Immunoprofiling of Fasciola gigantica infected adult bovines: 41kDa protein as potential immunodiagnostic target

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**ARTICLE INFO**

**Keywords:**
- Bovines
- Fasciolosis
- Immunoblot
- Sensitivity and Specificity

**ABSTRACT**

Fasciolosis is emerging as a serious medical and veterinary problem. Etiological agent for this economically important zoonotic disease are Fasciola hepatica and Fasciola gigantica which coexist in bovine hosts reared in Kashmir province because of overlap in the boundaries of their respective intermediate snail hosts. But there is predominance of Fasciola gigantica which incur heavy losses in bovine rearing industry. Present investigation was carried out for detection of Fasciola gigantica soluble antigens readily diagnosed by Fasciola gigantica infected bovine sera using Western blot analysis.

1. Introduction

Fasciolosis gains public concern not only due to its prevalence and economic significance to animal stock in all continents (Scheweizer et al. 2005, Mungube et al. 2012) but also to its zoonotic aspect. Two species of genus Fasciola i.e. Fasciola gigantica (Tropical liver fluke) and Fasciola hepatica (Temperate liver fluke) are the etiological agents of Fasciolosis. These two species are recognized as sister species as they hybridize in nature (Agatsuma et al., 2000). Molecular data using Cathepsin L and Glutathione S transferase (GST) revealed that Fasciola gigantica and Fasciola hepatica species have separated nearly 19 million years ago (Irving et al. 2003) which coincides with time scale (16MYA and 28MYA) when divergence of modern day pecoran lineages including ancestors of cow, gazelle, goat sheep, giraffe etc has occurred (Allard et al. 1992). This reveals the co-speciation and co-adaptation of Fasciola genus with the radiation of its host. Farm animals such as sheep and cattle are most commonly affected by fasciolosis and act as the main mammalian end hosts. Thus, fasciolosis is an impendent in profitable bovine farming and for butchers and consumers too. Fasciolosis leads to heavy economic losses (both direct and indirect) which include mortality, reduction in milk yield and meat production, secondary bacterial infections, expensive anthelmintic treatment and condemnation of livers at slaughter and drifted carcasses (Phiri et al. 2005). About 350 million cattle are currently at risk for fasciolosis (Hillyer and Apt. 1997). In this regard there is need of hour to devise the techniques for proper diagnosis so as to frame control measures. Fasciolosis can be controlled by adopting diversified and integrated approaches to combat it which is represented graphically below:

Among all, vaccine development is by far the most reliable control strategy. Proteins are often the targets for therapeutic agents (Barret et al. 2000). Information regarding parasitoproteomics broaden our knowledge regarding parasites and in developing effective strategies to deal with same. Immunodiagnosis is an important adjunct to clinical findings (Hillyer 1999). Though ELISA is the earliest technique but the main flaw of it is that it is quantitative analysis though sensitivity more but specificity is less as the cross reactivity of any antibody cannot be visualized in ELISA but with Western Blotting. During last few years, Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting have initiated new era in immunodiagnosis with greatly reduced cross reactions (Sarmehmetoglu 2002, Sharma et al. 1987). Due to lack of commercially available vaccines against fascioliosis, McManus and Dalton, 2006 has proposed four main antigens as leading vaccine candidates for Fasciola infections i.e. leucine aminopeptidase,
amicrobial drugs in the community have been major contributors. Resistance factors can spread rapidly, not only locally but also, with greater movement of people around the world, globally. Microorganisms and their resistance factors may also be transferred from country to country in animals and commercially produced fruits and vegetables.

Hospitals are a critical component of the antimicrobial resistance problem worldwide. The combination of highly susceptible patients, intensive and prolonged antimicrobial use, and cross-infection have resulted in nosocomial infections with highly resistant bacterial pathogens. Resistant hospital-acquired infections are expensive to control and extremely difficult to eradicate (WHO, 2002). The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetic or natural (Nascimento, et al., 2000).

The use of medicinal plants all over the world predates the introduction of antibiotics. Herbal medicine has been widely used and formed an integral part of primary health care in China (Liu, 1987), Ethiopia (Desta, 1993), Argentina (Anesini and Perez, 1993) and Papau, New Guinea (Nick, 1995). Traditional medical practitioners in Southwest, Nigeria, use a variety of herbal preparations to treat different kinds of microbial diseases including MRSA associated diseases. Hence an attempt was made to screen selected indigenous plants and culinary spices for their antibacterial activity against clinical isolates of MRSA. So that new leads from the novel plant derived natural products could be developed for the treatment of infectious diseases.

