

ANTIGENIC CHARACTERIZATION OF PORCINE METACESTODES

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Abstract: The antigenic profiles of bladder worms collected from pigs slaughtered at Mumbai abattoir were studied by protein estimation, SDS-PAGE mobility and column chromatographic pattern of crude fluid antigens. The average protein content of fluid from *Cysticercus cellulosae*, *Cysticercus tenuicollis* and hydatid cysts was found to be 69.42, 37.3 and 67.2 mg%, respectively. SDS-PAGE analysis of crude fluid antigens of *Cysticercus cellulosae*, *Cysticercus tenuicollis* and hydatid cysts revealed 13, 12 and eight protein moieties with their molecular weights ranging from 200 to 14, 312 to 24 and 272 to 38 kDa, respectively. Crude fluid antigens of the three types of porcine metacestodes when subjected to column chromatography through Sephadex G 200, demonstrated two distinct peaks each. The cross reactivity studies to identify the parasite specific component demonstrated that Peak II antigen of *Cysticercus cellulosae* and Peak I antigen of *Cysticercus tenuicollis* and hydatid fluid were more specific to their respective metacestode as revealed by less number of cross reactivity with heterologous antisera as compared to that with Peak I antigen of *Cysticercus cellulosae* and Peak II antigen of *Cysticercus tenuicollis* and hydatid fluid, respectively. Thus Peak II antigen of *Cysticercus cellulosae* and Peak I antigen of *Cysticercus tenuicollis* and hydatid fluid can be exploited further for improving the immunodiagnostic efficacy of porcine metacestode infections.

Keywords: Pig, *Cysticercus cellulosae*, Hydatid, Antigenic characterization, Gel filtration chromatography.

Introduction

Indian economy is agriculture oriented where in livestock industry contributes significantly. According to 19th livestock census (2012), livestock sector contributes 4.11% at current price to total GDP of India. Amongst different livestock pig is the most prolific animal with respect to both feed conversion and breeding potentiality. A sow attains puberty earlier and thus can be bred at the age of eight months after which it produces 12 - 24 piglets in two litters per annum. Further, the carcass yield varies between 65 and 80 % which is highest amongst the livestock species. Majority of piggery holders in India belong to socioeconomically weaker strata of the community, hence pigs not only suffer from malnourishment but also harbour different infections particularly those with parasitic

etiologies. Pigs act as intermediate host of larval tapeworms like *Cysticercus cellulosae*, hydatidosis and *Cysticercus tenuicollis*. These metacestodes along with causing huge economic losses to piggery industry through condemnation of carcasses and viscera also poses tremendous zoonotic risk to humans (Flisser, 2002; Okello *et al.*, 2015). Owing to their histozoic locations in the intermediate host, these cannot be diagnosed by conventional parasitological techniques and majority of the cases are detected only at the time of meat inspection at the abattoir level leading to economic losses to meat industry as a whole. Between the two other diagnostic options, imaging techniques such as radiography, sonography, CT scan, etc. are not feasible and one has to rely on immunodiagnostic tools for detection of bladder worms before slaughter.

The major hurdles in the immunodiagnosis of bladder worms in animals are low sensitivity and specificity. Since the antibody titres in infected animals are invariably low, a highly sensitive immunodiagnostic assay such as ELISA is necessary. On the other hand, the problem of specificity is mainly due to sharing of antigens by not only different species of bladder worms but also by different species of helminths (Poretti *et al.*, 1999; Dorny *et al.*, 2003) and thus cross reactivity leads to false positive results. Hence in order to minimize cross reactivity *vis-à-vis* false positivity, it is imperative to identify the proteins which are specific to each species of metacestodes. Use of these protein moieties as an antigen in a sensitive immunodiagnostic might drastically improve the sensitivity and specificity of the test. When such tests are employed for screening of pigs in endemic regions, the positive cases can be detained and treated with suitable anthelmintics prior to slaughter to curb economic losses to meat industry. In view of the above, a study was undertaken to characterize different antigenic components of fluid antigens of each metacestode and to assess their cross-reactivity.

