PCR results analysis. The C_t values were recorded for each gene expression assayed in real-time PCR either using the TaqMan or Sybr Green I chemistry. All the C_t values were mean of triplicate samples tested. The ΔC_t values indicated the difference in the C_t values between the target gene and the endogenous gene. The $\Delta\Delta C_t$ value indicated the difference between the ΔC_t of sample and the ΔC_t of calibrator. The calibrator was such a species of animal or breed of goat that exhibited the highest C_t value (e.g. lowest expression of target gene). The change in the gene expression is calculated as $2^{-\Delta\Delta Ct}$. Correlation coefficients were calculated between these changes using MS Excel package.

Results

SLAM expression in PBMCs of various ruminant species and effect of virus infection

The change ($2^{-\Delta\Delta Ct}$ values) in the basal SLAM expression in the different ruminant species of animals was showed (Table 2). Cattle had similar SLAM expression as buffaloes (1.1-fold higher). Sheep had 1.4-fold higher SLAM expression while goats had 2.2-fold higher levels as compared to buffaloes. PPRV H expression was highest in goat PBMCs with 3.9-fold higher replication levels than in buffalo PBMCs, while sheep PBMCs supported 1.6-fold higher replication (Table 3). Virus titers in various PBMCs ranged from $10^{-4.6}$ to $10^{-5.4}$ $TCID_{50}$/ml. There was high correlation coefficient of 0.986 between fold changes in basal

Table 2. Basal SLAM expression in PBMCs of various ruminant species

Animal (n = 3)	C_t for ß-actin (mean ± SD)	C_t for SLAM (mean ± SD)	ΔC_t	$\Delta\Delta C_t$ (fold increase)	$2^{-\Delta\Delta C_t}$
Buffalo	19.113 ± 0.106	28.720 ± 0.186	9.607	0	1
Cattle	20.213 ± 0.044	29.744 ± 0.431	9.531	-0.076	1.054
Sheep	22.710 ± 0.206	31.804 ± 0.217	9.094	-0.513	1.427
Goat (Telicherry cross)	20.127 ± 0.079	28.625 ± 0.005	8.498	-1.109	2.157

Table 3. PPRV H expression and PPRV titers in PBMCs of various ruminant species

Animal (n = 3)	C_t for ß-actin (mean ± SD)	C_t for PPRV H (mean ± SD)	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$ (fold increase)	Log virus titer (log $TCID_{50}$/ml)
Buffalo	19.165 ± 0.015	30.585 ± 0.500	11.42	0	1	4.6
Cattle	19.480 ± 0.038	30.894 ± 0.192	11.41	-0.01	1.006	4.8
Sheep	20.17 ± 0.057	30.930 ± 0.235	10.76	-0.66	1.580	5.2
Goat (Tellicherry cross)	21.973 ± 0.172	31.447 ± 0.401	9.47	-1.95	3.863	5.4

Table 4. SLAM expression in PBMCs of various goat breeds

Goat breed (n = 3)	C_t for ß-actin (mean ± SD)	C_t for SLAM (mean ± SD)	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$ (fold increase)
Barbari	21.692* ± 0.003	29.812 ± 0.252	8.120	-4.71	26.172
Tellicherry	20.127 ± 0.079	28.625 ± 0.001	8.498	-4.33	20.112
Jamunapari	20.348 ± 0.034	30.590 ± 0.247	10.242	-2.58	5.979
Kanni Cross	18.13 ± 1.826	30.96 ± 0.529	12.83	0	1

Table 5. Effect of con A stimulation on SLAM expression in PBMCs of various goat breeds

Goat breed (n = 3)	Non-stimulated PBMCs			Stimulated PBMCs			$\Delta\Delta C_t$ for SLAM A-B	$2^{-\Delta\Delta C_t}$ for SLAM (fold increase)
	C_t for ß-actin (mean ± SD)	C_t for SLAM (mean ± SD)	ΔC_{tA}	C_t for ß-actin (mean ± SD)	C_t for SLAM (mean ± SD)	ΔC_{tB}		
Barbari	21.69 ± 0.003	29.81 ± 0.252	8.12	19.44 ± 0.058	25.56 ± 0.068	6.12	-2.00	4.000
Telicherry	20.12 ± 0.079	28.62 ± 0.001	8.49	20.31 ± 0.186	26.16 ± 0.102	5.84	-2.65	6.276
Jamunapari	20.34 ± 0.034	30.58 ± 0.247	10.23	20.27 ± 0.057	27.41 ± 0.184	7.13	-3.10	8.574
Kanni cross	18.13 ± 1.826	30.96 ± 0.529	12.83	17.12 ± 1.82	25.93 ± 1.301	8.81	-4.02	16.223

Table 6. Effect of con A stimulation on PPRV H expression in PBMCs of various goat breeds