Materials and Methods

Fresh indigenous plant material of Achyranthes aspera L. (whole plant), Amaranthus gangeticus Linn (leaf), Anacardium occidentale L. (cashew nut shell), Calotrops gigantea R. Br. (Leaf), Gloriosa superba L. (tubers), Mangifera indica L. (seed kernel), and Sesbania grandiflora L. Pers (Leaf) were collected from different locations of Krishna District, Andhra Pradesh, India. Whereas culinary spices: Cinnamomum zeylanicum Bl. (bark), Cuminum cyminum L. (seed), Illicium verum (hook fruit), Menthe piperata L. (leaf), Myristica fragrans, Houtt.(seed), Nigella sativa Linn. (seed), Pimpinella anisum L. (seed) and Terminalia chebula Retz. (seed), were brought from Kaleswara Rao Market Vijayawada, Krishna District, Andhra Pradesh, India. Their botanical identities were determined and authenticated by A. Rama Krishna MSc, MPhil. Sr. Lecturer, Head of the Department of Botany and Principal of Vemulapalli Kodanda Ramah College, Gannavaram, Krishna District, Andhra Pradesh, India. Samples were deposited in the department herbarium.

EXTRACTION PROCEDURE

Shade dried plant parts were reduced to fine powder and 10g of powder was taken into a 250 ml conical flask and 100 ml of acetone was added. After thorough mixing the flask was kept on a rotary shaker at 190 – 220 r/min for 48 hours and it was filtered through whatman filter paper No1. (WHATMAN Ltd, England). The filtrate was evaporated until dry in a water bath at 80°C temperature. The crude extract was prepared by dissolving known amount of the dry extract in dimethyl sulfoxide (DMSO; Merck Specialties Private Limited, Mumbai, India), to have a stock solution of 10 mg/ml concentration and stored at 4°C.

Bacterial culture

The MRSA strains used in this study were clinical isolates from blood, urine, pus, ear swabs, eye swabs and nasal swabs of patients in the diabetic care centers and intensive care units of various corporate hospitals in East Godavari, West Godavari and Krishna districts of Andhra Pradesh, India. The swabs and body fluids of patient’s samples were inoculated onto blood agar plates. The inoculated plates were incubated at 37°C for 18-24 h. After inoculation on blood agar, the swabs were placed in brain heart infusion (BHI) with 7.5% sodium chloride, which were also incubated at 37°C for 18-24 h. Inoculated BHI broth was sub cultured onto blood agar plates. From these blood agar plates, the colonies which were opaque, circular, pigmented with β hemolysis were identified as S. aureus by the Grams staining, catalase and coagulase (slide and tube) tests (Cadness, 1943). A total of 153 coagulase positive S. aureus strains were isolated and identified from 478 clinincal samples. Antibiotic sensitivity pattern was determined by standard antibiotic disc diffusion technique using, oxacillin (1 µg), gentamicin (10 µg), erythromycin (15 µg), co-trimoxazole (25µg), vancomycin (30 µg) discs (Hi-media, India) with quality control strain of S. aureus (ATCC 25923) as per clinical and laboratory standards institute (CLSI) former National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Bacterial suspension matching 0.5 Mc Farland turbidity standards were inoculated on Muller-Hinton agar containing 4%NaCl and 6 µg/ml oxacillin (Cipla pharmaceutical Pvt. Ltd, India). Isolates showing visible growth after 24h incubation at 33-35°C were identified as MRSA. For further confirmation of MRSA phenotypically, the isolates were inoculated on MeReSa Hi-chrome agar supplemented with cefoxitin and incubated at 370C for 24 h. Isolates producing bluish green colour colonies on MeReSa agar were considered as positive for MRSA. Oxford strains of S. aureus (ATCC 25923) sensitive to methicillin and S. aureus (ATCC 33591) resistant to methicillin were used as control organisms. In the oxacillin resistant coagulase positive S. aureus isolates, PCR for the amplification of the mecA gene was performed using specific primers F 5’- TGGCTAATGGTGTCTAACAT CGG- 3’ and R 5’- CTGGAACTTGTTGAGCAGAG- 3’. PCR was carried out in 25 µl volume with 10x PCR buffer, 20 pM primers, 1.5 mM MgCl2, 10 mM DNTPs, 1.25U Taq DNA Polymerase and 2 µl of template DNA. The cyclic conditions were initial denaturation 3 min at 920C, followed by 30 cycles of 1 min at 920C, 1 min at 56°C, and 1 min at 72, with a final3
fatty acid-binding protein (FABP), glutathione S-transferase (GST) and cysteine protease (Cat L1/Cat L2). Since the major cause of bovine fasciolosis is Fasciola gigantica in Kashmir province (Gul et al. 2013), the current study deals with the same aetiological agent. In the present study, the whole worm extract of adult Fasciola gigantica has been analyzed for an antigenic profile using the immunoblotting technique with sera from Fasciola-infected cattle.

