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REGULAR ARTICLES

Immunomodulating dose of levamisole stimulates innate immune response and prevents intestinal damage in porcine rotavirus diarrhea: a restricted-randomized, single-blinded, and placebo-controlled clinical trial

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Abstract

A restricted-randomized, single-blinded, placebo-controlled clinical trial was conducted to examine whether immunomodulating dose of levamisole (LMS) can stimulate certain antiviral immune markers by measuring the concentrations of interferon-γ (IFN-γ), nitric oxide (NOx), and total immunoglobulin G (IgG); prevents the gut injury; and reduces fecal consistency and dehydration scores in rotavirus type A (RVA)-positive piglet diarrhea. The trial was executed between November 2015 and May 2016 in an institute owned experimental swine production farm. The naturally RVA-exposed diarrheic piglets were used in the study. The piglets born between November 2015 and May 2016, age group of 0 to 2 weeks and confirmed for RVA-positive diarrhea, were randomized to receive supportive treatment (ST) or ST along with levamisole (LMS + ST) at immunomodulating dose. Simultaneously, six piglets were randomly selected from healthy population and kept as placebo control. The primary outcome was reduction of fecal consistency and dehydration scores (≤ 1) over the trial period. The secondary outcome was reduction of concentration of gut injury marker and stimulation of immunomodulatory function. The LMS + ST treatment progressively improved the total leukocyte, neutrophil count, IgG concentration ($p < 0.05$), and reduced the intestinal fatty acid-binding protein 2 (IFABP-2) concentration in RV-positive diarrheic piglets than ST only. Although NOx and IFN-γ concentrations were enhanced initially on day 3, however, the values reduced significantly on day 5 in response to LMS + ST compared to ST. Interestingly, the scores of fecal consistency and dehydration of RVA-positive diarrheic piglets were dropped much earlier (on day 3) in response to LMS + ST than ST alone. The results indicate that LMS along with supportive treatment non-specifically modulated innate immunity and restored intestinal gut health, and thus, LMS may represent an additional therapeutic agent for management of RVA-inflicted piglet diarrhea.

Keywords Rotavirus · Levamisole · Intestinal fatty acid-binding protein 2 · Nitric oxide · Interferon-gamma · Immunoglobulin G

Abbreviations

LMS	Levamisole
IFN-γ	Interferon-γ
NOx	Nitric oxide
IgG	Immunoglobulin G
IFABP-2	Intestinal fatty acid-binding protein 2

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RVA	Rotavirus type A
ST	Supportive treatment
NSP1	Non-structural protein 1
RNA-PAGE	Ribonucleic acid-polyacrylamide gel electrophoresis
RT-PCR	Reverse transcription polymerase chain reaction
PBMCs	Peripheral blood mononuclear cells
cDNA	Complimentary DNA

Introduction

The success of commercial pig farm depends on a reliable supply of healthy piglets with good genetic potential for quality pork production. Several infectious, non-infectious, and managemental factors have been identified for increased neonatal piglet mortality. Porcine rotavirus A diarrhea is one of the principal causes of mortality in neonatal piglets and causes huge economic loss in swine production industry (Saurabh et al. 2018). Although the ubiquitous systemic infection of rotavirus is evident from recent study, small intestinal epithelium is the primary target of rotavirus in neonatal animals (Boshuizen et al. 2003; Kim et al. 2011). The major pathological changes attribute to ischemia and damage of gut epithelium and consequently lead to the structural and functional abnormalities of enterocytes and crypt cells (Osborne et al. 1991). Gut barrier dysfunction and hyper-permeability of the intestine play a pivotal role in pathogenesis of rotavirus-associated enteritis. Among the various physiological responses, the activation of host's innate immune response is essential to act as a first line of defense to suppress the virus multiplication (Koyama et al. 2008).

For therapeutic management of porcine rotavirus diarrhea, there is no specific antiviral drug in clinical practice and symptomatic treatment is the only choice (De et al. 2014). Further, there is no prophylaxis against porcine rotavirus in India. Therefore, when disease prevention remains the long-term goal, improvement of existing supportive therapy-based treatment strategies are still needed to reduce the mortality and increase the economic return of swine farming. A number of immunomodulators have been tried to improve the existing therapeutic response by stimulating the host immunity to virus infection (Malemud 2017). Previously, anti-rotaviral effects of *Lactobacilli* and *Bifidobacterium* strains and hyper-immune egg yolk have been reported in calves, mouse, and porcine models owing to their stimulatory effect on the immune system (Bilbao et al. 2006; Kang et al. 2015). Recently, we demonstrated immunomodulatory effect of β-glucan in porcine rotavirus infection with significant reduction in severity of diarrhea (Chethan et al. 2017a). Levamisole (LMS), a synthetic and soluble phenylimidazothiazole (2,3,5,6-tetrahydro-6-phenylimidazole (2,1-b)thiazole), is a known anthelmintic drug with potent immunostimulating activity and has been used in farm animals as immunomodulator or growth promoter (Kumar et al. 1999). Stimulation of T helper-1 cells with subsequent upregulation of interleukin-2, interleukin-12, and interferon-γ by LMS has been reported. Further, it inhibits the action of endogenous immunosuppressive factors like soluble immune response suppressor (Gupta 2016). Owing to its immunomodulatory and antiviral activity, levamisole has been used in the management of some viral diseases in human beings (Gupta 2016). It can enhance the functional activity of leukocytes and accelerates the antimicrobial function of mononuclear cells and neutrophils (Wright et al. 1977). However, its immunomodulatory effect has not been explored in pig rotavirus diarrhea. The purpose of the investigation reported herein was to compare the therapeutic efficacy of supportive treatment with or without LMS, administered at immunomodulating dose for the treatment of piglet diarrhea naturally exposed to rotavirus infection in farm condition. We hypothesize that administration of immunomodulating dose of LMS along with supportive treatment would stimulate certain antiviral immune indicators and prevent the gut injury in piglets naturally infected with porcine rotavirus type A (RVA).

