Standardization and Application of Modified Counter Immuno-Electrophoresis for the Detection of Antigenic Response against *Haemonchus contortus* in Rabbits

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Abstract
Present paper reports the standardization and application of modified counter immuno-electrophoresis (MCIE) technique to detect the antigenic response of *Haemonchus (H.) contortus* in rabbits. Sonicated somatic antigen prepared from the freshly collected worms was used to raise hyper immune sera in Albino rabbits. Optimum conditions for MCIE were determined by using hyper immune sera in comparison with the negative control. Optimum time for MCIE was 30 minutes at which visible precipitation band(s) were observed with hyper immune sera samples. A flow of 3 volts for 30 minutes was best for obtaining clear results. The quantity of the band(s) was dependent upon the concentration of the antigen; and the amount of antigen and antibody on each side of MCIE tube. MCIE is a simple, rapid, and inexpensive test and can be used for preliminary screening of haemonchosis and other parasitic infections in sheep/goats.

Keywords: *Hemonchus contortus*, Modified counter immuno-electrophoresis, somatic antigen, rabbits

Introduction
*Haemonchus (H) contortus* is the most pathogenic nematode parasite of sheep and goats that cause marked decrease in productivity due to its voracious blood sucking habit and has been reported to result in 23.8 and 40.0 per cent reduction in meat and wool production, respectively (Mortensen et al., 2003). The adult worms affect the abomasum of the host and feed on blood, resulting in severe damage, such as poor growth rate, loss of weight, anemia and, occasionally, death (Githigia et al., 2001; Vatta et al., 2001). Haemonchosis is prevalent throughout the world where sheep and goats are raised but it exerts greatest economic losses in the temperate and tropical region (Wakelin, 1996). The economic losses due to haemonchosis in sheep and goat at Faisalabad (Pakistan) have been estimated to be Rs. 32 billion per anum (Iqbal et al., 1993). Most frequently described protective immune response against *H. contortus* in sheep is due to the self/spontaneous reaction where expulsion of already established worms is participated by the intake of ineffective larvae (Wakelin, 1996). However, other forms of acquired immunity may regulate the number of *H. contortus* in sheep. For instance, by preventing the establishment of infection in the host and lowered worm burden followed by re-infection in sheep due to series of the previous infections (Koski and Scott, 2001).

The diagnosis of haemonchosis is usually based upon clinical signs/symptoms and coprological examinations. Clinical signs usually appeared during serious infection and eggs passed in feces after the prepatent period of approximately 3–4 weeks in which infection firmly established and damage has already been done. Under the circumstances, any reliable serological assay, which enables the detection of subclinical infection has paramount importance (Almazan et al., 2001). In this regard, several techniques, including enzyme linked immunosorbent, radio-immuno and fluorescent antibody techniques are being used to demonstrate the various parasitic infections including *H. contortus* (Charely et al., 1981; Eysker et al., 2000) although all these methods have been reported to be expensive and cumbersome. Present paper describes the standardization and application of modified counter immuno-electrophoresis to detect the antigenic response against *H. contortus* in rabbits.

Materials and Methods
The Parasite
Adult *H. contortus* worms were collected from the abomasums of the sheep from the local slaughter house, Faisalabad, Pakistan. Collected worms were given several washings with phosphate-buffered saline (PBS; pH 7.2).
Preparation of sonicated somatic antigen
Antigen was prepared following the method described by Jan (1993) with minor modifications. Worms (5gm/100mL) were subjected to ultrahomogenization for 3 minutes (1x 3) at 4°C followed by sonication for 60 seconds (30 seconds x2) in a jacketed vessel containing cool water. The sonicated suspension was centrifuged (5,000 ×/g 15 minutes). Supernatant thus collected was used as antigen and its protein concentration was measured (Bradford, 1976). The antigen was stored at -20°C until further use.

Experimental Animals
A total of 20 male Albino rabbits were purchased from local market and maintained at experimental station, Department of Parasitology, University of Agriculture, Faisalabad, Pakistan. Before experimentation, all the rabbits were subjected to deworming using Oxfendazole (0.5 ml/ rabbit) to make them parasite free. Rabbits were divided into two equal groups A and B. Rabbits in group A were injected with H. contortus antigen to raise hyper immune sera (Gomez-Munoz et al., 2000) and those in group B served as control.

