IMMUNO-STIMULATORY EFFECT OF LEVAMISOLE HYDRO CHLORIDE AND TRICLABENDAZOLE ON THE IMMUNE RESPONSE OF PESTE DES PETITS RUMINANTS (PPR) VACCINATION IN GOATS

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CERTIFICATE

This is to certify that the thesis entitled ***IMMUNO-STIMULATORY EFFECT OF LEVAMISOLE HYDROCHLORIDE AND TRICLABENDAZOLE ON THE IMMUNE RESPONSE OF PESTE DES PETITS RUMINANTS (PPR) VACCINATION IN GOATS"*** *submitted to the Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfilment of the requirements for the degree of* ***Master of Science in Parasitology****, embodies the result of a piece of bona fide research work carried out by* ***Nupur Dhar****, Registration No.* ***19-10157*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dated:
Place: Dhaka, Bangladesh

Dr. Uday Kumar Mohanta
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Dedicated

to

My beloved Parents

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ACRONYMS AND ABBREVIATIONS

Abbreviation		Full meaning
et al.	=	And others/Associates
HCl	=	Hydrochloric acid
M.S.	=	Master of Science
n	=	Sample number
No.	=	Number

CHAPTER 1

INTRODUCTION

Bangladesh is an over populated country, and facing problems to supply safe animal protein to the people. Total goat population in Bangladesh is 26.1 million (DLS, 2019). The contribution of goat production to the national economy is valued for its high quality meat, milk, skin and also for poverty alleviation through creation of employment opportunity (Debnath, 1995). Many commercial goat farms are already established in addition to the small and marginal farmer. Unfortunately, Peste des Petits Ruminants (PPR) is the most destructive viral disease in goat, affecting the young ranging from 3 to 18 months.

PPR is a highly contagious viral disease with its transboundary importances to small ruminants which was first reported in Ivory Coast in 1942 (Gargadennec *et al.,* 1942). PPR is popularly known as goat plague and has been put as an important OIE listed transboundary animal disease (TAD) of small ruminants in Bangladesh (FAO, 2015). In Bangladesh, PPR was confirmed first in Meherpur in 1993 (Sil *et al*., 2001) and the disease has high morbidity as much as 100%, with a mortality rate reaching upto 90% (Salih *et al.,* 2014). PPRV is a member of the genus *Morbillivirus* under the family *Paramyxoviridae* and order *Mononegavirales* (Braide, 1981). PPRV can be classified into four groups: three from Africa and one from Asia. The Asian strain was introduced to some African countries, including Cameroun, Central Africa Republic, Sudan, Morocco, Egypt, Tunisia, Algeria, and Uganda (Kwiatek *et al.*, 2011). Paramyxoviruses are enveloped viruses which are found as nucleocapsid structures. Characteristically, PPRV common with the other members of the group by its structural, biological, antigenic and molecular features. PPRV closely related to the rinderpest virus (RPV) of cattle, the measles virus (MV) of humans, the canine distemper virus (CDV) of dogs and some wild carnivores, and the morbilliviruses of aquatic mammals. The virus is very fragile and cannot survive for a long time outside susceptible host. Both crowding of animals in market places and close housing can increase risk for transmission of virus when it is introduced (Rahman *et al.,* 2011). The wet season also predispose to secondary bacterial infections, inducing the viral pneumonia (Abu *et al.,* 2008). The half-life of PPRV has been already estimated to be 22 minutes at 56°C and 3.3 hours at 37°C. The serological relationship

between PPRV and RPV is close but viruses are not identical; cross protection and neutralization tests may be employed to differentiate them (Hamdy *et al.,* 1976). Gibbs *et al.,* 1979 noted serological overlaps with measles, canine distemper and rinderpest viruses. PPRV is an acute disease with high fever, anorexia, ulcerative stomatitis, diarrhoea, purulent oculo-nasal discharges, and pleuropneumonia, consequently respiratory distress, cough and pneumonia. PPRV can be shed in nasal and ocular secretions, saliva, urine, feces and may pass through milk. Water, feed troughs and bedding can also probably transmit PPRV for a short time, but do not remain infectious for long periods (Shadmanesh, 2014).

PPR mostly affects the safe food production as well as reduces meat and milk production and thereby reduces income from the goat rearing. PPR severely affects rural economy, reduces genetic resources and ultimately endangers for breeding policies. Livestock Research Institute (LRI) produces a live attenuated PPR vaccine to control PPR in Bangladesh, and the vaccination program has been also continued in many parts of the country. However, PPR outbreaks continue to occur among vaccinated and non-vaccinated goats reported by Ahamed *et al*., 2019. Immune response after vaccination in goat may be a factor for the efficacy of vaccine.

Immune response of goats to vaccination can be accelerated by employing the simultaneous administration of immune stimulant along with PPR vaccine. Levamisole HCl is an anthelmintic drug with immune stimulant properties through stimulating T helper cells. Cytokines has been reported to help in stimulating the immune system, sustains the immune system and animal's response to a vaccine without having any specific antigenic effect in itself (Moertel *et al.,* 1990). Gastro-intestinal helminthiasis, mainly *Fasciola* spp. affects 75 to 88 % goats (Agrawal, 2003). Triclabendazole is a benzimidazole derivative which was reported to be 98-100% effective against fascioliasis (Wolff *et al*., 1983). Efficacy of vaccination mostly depend on the host's extrinsic and intrinsic factors. Parasitic infections induce low immune response and effect on vaccine efficacy. In addition, the combination of triclabendazole and levamisole HCl has been frequently used for the treatment and control of fascioliasis, gastro-intestinal round worms and lung worm in goat and sheep. Finally, this study was designed to determine the immune stimulatory effect of use of levamisole HCl and triclabendazole with local PPR vaccine in goats. Therefore, this study was undertaken for the very first time in Bangladesh, to evaluate the immune response of host body

against PPR when vaccine is used along with the combined use of levamisole HCl and triclabendazole.

Aims of the study

Considering the above facts the research work was undertaken to investigate the immune stimulatory effects of levamisole HCl and triclabendazole when used in combination or singly with homologous PPR vaccine in goats.