Material and Methods

Processing of parasitic material in laboratory

The parasitic material (*Cysticercus cellulosae*, hydatid and *Cysticercus tenuicollis* cysts) was collected from pigs slaughtered at Mumbai abattoir and transported to laboratory in thermocol boxes having enough ice packs. Cysticercii in the mealy pork were separated by blunt dissection to recover them in intact condition. The cysts were washed twice with PBS. The fluid from individual cyst was collected using sterile insulin syringe into sterile container and cystic fluid antigen (CFA) was prepared as described by Yang *et al.* (1998).

Hydatid fluid (HF) was aspirated after cleaning the cyst wall with spirit swab. The fluid was then centrifuged (5000 rpm for 5min), supernatant was used as HF antigen and sediment was examined under microscope to determine the fertility of each cyst.

Fluid of *Cysticercus tenuicollis* cysts was aspirated and centrifuged to obtain a clear fluid which was stored in well labelled sterile containers and used as tenuicollis fluid antigen (TFA). All the fluid antigens (CFA, HFA and TFA) were frozen at -20°C after adding 0.1% sodium azide as preservative.

Protein estimation

Cystic fluid from representative samples of each type of metacestode was subjected to protein quantification as described by Lowry *et al.* (1951).

Dialysis and concentration of crude antigens

All the fluid antigens were dialyzed against distilled water at 4°C in separate dialysis tubing (Hi-Media) for 44 hours, during which the water was agitated intermittently and replaced after every four hours during first three changes and after eight hours intervals between last four changes. After dialysis, all the crude antigens were concentrated in the same dialysis tubing, at 4°C in the refrigerator by per evaporation method as described by Hamm (1966). The concentration procedure was continued till the quantity of dialysis tubing reduced approximately to 10%.

Raising hyper-immune sera in rabbits

The hyper-immune sera were raised against three fluid antigens (CFA/HFA/TFA) in duplicates in six New-Zealand white rabbits. Each parasitic antigen (0.25 ml) was emulsified in equal volumes of Freund's complete adjuvant and injected intramuscularly (thigh muscle) in the rabbits. Booster injections were administered at 14, 21, 28 and 35 days, post initial inoculation, with Freund's incomplete adjuvant (Alkarmi and Faubert, 1985). Serum samples were collected 7 days after the last booster injection.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Slab gel SDS-PAGE was performed as per the method described by Laemmli (1970) in 10 % polyacrylamide gel and 4.5 % stacking gel. A protein amount of 50 μg of each sample was loaded. The proteins were allowed to migrate in the slab gel under the constant current of 26mA till the tracking dye, bromophenol blue reached the lower margin of the slab. Gels were then stained with 0.1 % Coomassie brilliant blue (R-250) for four hours and destaining was done till the background became clear.

Fractionation of CFA, TFA and HFA antigens by gel filtration column chromatography

Fractionation of concentrated *Cysticercus cellulosae*, *Cysticercus tenuicollis* and hydatid fluid antigens was done in Sephadex G-200 (Superfine Pharmacia) column as per the protocol specified in Pharmacia manual entitled "Gel Filtration-Theory and Practices (1981)." Briefly, column was loaded with 70 mg of antigen (CFA, TFA or HFA) reconstituted in five ml of eluent buffer (PBS, 0.1M, pH7.2). A total of 80 fractions of 2.5 ml each were collected in test tubes by using automatic fraction collector (Bio-Rad). The optical density of each tube read on double beam UV spectrophotometer (Shimadzu, Japan) at 280 nm. Before switching over from one antigenic type to the other type, eluent buffer equal to five volumes of bed was run through the column. The antigenic fractions obtained under each peak were pooled, dialyzed and concentrated as per the method described earlier.

