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Studies on antigenic profile of *Cysticercus cellulosae* in pig to develop specific immunodiagnostic test

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Abstract

A study was conducted to standardize an immunodiagnostic assay (ELISA) with *Cysticercus cellulosae* specific purified antigen for ante-mortem detection to study epidemiology of porcine cysticercosis. *Cysticercus cellulosae* cysts were exploited for preparation of whole cysts antigen (WCA) and scolex antigen (SA). The average protein content of whole cyst and scolex were 8.5 mg/cyst and 5.92 mg/scolex, respectively. SDS - PAGE analysis of WCA and SA revealed 18 and 16 fractions with their molecular weights ranging from 103 to 12 and 98 to 12 kDa, respectively. The comparison of the band patterns revealed that protein fractions of lower molecular weights (29, 26, 24, 18, 14 and 12) were strikingly common in WCA and SA. Chromatographic analysis of crude antigen revealed two identical peaks of WCA and SA. SDS-PAGE of peak I antigen revealed two immunoreactive components, viz. 29-26 kDa and 14 kDa. Hence, Peak I antigen was used for standardization of indirect ELISA for detection of porcine cysticercosis. The assay revealed sensitivity of 98.35%, specificity of 100% and accuracy rate of 98.58%. The predictive values for positive and negative readings were 100 and 91.91%, respectively and percentage of false positive and false negative was found to be zero and 1.65, respectively. Cross reactivity of 4.55% and 15.0% was noticed between the peak I antigen and hydatid and *Cysticercus tenuicollis* positive test sera, respectively. Thus antibody-ELISA with peak I antigen of WCA is strongly recommended for sero-epidemiological study of porcine cysticercosis.

Keywords: *Cysticercus cellulosae*, whole cyst antigen, scolex antigen, immuno-diagnosis

Introduction

Among the different helminths of pigs, *Cysticercus cellulosae* is considered to be the most economically and zoonotically significant helminthic parasite that settles in musculature and viscera. Man acquires the infection of *Taenia solium* through consumption of uncooked or partially cooked pork containing these *C. cellulosae* cysts. Occasionally humans are also infected with these cysts by consuming eggs of *T. solium* through contamination of food (Uncleaned green vegetables) and water. The disease taeniasis and cysticercosis caused by this parasite is considered as neglected tropical disease ^[1].

For prevention of public health hazard, inspection of pork for presence of *C. cellulosae* is the key and infected carcasses have to be condemned completely or partially depending on intensity and distribution of the cysts, leading to a great economic loss. *C. cellulosae* are Located in the musculature and viscera of pigs and thus cannot be diagnosed by conventional parasitological methods. Hence serological methods have been and are being employed for anti-mortem detection of porcine cysticercosis. The efficacy of sero-diagnostic methods is greatly influenced by antigenic preparation. Thus there is a need to standardize an immunodiagnostic tool which is very sensitive and reasonably specific to generate epidemiological data. Hence the present study was conducted to identify and characterize parasite specific antigenic component of *C. cellulosae* that can improve the efficacy of immune-diagnosis of porcine cysticercosis. *C. cellulosae* whole cyst antigen and scolex antigen were exploited in the present study with SDS- PAGE analysis and column chromatography to evaluate their suitability for development of specific immunodiagnostic test.

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Materials and Methods

Collection of material

Cyst sample were collected at Deonar abattoir Mumbai from the carcass showing pork measles and placed in normal saline. Few specimens were used for morphological identification of *Cysticercus cellulosae*. Blood samples of pigs were collected at the time of sticking and known positive and known negative status of the animal was registered during the process of slaughter. The serum samples collected from blood were stored at -20°C after adding merthiolate as preservative till further use. Sera samples from 20 farm pigs below six months of age were also collected and used as a panel of known negative sera.