Materials and methods

Care and use of animals

The animal experimentation during the present study was conducted following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (approval number F.25/08/2016-CPCSEA).

Trial facilities

The present trial was conducted in an experimental swine production farm (28° 24′ N and 79° 26′ E, 179 m above sea level) in the northern Indian state, which had a capacity of 160 pigs. The institute maintains the elite herd of pigs for research, standardization of management practices for the benefit of researchers, pig farming professionals, and farmers. All animals were maintained under standard managemental conditions. The pigs were housed in concrete pig sties constructed as per the standard space requirements. The sows were housed in individual pens. A herd health program for an organized herd was followed; it involved routine prophylactic and therapeutic measures including treatment of clinical cases, supplementation of multivitamins, vaccinations against foot-and-mouth disease and swine fever, and deworming at regular interval as per the standard recommendations. There was one teaching veterinary clinical complex which is located approximately 1.5 km away from the herd.

Trial animals

The animals utilized in the study were recently born piglets from sows present in the farm. The piglets allocated to the study were approximately 0 to 2 weeks of age and weighed between 1.2 to 2.3 kg. After birth at the farm, the piglets were weighed; teeth of piglets were clipped, marked with individual animal identification number, and reared with sows until weaning at 42 days of age. The piglets were injected iron injection on the 3rd and 14th day of age to prevent piglet anemia.

Experimental design

The present study was an interventional study. Although the variables are quantitative data, the prerequisites required for sample size calculation by power analysis like standard deviation were not available. The sample size calculation could not be performed by the power analysis method. Therefore, the resource equation method was applied to decide the sample size (Festing and Altman 2002).

The piglets born in the farm between November 2015 and May 2016 were participants for the study. Piglets were reared with sows in their designated farrowing pen until weaning. Each pen was checked twice daily by expert animal health specialist for evidence of diarrhea. In this study, the cases of piglet diarrhea were defined on the basis of fecal consistency and dehydration status (Pereira et al. 2002; McLamb et al. 2013) with or without elevated rectal temperature. In the study period, a total of 42 diarrheic piglets (0 to 2 weeks) had been identified. About 1.0-g fecal material was collected from each case and subjected to RNA-polyacrylamide gel electrophoresis (RNA-PAGE) for diagnosis of rotavirus type A (RVA). Based on specific genome migration pattern by RNA-PAGE, a total of 14 samples were found positive for type A rotavirus infection. Positive samples by RNA-PAGE were further confirmed by VP6 gene-based reverse transcription polymerase chain reaction (RT-PCR). Out of 14 RV-positive diarrheic piglets, 12 diarrheic piglets were randomly assigned equally to 2 experimental groups as group ST, which received supportive treatment (ST) consisting of antibiotic (ceftriaxone @ 10 mg/kg body weight, intramuscular route, twice a day for 3 days), fluid (25.0 mL Ringer's lactate in 3 divided doses orally per day) as well as anti-inflammatory drug (meloxicam @ 0.4 mg/kg body weight, intramuscular route, once a day) whenever indicated and group LMS + ST, which received above mentioned supportive treatment and levamisole (LMS @ 2.5 mg/kg body weight, subcutaneous route, single dose on day 0). Simultaneously, six piglets were randomly selected from healthy population and kept as placebo control and received only normal saline orally twice a day for 3 days. The piglets were returned to their designated pens on the same day after allocation and treatment. Individual piglet was considered as study unit. The treatment events, including treatment date, presumptive diagnosis, and observations were executed under the supervision of experienced animal health personnel. The entire treatment regimen was completed for all the participating piglets and no piglet died during the study period. The primary outcome is the reduction of fecal consistency and dehydration scores (≤ 1) over the trial period. The secondary outcome is the reduction of concentration of gut injury marker and stimulation of immunomodulatory function. This study was a single-blinded, restricted-randomized (randomization within the healthy and RVA-positive diarrheic piglets), placebo-controlled clinical trial. The trial flowchart and trial events schedule of the study are depicted in Fig. 1 and Table 1.

Laboratory investigation

Detection of rotavirus in piglets stool samples

Fecal samples (approximately 1.0 g) from diarrheic piglets were collected and transported to a rotavirus laboratory for RVA confirmation. Initially, RVA was detected on the basis of typical genome segments electrophoretic pattern in RNA-polyacrylamide gel electrophoresis (RNA-PAGE) and later VP6 gene-based reverse transcription polymerase chain reaction (RT-PCR) amplification.

For RVA screening by RNA-PAGE, samples were processed by preparing a 10% (*w*/*v*) fecal suspension in phosphate-buffered saline (PBS; pH 7.2). For viral RNA extraction, standard protocol was followed as used in our earlier study (Malik et al. 2012). For electrophoresis, resolving gel (10%) and staking gel (5%) were prepared as per standard method (Laemmli 1970). The migration of dsRNA genomic segments followed by silver staining was carried out following method described earlier (Svenson et al. 1986; Malik et al. 2012). Post-staining gel was documented and preserved in 10% ethanol.

For RT, the protocol described earlier was followed (Malik et al. 2012) and complimentary DNA (cDNA) was stored at − 20 °C for further use. Diagnostic PCR targeting the partial group-specific VP6 gene to identify RVA was used to detect rotavirus infection (Mondal et al. 2013). The PCR reaction was carried out as described in an earlier publication (Malik et al. 2012).

