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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 17 (4): 578-581, 2014
ISSN 1028-8880 / DOI: 10.3923/pjbs.2014.578.581

Evaluation of *Gastrothylax cruminifer* Antigenic Preparation in Serodiagnosis of Paramphistomiasis in Sheep

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Abstract: An evaluation of *Gastrothylax cruminifer* crude antigen preparation viz., Somatic Antigen (SAg), Exerelory Secretary Antigen (ESAg) and Egg Antigen (EAg) in serodiagnosis of disease was undertaken. Test sera samples were obtained from 30 Paramphistomiasis Positive and 30 Gastrothylax free sheep slaughtered at Hazratbal Kashmir. The referral antigenic preparation were evaluated against Paramphistomiasis positive sera, via., control negative sera, using double immunodiffusion test (DID), (IEP) Immunoelectrophoretic assay and ELISA. The performance of referral antigens, as assessed from percent sensentivity and specificity, revealed an increasing trend from DID (Double immunodiffusion-An immunological technique used in the detection, identification and quantification of antibodies and antigens) to IEP (immunoelectrophoresis-A general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies), followed by ELISA, detecting higher number of sheep positive for paramphistomisis. In ELISA the ESAg and SAg were evaluated as most reactive antigens with no significant difference and EAg was the least antigenic. In IEP, EAg had the higher sensitivity (60%) and analogous specificity of SAg and ESAg. The formation of the preceptin lines in the proximity to EAg containing wells (cathode end) in IEP was suggestive of higher molecular weight of *G. cumnifer* specific protein molecules with slower rate of migration. Purification and characterization of *G. cumnifer* and identification of specific antigenic molecules, particularly in EAg has been suggested for qualitative improvement of diagnostic value of the antigens in the tests used here in.

Key words: Sheep, *Gastrothylax cruminfer*, rabbit, antigen and ELISA

INTRODUCTION

Paramphistomum cervi and *Gastrothylax cruminifer* (Stomach fluxes) are economically important parasities that infect the wide range of live stock species including cattle, sheep and buffalos. Both the Amphistome parasites (disease Amphistomosis) causes high morbidity and mortality resulting in great economic losses through reduced productivity (Mukherjee and Chauhan, 1965). Immature stages of *P. cervi* are highly pathogenic (Horal, 1971) but the routine parasitological diagnosis is difficult, under the circumstances immunological test resorted to the present study, is aimed at investigating the relative immunodiagnostic reliability and sensitivity of the Ouchterlony gel diffusion test and ELISA in paramphistomiasis. *Paramphistomum* is a member of a family Paramhistomidae which constitutes one of the most common and abundant groups of digenetic trematodes of domesticated livestock, especially in tropical and subtropical regions of the world. The disease paramphistomiasis caused by massive infection of small intestines with immature paramphistomes, is characterized by sporadic epizootic outbreaks of acute parasitic gastroentereritis with high morabity and morality rates, particularly in young stock (Horal, 1971). Diagnosis of paramphistomiasis during sub-clinical phase is of immense importance for the early detection of disease so that the mortalities can be reduced by timely intervention and treatment. However, early detection of the disease is difficult applying coprological techniques. There is an urgent need for the development of a reliable diagnostic test and an attempt is present investigation for detection of anti *Paramphistomum cervi* antibodies by indirect ELISA.

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MATERIALS AND METHODS

Preparation of antigen: Adult *P. cervi* were collected from the rumen of infected sheep. The worms were washed with physiological saline and stored at -20°C. until use. Somatic antigen of adult fluke was prepared by the technique of Yadav and Gupta (1966). The paramphistome fluke were blotted over sterile filter paper, fluke was immersed in 5 mL of PBS and then homogenized for 30 min, followed by sonication for 1 min at 4°C.

The emulsion was then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was collected and stored at -20°C. The protein concentration was determined by the method of Spector (1978). Finally 0.1% of thiomersol preservative was added to each antigen and stored in refrigerator.