Preparation of crude antigens

Crude whole cyst antigen (WCA) was prepared as per the procedure described by Mahajan *et al.* [12] with few modifications. Ten grams of *C. cellulosae* cysts were washed in PBS pH 7.2 containing antibiotics and suspended in 20 ml of PBS containing phenyl methyl sulphonyl fluoride (PMSF) as protease inhibitor (20 µg/ml) and 0.01% sodium azide as antifungal agent. The cysts were homogenised at 4°C for 30 minutes and then sonicated four times. The homogenate was then centrifuged at 4°C in a refrigerator centrifuge and supernatant was separated as crude whole cyst antigens and preserved in small aliquots at -20°C. Ten gram of scolices were used for preparation of scolex antigen (SA) and 10 grams of skeletal muscle piece from healthy pig was used for preparation of crude muscle antigen as per the method described for WCA.

Protein estimation, dialysis and concentration of crude antigens

Each aliquot of crude WCA and SA was subjected to protein estimation by Lawry's method as modified by Hartree [13]. The crude antigens *viz.* WCA and SA were concentrated in dialysis tubing as per the method described by Kumar *et al.* [14]. The dialysis tubing was placed in petri dish containing hot 2% sodium bicarbonate and EDTA solution for 10 minutes and then thoroughly rinsed in distilled water for 30 minutes. The filled dialysis tubing then suspended in a beaker containing distilled water in refrigerator 4°C for 32 hrs. After dialysis both the crude antigens were concentrated in the same dialysis tubing at 4°C in the refrigerator by per evaporation method as described by Hamm [15]. The concentrated crude antigens were subjected to protein estimation before lyophilisation.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weights of different protein components of WCA and SA were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis [16] with few modifications, in 10 % separating gel and 4.5% stacking gel. The lyophilized crude antigens were reconstituted in phosphate buffer pH 7.2 just before their application to obtain concentration of each antigen at 50mg per millilitre. The gels were stained with Coomassie brilliant blue stain and the molecular weights were determined by using standard molecular weight marker and the gel scanner software.

Fractionation of WCA and SA by column chromatography

Fractionation of concentrated WCA and SA was done in Sephadex G-200 column as per the protocol of manufacturer.

The column was loaded with 50 mg of antigen (WCA or SA) reconstituted in 5ml of eluent buffer (PBS 0.1M, PH 7.2) and then connected to the reservoir containing eluent buffer. The flow rate was adjusted to 10ml per hour. A total of 80 fraction of 3 ml each were collected in a test tube by using automatic fraction collector. The optical density of each tube read on double beam UV spectrophotometer at 280 nm.

Preparation of hyperimmune sera in pigs

Hyperimmune sera were raised in two pigs against crude WCA by injecting 400 µg antigen mixed with equal volume of Freund's complete adjuvant [17]. Booster inoculations with Freund's incomplete adjuvant were repeated fortnightly. Both the pigs were test bled after 5th and 6th inoculations and sera were separated and kept in aliquots at 20°C by adding 0.01% merthiolate as preservative.

Identification of *C. cellulosae* specific components in fractionated WCA and SA

Different peaks obtained by fractionating WCA and SA through gel filtration were dialysed and concentrated by per evaporation method. The purified antigens under each peak were then tested against hyper immune sera by employing double diffusion test. Double diffusion test was performed as per the standard procedure described by Varela-Diaz and Coltorti [18]

Indirect enzyme linked immuno sorbent assay (Indirect ELISA)

The assay was performed with purified peak II antigen of WCA/SA. Affinity isolated anti pig IgG peroxidase conjugate and ortho-phenylenediamine were used to develop colour reaction indicating presence of antigen antibody complexes. The colour intensity was read at 490 nm. Optimum concentration of the antigen and dilution of secondary antibody conjugate and test sera were decided by checkerboard titration.

After the standardization the test sera was run in duplicate along with positive and negative control, buffer control and substrate control. A total 283 pigs sera (221 pig sera including 121 from pigs with *Cysticercus cellulose* in the musculature and 100 from pigs without *C. cellulose* cysts, 22 known positive sera for hydatid cyst, 20 known positive sera for *C. tenuicollis* and 20 known negative sera) were tested by ELISA.