Blood sampling and processing

Before the initiation of treatment, 3.0 mL of blood was drawn from each piglet and kept in sterile vials with or without K_2EDTA. Subsequently, blood was drawn on days 3 and 5. Out of 3.0 mL blood, 0.5 mL was taken for hematological parameters, 1.0 mL for separation of peripheral blood

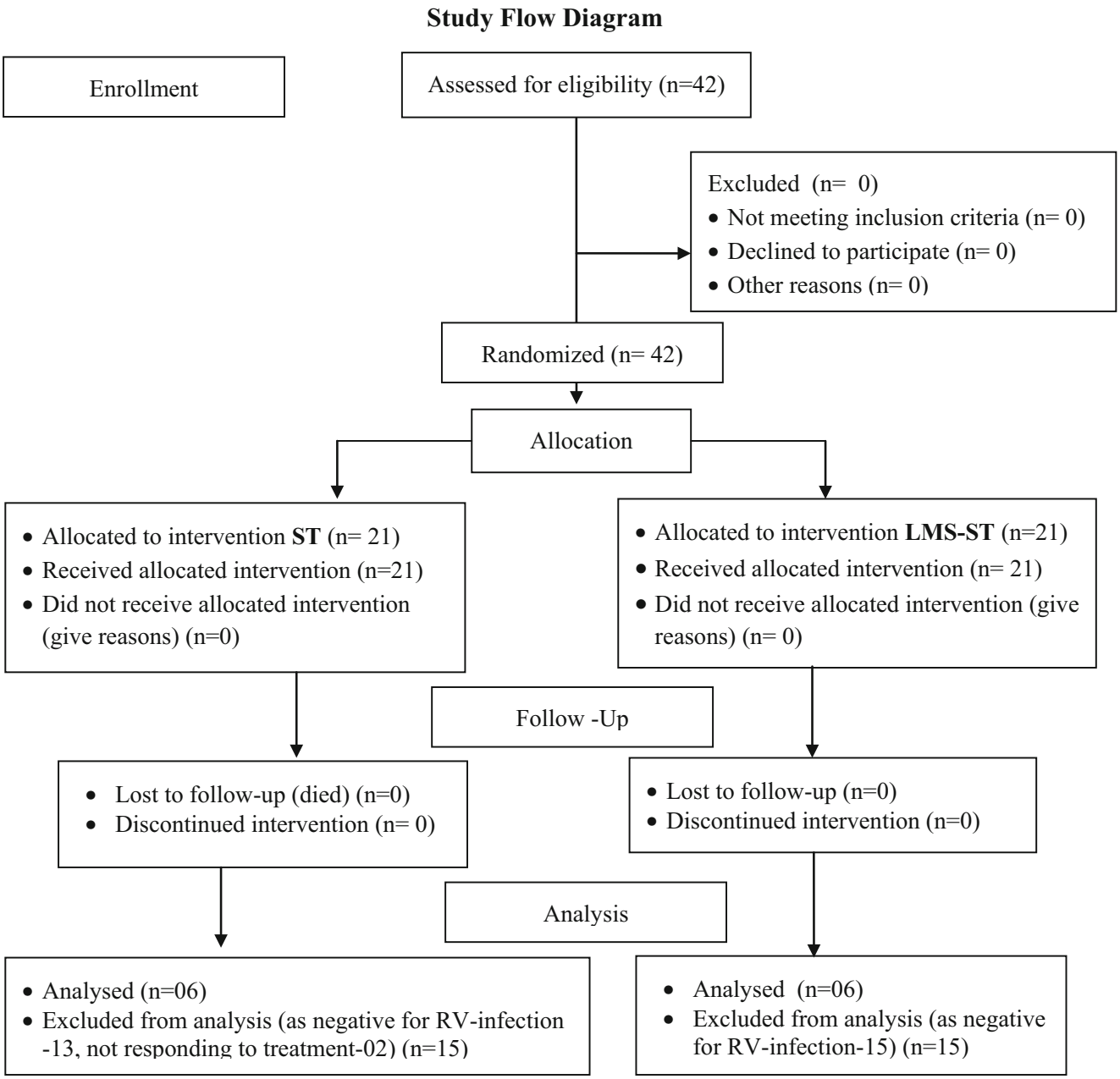

Fig. 1 Study flow diagram

mononuclear cells (PBMCs), and the remaining volume (1.5 mL) was centrifuged at 1500 rpm for 10 min to retrieve the serum. It was kept at − 20 °C until examination of intestinal fatty acid-binding protein 2 (IFABP-2), serum immunoglobulin G (IgG), and interferon-γ (IFN-γ).

Hemogram

The hemogram (total erythrocyte count, TEC; total leukocyte count, TLC; hemoglobin, Hb; and differential leukocyte count, DLC) analysis was evaluated manually following standard method (Jain 1986) before treatment (day 0), day 3, and day 5 of treatment.

Isolation of PBMC and measurement of NOx

Two milliliter of histopaque (density 1077, Sigma Chemicals, St. Louis, MO, USA) was layered over 1.0 ml of blood which was diluted in 1.0 mL of PBS (10 mM, pH 7.4) and centrifuged at 400×*g* for 40 min. The layer in between plasma and histopaque containing PBMCs were separated in a sterile tube and rinsed with PBS twice at 300×*g* for 10 min at 4 °C. After viability checking of the cells, cell number was adjusted to

Table 1 Trial events schedule

	On-study period screening/baseline			Short-term follow-up		Long-term follow-up	
Procedures	Day 1	Day 2	Day 3	Day 4	Day 5	Day 15	Day 42
Informed consent	X						
Eligibility assessment	X						
Demographics	X						
Medical history	X						
Enteritis symptom history	X						
Stool sample	X						
Results from routine laboratory tests	X						
Randomization	X						
Trial drug administration (twice daily)	X	X	X				
Overnight nursing assessment of readiness for discharge (once daily)*	X	X	X	X	X		
Medical assessment by study doctor (twice daily)*	X	X	X	X	X		
Medically significant adverse event assessment						X	X
Concomitant medications	X	X	X	X	X		

*During the period of trail

1.0×10^6 cells/mL. Twenty five micrograms of lipopolysaccharide (1.0 mg/mL, lipopolysaccharide, *E. coli* 0111: B4 Cat. #0219933405, MP Biomedicals, Santa Ana, California, USA) were used to stimulate cells at 37 °C for 24 h following which nitric oxide was measured. The concentration of NOx in stimulated cells was measured following protocol outlined in previous study (Yucel et al. 2012).