Determination of parasite specific component

To determine the parasite component of different metacestode, the column chromatographically fractionated antigens were subjected to double diffusion (Varela-Diaz and Coltorti, 1974) and indirect haemagglutination (Zamani *et al.*, 2001 and Selvam *et al.*, 2004) tests with each type of antisera raised separately in duplicates against crude fluid antigens of the three bladder worms. Two rabbit sera not immunized with fluid antigens constituted known negative sera.

Results

Protein content of cystic fluid

The protein content of fluid samples of 30 cysts of *Cysticercus cellulosae* collected from 10 different pigs ranged from 13 - 148 mg% with an average of 69.42 mg%. Fluid samples from 20 *Cysticercus tenuicollis* cysts recovered from 15 pigs revealed protein content of 16 to 78 mg% with an average of 37.3 mg%. Average protein content of HF (**Table 1**) recovered from the cysts encountered in the pigs was 69.88 mg% with higher protein content in the HF from hepatic cysts (78.15 mg%) than that from pulmonary cysts (68.28 mg%) and in cysts encountered in spleen it was lowest (42.60 mg%). In general protein content of HF from fertile cysts (76.03 mg%) was higher than that from sterile cysts (58.40 mg%).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of cystic fluids

As shown in **Figure 1**, in CFA at least 13 protein bands were recognized with their molecular weights ranging from 220 to 14 kDa (220, 160, 97, 90, 84, 70, 65, 58, 54, 38, 29,

24 and 14 kDa); bands of 84, 65, 54 and 38 kDa were prominent. TFA revealed 12 bands in the molecular weight range of 312 to 24 kDa (312, 298, 256, 220, 160, 114, 104, 70, 58, 50, 44 and 24 kDa) of which 220, 160 and 24 kDa were prominent as demarcated by sharp and dense bands. HF showed eight distinct fractions with their molecular weights evidenced as, 272, 237, 180, 90, 70, 58, 48 and 38 kDa with an increasing mobility; protein components of 90, 70 and 58 kDa were found to be prominent.

Comparison of band pattern of crude fluid antigens revealed that fractions having molecular weights of 70 and 58 kDa were common to all the three metacestodes encountered in the present study. The components having molecular weights 220, 160 and 24 kDa were common between *C. cellulosae* and *C. tenuicollis* and proteins with molecular weights of 90 and 38 kDa were common between *C. cellulosae* and hydatid cyst.

Column chromatography of crude cystic fluid antigens

Column chromatographic analysis of all the three crude antigens revealed same pattern i.e. two distinct peaks representing high and low molecular weight proteins, respectively.

Identification of parasite specific component

From the **tables 2 and 3**, it is evident that none of the peaks i.e. partially purified antigens, is absolutely specific to the metacestodes as cross reactivity was exhibited by all the six partially purified antigens. At the same time, both the negative rabbit sera did not show positive reaction with all the six antigens in DD as well as IHA tests. However it is amply evident from both the tables that Peak II antigen of *Cysticercus cellulosae* fluid and Peak I antigen of *Cysticercus tenuicollis* and hydatid fluid exhibited better specificity than Peak I antigen of *Cysticercus cellulosae* fluid and Peak II antigen of *Cysticercus tenuicollis* and hydatid fluid for immunodiagnosis of *Cysticercus cellulosae*, *Cysticercus tenuicollis* and hydatidosis, respectively. Peak II antigen of *Cysticercus cellulosae*, although did not reveal cross reactivity with antisera raised against *Cysticercus tenuicollis* and hydatid fluid crude antigens in DD test, one antiserum raised against hydatid antigen showed mild positive reaction (1:16 titre) in IHA. Similar trend of more number of cross reactivity with IHA than that with DD was also evinced with Peak I antigen of *Cysticercus tenuicollis* and hydatid fluid.