Statistical analysis

The data generated during the study were subjected to completely randomized design for serological findings as per the method described by Snedecor and Cochran [19].

Results

Antigenic preparation and antigenic characterization of *Cysticercus cellulosae*

Protein content of *C. cellulosae* antigens

The protein content of whole cyst antigen varied from 6 to 14 mg per cyst with an average of 8.5 mg/cyst. Protein content of scolex antigen ranged from 3 to 9 mg /cyst with an average of 5.92 mg /cyst. The average weight of each cyst and scolex was found to be 514 and 37.5 mg, respectively and hence percentage of soluble protein calculated on w/w basis in whole cyst and scolex was found to be 1.65 and 15.79, respectively. Thus 69.64 % of the total proteins of *C. cellulosae* cysts was calculated to be derived from scolices.

SDS - PAGE analysis of WCA and SA

SDS - PAGE analysis of WCA showed total 18 differentiated bands with molecular weight ranging from 103 to 12 kDa; while SA revealed 16 components, the molecular weight of which ranged from 98 to 12 kDa. The protein moieties of WCA expressed on the slab gel were 103, 84, 74, 69, 57, 50, 45, 40, 38, 37, 35, 29, 28, 26, 24, 18, 16, 14 and 12kDa. In SA, protein fractions of 98, 80, 72, 67, 55, 48, 44, 39, 37, 34, 29, 26, 24, 18, 14 and 12kDa were separated according to electrophoretic mobility. However, the fractions of WCA and SA, in the zone below 28kDa marker protein appeared similar in their electrophoretic mobility and density when stained with silver nitrate. Amongst these low molecular weight proteins the bands of 29 and 26 kDa were prominent and sharp. Comparatively bands between 26-12 kDa were less prominent in terms of density and sharpness. (Fig.1).

Column chromatography of crude antigens

Gel filtration column chromatography graph revealed two distinct peaks of WCA as well as SA. The first peak of WCA and SA yielded 18 and 17 mg of proteins and distinctly smaller than the second peak which yielded 28 and 27 mg of proteins respectively. Thus the first peak antigen constituted about 34 to 36% and second peak antigen constituted 54 to 56 % of total proteins.

Identification of *C. cellulosae* specific immunodominant protein component

Peak I antigen from WCA and SA showed positive precipitation reaction with hyper immune sera raised in pigs against whole *Cysticercus cellulosae* crude antigen but did not react with other three types of sera. As regards IHA test, similar trend was obtained except one serum from hydatid

positive pig showed mild positive IHA titre (1:16). The cut off titre based on results of known negative sera (1:4) was decided as 1:8. Performance of Peak II proteins of both antigens was also identical. (Table 1 and Fig. 2).

SDS-PAGE analysis of Peak I and Peak II fractions of both the sources revealed identical results. Peak I antigen contained 12 and 10 protein bands with molecular weights lower than 45 and 44 kDa of WCA and SA, respectively; while Peak II antigen possessed higher molecular weight proteins equal to and more than 50 and 48 kDa expressed in 6 bands each in WCA and SA, respectively.

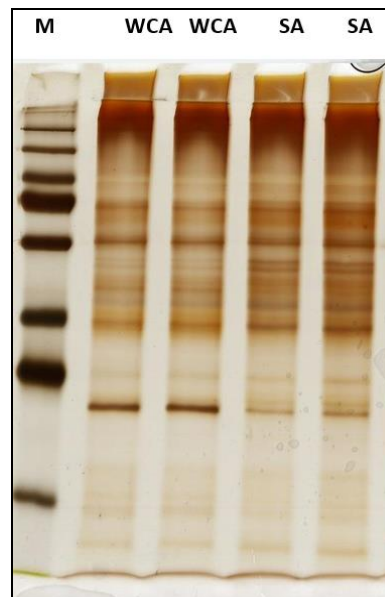


Fig 1: SDS-PAGE band patterns of SA and WCA by silver staining

Table 1: Reactivity of Peak I and Peak II proteins of WCA and SA against *C. cellulosae*, *C. tenuicollis*, hydatid known positive and known negative sera in DD and iHA