Assay of interferon-gamma, immunoglobulin G, and intestinal fatty acid-binding protein 2 in serum

All the stored serum samples were brought to room temperature before analysis. The concentrations of IgG, IFN-γ, and IFABP-2 were estimated employing porcine Pig IgG ELISA kit (KOMABIOTECH, Komabiotech Inc. Korea), IFN-γ ELISA kit (Quantikine ELISA, R&D Systems, Inc. Minneapolis, USA), and ELISA kit for fatty acid-binding protein 2, intestinal (FABP-2) [Cloud-Clone Corp. Houston, USA], respectively following manufacturer's guidelines.

Clinical observation

On days 0, 3, and 5 of treatment, fecal consistency and dehydration scores were evaluated to assess the clinical recovery (Pereira et al. 2002; McLamb et al. 2013).

Data analysis

Data were analyzed with statistical software (package SAS v 9.3 (SAS Institute, Inc. 2011, Cary, NC, USA) to determine whether the variables were significantly different in LMS-treated piglets when compared to ST-treated and control piglets over the time. The data were analyzed using repeated measurement model with piglet as subject and period as repeated measurement. The one-way ANOVA was used to compare the treatments with respect to hematology, NOx, IgG, IFN-γ, IFABP-2, fecal consistency, and dehydration scores, when an interaction was found, Tukey's post hoc test was used to determine statistical significance between the different treatment groups. An individual piglet was considered the experimental unit. When an interaction was present between the treatment and variables as determined by one-way ANOVA, the p value obtained by Tukey's post hoc test for the relevant treatment groups was presented, when an interaction was not present, the p value obtained from the one-way ANOVA was presented. Results are expressed as means ± SEM. The p value of 0.05 was considered as statistical selection limit for all tests.

Results

Diagnosis of RV infection based on symptoms and molecular techniques

Dehydration and watery diarrhea of yellow color were the prominent clinical symptoms of RV-infected diarrheic piglets. Out of 42 fecal samples from diarrheic piglets, 14 and 19 samples were found positive for mammalian RVA based on RNA-PAGE (Fig. 2) and RT-PCR (Fig. 3), respectively. No clinical symptoms of diarrhea were noted in healthy piglets.

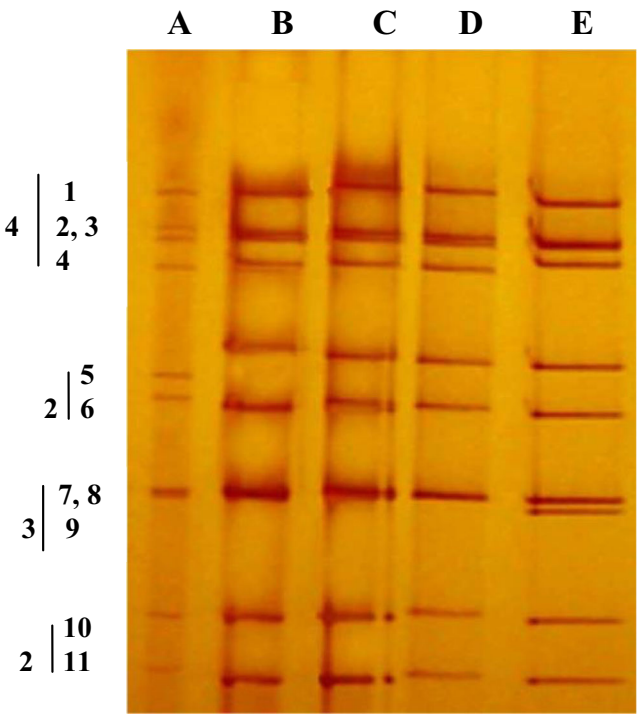

Fig. 2 Genomic segments migration pattern of mammalian rotavirus in RNA-PAGE. Lanes A, B, C, D, and E showing typical pattern of mammalian rotavirus type A (4–2–3–2). RNA segments were numbered according to the electrophoretic mobility in polyacrylamide gel, and visualization was done using silver staining

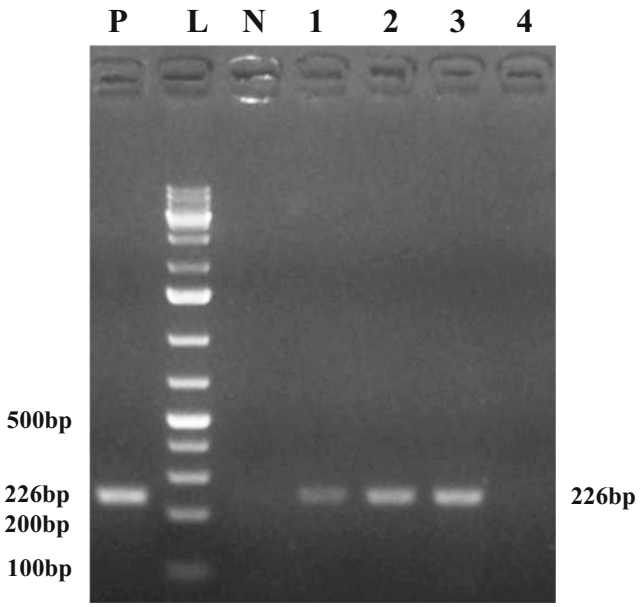

Fig. 3 Agarose gel electrophoresis of the RT-PCR products for identification of porcine rotavirus type A using primers RVA-D-226-FP and RVA-D-226-RP in fecal samples collected from piglets suffering from diarrhea. Lane L denotes 1 kb plus DNA ladder, lane P denotes positive control (product size 226 bp), lane N denotes negative control (no template), lanes 1, 2, and 3 denote positive for porcine rotavirus type A, and lane 4 denotes negative for porcine rotavirus type A in fecal samples