CHAPTER 2

REVIEW OF LITERATURE

Immune suppressed animal are to prone to any viral infection. Parasitic worms often affect the immune system's activity on efficaciously respond to a vaccine. Parasitic worms induce a Th^2-based immune response that is less responsive than normal to antigens and it's a major concern in developing countries where parasitic worms and the need for vaccinations exist in large number. It may be a cause that vaccines are often ineffective in developing countries (Kamal *et al*., 2006). Parasitic infestation causes a major constraint to livestock productivity across all agro zones and production systems, among them gastrointestinal nematodes having major economic importance in domesticated livestock rearing country.

Many types of synthetic anthelmintics are used for parasitic disease. Levamisole HCl, triclabendazole and combination of levamisole HCl and triclabendazole using against most of helminth infection (Lacey, 1990).

Levamisole HCl used as an anthelmintic to treat worm infections in animals. Levamisole HCl acts on the nematode muscle, interfering with the activity of a nicotinic acetylcholine receptor and, eventually depolarizes the muscle membrane and paralyzes the worm. Resistance appears to be due to ion channel desensitization in the nicotinic acetylcholine receptor (Barragry, 1994).

Triclabendazole, a narrow-spectrum agent with its highly specific activity against *Fasciola* spp. and *Paragonimus* spp. Like other benzimidazoles, the mechanism of action of triclabendazole results from inhibition of microtubule formation of parasite and also boost the immunity of host (Regenmortel *et al*., 1983). When the combination of triclabendazole and levamisole HCl is utilized for the treatment and control of fascioliosis, gastro-intestinal round worms and lung worm in cows, sheep and goat. Triclabendazole restrains energy digestion and levamisole HCl hinders neuromuscular transmission of helminths.

Levamisole HCl is an antiparasitic and immune stimulant that is used to treat many kinds of nematodes in cattle, sheep, and goats. It is also used in swine to treat *Ascaris* spp.,

Oesophagostomum spp., *Strongylus* spp., *Stephanurus* spp., and *Metastrongylus* spp. This drug has also been used in dogs as a microfilaricide for treating *Dirofilaria immitis*. Levamisole (Ergamisol) is also been found to enhance T-cell function and cellular immunity. The T and B lymphocytes (T and B cells) are involved in the acquired or antigen-specific immune response given that they are the only cells in the organism able to recognize and respond specifically to each antigenic epitope. The B cells have the ability to transform into plasmocytes and are responsible for producing antibodies (Abs). Thus, humoral immunity depends on the B cells while cell immunity depends on the T cells. In the present chapter, the processes of ontogeny are summarized for each type of lymphocyte together with their main characteristics, the different subpopulations described to date, the signalling mechanisms employed for their activation, and their main functions based on the immunological profile that they present. From the morphological point of view, T and B lymphocytes are indistinguishable since they are both small cells (8–10 microns in diameter) and each possesses a large nucleus with dense hetero-chromatin and a cytoplasmic border that contains few mitochondria, ribosomes, and lyzosomes. When they are activated by the antigenic stimulus, they may enlarge, thus increasing their cytoplasm and organelle number. Lymphocytes present receptors for antigen (Ag) recognition (TCR and BCR respectively) with different specificities on their surfaces. The genes that encode for these structures undergo a series of DNA recombinations, which provides them with immense phenotypic diversity. (Pelletier *et al.*, 1978) was believed that levamisole did not affect B-lymphocytes directly, but humoral response was influenced indirectly by affecting macrophages and T-lymphocytes.

Immunomodulators are substances that are able to regulate or modulate immune responses and (Blecha, 1988) reported it as Biological response modifiers. Immunomodulators may either augment or suppress immune response, though the term is often used to refer to substances that enhance the immune response.

The use of immunomodulators increases in poultry production by mitigating infectious bursal disease (IBD) and chicken infectious anemia (CIA). The immune system can be overwhelmed by aforementioned factors; hence, the need for routine usage of immunomodulators (Calnek *et al*., 1994; Karnatak *et al*., 1993; Porchezhian and Punniamurthy, 2006).

Levamisole is able to enhance both humoral and cellular immune responses in normal chickens reported by (Soppi *et al*., 1979). And its immunumodulatory property was later substantiated in diseased and stressed birds (Giambrone and Klesius, 1985; Emikpe *et al*., 2010).

Levamisole HCl has been used to boost immunity in infectious diseases, leprosy and cancer in humans (Kar *et al*., 1986; Mutch and Hutson, 1991; Katoch, 1996).

Symoens *et al*., (1977) summarized levamisole as a drug that enhances the immune response by restoring phagocyte and T-lymphocyte functions in immune deficient hosts, but does not increase immune response above normal level in the immunologically competent host. They also described it as protective effects of some vaccines and its potential advantage in various chronic and recurrent infections, immune deficient conditions and neoplastic diseases in man and animals.

Although various avian species have been reported to respond to the immune modulatory effect of levamisole, most of the studies in domestic poultry involved the immunomodulatory effect of the simultaneous administration of levamisole HCl with various vaccine types (Kulkharni *et al*., 1973).

A study conducted by (Sanda *et al*., 2008) on the immune modulatory property of levamisole in cockerels in a tropical environment showed that it was efficient immunomodulator. Moreover, another study by (Emikpe *et al*., 2010) showed that levamisole enhanced humoral immune response in chemically-immunosuppressed broilers.

Levamisole, itself as a compound capable of increasing defective immune responsive when treatment of Brucella abortus-vaccinated mice that reported by (Renoux and Renoux, 1972). Levamisole possesses anti-anergic properties and restores immune responses in both animals and humans stated by (Janssen *et al*., 1976; Symoens *et al*., 1979). Levamisole HCl also act on the chronic or inflammatory diseases such as herpes, aphtous stomatitis, rheumatoid arthritis and minimal change nephrotic syndrome and also exert antimetastatic effects as an adjuvant to conventional antineoplastic therapies (Amery *et al*., 1977; Spreafico *et al*., 1980).

DNA vaccination is useful for the the cell-mediated immune response It can reach expected level through the use of a chemical adjuvant Levamisole and elevated antibody production, with low level of Ig2a (Jin *et al*., 2004: Szeto *et al*., 2000).