Discussion

In the pursuit of antigenic study, several workers have employed electrophoretic mobility pattern as a criterion for identification of parasite specific components of *Cysticercus cellulosae* and reported fractions from 5 to 19 with their molecular weights

ranging from 14 to 200 kDa (Cheng and Ko, 1992 and Ko and Ng, 1998). The banding pattern of SDS-PAGE analysis of fluid of *Cysticercus cellulosae* obtained in the present study is more or less matches with the findings of Shiguekawa *et al.* (2000). Among the different fractions, components having lower molecular weights ranging between 32 to 8 kDa identified as *Cysticercus cellulosae* specific immuno-reactive proteins by several workers (Kong *et al.*, 1992; Ko and Ng, 1998 and Shiguekawa *et al.*, 2000) have also been detected in the present study (29, 24 and 14 kDa).

Owing to non zoonotic nature and comparatively lesser economic importance the number of studies reporting antigenic profiles of *Cysticercus tenuicollis* are relatively less in the available literature. Further, the pattern of electrophoretic mobility of various components of *Cysticercus tenuicollis* fluid from pigs has not been cited in the available literature. However, observations on the same from the material derived from the intermediate hosts other than pigs reported by Kara (2005), Kordafshari *et al.* (2010) and Goswami *et al.* (2013) showed variation in the number of protein components (5 to 8) and their molecular weights (149.4 to 9.6 kDa).

Very few workers have investigated HF of pig origin as a source of antigen for immunodiagnosis of hydatid disease (Gatne, 2001). Protein fractions in the range of 70 to 50 kDa obtained in the present study conforms to electrophoretic mobility of a well known “Antigen 5” which is considered as hydatid specific proteins (Felice *et al.*, 1986 and Hamrioui *et al.*, 1988). However, three bands with high molecular weights (272, 237 and 180 kDa) noticed in the present study have not been reported in HF from pigs in the available literature. This could be attributed to strain differences of *Echinococcus granulosus* as also revealed during molecular studies conducted by Pednekar *et al.* (2009) who reported G3 (buffalo strain) and G5 (cattle strain or *Echinococcus ortleppi*) strains from the infected pigs slaughtered at Mumbai abattoir.

Several workers have used column chromatography with different grades of Sephadex and Sephacryl, as a tool to separate protein fractions of *Cysticercus cellulosae* (Kumar *et al.*, 1987; Kumar and Gaur, 1989; Cheng and Ko 1992 and Kong *et al.*, 1992) and hydatid fluid (Gatne, 2001) and reported 4 to 7, and two distinct peaks, respectively. In contrast, Saha *et al.* (2011) observed several peaks of HF proteins recovered from the cysts in sheep. However, majority of workers have reported two well separated peaks in the cystic fluid of *Cysticercus cellulosae* (Kumar and Gaur, 1989) and hydatid fluid (Gatne, 2001). The information on column chromatographic pattern of *Cysticercus tenuicollis* fluid antigen is surprisingly not

cited in the available literature to confirm the trend. The present observation thus seems to be the first report of chromatographic behaviour of cystic fluid antigen of *Cysticercus tenuicollis*.

Peak II of *Cysticercus cellulosae* fluid antigen most probably contained protein fractions of low molecular weights (MW < 32 kDa). Xuepeng *et al.* (2006) in their exhaustive review article on immunodiagnosis of cysticercosis, emphatically stated that high molecular weight proteins (105 to 85 kDa) of *Cysticercus cellulosae* fluid antigen are responsible for non specificity owing to cross reactivity with individuals harbouring other helminthic infections and more particularly *Cysticercus tenuicollis* infection. Correspondingly, several workers (Cho *et al.*, 1988; Chung *et al.*, 1999 and Espindola *et al.*, 2005) have reported high specificity of low molecular weight (LMW) proteins of the pork measles using number of serodiagnostic methods in pigs and man. However, cross reactivity of these components with the sera of the individuals infected with other bladder worms such as hydatid cyst (Cho *et al.*, 1987 and Yang *et al.*, 1998) and *Cysticercus tenuicollis* (Kumar *et al.*, 1987 and Cheng and Ko, 1991) have also been reported in the literature. Further cross reactivity of these antigens with the sera of individuals with other helminthic infections such as ascariasis (Cheng and Ko, 1991), *Metastrongylus apri* (Cheng and Ko, 1991) and *Fasciolopsis buski* (Cheng and Ko, 1992) have also been cited in the literature.