Materials and Methods
Collection of flukes: Naturally infected livers of Cattle were collected from local slaughterhouses past midnight. In order to obtain flukes, liver was incised and then bile ducts were opened, starting from common bile ducts to smaller ones at the periphery of the liver. The infected livers were squeezed manually to macerate the parenchyma and the flukes were carefully removed and placed in petridish containing 0.15M PBS (pH 7.3) for initial washing to remove host material and allow regurgitation of gut contents. The flukes were stored in collection vials containing PBS and were transported to the Parasitological laboratory for further processing.

Fasciola species identification:
Fascioids were identified primarily on variation in shape and size of the adult fascioids, with the smaller F. hepatica exhibiting wide and defined shoulders compared to the slender F. gigantica having less defined shoulders and shorter cephalic cones (Soulsby, 1986). Identification of Fasciola gigantica was also carried out by taking in consideration of three statistically significant morphometrical parameters as proposed by Gul et al. 2013 i.e. Body length (BL: 27-45 mm, 33.6±4.42), body width (BW: 4-9 mm, 5.48±0.92) and ratio of BL/BW (3.7-10.5 mm, 6.27±1.20) to properly segregate the coexisting Fasciola species in infected bovine livers.

Preparation of Fasciola gigantica soluble protein extracts:
Freshly collected Fasciola samples were washed with ice cold PBS several times so as to reduce muscle constriction of the acetabulum and oral sucker to avoid release of excretory secretory products and enzymes of fascioloids which otherwise may become attached to the surface of adult Fasciolids as described by Morales and Espino, 2012. Protein extracts were obtained by homogenization of 5 flukes in 5ml of ice cold lysis buffer (pH 7.2) I and II separately so as to compare the maximum yield between two lysis buffer using tissue homogenizer (Wise Tis HG 15D). Lysis Buffer I comprised of 50mM Tris, 50mM NaCl, 0.5% TritonX-100, 0.5mM EDTA and 2mM PMSE. Buffer II contained 5 mM MgCl2 (pH 7.4), 100mM DTT, 31mM EDTA, 30mM Tris Cl and 2mM PMSE. Homogenization was carried out for 10 minutes at 1300 rpm taking pause of 2 minutes after every one minute of homogenization. To 5 ml of lysis buffer 200µl of cocktail of protease inhibitors (Roche) was added. After homogenisation, the mixture was allowed to stand for half an hour on ice till the foam settles down. Samples were then sonicated for 10 minutes with pulse 10 lapse 30 and amplitude 70% on ice and centrifuged at 14000 rpm for 20 minutes at 40C. The supernatant protein fraction was concentrated by AMICON® ultra using a YM-3 membrane (cutoff, 3 kDa) for 20 minutes at 14g. The protein content was determined by Nano Drop.

Collection of Bovine Sera: A 10 ml blood sample was taken from the jugular vein of each animal into evacuated tubes, and transported to the Parasitology Laboratory of the Faculty of Biological Science Hazratbal. Serum was removed from the clotted blood samples by centrifugation at 4 000 rpm for 10 minutes, and stored at –20 °C.