Goat breed (n = 3)	Non-stimulated PBMCs			Stimulated PBMCs			$\Delta\Delta C_t$ for PPRV H A-B	$2^{-\Delta\Delta C_t}$ for PPRV H (fold increase)
	C_t for ß-actin (mean ± SD)	C_t for PPRV H (mean ± SD)	ΔC_{tA}	C_t for ß-actin (mean ± SD)	C_t for PPRV H (mean ± SD)	ΔC_{tB}		
Barbari	20.16 ± 0.003	30.66 ± 0.602	10.50	20.12 ± 0.058	29.86 ± 0.327	09.74	-0.76	1.693
Telicherry	20.12 ± 0.079	30.93 ± 0.225	10.81	20.88 ± 0.186	30.54 ± 0.229	9.66	-1.15	2.219
Jamunapari	20.34 ± 0.034	31.53 ± 0.274	11.19	20.57 ± 0.057	29.98 ± 0.247	9.41	-1.78	3.434
Kanni cross	20.03 ± 0.025	32.39 ± 0.384	12.36	20.96 ± 0.045	31.38 ± 0.157	10.42	-1.94	3.837

Table 7. Effect of con A stimulation on PPRV titer in PBMCs of various goat breeds

Goat breed (n = 3)	Log virus titer in non-stimulated PBMCs	Log virus titer in con A-stimulated PBMCs	Increase in log virus titer due to stimulation
Barbari	5.0	5.4	0.4
Tellicherry	6.0	6.4	0.4
Jamnapari	6.1	7.0	0.9
Kanni cross	5.2	6.56	1.36

SLAM expression and PPRV H expression. A correlation coefficient of 0.920 was observed between SLAM fold changes and virus titers. PPRV H expression and virus titers also had a high correlation coefficient of 0.845 indicating that PPRV H expression reflected the virus titers properly.

Table 4 shows the changes in the basal SLAM expression in the PBMCs of different breeds of goats. Jamunapari, Tellicherry, and Barbari goats had 6.0-, 20.1-, and 26.2-fold increase respectively, in the basal SLAM expression over that observed in Kanni cross goats.

Effect of the con A stimulation on SLAM expression and virus infection

SLAM expression was increased following con A stimulation in all animals. The ΔC_t values decreased from 8.1-12.8 in non-stimulated culture to 5.8–8.8 in con A-stimulated one, what was reflected as 4.0 to 16.2-fold increase in SLAM expression over non-stimulated PBMCs (Table 5).

PPRV H expression was increased in the con A-stimulated PBMCs derived from all breeds. The ΔC_t values decreased from 10.5–12.4 in non-stimulated cultures to 9.4-10.4 in con A-stimulated ones, what was reflected as a 1.7- to 3.8-fold increase in PPRV H expression over non-stimulated PBMCs (Table 6). Viral titers also increased from $10^{0.4}$ to $10^{1.4}$ $TCID_{50}$/ml after infection of con A-stimulated PBMCs (Table 7).

A correlation coefficient of 0.894 between fold increase in SLAM expression and increase in PPRV H expression was seen. Similar correlation coefficient between the increase in SLAM expression and increase in virus titers was 0.961. As before (0.845), there was a high correlation (0.946) between the increase in PPRV H gene expression and increase in the virus titers.

Discussion

The presence or expression levels of specific viral receptors could be a major determinant of the host specificity. To determine the possible reason(s) for the species-specific occurrence of PPR disease, PPRV replication and virus titers were determined in PBMCs of ruminant species and compared with SLAM expression. PPRV replicated to a high level in sheep and goat PBMCs that is in agreement with the status of infection/disease in these animals. Similarly, SLAM expression was also found to be highest in goat PBMCs followed by sheep. Cattle and buffalo had lower expression levels that were similar. Although SLAM expression in different species of animals may not be comparable because of differences in age, nutrition, and managerial conditions that might play a role in an variation of these levels, this study was an indicator that lower SLAM expression in PBMCs of cattle and buffalo may be related to lowered PPRV replication in these species and absence of clinical signs in the case of infection.

When SLAM expression was compared between different breeds of age-matched male goats, Barbari breed was found to have the highest SLAM expression. On the other hand, Kanni crossbreed had the lowest levels. However, whether these levels directly translate into higher disease susceptibility for Barbari breed of goats or higher disease resistance for Kanni crossbreed needs to be established through experimental infections, although subjective evidence suggests that indigenous Kanni cross breed do not contract PPR disease commonly.

Con A stimulation of PBMCs increased SLAM expression 4- to 16-fold. Highest fold increase was observed in Kanni cross breed of goat. This increased level of SLAM expression correlated with increase in PPRV replication and virus titer. Although basal SLAM expression in Kanni cross breed of goat was low, after con A stimulation it had the highest increase in SLAM expression and also the highest increase in PPRV replication and virus titer. This clearly confirmed that increase of SLAM levels made PPRV replication and titers higher. Sreenivasa *et al*. (2006) showed that PPRV virus grew to higher titers in B95a cells, which express SLAM in comparison to Vero cells, which does not express SLAM. Our previous study using small interfering RNA to suppress SLAM confirmed that SLAM acted as a co-receptor for PPRV (Pawar *et al*., in press). However, it is possible that during an outbreak of the disease, the susceptibility of particular breeds of goats to PPR may be in relation to their levels of SLAM expression (in addition to the virus factors such as dose) at the time of virus entry.

The results of this study indicated that SLAM mRNA expression levels could be one of the determinants of different susceptibility of ruminant species to PPR. However, further effort with experimental infections of different breeds of animals may elucidate this mechanism in more detail.

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