Lejan *et al*., (1981) reported levamisole appeared to have an immunostimulating effect on the response of sheep to Blue Tongue vaccination with at repeated doses of 2.5 mg/kg prior to a vaccine being administered. In this experiment, four groups of unvaccinated pregnant sheep (8 sheep per group) were used. Group A received vaccine only; Group B received levamisole+vaccine; Group C received levamisole only; Group D was a non-treated control. Levamisole (Citarin L-10%) was administered three times weekly at an initial dose of 5.0 mg/kg of body weight and subsequently at 2.5 mg/kg of body weight. At 0 days of vaccination, all animals were serologically negative for Blue Tongue antibodies but after vaccination, there was a difference in antibody response in animals in the treated groups. Significantly, more animals in Group B developed Blue Tongue antibodies following vaccination than those in Group A.

Mojzisova *et al*., (2004) stated that the specific and non-specific immune response of levamisole were studied in dogs with altered immune function due to Giardiasis and vaccinated against canine parvovirus (CPV) infection. In immunosuppressed dogs combination of vaccine with levamisole treatment enhanced depressed phagocytic ability and stimulated proliferation activity of lymphocytes. Mojzisova and his team found higher titer of CPV antibodies by using levamisole

Babiuk *et al*., (1981) studied on thirty Holstein dairy calves, designed into two groups: test (n=15) and control (n=15) and levamisole HCl was administrated orally at a dose of 2 mg/kg body weight. Sampling from all the calves was continued at days 7, 14, 21 and 28 and remarkable change found in hematological test.

Das *et al*., (2016) examined that elevated antibody titer was obtained when levamisole was used prior to the PPR vaccination in treatment groups. In the present study, when the vaccine was administered with levamisole then the activity of immune effector cells also increased.

Anilkumar *et al*., (1986) studied in vivo immune stimulatory effect of levamisole for the first time in goats. Kids, spontaneously affected with pneumonia and clinically healthy kids were used for the study. On the basis of the leucocyte count, lymphocyte count, neutrophil count, neutrophils in the peripheral blood and concentration of gamma globulin in the serum, it was concluded that levamisole at the dose rate of 3.75 mg/kg body weight, caused immune stimulation in healthy kids. No immunomodulation was observed when there was sufficient immune stimulation due to infections. Levamisole at 3.75 mg/kg body weight was recommended for stimulating the immune system of healthy goats when outbreak of infections is prevalent or expected in a herd.

Johnkoski *et al*., (1997) found that levamisole increases the cytotoxic, cytostatic, and proliferative activity of murine non parenchymal liver cells in vitro. Their findings suggested that IFN alpha/beta, IL-6, and IL-1 play important regulatory roles in controlling the proliferative response of murine liver-associated T lymphocytes to levamisole. Finally, the proliferation of bone marrow cells was increased in mice given 5-fluorouracil.

Kang *et al*., (2005) investigated the usefulness of levamisole (LMS) as one such adjuvant for two different preparations of killed viral vaccines, derived from the foot mouth disease virus (FMDV) or the porcine respiratory reproductive syndrome virus (PRRSV) and tested respectively in BALB/c or C57 BL/6 mice. The results showed that LMS induced different types of immune responses in the host, depending on its dosage. Authors established that Levamisole can be used to induce Th1-biased immune responses when combined with killed-virus-based antiviral vaccines and that such adjuvant effect depends on the optimal LMS dosage.

Zhang *et al*., (2014) reported that to promote the development of immune systems, restore the function of depressed phagocytes and lymphocytes, and enhance both cell-mediated and humoral immune responses.

Chen *et al*., (2008) examined that the molecular mechanisms of levamisole HCl and triclabendazole in the activation and maturation of human monocyte-derived dendritic cells (DC) and human T cells. Treatment of DC with levamisole increased the presentation of CD80, CD86, CD83 and human leucocyte antigen D-related (HLA-DR) molecules on the cell membrane, as well as the production of interleukin (IL)-12 p40 and IL-10.Levamisole-treated human DC also enhanced T cell activation towards type 1T helper immune response by inducing interferon

secretion. Neutralization with antibodies against Toll-like receptor (TLR)-2 inhibited levamisole-induced production of IL-12 p40 and IL-10, suggesting a vital role for TLR-2 in signalling DC upon incubation with levamisole. The inhibition of nuclearfactor-kB, extracellular signal-regulated kinases 1/2 or c-Jun N-termina lkinases pathways also prevented the effects of levamisole on DC in producingIL-12 p40 or IL-10. Treatment with Levamisole at birth and at weaning is associated with an increase in average daily gain (Kumar *et al*., 1999).

Mohamed *et al*., (2016) examined the effects of levamisole and garlic oil singly and in combination on the immune response of Wistar rats. A total of 24 male Wistar rats were allocated into four equal groups: Control group, which was given ad libitum access to food and water; and groups 2-4, which were orally administered levamisole at 2.5 mg/kg body weight Garlic oil at 5 ml/kg and combination both drugs respectively for 4 consecutive weeks. Serum immunoglobulin IgG and IgM levels were measured using a radial immunodiffusion assay. Serum cytokine levels, including interferon (IFN)-γ, interleukin (IL)-5 and tumor necrosis factor (TNF)-α, were measured using enzyme-linked immunosorbent assay kits. Total blood counts were measured automatically using a cell counter. Serum lysozyme enzymatic activity was determined by measuring the diameters of the zones of clearance relative to lysozyme. Immunohistochemical detection of CD4 and CD8 was carried out using the streptavidin-biotin-peroxidase method. Furthermore, the mRNA expression levels of IL-4, IL-5 and IL-12 were measured in the leukocytes and thymus gland by semi-quantitative polymerase chain reaction. The results revealed that levamisole HCl increased serum levels of IFN-γ, IL-5 and TNF-α cytokines, At the cellular level, in the spleen, levamisole HCl increased immune reactivity of CD4 and CD8 and regulated the mRNA expression levels of IL-2, IL-4 and IL-5 (Abbas *et al*., 2005).