Effect of treatment on hemogram

The RV infection significantly ($p < 0.05$) increased Hb and TEC and decreased absolute leukocyte and neutrophil counts in blood when compared with healthy control piglets before treatment. The LMS + ST reduced ($p < 0.05$) the mean Hb and TEC values on days 3 and 5 in RV-infected piglets, with a significant interaction between treatment and TEC ($p = 0.002$) but not for Hb ($p = 0.220$). The TLC and neutrophil count were significantly ($p < 0.05$) improved on days 3 and 5 in the LMS + ST group but no such improvement was noticed in the ST group, whereas the mean lymphocyte count was significantly lower on day 3 and thereafter the value (day 5) did not differ statistically from pre-treatment value in RV-infected piglets received LMS + ST. When the mean TLC and DLC were compared between RV-infected piglets (LMS + ST vs ST), it was observed that the TLC and neutrophil count were significantly ($p < 0.0.5$) higher on day 5 in the LMS + ST group than the ST group, with a significant interaction between treatment and neutrophils count ($p = 0.030$) (Table 2).

Effect of treatment on IFN-γ and NOx

The changes of IFN-γ and NOx concentrations among all groups during the treatment period were shown in Fig. 4. The RV infection significantly ($p < 0.001$) increased the IFN-γ and NOx concentrations in diarrheic piglets than healthy piglets before institution of any treatment. The concentrations of IFN-γ and NOx were persistently elevated until day 5 in the ST group, whereas the values reduced significantly on day 5 ($p < 0.001$) in the LMS + ST group, with a significant treatment effect on IFN-γ ($p < 0.001$) and NOx ($p < 0.001$).

Effect of treatment on IgG and IFABP-2

A significantly ($p < 0.001$) decreased concentration of IgG and increased concentration of IFABP-2 was noticed before treatment in RV-infected diarrheic piglets than healthy piglets (Fig. 5). A pronounced enhancement of IgG and drop of IFABP-2 concentrations was observed on days 3 ($p < 0.01$) and 5 ($p < 0.001$) in RV-infected piglets regardless of treatment. The IgG concentration was significantly ($p < 0.001$) higher and IFABP-2 concentration was significantly ($p < 0.001$) lower on day 5 in the LMS + ST group when compared with the ST group. The effect of treatment was significant ($p < 0.001$) for both IgG and IFABP-2.

Effect of treatment on fecal consistency score and dehydration score

The scoring for fecal consistency and dehydration was performed for all groups on days 0, 3, and 5 and the results were

Table 2 Changes of hemogram and leukogram kinetics over time in six healthy piglets (control), six rotavirus-infected piglets treated with supportive treatment ST (ST), and six rotavirus-infected piglets treated with supportive treatment plus levamisole (LMS + ST). The data were analyzed by Tukey's post hoc test using repeated measure analysis. A statistically significant interaction was found between treatment and TEC ($p < 0.01$) and neutrophils count ($p < 0.05$) but such interaction was not present for Hb ($p = 0.220$), TLC ($p = 0.868$), lymphocytes count ($p = 0.935$), monocytes count ($p = 0.916$), eosinophils count ($p = 0.704$), and basophils count ($p = 0.576$). The values were expressed as mean ± SEM