Immunoreactivity of antigens								
Peak I				Peak II				
	DD	WCA		SA		WCA		SA
		IHA	DD	IHA	DD	IHA	DD	IHA
		Titre		Titre		Titre		Titre
Hyper immune sera (1)	+	1:1024	+	1:1024	+	1:2048	+	1:2048
Hyper immune sera (2)	+	1:512	+	1:512	+	1:512	+	1:512
Known negative serum (1)	—	1:4	—	1:4	—	1:4	—	1:4
Known negative serum (2)	—	1:4	—	1:4	—	1:4	—	1:4
Serum from pig with hydatidosis (1)	—	1:16	—	1:16	—	1:128	—	1:128
Serum from pig with hydatidosis (2)	—	1:4	—	1:4	—	1:32	—	1:32
Serum from pig with <i>C. tenuicollis</i> (1)	—	1:4	—	1:4	—	1:16	—	1:16
Serum from pig with <i>C. tenuicollis</i> (2)	—	1:4	—	1:4	—	1:4	—	1:4

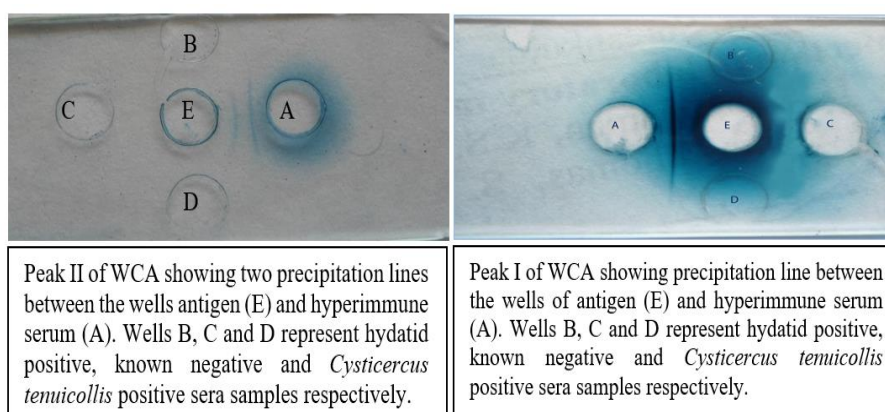


Fig 2: Double diffusion test

Immunodiagnosis of porcine cysticercosis by ELISA

In the present work, antibody ELISA was standardized with peak I antigen of WCA using known positive hyperimmune and known negative pig sera. The results of the ELISA of 121 *Cysticercus cellulosae* confirmed cases as per meat inspection and 20 assured known negative cases are depicted in (Table 2). These findings revealed that the assay was 98.35% sensitive and 100% specific with a very high accuracy rate of 98.58%. The predictive values for positive and negative readings were 100 and 91.91%, respectively and percentage of false positive and false negative was found to be zero and 1.65, respectively. The results of ELISA with panel of other

three categories of sera viz. 100 from pigs without apparent cysticerci, 22 from porcine hydatidosis and 20 *Cysticercus tenuicollis* infected pigs, are furnished in Table 2. When OD values of sera of pigs belonging to different groups as stated in the table were subjected to completely randomized design of statistic, highly significant difference was obtained between two OD values of sera from pigs with *Cysticercus cellulosae* in the meat and the OD values of sera from the other three groups (Table 3). Thus statistical analysis clearly indicated distinctly higher antibody response in the pigs with detectable *Cysticercus cellulosae* in the pork.