SDS-PAGE and immunoblot analysis: The Fasciola gigantica soluble protein fraction was analyzed under reducing conditions in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protein II electrophoresis apparatus (Bio-Rad, Hercules, CA). The gels were then stained with Coomassie blue R-250/G-250 (Sigma-Aldrich, St. Louis, MO) for protein visualisation. For antigenic analysis proteins on gels instead of staining were transferred according to standard procedures using Bio-Rad criterion blotter for 2 hours on ice to a PVDF membrane (Immobilon.) for 2 hours. The membrane was blocked with 3% BSA w/v in TTBS (20 mMTris·HCl, 154 mM NaCl, 0.1 % v/v Tween 20, pH 7.2) overnight at 200C. Strips containing Fasciola gigantica soluble antigens were incubated overnight with bovine serum samples. All sera were diluted 1:100 in PBS containing BSA and incubated at 4°C overnight with gentle shaking. The membrane strip was washed thrice with TBST for 5 min each, and then further incubated with alkaline phosphatase-conjugated anti-bovine IgG (Bio-Rad) diluted at 1:10,000 in TBST containing BSA for 2 hours. After 3 washes with TBST, the PVDF membrane were incubated in a solution of BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) liquid substrate system with gentle shaking for 15 minutes. The development reaction was terminated by briefly washing the membranes with distilled water. Gels were then visualized under Bio Rad gel documentation system.

Experimental Design: For immunodiagnosis via western blotting, 27 bovine sera were selected in the study and categorized into the following groups:

1. Group I (Sera of bovines with patent Fascioliasis): 16 infected animals with fasciolosis, all of them reported by necropsy examination.

2. Group II (Sera of infected control group): Six cases infected Paramphistomewere included as infected control group

3. Group III (Sera of normal control group): 5 apparently healthy animals confirmed via stool examination and postmortem study analysis

Evaluation of the diagnostic sensitivity of above antigens: Sera from 16 cattle with natural infections of the most Prevalent Causal Agent of Fasciola gigantica and 5 uninfected hosts were tested, together with sera from cattle with other trematode infection i.e Paramphistome infection was carried out so as to record sensitivity and cross reactivity if present.
Sensitivity=\([A/(A+C)\]×100

Accuracy=\([(A+D)/(A+B+C+D)]\]×100

Where A= True positive, B= False positive, C= False negative, D= True negative.

Results

Electrophoretic analysis of referral antigens i.e. Fasciola gigantica Crude worm protein extract resolved into 14 polypeptides with molecular weight ranging between 17-200 kDa under reducing conditions in 12% SDS-PAGE. The probable molecular weight of these protein bands were 18, 22, 24, 25, 31, 45, 46, 52, 57, 70 kDa respectively and multiple bands between 80 to 175 kDa (Fig. 1A). Moreover, the concentration of protein yield increased using Lysis Buffer II (Fig. 1B). The western blot analysis of Fasciola gigantica Antigens revealed 4 most prevalent immunodominant polypeptides of the mol wt 41, 45, 60-69 and 70kDa when reacted with sera of animals infected with Fasciolosis (Fig. 2). Further confirmation was done by western blotting on probing with Paramphistome sera and negative control sera against FgAg to determine the cross reactivity and specificity of the polypeptide bands (Fig. 3). The sensitivity and specificity of four antigenic bands is shown in Table 2. Though 70kDa antigen showed highest sensitivity (93.75%) among the 4 most prevalent bands but it showed lesser specificity(63.33%) by showing crossreactivity with 4 false positive cases(actually infected with Paramphistome) and thus cannot be used as suitable specific tool for immunodiagnosis of Fasciolosis. Another immunodominant band of M.Wt. 45kDa was 100% specific but the sensitivity was too low, i.e., 62.5%. Among the immunodominant bands 41kDa antigen showed 87.5% of sensitivity with 100% specificity and accuracy of 92.5% and proved to be potential band for diagnosing Fasciolosis.

Discussion

Western blotting is a good confirmation test for immunodiagnosis of fasciolosis by decreasing the risk of cross reaction (Sarimehtetoglu, 2002). While resolving somatic protein of F. gigantica by SDS PAGE, Gupta et al.(2003) reported 6 protein bands with M.Wt 27.7-37.5 kDa. Meshgi et al. (2008)
which revealed 11 major bands having range between 18-68kDa for somatic antigens of Fasciola hepatica while only 5 and 7 protein bands of range 27-57.6kDa and 6.5-205kDa were reported by Allam et al. (2002) and Gonen et al. (2004) respectively for same protein extract. Upadhyay and Kumar (2002) reported 7 bands of 16-62kDa M.Wt while as Yadav and Gupta (1995) reported protein bands of range12-95kDa. Different workers have showed variable number of diagnosable protein bands which could be attributed to existence of different isolates from different host species or geographic variations and handling errors while homogenization and protein extraction cannot be discounted too. The other reason could be the presence of both endogenous and host derived proteins primarily during isolation of flukes from thawed livers as suggested by De Vera et al. (2009). Moreover the pooled extract of grouped flukes based on morphological characters may have included potential hybrid within the purportedly F. gigantica samples.