Groups ($n = 6$)	0d	3d	5d
Hb (g/dL)			
control	$8.681 \pm 0.115^{a,A}$	$8.676 \pm 0.111^{a,A}$	$8.680 \pm 0.105^{a,A}$
ST	$9.995 \pm 0.105^{a,B}$	$9.761 \pm 0.090^{b,B}$	$9.361 \pm 0.227^{b,A}$
LMS + ST	$9.625 \pm 0.172^{a,B}$	$9.425 \pm 0.148^{b,B}$	$9.105 \pm 0.123^{c,A}$
TEC ($\times 10^6$ cells/μL)			
control	$5.033 \pm 0.063^{a,A}$	$5.033 \pm 0.072^{a,A}$	$5.046 \pm 0.058^{a,A}$
ST	$6.038 \pm 0.064^{a,B}$	$5.730 \pm 0.049^{b,B}$	$5.366 \pm 0.036^{c,B}$
LMS + ST	$5.748 \pm 0.123^{a,B}$	$5.613 \pm 0.102^{b,B}$	$5.416 \pm 0.066^{c,B}$
TLC ($\times 10^3$ cells/μL)			
control	$10.328 \pm 0.250^{a,A}$	$10.293 \pm 0.209^{a,A}$	$10.430 \pm 0.208^{a,A}$
ST	$6.915 \pm 0.303^{a,B}$	$6.966 \pm 0.283^{a,B}$	$7.116 \pm 0.247^{a,B}$
LMS + ST	$7.050 \pm 0.281^{a,B}$	$7.223 \pm 0.258^{b,B}$	$7.685 \pm 0.263^{c,C}$
Neutrophils ($\times 10^3$ cells/μL)			
control	$4.083 \pm 0.149^{a,A}$	$4.008 \pm 0.068^{a,A}$	$4.068 \pm 0.103^{a,A}$
ST	$1.507 \pm 0.117^{a,B}$	$1.755 \pm 0.097^{b,B}$	$1.771 \pm 0.112^{b,B}$
LMS + ST	$1.649 \pm 0.094^{a,B}$	$1.983 \pm 0.071^{b,B}$	$2.306 \pm 0.086^{c,C}$
Lymphocytes ($\times 10^3$ cells/μL)			
control	$5.196 \pm 0.113^{a,A}$	$5.272 \pm 0.792^{a,A}$	$5.301 \pm 0.114^{a,A}$
ST	$4.614 \pm 0.177^{a,A}$	$4.422 \pm 0.188^{a,B}$	$4.550 \pm 0.155^{a,B}$
LMS + ST	$4.639 \pm 0.184^{a,A}$	$4.483 \pm 0.186^{b,B}$	$4.585 \pm 0.160^{ab,B}$
Monocytes ($\times 10^3$ cells/μL)			
control	$0.755 \pm 0.115^{a,A}$	$0.720 \pm 0.038^{a,A}$	$0.782 \pm 0.046^{a,A}$
ST	$0.610 \pm 0.033^{a,B}$	$0.569 \pm 0.036^{a,B}$	$0.582 \pm 0.035^{a,B}$
LMS + ST	$0.585 \pm 0.024^{a,B}$	$0.577 \pm 0.032^{a,B}$	$0.599 \pm 0.022^{a,B}$
Eosinophils ($\times 10^3$ cells/μL)			
control	$0.274 \pm 0.020^{a,A}$	$0.257 \pm 0.023^{a,A}$	$0.296 \pm 0.020^{a,A}$
ST	$0.169 \pm 0.009^{a,B}$	$0.207 \pm 0.016^{a,B}$	$0.200 \pm 0.022^{a,B}$
LMS + ST	$0.175 \pm 0.016^{a,B}$	$0.179 \pm 0.014^{a,AB}$	$0.179 \pm 0.014^{a,B}$
Basophils ($\times 10^3$ cells/μL)			
control	$0.018 \pm 0.018^{a,A}$	$0.034 \pm 0.022^{a,A}$	$0.000 \pm 0.000^{a,A}$
ST	$0.012 \pm 0.012^{a,A}$	$0.011 \pm 0.011^{a,A}$	$0.011 \pm 0.011^{a,A}$
LMS + ST	$0.00 \pm 0.000^{a,A}$	$0.00 \pm 0.000^{a,A}$	$0.000 \pm 0.000^{a,A}$

0d, before initiation of treatment; *3d*, day 3 of initiation of treatment; *5d*, day 5 of initiation of treatment

^{A,B,C} Represents significant difference among the groups within a day ($p < 0.05$)

^{a,b,c} Represents significant difference among the days within a group ($p < 0.05$)

presented in Table 3. A significant drop of fecal consistency score on days 3 ($p = 0.001$, 0.004) and 5 ($p = 0.001$, < 0.001) was noticed in all RV-infected groups received irrespective of treatment. As regards to dehydration score, it was reduced on day 5 only in the ST group, whereas it was from days 3 to 5 in the LMS + ST group. Interestingly, the scores of fecal consistency ($p = 0.004$) and dehydration ($p = 0.017$) were significantly lower on day 3 in the LMS + ST group when compared with the ST group. Both scores of the LMS + ST group of day 5 did not vary significantly with the healthy group. An interaction between the treatment and fecal consistency score ($p < 0.001$) and dehydration score ($p = 0.002$) was significant.

Discussion

In case of most viral diseases, the therapy is primarily based on supportive care. Sometimes, supportive treatment is not sufficient to effectively treat the viral diseases. A broad range of immunomodulators has been tried as adjunctive therapy to improve the therapeutic response in viral diseases where the immune response plays a critical role (Chethan et al. 2017a; Malemud 2017). In this study, we investigated whether the immunomodulating dose of levamisole as adjunctive therapy stimulates innate immune response, prevents intestinal injury, and reduces fecal consistency and dehydration scores in piglet diarrhea caused by type A rotavirus by a single-blinded,

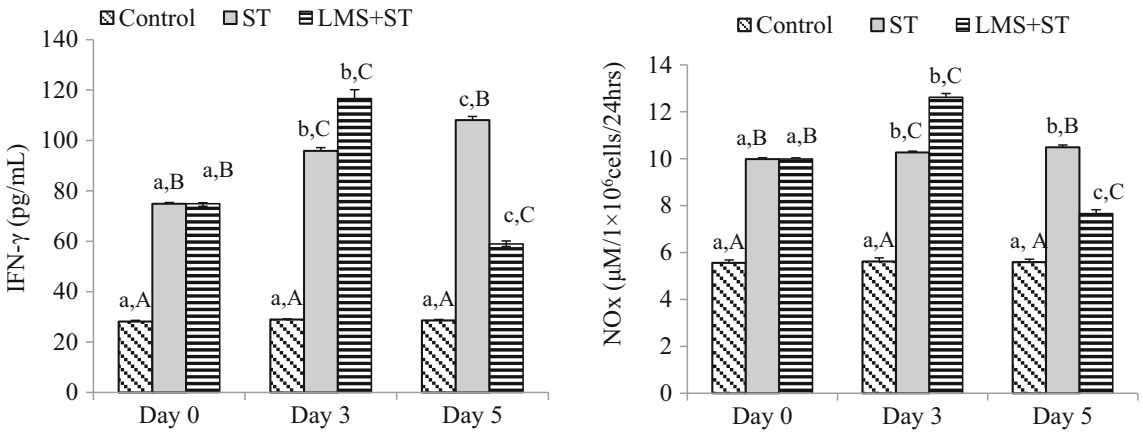

Fig. 4 Changes of IFN-γ and NOx concentration over time in six healthy piglets (control), six rotavirus-infected piglets treated with supportive treatment ST (ST), and six rotavirus-infected piglets treated with supportive treatment plus levamisole (LMS + ST). The data were analyzed by Tukey's post hoc test using repeated measure analysis. The IFN-γ and NOx concentrations progressively increased from day 0 ($p < 0.001$, < 0.01) to 5 ($p < 0.001$, < 0.01) in rotavirus-infected piglets in response to ST and the values remained persistently higher than the control group. LMS + ST treatment significantly increased the IFN-γ and NOx production on day 3 ($p < 0.001$) and thereafter, the values significantly reduced on day 5 ($p < 0.001$) from pre-treatment value. Statistically significant interaction was found between the treatment and IFN-γ ($p < 0.001$) and NOx concentrations ($p < 0.001$)