Stelletta *et al*., (2004) stated that levamisole act as an immune stimulant when it administered at repeated doses of 2.5 mg/kg prior to a vaccine being administered. The author and his team found immune response of sheep to Blue Tongue vaccination.

The therapeutic efficacy was evaluated by (Khair *et al*., 2010) through determination of parasitological, body weight gain and haematological findings and showed that a combination of triclabendazole and levamisole HCl acted against the parasites and immune response of the host.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling area

Blood samples were collected from Daspara village, Damodarpur union, Badarganj, Rangpur. The study was conducted at the laboratory of Microbiology and Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka-1207 during the period of July, 2019 to June, 2020.

Fig. 1: Geographical Location of Daspara village, Damodarpur Union, Badarganj

3.2 Animal selection

A total of sixty goats, aged two months or above were randomly selected from same management flocks belonging to small and marginal farmers. Then all the goats were grouped into four groups containing 15 goat in each group. Anthelmintic treatment fifteen (15). Groups A, B and C were used as treatment groups while the rest group D was kept as a control. All the experimental goats were fed with green leaves, wheat bran, and fresh water in clean water pot. Goats in Group A were treated with oral levamisole HCl and triclabendazole, and subsequently vaccinated with PPR vaccine. In

Group B, goats were treated with oral triclabendazole only, and subsequently vaccinated with PPR vaccine. Goats of Group C were treated with oral levamisole HCl only, and subsequently were vaccinated with PPR vaccine. On the contrary, control group D was only vaccinated with PPR vaccine.

Fig. 2: Experimental animals in the study area

3.3 Administration of levamisole HCl and triclabendazole:

Treatment group A was dewormed with a combination of triclabendazole and levamisole HCl drug at 18 mg/kg body weight before 15 days of vaccination. Treatment group B was dewormed with triclabendazole drug at 12 mg/kg body weight before 15 days of vaccination. Goats in treatment group C was dewormed with levamisole HCl only at 8 mg/kg body weight before 15 days of vaccination.

Table 1. : Anthelmintic drugs with their trade name, composition and dose

Sl. No.	Composition	Dose
01.	Levamisole Hydrochloride BP 600 mg and Triclabendazole INN 900 mg.	18 mg/kg body weight
02.	Levamisole Hydrochloride BP 300 mg per gram powder	8 mg/kg body weight
03.	Triclabendazole INN 900 mg.	12 mg/kg body weight

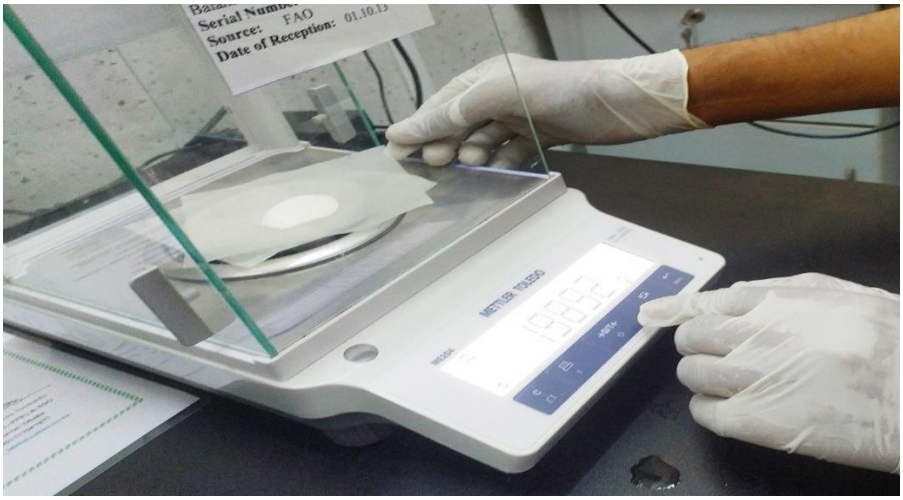

Fig. 3: Medicine preparation according to live weight of goats by electronic balance

3.4 PPR vaccination:

Goats of treatment groups and control group were vaccinated with live attenuated PPR vaccine at the rate of 1 ml/goat subcutaneously.

3.5 Blood sample collection:

For both treatment and control group, 2 ml blood/goat sample was collected after 0, 21 and 28 days of vaccination.

Fig.4: Administering PPR vaccine

Fig. 5: Blood collection from Jugular vein

3.6 Transportation and storage of samples

A total of 180 serum samples were collected from goat in the study period. The aim was to determine the level of antibody in the serum to assess the herd immunity in vaccinated and non-vaccinated goats. Initially, blood samples were collected from jugular-vein through puncturing with 5 ml sterile syringe. The suspected nasal swabs were also collected for molecular study. Finally, the sera samples were transported in an ice box to the laboratory where serological analysis was carried out using cELISA kit, and the collected samples were stored at −20°C until processed. For determination of goat blood profile, blood samples were transported in collection tube containing anti-coagulant (Sodium-EDTA).

Fig. 6: Serum samples were stored at −20°C temperature

3.7. Questionnaire based survey

To collect epidemiological data, a questionnaire based survey was conducted to reveal information regarding flock size, age and sex, health status, grazing management, yearly income, introduction of new animals, access to veterinary services, clinical signs of disease encountered, number of diseased and dead animals.

3.8. Laboratory Tests:

3.8.1 Total Leucocyte Count (TLC)

Neubauer counting chamber is widely used to count cells in body fluid, and its result is generally considered to be “gold standard.” A Neubauer counting chamber, there are usually a lot of border cells on the four outer lines of each counting square. The conventional method only counts cells

on one side of the upper and the lower boundaries and one side of the left and the right boundaries. The sum of cells on the two outer lines is used as the total number of cells on border. (Fuentes and Dot, 2009)

The procedure of TLC:

1. The counting chamber was placed on the microscope stage. The illumination is adjusted and the right upper groups of 16 squares for WBCs were focused. One can see all the squares in alone field.
2. 1 ml of Turk's fluid was taken in a watch glass.
4. The blood was diluted with Turk's fluid up to 11 mark by sucking the diluting fluid by the pipette kept in a watch glass. When the blood was taken up to the mark of 0.5, the blood was diluted only 20 times. The contents inside the bulb of the pipette was mixed for 4 minutes.
5. The first two drops of diluted blood from the pipette were discarded and then this mixture was run on the hemocytometer slide on both the chambers on both sides under a special coverslip.
6. The corpuscles were allowed to settle down for 4 minutes and then the number of WBCs are counted under low power of the microscope in the four counting areas.
7. The white cells were recognized by the retractile appearance, and by the slight colour given to them by stain contained in the diluting fluid.
8. The cells were counted under high power lens. WBCs can be counted in 16 squares under low power, and then under high power for comparing the results. The cells in four groups of 16 squares each (a total of 64 squares) were counted.