Table 2: Results of ELISA for diagnosis of porcine cysticercosis

Elisa results diagnostic OD 0.140	Status of <i>C. cellulosae</i>		Total
	No. of known positive sera	No. of known negative sera	
Positive OD	119	0	119
Negative OD	02	20	22
Total	121	20	141

Table 3: Details of ELISA ODs in diagnosis of swine cysticercosis with sera from different groups of pigs

Description of sera Diagnostic OD = 0.140	Number of sera	OD range (Average)	Sera with positive OD (%)	Sera with negative OD (%)	Remarks
Pigs with <i>C. cellulosae</i> in musculature	121	0.127 – 0.865 (0.382) ^a	119 (98.34)	02 (1.65)	Confirmed positive
Pigs apparently free of cysticerci during meat inspection	100	0.055 – 0.230 (0.096) ^b	09 (9.0)	91 (91.0)	Limitation of meat inspection
Pigs with hydatid cyst	22	0.066 - 0.232 (0.099) ^b	01 (4.55)	21 (91.45)	Cross reactivity with hydatid sera
Pigs with <i>C. tenuicollis</i>	20	0.060 - 0.292 (0.128) ^b	03 (15.0)	17 (85.0)	Cross reactivity with <i>C. tenuicollis</i> sera

OD average with different superscripts indicates significant difference at 1% level of probability (CD = 0.155).

Correlation between ELISA OD and intensity of *Cysticercus cellulosae*

When the OD values of ELISA arranged as per the number wise intensity of the cysts were subjected to statistical analysis by completely randomized design, a highly

significant difference was noticed between all the three groups. Sensitivity of the assay in detection of mild intensity cases was lowered to 96.72% as compared to 100% for detection of cases with moderate or heavy intensity of the cysts. (Table 4).

Table 4: Correlation between ELISA OD and intensity of *Cysticercus cellulosae*

Intensity of the cysts in meat/unit area Diagnostic OD = 0.140	Number of Sera	OD range (Average)	No. of false negative	Sensitivity (%)
Mild (up to 4 cysts)	61	0.127-0.382 (0.256) ^a	02	96.72
Moderate (5-8 cysts)	48	0.382-0.580 (0.458) ^b	Nil	100
Heavy (9 & above cysts)	12	0.601-0.865 (0.718) ^c	Nil	100
Total	121	0.127-0.865 (0.382)	02	98.35

OD average with different superscripts indicates significant difference at 1 % level of probability (CD = 0.097).

Discussion

SDS - PAGE analysis of WCA and SA

There could be masking effect of scolex derived proteins over WCA proteins in SDS-PAGE. The band pattern of WCA and SA obtained in the present study principally coincides with the pattern described by Tsang *et al.* [10], Ko and Ng [11] Sreenivasamurthy *et al.* [12], Dhanalakshmi *et al.* [13] and Sreedevi [14] particularly in relation to molecular weight range of fractionated proteins. However, number of protein components and their representative molecular weights in different antigenic preparations (membrane / scolex / fluid / whole cyst and extracts) of *Cysticercus cellulosae* shows vast difference, may be due to method of antigen preparation, sensitivity of the fractionation technique employed, strain difference of *T. solium* in different parts of the world and development stage of the parasitic cysts.

Column chromatography of crude antigens

The chromatographic behaviour of WCA and SA observed in this study is in full agreement with the finding of Kumar and Gaur [15] and Kaur *et al.* [16]. However Kumar *et al.* [4] and Cheng and KO [17] obtained respectively three and four distinct peaks of crude *Cysticercus cellulosae* antigens subjected to gel filtration chromatography respectively.

Identification of *Cysticercus cellulosae* specific immunodominant protein component

From the result of DD and IHA tests with Peak I and Peak II fractions of WCA and SA, it was evident that Peak I and Peak II of WCA were identical to Peak I and Peak II of SA, respectively as far as immunoreactivity was concerned. Further Peak I and Peak II, irrespective of the source were

equal in sensitivity but specificity of Peak I was better than that of Peak II. The result of DD and IHA using Peak I and Peak II components of WCA and SA against sera samples from pigs with known status of bladder worm infections revealed superiority in terms of specificity of Peak I antigen over Peak II antigen of either source.