Peak I antigen of HF probably contain 'Antigen 5' the molecular weights of which reported to be in the range of 70 to 50 kDa. Although it is considered as specific to hydatid, references revealing cross reactivity with the sera of the subjects infected with *Cysticercus cellulosae* (Shepherd and MacManus, 1987 and Kong *et al.*, 1989) are not uncommon in the literature.

As regards *Cysticercus tenuicollis*, the information pertaining to antigenic profiles in general and cross reactivity in particular is not adequately available in the literature to confirm the trend observed in the present study.

Conclusion

In conclusion, the present study throws adequate light on the cross reacting components in cystic fluid of commonly occurring porcine metacestodes. The study reveals that Peak II antigen of *Cysticercus cellulosae* (7-32 kDa proteins) and Peak I antigen of *Cysticercus tenuicollis* and hydatid fluid are more specific than the proteins covered under other peaks and hence can be exploited further for standardization of sensitive and specific immunodiagnostic assay for screening of sera sample prior to slaughter to curb the economic

losses and public health hazard (except *Cysticercus tenuicollis*) associated with this group of parasites. The antigens, if refined further can also be used in seroepidemiological surveys.

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List of Tables

Table 1: Organ-wise protein content of hydatid fluid from sterile and fertile cysts in pigs

Organ	Type of cyst	No. of HF samples	Protein range (mg%)	Average protein content (mg%)	Organ-wise protein content (mg%)
Lungs	Fertile	12	16 -156	72.33	14 – 156 (68.28)
	Sterile	06	14 -139	60.16	
Liver	Fertile	13	21 -168	85.85	16 – 168 (78.15)
	Sterile	07	16 -148	63.85	
Spleen	Fertile	03	19 – 72	48.33	15 – 72 (42.60)
	Sterile	02	15 & 53	34.00	
Total	Fertile	28	16 -168	76.03	14 – 168 ^{NS} (69.88)
	Sterile	15	14 – 148	58.40	

Figures in parentheses indicate averages. NS-Non significant

Table 2: Results of Double Diffusion (DD) test showing cross reactivity

Bladder Worm	Peak	Antisera raised against bladder worm					
		<i>Cysticercus cellulosae</i> (2)		<i>Cysticercus tenuicollis</i> (2)		Hydatid Cyst (2)	
		1	2	1	2	1	2
<i>Cysticercus cellulosae</i>	I	P	P	N	P	P	N
	II	P	P	N	N	N	N
<i>Cysticercus tenuicollis</i>	I	N	P	P	P	N	N
	II	P	N	P	P	N	P
Hydatid cyst	I	P	N	N	N	P	P
	II	P	N	N	N	P	P
Known Negative sera (2)		All the six peaks revealed negative results					