The number of WBC per mm^3 was calculated as shown below:

The WBCs counted in 16 squares and the volume of one square being 1/64 mm^3.

Volume of 16 square = 1/160 x 64 = 4/10 mm^3.

Thus, the total volume of diluted blood in which WBCs were counted = 4 / 10 mm^3

Let, the count in 4/10 mm^3 = 'X' WBCs.

Then 1 mm^3 of diluted blood will contain = 'X' x 10/4 white cells of WBCs.

As dilution is 20 times,

1 mm^3 of undiluted blood will contain = 'X' x 10/4 x 20, = 'X' x 200/4, = 'X' x 50 WBCs.

Fig. 7: Leucocyte count conducting under Microscope

3.8.2 Serological study

A monoclonal antibody (MAb) based cELISA (Diallo *et al.* 2007) was used for the detection of antibodies directed against the nucleoprotein of the PPR virus using approved competitive ELISA kit (ID vet. Innovative Diagnostics, France). The resulting coloration is depended on the quantity of specific antibodies present in the sample to be tested. In the absence of antibodies, a blue solution appeared which becomes yellow after the addition of the stop solution whereas the presence of antibodies, no coloration appeared.

Test procedure of cELISA

All the reagents were allowed to come to room temperature (25℃) before use. All reagents were homogenized by inversion or vortex.

a) 25 μl of dilution buffer were added to each well of the ELISA micro plate.
b) Then 25μl of the positive control were added to wells (A1 and B1), and 25 μl of the negative control were added to wells (C1 and D1).
c) 25 μl of the each sample were added to the remaining wells to test.
d) Then the plate was incubated at 37℃, 45 mnutes.
e) The plate was washed 3 times with approximately 300μl of the wash solution, and to avoid drying of the wells between washings.
f) After washing then the conjugate was prepared 1X by diluting the conjugate in 10X to 1/10 in dilution buffer 4 and again 100 μl of the conjugate 1X was added to each well.

g) The plate was incubated at 21℃, 30 minutes.

h) Then the plate was washed 3 times with approximately 300 μl

i) 100 μl of the substrate solution was added to each well.

j) Then the plate was incubated at 21℃ in the dark place for 15 minutes.

k) 100 μl of the stop solution was added to each well in order to stop the reaction.

l) Finally, the micro plate was read for OD values with multichannel spectrophotometric ELISA plate reader with interference filters of 450 nm, and the reading data was placed into data sheet of Microsoft® Excel program.

Test Validation of cELISA

The test was validated if:

a. The mean value of the negative control O.D (OD_{NC}) is greater than 30% of the OD.

$$OD_{NC} > 0.700$$

b. The mean value of the positive control (OD_{PC}) is less than 30% of the OD.

$$OD_{PC} / OD_{NC} < 0.3$$

Interpretation of test result

For each sample, the competition percentage was calculated using the following formula

$$S/N\ \% = \frac{OD_{sample}}{OD_{NC}} x\ 100$$

Sample presenting an S/N%:

- Less than or equal to 50% are considered positive
- Greater than 50% and less than or equal to 60% are considered doubtful.
- Greater than 60% are considered negative.