Exceratory secretory antigen (ESAg): ESAg was prepared as described earlier (Yadav and Gupta, 1966). Live *G. cuminifer* flukes recovered at necropsy were suspended at 30 flukes/10 mL of PBS after sepontanous washing in sterile and chilled PBS and through removal of host origin materials and were finally incubated for 3-6 h at 30°C under sterile conditions. The incubated flukes were removed and exceratory/secretory products in PBS was centrifuged at 12000 rpm for 40 min at 4°C. The supernatant was separated and subjected to lyophilization until desired concentration was achieved. This was designed as ESAg.

Egg antigen: Method of Boros and Warren (1970) as modified by Mandal (1997) was used. Stored *G. cruminifer* eggs suspended in equal quantity (w/v) of chilled PBS were twice sonicated for 40 min at maximum amplitude 6-8 h, MSE-100 watt ultrasonic disintegrator. The suspension was finally centrifuged at 12000 rpm in refrigerated centrifuge. The supernatant was separated, lypholized to desired concentration using centrifugal freeze drier and the ultimate product was designated as EAg. An optical density (O.D 492 nm) values, 2 times of the mean $O.D^{+}$-S.D of negative control sera was read as positive reactants and those with values below it were taken as negative reactants.

In order to evaluate sensitivity and specificity of *G. cumenifer* antigen with test sera for each method was calculated as follows:

$$\text{Specificity (\%)} = \frac{\text{True negative-False positive}}{\text{True negative}} \times 100$$

$$\text{Sensitivity (\%)} = \frac{\text{True positive-False negative}}{\text{True positive}} \times 100$$

Sera samples: Blood samples were collected at necropsy from the sheep, positive for *P. cervi* infection. The serum was separated, respectively labeled as positive and negative (control) sera.

Hperimmune sera: Hyperimmune sera was raised in white new Zealand rabbits against respective antigens, by following standard protocol (Yadav and Gupta, 1995). The injection was given subcutaneously with actual quantity of frauds completed adjuvant at 5 days intervals. Rabbits were bled after 7 days of last injection. The serum was separated and keep at -20°C for further use.

Agar gel diffusion test: Immunodiffusion test was carried out as per the method described by Ouchterlony (1958). The test was carried out using 5 mL of 1% agar solution (defco) prepared in 100 mL of baritone buffer (pH 8.6). The agar solution was poured on to slides at 5 mL $slide^{-1}$. Then slides were kept at room temperature so that gel solidifies. At the centre of gel plate, one well were formed at a distance of 3 mm between the central and peripheral wells. The wells were charged with antigen and antibody, the diffusion allowed in moist chamber at a 37°C for 48 h, two precipitin lines were noted after staining the gel with coomasie brilliant blue R-250 stain.

ELISA: Micro titration plates (Nunc) were coated with antigen diluted (2 μg mL^{-1}) in carbonate buffer (pH 9.6) and incubated at 37°C for 1 h before keeping overnight at 4°C, coated plates were blocked with 3% skimmed milk in PBS for 2 h at 37°C. After washing the plate with phosphate buffer (3 times),add test sera (in appropriate 1:1000, 1:2000, 1:4000 etc.) and normal sera into each well at 100 mL $well^{-1}$. Serum was diluted in PBS-1% skimmed milk and then incubated at 37°C for 2 h. Then the plates were again washed and 100 mL of anti-ovine and anti-rabbit IgG-peroxidase conjugate (1:5000 dilution, sigma USA) was added and the plate were kept at 37°C for 2 h After washing 5 times, substrate (ortho phenylenediamine) in 100 mL of phosphate citrate buffer (pH 9.6) and 40 mL H_2O_2 was added and then plates were kept in dark for 7 min (Ghosh *et al*., 2005; Tariq and Reshi, 2011). The reaction were stopped by adding 50 mL of 3 NHCL per well and the optical desity was measured at 492 nm using ELISA reader (Labsystem Multiskan).

Statical analysis: The data obtained were analyzed by using simple descriptive statical analysis like (Mean±SD, percentage and frequency).