restricted-randomized, placebo-controlled clinical trial. We found marked hemoconcentration characterized by elevated Hb% and TEC levels with leukopenia and neutropenia in rotavirus-positive diarrheic piglets than healthy piglets. Hemoconcentration in the present study is the outcome of fluid deficit due to the episodes of diarrhea (Sastry 1985; Malik et al. 2014), whereas pronounced leukopenia and neutropenia in diarrheic piglets might be either due to systemic infection by rotavirus or drain of leukocytes and inadequate availability of leukocytes in the inflamed gastrointestinal tract with the massive demand (Goddard et al. 2008; Greenberg and Estes 2009; Shim et al. 2012; Chethan et al. 2017b). However, the leukocyte count was significantly ($p < 0.05$) improved on days 3 and 5 in rotavirus-positive diarrheic piglets treated with LMS + ST. Although the effect of levamisole on leukocyte subpopulation in different stress conditions is not completely understood, it has been reported that levamisole stimulates the immune system especially some components of the non-specific immune system and proliferation of leukocytes (Perera and Pathiratne 2008). The levamisole treatment has been reported to improve the leukocyte and neutrophils counts in boars and immunosuppressant rats (Bilandzic et al. 2010; Mohamed and Abdou 2014).

The innate immune response of host against virus infection is mostly subjugated by the production of interferon and interferon-stimulated genes (Mehta et al. 2012) and nitric oxide in association with an intact interferon system plays a vital role in antiviral protection (Reiss and Komatsu 1998). In viral

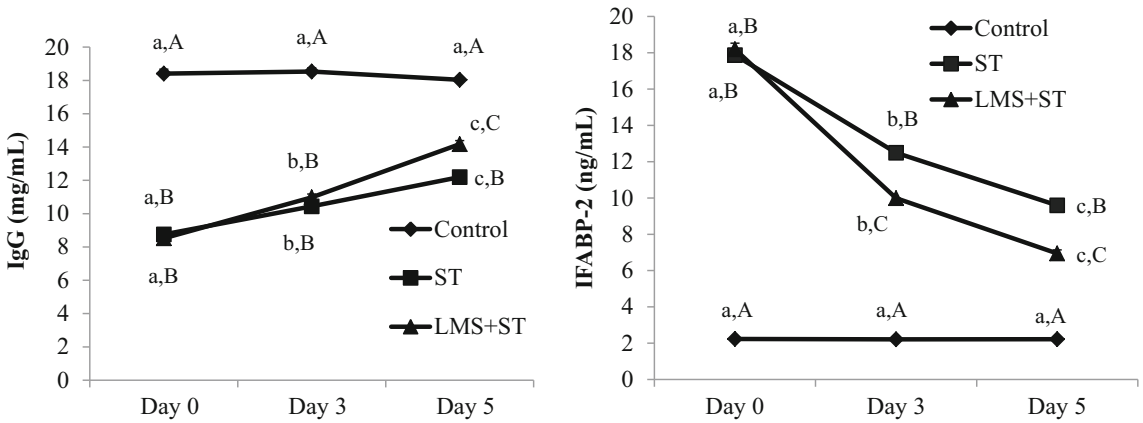

Fig. 5 Changes of IgG and IFABP-2 concentration over time in six healthy piglets (control), six rotavirus-infected piglets treated with supportive treatment ST (ST), and six rotavirus-infected piglets treated with supportive treatment plus levamisole (LMS + ST). The data were analyzed by Tukey's post hoc test using repeated measure analysis. The significant enhancement of IgG concentration on days 3 ($p < 0.01$) and 5 ($p < 0.01$, < 0.001) and significant reduction of IFABP-2 on day 3 ($p < 0.001$) and 5 ($p < 0.001$) was observed compared to pre-treatment value in rotavirus-infected piglets either treated with ST or LMS + ST. Statistically, a significant interaction was found between the treatment and IgG concentration ($p < 0.001$) and IFABP concentration ($p < 0.001$)

Table 3 Changes of clinical observation of animals (fecal consistency score, dehydration score) over time in six healthy piglets (group A), six rotavirus-infected piglets treated with supportive treatment ST (ST, group B), and six rotavirus-infected piglets treated with supportive treatment plus levamisole (LMS + ST, group C). The data were analyzed by Tukey's post hoc test using repeated measure analysis. The fecal consistency score was significantly reduced on days 3 ($p < 0.01$) to 5 ($p < 0.001$) whereas dehydration score was significantly reduced on days 5 ($p < 0.01$) in rotavirus-infected piglets in response to ST. However, LMS + ST treatment significantly reduced fecal consistency and dehydration scores on days 3 (< 0.01) to day 5 (< 0.01). Statistically, a significant interaction was found between the treatment and fecal consistency score ($p < 0.001$) and dehydration score ($p < 0.001$). The values were expressed as mean ± SEM