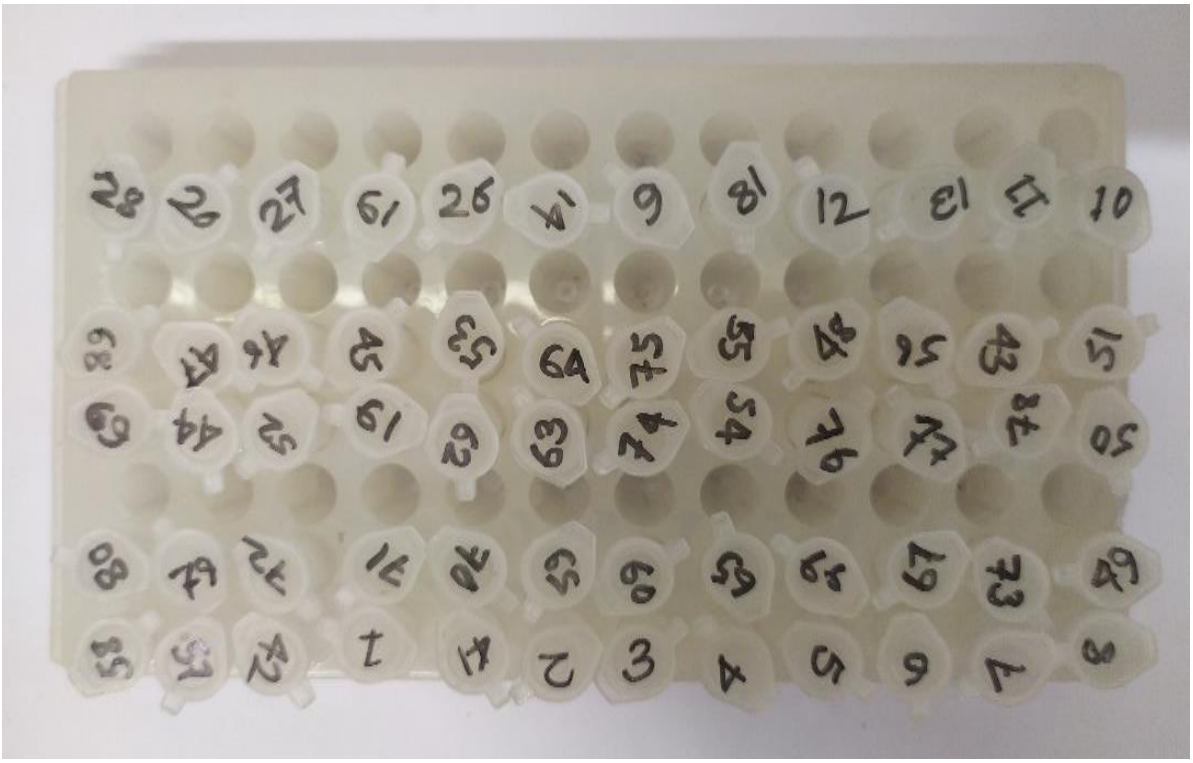

Fig. 8: Serum samples prepared for c-ELISA test

Fig. 9: Conducting c-ELISA test

Fig. 10: OD Values interpretation

3.9 Data Analysis

All the data were expressed with Statistical Package for the Social Sciences (SPSS) Software. *p-value* was determined by comparing means through univariate analysis at a 5% level of significance.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 RESULTS

In the experiment, TLC and c-ELISA value were studied to know the immune modulating activity of PPR vaccine in goats after treatment with a combination of levamisole HCl and triclabendazole, triclabendazole alone , levamisole HCl alone, and the control group D was not treated with any anthelmintic before PPR vaccination.

4.1.1 Total Leucocyte Count Value

Table 2. TLC value in different days of PPR vaccination

Group	**Day 0** (WBC/mm^3) (Mean ± SD)	**Day 21** (WBC/mm^3) (Mean ± SD)	**Day 28**(WBC/mm^3) (Mean ± SD)
A (levamisole HCl and triclabendazole)	6570.00 ±1136.065	8393.33 ±845.549	10016.67 ±1062.623
B (triclabendazole)	7083.33±612.275	8083.33 ±548.591	8716.67±525.991
C (levamisole HCl)	6380.00±783.946	7233.33±749.206	8630.00±755.645
D (Control)	6987.00±913.548	7439.82±687.251	8375.317±793.894

The TLC Values of Group A (n=15) at 0 day, 21 days and 28 days were 6570.00, 8393.33 and 10016.67, respectively. The TLC Values of Group B (n=15) at 0 day, 21 days and 28 days were 7083.33, 8083.33 and 8716.67, respectively. The TLC Values of Group C (n=15) at 0 day, 21 days and 28 days were 6380.00, 7233.33 and 8630.00, respectively. The TLC Values of Group D (n=15) at 0 day, 21 days and 28 days were 6987.00, 7439.82 and 8375.317, respectively.

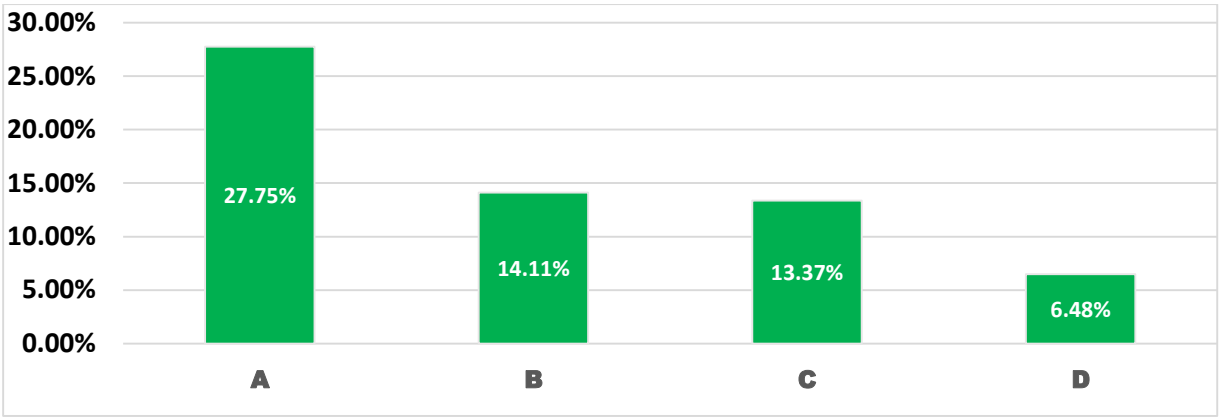

Fig. 11: Comparison of TLC Values in percentage increased among the experimental groups from the day of vaccination at the 21 days of vaccination.

The above values express the level of percentage increased at 21 days post vaccination in goats among the groups. In Group A, Group B, Group C and Group D, the level of TLC increased which followed by 27.75 %, 14.11 %, 13.37 % and 6.48 %, respectively.

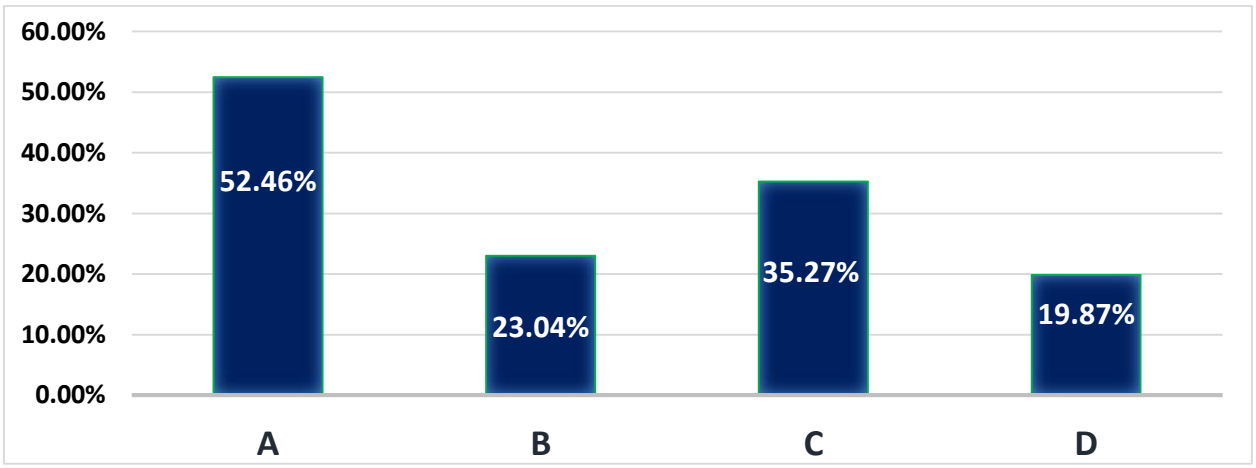

Fig. 12: Comparison of TLC Values in percentage increased among the experimental groups from the day of vaccination at the 28 days of vaccination.