The detected protein band during current study with apparent molecular weight 18kDa may correspond to 17.5kDa protein studied by Kim et al. 2000 and recognized it as Cu/Zn superoxide dismutase (SOD) which develops antibodies against sera from Fasciola infected humans and cattle. Two forms of Nitric oxide synthase (NOS I and NOS II) in Fasciola gigantica have been detected via chromatography on CM cellulose. NOS II showed highest specific activity and was detected as 18kDa band using Sephacryl S-200 and SDS-PAGE (Hamdy and Ali, 2009). NOS II of F. gigantica is thought to play a vital role in arginine metabolism rather than urea cycle pathway.

affinity chromatography successfully isolated GST proteins from F. hepatica and confirmed a band with Molecular weight of 26 kDa as F. hepatica GSTs using SDS-PAGE (Farahnak and Brophy 2004). At least 7 GST isoenzymes in the size range of 23-26.5kDa are distributed in Fasciola hepatica tissues (Rossjohn et al.1992).

The 45 kDa protein was also identified in excretory secretory (E/S) product of parasite by Goreish et al. (2008) who stated that it may correspond to the 48kDa protein in E/S product of adult parasite identified by Ajannusi et al. (1993). Estuningsih and Widjajanti (1999) reported the role of antigens with M.Wt 46 and 47 kDa for serological evaluation of Fasciolosis in sheep. These 2 bands may correspond to the band 45kDa of the current study which showed no cross reaction with both positive and negative control groups. Among the seven detected protein bands reported by Chauvin et al. (1995) there was 46kDa protein detected by Fasciola hepatica infected sheep sera. 46kDa protein was isolated by Espino and Hillyer 2003 and identified it as protein belonging to Saposin family (SAP). Gram et al. (2006) detected 46kDa as SAP2 in adult Fasciola gigantica located on gut epithelium whose primary function is strong activity against host erythrocytes and peripheral mononuclear blood cells.

In the current study 60-69kDa bands were revealed in the sera of 12 out of total 16 positive cattle for F. gigantica. Ortiz et al. (2000) also reported that dairy cattle naturally infected with Fasciola hepatica develop antibody response against 60-66kDa in E/S antigen which is in close proximity to our findings. El Ridi et al. (2007) and Morphew et al. (2007) have suggested ESP derived 62-60kDa molecules by young worms as strongly immunogenic both in vivo and ex host within ovine bile. Several researchers have reported that 63kDa antigenic polypeptide is recognized as sharp band by serum samples from humans, rabbits, cattle and sheep with fasciolosis (Sarimehtmetoglu 2002). De Almeida et al. (2007) showed that 60-63kDa FhAWV (Antigen of vomit of adult worm of F. hepatica) as the most nonspecific band as was recognized by current study showing lower specificity (63.63% only). Though thioredoxin reductase (TrxR as 60kDa and 55kDa band) has been isolated from F. hepatica adult deoxycholate-soluble extract but the same has not yet described in Fasciola gigantica (Jefferies et al. 2001 and Rioux 2011). But the current study has revealed 60kDa reactive band in Fasciola gigantica and thus can be ascribed to TrxR.

Estuningsih et al. 1997 has identified 94kDa as paramyosin and isolated it from adult Fasciola gigantica where it is located within the musculature and tegument. It tends to increase the antibody titre in infected cattle sera. However, same protein from Fasciola hepatica migrate at 97kDa under reducing conditions, and 200 kDa under non-reducing conditions (Cancela et al. 2004).

Hoyle et al. (2003) found that the immune response against higher molecular mass proteins (>50kDa) was associated with a significant resistance to reinfection in cattle.

Gonen et al. (2004) mentioned 39.5 and 42kDa protein band as specific for diagnosis of sheep fasciolosis caused by Fasciola hepatica which may correspond to the 41 kDa of current study. 43kDa metacercarial antigen was reported as immunoreactive protein recognized by Fasciola gigantica infected bovine calves (Velusamy et al. 2006)

References