Sensitivity of ELISA in detection of porcine cysticercosis

In the present study average OD of ELISA was found to be lowest in the pigs carrying mild intensity of *Cysticercus cellulosae* and it was highest in the pigs with heavy intensity of the cysts in the musculature. Both the sera showing false negative ELISA OD were from pigs with mild intensity of porcine cysticercosis. The difference in ELISA titers (OD) of the three groups of known positive Pigs viz. mild, moderate and heavy, was found to be statistically significant. This variation in the sensitivity of serodiagnostic procedure could be due to difference in the avidity of antibodies induced during immunostimulation of host by the bladder worms at different stages of development. Furthermore, ELISA based on IgG detection has been reported to be more sensitive than ELISA based on IgM detection [18]. This is obviously due to variation in the half-life of immunoglobulins of different classes in the circulation.

Although there exists a wide variation in the sensitivity of ELISA and other serodiagnostic tests in detection of porcine cysticercosis, it is definitely a better option than the conventional methods such as antemortem tongue inspection [19, 20] and post mortem meat inspection in the slaughter house [21, 22]. In the present study as well, the prevalence of porcine cysticercosis based on meat inspection in abattoir revealed only 1.26%. However, out of 100 sera of the pigs found free of *Cysticercus cellulosae* during meat inspection in the abattoir, 9 (9.0%) samples showed positive OD (Table 4). Thus seroprevalence was found to be almost seven times higher than the prevalence rate detected during meat inspection. This discrepancy in the prevalence rate could be due to either presence of either very few deeply seated cysts or presence of degenerated/calcified indistinguishable cysts or presence of migrating oncospheres in the body prior to transformation into the cysts. Interestingly, OD values of all the nine cases were in the range (Avg.) of mild intensity group of 121 known positive pigs inspected during the study.

Specificity of ELISA in detection of porcine cysticercosis

As regards specificity of the assay, cent percent results obtained in the present study tallies precisely with observations of D'Souza and Hafeez [21], Biondi *et al.* [23], Pinto *et al.* [24] and Selvam *et al.* [22]. In the present study, specificity of ELISA was determined exclusively based on known negative sera. However, present study also revealed cross reactivity with sera from porcine hydatidosis (4.55%) and *Cysticercus tenuicollis* (15.0%). Although prima facie it appears to be cross reaction, there exists a possibility of concomitant *C. cellulosae* infection with very light intensity of the cysts located deeply in the musculature or presence of migrating oncospheres or disintegrating indistinguishable cysts that could not be detected during meat inspection. Specificity of 90.48% based on results of these 42 cases (22 hydatid and 20 *Cysticercus tenuicollis*) in the present study is by no means a rejectable proportion.

Conclusion

Thus in the present investigation, partially purified antigen

was used for sero-diagnosis of porcine cysticercosis without under going further purification of 29 and 14 kDa proteins as identified by immunoblot and the composition of ELISA reagents gives more than adequate confidence level in the diagnosis of porcine cysticercosis. As also discussed earlier, porcine cysticercosis in India owing to below par managerial standards will always be detected on drift basis for which sensitivity is more important than the specificity. In endemic area with every possibility of having substantial number of lightly infected cases, a sensitive diagnostic procedure has to be adopted. In the present study as well, there was more percentage of lightly infected cases as compared to the other two categories encountered during meat inspection. Thus the ELISA with the composition of reagents used in the present study offers a reliable tool in the sero-epidemiology of porcine cysticercosis and hence is of a great support in the comprehensive porcine cysticercosis control program designed towards curbing economic losses and public health hazards. Use of antibody ELISA with HRPO enzyme system and partially purified peak I of whole cyst antigen of *Cysticercus cellulosae* is therefore recommended for sero-epidemiological studies of porcine cysticercosis in India.

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