The above values express the level of Percentage increased from the day of vaccination to the 28 days after the vaccination in goats among the groups. In Group A, Group B, Group C and Group D, the level of TLC increased by 52.46 %, 23.04 %, 35.27 % and 19.87 %, respectively.

4.1.2 Competitive screening ELISA Value

The antibody level of PPR virus in the goats was determined by Competitive screening ELISA test following manufacturer's protocol.

Table. 3 On the day of vaccination, group wise antibody titre (%) in the tested samples.

Group	Day 0			Day 21			Day 28		
	Sero-positive	Sero-negative	Sero-positivity (%)	Sero-positive	Sero-negative	Sero-positivity (%)	Sero-positive	Sero-negative	Sero-positivity (%)
A (n=15)	0	15	0	11	04	73.33	14	01	93.33
B (n=15)	0	15	0	09	05	60.00	09	06	60.00
C (n=15)	0	15	0	10	05	66.67	12	03	80.00
D (n=15)	0	15	0	08	07	53.33	11	04	73.33

All the goats selected for the experiment had non-vaccinated history.

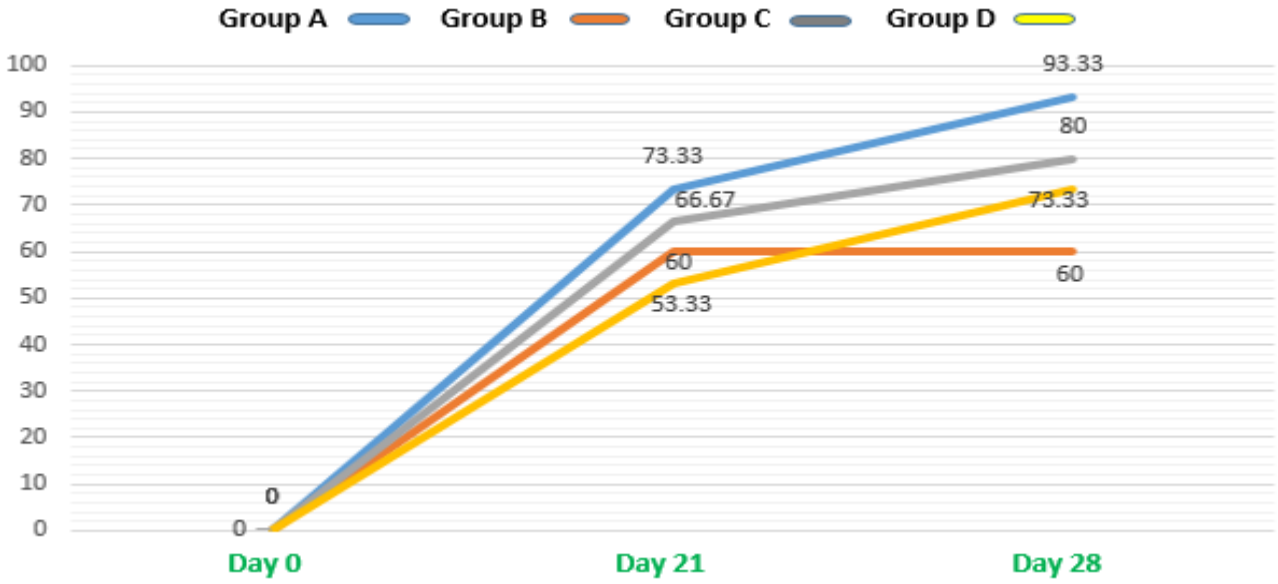

Fig. 13: Comparison among the antibody titre at 0 day, 21 days and 28 days of vaccination