Groups	0d	3d	5d
Fecal consistency score			
control	$0.500 \pm 0.223^{a,A}$	$0.333 \pm 0.210^{a,A}$	$0.333 \pm 0.210^{a,A}$
ST	$3.500 \pm 0.223^{a,B}$	$2.667 \pm 0.210^{b,B}$	$1.667 \pm 0.210^{c,B}$
LMS + ST	$3.500 \pm 0.223^{a,B}$	$1.500 \pm 0.223^{b,C}$	$1.167 \pm 0.307^{b,AB}$
Dehydration score			
control	$0.333 \pm 0.210^{a,A}$	$0.333 \pm 0.210^{a,A}$	$0.167 \pm 0.166^{a,A}$
ST	$2.833 \pm 0.166^{a,B}$	$2.167 \pm 0.307^{a,B}$	$1.167 \pm 0.166^{b,B}$
LMS + ST	$2.667 \pm 0.210^{a,B}$	$1.167 \pm 0.166^{b,C}$	$0.667 \pm 0.210^{b,AB}$

0d, before initiation of treatment; *3d*, day 3 of initiation of treatment; *5d*, day 5 of initiation of treatment

^{A,B} Represents significant difference among the groups within a day ($p < 0.05$)

^{a,b,c} Represents significant difference among the days within a group ($p < 0.05$)

infection, IFN-γ induces the expression of inducible nitric oxide synthase (iNOS) that synthesizes nitric oxide from wide variety of cells (Kitasato et al. 2007). In the current study, the elevated concentrations of IFN-γ and NOx in rotavirus-positive diarrheic piglets seemed to be an antiviral immune response guided compensatory mechanism. Further, accumulating evidences suggest that enterotoxigenic NSP 4 of rotavirus promotes iNOS expression by increasing intracellular calcium concentration through activation of phospholipase C and IP3 mobilization (Berkes et al. 2003; Borghan et al. 2007). The average concentrations of IFN-γ and NOx were significantly higher on day 3 and then the values decreased significantly on day 5 in diarrheic piglets received LMS + ST than ST alone. Levamisole can enhance both humoral and cellular immune responses with strong production of IL-2, IL-12, and IFN-γ in poultry, rat, and mice (Szeto et al. 2000; Jin et al. 2004; Habibi et al. 2012; Sinha et al. 2015). Further, the antimicrobial action of levamisole is mediated through increased nitric oxide production (Kumar et al. 2014). It seems that the marked increase of IFN-γ and NOx concentration on day 3 could be due to immunostimulatory property of levamisole and subsequent decrease on day 5 might be a result of the reduction of viral concentration in the system. It has been reported that the concentration of PRRS virus in tonsil decreases with elevated concentration of serum IFN-γ in pigs (Ladinig et al. 2014).

Intake of sufficient quantity of high quality colostrum is essential to provide systemic immunoglobulin protection through absorption of IgG from enteric infection in neonatal animals (Besser et al. 1988; Cabrera et al. 2012). Serum IgG prevents rotavirus infection by providing appropriate mucosal immunity in humans (Westerman et al. 2005). In the current study, a significantly ($p < 0.05$) lower IgG concentration in rotavirus-positive diarrheic piglets than healthy piglets could be due to compromised suckling ability of affected piglets or intestinal loss of immunoglobulins with consequent hypoproteinemia as evident in rotavirus infection. Comparatively, lower IgG concentrations were reported in rotavirus-positive diarrheic calves than rotavirus-negative diarrheic calves (Rocha et al. 2016). They further opined that an optimal passive immunity transfer of immunoglobulins decreases the possibility of rotavirus-associated calf diarrhea. The average concentration of IgG was significantly ($p < 0.01$) improved on days 3 and 5 compared to day 0 in RV-positive diarrheic piglets following LMS-ST rather than ST alone. It has been reported that levamisole increases serum IgG level and non-specific humoral immune response in young boars, newborn calves, and weanling pigs (Pekmezci and Cakiroglu 2009; Bilandzic et al. 2010; Valpotic et al. 2016).

Rotavirus has a tendency of marked tropism for upper epithelium of the small intestine, resulting in destruction of infected enterocytes and secretion of numerous biomarkers in the system (Bugarcic and Taylor 2006). One of such potential biomarkers is I-FABP, which is released into circulation during intestinal damage of animals and human being (Dundar et al. 2012). In our study, significantly ($p < 0.05$) elevated concentration of IFABP-2 in rotavirus-infected diarrheic piglets may be the result of intestinal damage as rotavirus is reported to cause destruction of enterocytes and villus ischemia of small intestine (Berkeveld et al. 2008; Kittaka et al. 2014). The LMS treatment significantly decreased the IFABP-2 concentration in post treatment and the value of IFABP-2 was much lower on days 3 ($p < 0.001$) and 5 ($p < 0.001$) in the LMS + ST group than ST alone. It has been reported that LMS improves favorable gut health and intestinal functions by its immunostimulatory effect in weaned pigs (Bozic et al. 2002; Valpotic et al. 2013). The average scores of fecal consistency and dehydration scores on day 3 were significantly lower in rotavirus-positive diarrheic piglets treated with LMS + ST than ST alone. Valpotic et al. (2013) reported that levamisole reduces severity of diarrhea in pigs with enterotoxigenic *E. coli* infection by increasing proliferation of circulating and intestinal immune cells.

From the results of the present study, it is concluded that the incorporation of levamisole at immunomodulating dose in standard treatment schedule stimulate antiviral innate immune response and favors gut health recovery in piglet diarrhea caused by type A rotavirus. Levamisole may be used as a new candidate method for additional treatment of RV-infected piglet diarrhea. However, the immunotherapeutic

potential of levamisole needs to be further examined involving large number of piglets with other enteric diseases including rotavirus infection.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

Animal studies Animal studies were carried out humanely and according to national and international Animal Care and Use Committee protocols and following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (approval number F.25/08/2016-CPCSEA).

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