RESULTS AND DISCUSSION

The prevalence of *G. cuminifer* infection was 47% as assessed from necropsy fluke recovery of sheep

slaughtered at various abattoirs. The mean necropsy fluke recovery was 51±23. The protein concentration of Somatic Antigen (SAg), Exceratory Secretory Antigen (ESAg and Egg Antigen (EAg)), respectively estimated as 2.5, 2 and 1.5 mg mL^{-1}. The referral antigens (SAg, ESAg, and EAg) were evaluated against positive sera vis-avis, control negative sera, using DID, IEP and ELISA after their standardization with hyper immune sera raised in rabbits. The referral antigens in homologues systems showed 3 black perception brands. The positive reactions of naturally infected sheep along with hyper immune serum as control. In IEP it was interesting that EAg reacted with positive sera and formed precipitin lines in proximity of cathode, contrary to the SAg and ESAg, each forms precipitin lines towards anode. Out of 30 positive test sera 10, 8, 6 reacted, respectively with SAg, ESAg and EAg and showed two preceptin lines in DID where as in IEP, referral antigen could perform better than DID and detected anti fluke antibodies in 12, 10, 14 positive sera tests, respectively using these antigens. Negative control showed no reaction in IEP Band gave false positive reactions in DID.

The mean ELISA OD Value±SD in negative control sera were 2.205±0.25.

For SAg, 0.210 ±0.02 for ESAg and EAg 0.212±0.03. Considering these mean optical density as standards . The test sera showing twice O.D were read as positive recants and those below it , as negative for *G. cumenifer* infection. The referral antigens exhibited the highest sero-reactivity and could demonstrate *G. cumnifer* antibodies in 18, 21 and 14 positive sera with SAg, ESAg and EAg, respectively. It has been seen that ELISA was the most sensitive as it demonstrated increased efficiency in detecting *G. cruminifer* antibodies in positive sera. Amongst the 3 referral antigens EAg had the lowest whereas ESAg has the highest reactivity. The SAg occupied position in between. However, ELISA gave 6-8% false positive reactions, resulting in reduced specificity of 85 and 95%, respectively for SAg, ESAg and EAg.

It is well known fact that helminth parasites during their development undergo antigenic polymorphism which induces drastic alteration in immune response, so use of these different developmental antigens in the immunodiagnostic is very important. Development of simple and specific immunological tests for the diagnosis of helminth infections has been a major goal for recent immunological research.

Ovine Paramphistomiasia, mainly caused by *G. cruminifer* in Indian subcontinent is the most wide spread and economically important disease. The pathological effects of the disease are essentially due to traumatic hepatitis caused by adolescercaria during 4-6 week post infection, resulting in field outbreaks of per acute/acute paramphiostiomasia. At this stage of infection, serodiagnosis of the disease seems to be the only alternate to coproscopic detection of the fluke eggs, as these are often seen only after 13 weeks post-infection, when adolescencia matures and lay eggs an *in situ* (Hammond, 1973; Herith, 1980; Bashandy *et al*., 1990; Gupta and Yadav, 1992). The DID, IEP and ELISA have been found suitable and test of choice for the serodiagnosis of fasciloiasis. The IgA and IgG, thus produced in bile of primary infected rats reacted intensely against ESAg in ELISA. The comparative low specificity of ELISA may be due to use of crude antigenic preparations the mosaic of proteins containing phosphocholoine epitope (Rao *et al*., 1996). Which is reported to be ubiquitous epitope detected in many other parasites as well. Due to their high specificity and stastificatory sensitivity. However, suitability and reliability of this test in this host-parasite system, it depends upon the quality of *G. cruminifer* preparation. The antigen used should be high quality in respect of specificity and sensitivity. The sensitivity of the test used also depend upon the clinical progress of the disease and *in situ* developing fluke population in sheep. Heavy infection could be detected with greater accuracy than low grade infections in buffalo (Swarup *et al*., 1987). Wang *et al*. (1987) opined that ELISA is the most suitable serodiagnostic test with greater reliability for seroepizootiological work in paramphistomeasis endemic areas.

CONCLUSION

This study of serodiagnostic evaluation of *G. crumnifer* by preparing different Antigenic preparations in sheep has revealed that the performance of these different antigens showed increasing trend from DID to IEP followed by ELISA in detecting higher number of *G. cruminifer* positive infections. In ELISA, ESAg (Execratory secretory antigen) has been found as most reactive antigen closely followed by SAg (somatic antigen) and the EAg (egg antigen) was found least reactive one, where as in IEP, the EAg showed higher sensitivity with analogous specificity than SAg or ESAg.