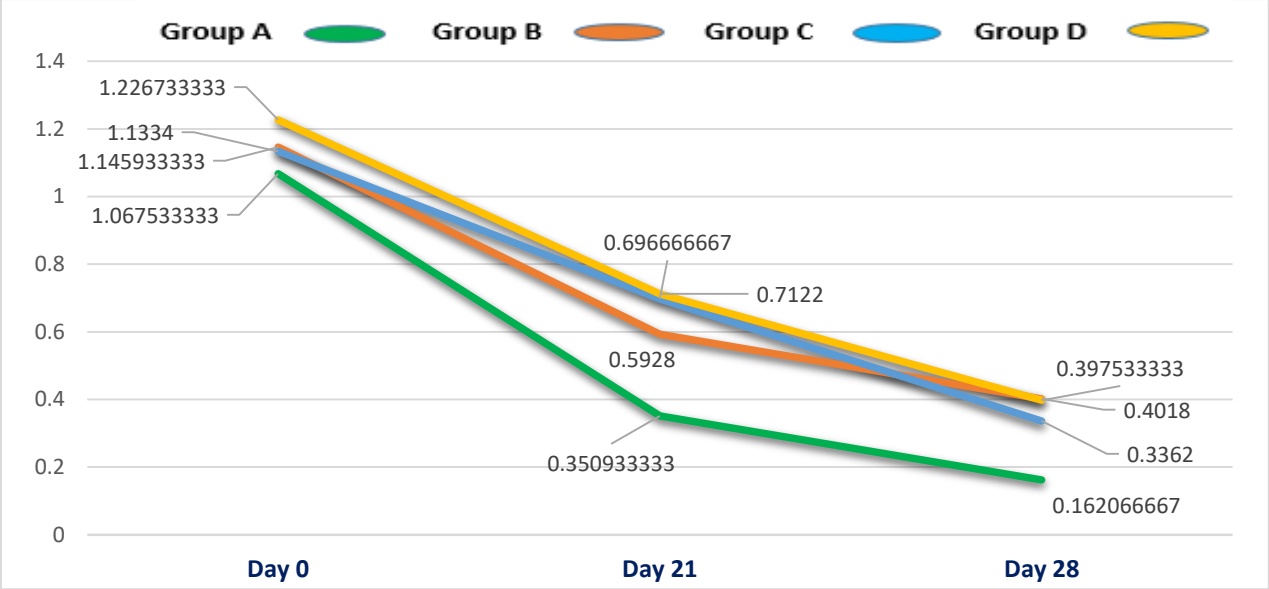

Fig. 14: Group wise OD values at 0 day, 21 days and 28 days of vaccination

4.2 DISCUSSION

PPR affect small ruminants (sheep and goat) in many countries of the world, specifically in Africa, the Middle East and parts of Asia. In Bangladesh, it became endemic from the year of 1993. To control and eradicate PPR by 2030, FAO has already announced a strategy and framework for all member countries (FAO, 2015).

Haematological data, TLC values in group A higher after 21 and 28 days of vaccination than the goats of Group B, Group C and Group D (Table. 2). Only levamisole HCl treated prior to PPR vaccine in Group C results higher TLC values than only triclabendazole treated before PPR vaccine in Group B. The level of TLC increase after 21 days of post vaccination in Group A, Group B, Group C and Group D was 27.75 %, 14.11 %, 13.37 % and 6.48 %, respectively (Fig. 11). These results agreement with that of Pelletier *et al.*, 1978, and the respective author stated that levamisole HCl acts on the macrophages and T-lymphocytes that initiate the humoral response and boost the immunity. Bozic *et al*., (2003) demonstrated that levamisole HCl exerts its immunostimulatory activities in pigs against colibaccillosis by recruitment and activation of cells that participate in cell-mediated immunity. On the other hand, only triclabendazole along with PPR vaccine results higher TLC values than only administering PPR vaccine in Group D. At the same time the sero conversion rate (Table. 3) in the goats of Group A, Group B, Group C and Group D, were 73.33%, 60%, 66.67% and 53.33% respectively in vaccinated goats through cELISA at 21 days after vaccination. These findings supported by Rahman *et al*., 2011 who found 62% goats were seropositive after 21 days by using the current PPR vaccine in Bangladesh. Anderson and McKay, 1994 also reported that about 60% to 70% goats became seropositive after PPR vaccination.

At 28 days post-vaccination, the TLC level increase in Group A was relatively higher than that in the goats of Group B, Group C and Group D by 52.46 %, 23.04 %, 35.27 % and 19.87 %, respectively (Fig. 12). Levamisole HCl was used prior blue tongue virus vaccine in sheep model, influenced the total number of leucocytes, lymphocytes and monocytes (Stelletta *et al*., 2004). levamisole HCl with Foot and Mouth Disease vaccine increased both cellular and humoral immunity (Jin *et al*., 2004). It was revealed that the TLC values increase percentage is higher in

Group B than in Group D. Treatment of the goats in group B with triclabendazole at 15 days of pre vaccination might be a cause in the increase of percentage.

On the other hand, at 28 days of vaccination the sero-conversion rate of Group A, Group B, Group C and Group D, were 93.33 %, 60 %, 80 % and 73.33 %, respectively in vaccinated goats (Table 3). Group D sero-conservion rate agreed with Gowane *et al.*, 2016 who reported the sero-conversion rate as 71.8% at 28 days of post-vaccination was. Group A showed a higher seroconversion rate compared to Group B, C and D. These results are similar to other trials (Krakowski *et al*., 1999) demonstrating an increase in specific immunoglobulin levels after treatment reported by. The results showed that the sero-conversion rate (Fig. 13) and OD values also decreased day by day except group B (Fig. 14). This finding is supported by the previous authors (Sil *et al*., 2001; Razzaque *et al*., 2005). It was observed that increase in leucocytes following administration of levamisole HCl possibly as a result of increase in the function of lymphoid organs such as the thymus and spleen resulting in increased proliferation of the bone marrow stem cells. Levamisole HCl, when administered in combination, enhanced antibody production against parvo virus (Mojzisova *et al*., 2004). In this study, levamisole HCl and triclabendazole acted as immunomodulator by increasing the humoral immunity and immunity induced blood cells number. The observed pattern of leucocyte change could have been as a result of increase in the function of the lymphoid organs due to levamisole HCl and triclabendazole. Its effect when combined with PPR vaccine as in goats in group A, resulted in high seroconversion and significant change in leucocyte value.

This experiment demonstrated the immunostimulatory effect of levamisole HCl and triclabendazole on PPR vaccination in goats which might due to an improvement in their general physical condition of goats with a decrease of helminthic infection.

CHAPTER 5

SUMMARY AND CONCLUSION

This study was done at Daspara village, Badarganj upazilla, Rangpur aimed to find out the immune modulatory activity of anthelmintic combination of levamisole HCl and triclabendazole, triclabendazole alone and levamisole HCl alone in prior to PPR vaccination in goat. The experiment was conducted at laboratory of Microbiology and Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka-1207. In this experiment, anthelmintic combination of levamisole HCl and triclabendazole, levamisole HCl studied in terms of knowing the parameter of Total Leucocyte Count and antibody titres and the immunological effects of applying combination of levamisole HCl and triclabendazole, levamisole HCl before vaccination. The treatment group A was recorded statistically significant for acting as immune stimulator than that of Group B, C and D. It showed that the immune stimulatory effect of combination of levamisole HCl and triclabendazole gives highest protective immune response in prior to administering PPR vaccine as well as the combination of levamisole HCl and triclabendazole drug having cost effectively, availability and low residual effect will intensify the PPR control and eradication programme. It is to be perceived through the experiment, levamisole HCl and triclabendazole modulating the humoral immune response of goats. As Bangladesh have the successful experience of eradicating Rinderpest and by this experience, the effective eradication programme of PPR disease can be enhanced by administration of current PPR vaccine with anthelmintic combination of levamisole HCl and triclabendazole. Further study is needed to determine the persistent of immunity.

CHAPTER 6

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