Collection of sera samples
Blood was collected from the rabbits of both the groups on day 14th post last injection of antigen and serum was separated by slant method. Serum thus separated was stored at -20 °C for further use in modified counter immuno-electrophoresis.

Modified Counter Immuno-electrophoresis (MCIE)
Modified counter immuno-electrophoresis was standardized and applied to detect the antigenic response against H. contortus (Tahir et al., 1998). Briefly, two mL of 1% melted Agarose (Sigma Inc., USA) in barbiturate buffer (pH 8.6) was dispensed in U-shaped glass tubing. Gel was allowed to solidify in a groove. A total of 10 µl of each serum and H. contortus antigen were dispensed in anodal and cathodal arms of U-shaped glass tubing (in triplicate) that was connected with aluminum wire. One end of the aluminum wire was pierced down into the gel about 0.5 cm in both arms of U-shaped glass tubing while the other ends were connected with battery cells (1.5 volts each). A constant current was applied up to 30 minutes and then it was switched off. Observations were recorded after every five minutes up to 30 minutes. Development of circular precipitate band(s) was observed in the form of visible precipitation band(s) when hyper immune sera samples were used while no precipitation band(s) were observed with control sera samples.

Results and Discussion
Various serological tests have been developed and used for the accurate and early diagnosis and serological screening of haemonchosis in sheep and goats but each with certain drawbacks (Li et al., 2007; Ndao et al., 2009). MCIE is not used for parasitic infections although it is a frequently used test for the detection of viral antigens (Tahir et al., 1998). In the present study, MCIE was used for the detection of antigenic response of H. contortus in rabbits.

Based upon the findings of present study, MCIE was found to be a rapid test for the detection of H. contortus antigen. It was a time saving technique in which the reactants move towards each other. At pH 7.8, the antibodies/immunoglobulin molecules carrying a slight positive charge, move toward the cathode. On the other hand, the H. contortus antigens having a negative ionic charge at alkaline pH displaced toward the anode (Barrett, 1988).

Serological testing is an important component of any programme to control the disease in epidemiological surveillance, vaccine testing and in the selection of an appropriate strain because of considerable antigenic variations. Techniques reported in the literature have their own merits and demerits. It is reported that MCIE is more sensitive and the specific than agar gel precipitation test (Obi and Patrick, 1984) and this could successfully be used under the field conditions to screen out a larger number of sera samples. Keeping in view the merits of the assay, MCIE was standardized with the following conditions:

i. It gave rapid and clear results within 30 minutes.

ii. Positive response was obtained in the form of visible precipitation band(s) when hyper immune sera samples were used while no precipitation band(s) were observed with control sera samples.

iii. The flow of current was considered a vital step in the MCIE. It was observed that precipitation band(s) were more obvious when 3 volts current was allowed to pass for 30 minutes. Although, the results were achieved within 20 minutes by passing the current of 6 volts but the bands were not clearly demarcated.

iv. It was also observed that the quality of the band(s) depend upon the concentration of the antigen; and the amount of antigen and antibody (serum) on each side of MCIE.

v. During the present study, different concentrations of antigens (50µg, 100µg, 150µg, 200µg, 300µg and 350µg) by diluting in PBS were used. It was observed that bands were much more clear when 150µg concentration of H. contortus (50µg, 100µg, 150µg, 200µg, 300µg and 350µg), supplying variable current at different time intervals.
antigen was used. It was also observed that an amount of 50µl each of antigen and serum on each side of the MCIE gave good results.

All the sera samples obtained from the experimental group processed through MCIE gave positive results in comparison with the control samples that showed no precipitation line(s)/band(s). Among ten sera samples, seven samples showed a thick precipitation band of antigen and antibody complexes while three samples showed two precipitation lines that indicated that H. contortus somatic antigen used in the study was complex in nature and contained two distinct antigen(s). These observations are contrary to the results of other workers (Munn et al., 1993; Cuquerella et al., 1991; Knox et al., 1999; Pena et al., 2004) who recognized several polypeptides/proteins from H. contortus antigen by using immunoblotting (Jasmer and McGuire, 1991), Thiol-Sepharose chromatography (Knox et al., 1999) techniques. Based upon the results of present study, it can be concluded that MCIE is a simple, rapid and inexpensive technique that does not require any sophisticated equipments and reagents. It can be used as a preliminary screening tool in sheep/goat flocks to detect the H. contortus infection.

References
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