ACKNOWLEDGMENT

The authors are thankful to the Department of Zoology University of Kashmir and Department of Microbiology (Parastology) and immunology SKIMS Kashmir for providing the necessary facilities for the work.

REFERENCES

Bashandy, M.M., M.M. Yassein lofty, A.M. El-Bhi. M. Mohammad and M.I. Dessouqyi, 1990. Hematalogical and Biochemical profiles in experimental Fasciolasis in Sheep. Egypet. J. Comp. Pathol. Clin. Pathol., 3: 357-374.

Boros, D.L. and K.S. Warren, 1970. Delayed hypersensitivity-type granuloma formation and dermal reaction induced and elicited by a soluble factor isolated from *Schistosoma mansoni* eggs. J. Exp. Med., 132: 488-507.

Ghosh, S., P. Rawat, S.C. Gupta and B.P. Singh, 2005. Comparative diagnostic potentiality of ELISA and dot-ELISA in prepatent diagnosis of experimental *Fasciola gigantica* infection in cattle. Indian J. Exp. Biol., 43: 536-541.

Gupta, S.C. and S.C. Yadav, 1992. Sexual maturity of F. gigantica in expermintally infected Rabbit, Goat and Buffalos. Ind. J. Paristol., 16: 133-134.

Hammond, J.A., 1973. Experimentally chronic Fasciola gigantic infection in Sheep. Trop. Anim. health. Prod., 5: 12-21..

Horal, I.G., 1971. Paramphistomiasis of domestic ruminants. Adv. Parasitol., 9: 33-72.

Herith. L.A., 1980. The incidence of the alternative intermediate host lymina trunca tula on the development and pathogensis of *F.gagantica* infection in Sheep. Sudan J. Anim. Sic. Anim. Husbandery, 21: 16-25.

Mandal S.C., 1997. Evaluation of Fasciola gigantic antigens for the immunodiagnostic of Fascilosis in ruminants. MVSC Thesis IVRI Izatnagar U.P, India.

Mukherjee, R.P. and B.S. Chauhan, 1965. Studies on the trematode fauna of India. Part V. sub-class digenea: Paramphistomidae fischoeder (1901). J. Zool. Soc. India, 17: 151-225.

Ouchterlony, O., 1958. Diffusion-in-gel methods for immunological analysis. Progress Allergy, 5: 1-78.

Rao, R., H.C. Yadav, G.C. Ram, P.R.S. Raghav and Lal, 1996. Common antigenic determinanat of *F. gagintica* as defined by monoclonal antibodies to Phosphocholine. J. Applied Res., 9: 95-104.

Spector. T., 1978. Refinment of the cosmio brillant blue method of protein quantification. Jou. Analy. Biochem., 86: 142-146.

Swarup, D., S.P. Pachouri and B. Sharma, 1987. Seriodiagnostic of Fascoliasis in Buffalos by Inmunoprecipitation using adult *Fascolia gagantica* antigens. Res. Vet. Sic., 22: 130-130.

Tariq, A. and M.L. Reshi, 2011. Dot-Enzyme Linked Immunosorbant Assay for detection of *Paramphistomum cervi* antibodies in Rabbits. Indian J. Applied. Pure. Bio., 26: 357-359.

Wang, P.Y., J.S. Jin, K.S.P. Cai, Z.O. Daun and W.Z. Yadav, 1987. Regional Trial of ELISA for diagnostic of Fascolsis in sheep and Goats. Chinease J. Vet. Sci. Technol., 7: 9-11.

Yadav S.C. and S.C. Gupta, 1966. Identification of immunodiagnostic antigen of Fasciola gigantica. Jou. Parasit Dis., 20: 201-202.

Yadav, S.C. and S.C. Gupta, 1995. Immunodiagnostic moieties somatic and excretory/secretory antigens of *F. gigantica*. India J. of Expt. Biol., 33: 828-834.