P= Positive reaction (precipitation band) and N= Negative reaction

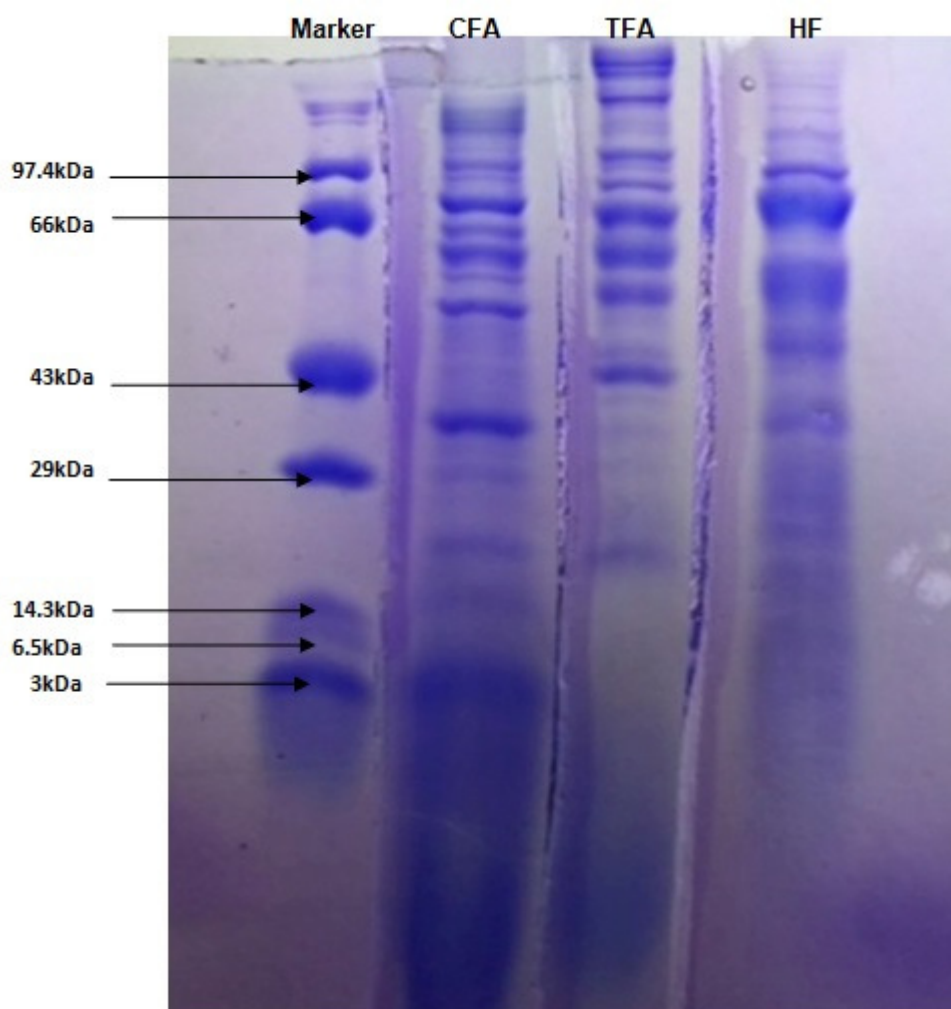
Table 3: Results of Indirect Haemagglutination (IHA) test showing cross reactivity

Bladder Worm	Peak	Antisera raised against bladder worm (IHA Titres)		
		<i>Cysticercus cellulosae</i> (2)	<i>Cysticercus tenuicollis</i> (2)	Hydatid Cyst (2)

		1	2	1	2	1	2
<i>Cysticercus cellulosae</i>	I	1: 256	1:128	1:4	1:32	1:16	1:8
	II	1: 256	1: 512	1:4	1:8	1: 16	1:8
<i>Cysticercus tenuicollis</i>	I	1:8	1:16	1:128	1:512	1:8	1:16
	II	1:32	1:8	1:64	1: 128	1:8	1: 16
Hydatid cyst	I	1:32	1:8	1:8	1:8	1: 256	1:256
	II	1:16	1:8	1:4	1:64	1: 128	1:512
Known Negative sera (2)	All the six peaks revealed negative results IHA titre less than 1:16 (cut off value)						

Titres 1:16 & above were considered as positive reaction

Figure 1: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Cystic Fluids of porcine metacestodes



CFA- cyst fluid antigen of *Cysticercus cellulosae*
TFA- tenuicollis fluid antigen

HF- hydatid fluid antigen
kDa- kilo Dalton