7. SUMMARY AND CONCLUSION
Cystic echinococcosis (CE), caused by *E. granulosus*, is a disease of high prevalence in developing countries including India. Though the actual country wide prevalence and incidence of human CE in India is not yet known, the increasing reports and information, obtained mostly from hospital-based studies, indicates the disease is still very active or is re-emerging and probable endemicity of CE in India, which is the subject of concern. Therefore, the development of immunodiagnosis tests that detects species-specific antibodies or antigens in serum or other body fluids is a necessity.

Hence, in the first phase of the present study, ZAP Express cDNA library was constructed from *E. granulosus* metacestode germinal membrane mRNA to identify and evaluate sensitive and specific antigen for the diagnosis of CE.

The *E. coli* expression cDNA library was immunoscreened using a pool of sera collected from patients with surgically confirmed CE and rabbit polyclonal hyperimmune serum to identify and isolate immunodominant diagnostic relevant recombinant antigen producing clones. Immunoscreening of $10^6$ (1.5 x $10^6$/10 cm plate) pfu of the ZAP Express cDNA library produced nine positive clones (HSP2, HSP4, HSP9, CWP4, CWP5, CWP9, CFP2, CFP3, CFP7) with high intensity.

Having shown the potential serodiagnostic value of their expression products, the cDNA inserts of HSP2, HSP4, HSP9, CWP4, CWP5, CWP9, CFP2, CFP3 and CFP7 plaques were sub-cloned into the pGEX-3X vector and expressed as GST fusion proteins. All nine clones produced GST-fusion proteins in *E. coli* cells which were well detectable by staining the gel with Coomassie blue R-250.

All the nine GST-fusion proteins were tested for their immunoreactivity towards human anti-*E. granulosus* antibodies by EITB. The four GST-fusion proteins which were found to be expressed in larger quantities (HSP2, HSP4, CWP5 and CFP7) gave strong positive signals with all 10 sera collected from surgically confirmed patients with CE. Other GST-fusion proteins (HSP9, CWP4, CWP9, CFP2 and CFP3) though recognized by anti-*E. granulosus* antibodies, showed weak signals with the surgically confirmed patient sera in westernblot analysis.

In the present study, therefore, the clones HSP2, HSP4, CWP5 and CFP7 producing
recombinant \textit{E. granulosus} antigens were selected to test their specificity to use as diagnostic antigens for the diagnosis of CE. The four clones, HSP2, HSP4, CWP5 and CFP7 produced expression proteins with a Mr of about 40 kDa, 24 kDa, 12 kDa and 10 kDa respectively, after cleaving the GST moiety (Mr of about 27 kDa).

In the present study, \textit{E. granulosus} recombinant antigen B8/2 was also produced by cloning and expression of corresponding genomic DNA fragment. The \textit{E. granulosus} recombinant antigen B8/2 showed a Mr of about 8 kDa, after cleaving the GST moiety.

For further immunological evaluation of these \textit{E. granulosus} recombinant antigens, a pannel of sera collected from 10 surgically confirmed patients with CE, 10 patients with NCC, 3 patients each with filariasis, schistosomiasis, malaria, amoebiasis, and 10 healthy individuals, were tested by both EITB and ELISA. Both EITB and ELISA using GST-fusion proteins produced by HSP4, CWP5 clones and Eg-AgB8/2 (designated as Eg-rCW24, Eg-rCW12 and Rec Eg-AgB8/2 respectively) showed 100 \% sensitivity and specificity in initial screening.

The recombinant proteins, Eg-rCW24, Eg-rCW12 and Rec Eg-AgB8/2 were further evaluated for their potential use as immunodiagnostic antigens by ELISA and Dot-ELISA for diagnosis of CE.

\textbf{Evaluation of ELISA and Dot-ELISA using \textit{E. granulosus} recombinant antigens to detect hydatid antibodies in serum for diagnosis of cystic echinococcosis}

The ELISA using \textit{E. granulosus} recombinant antigens, Eg-rCW12, Eg-rCW24, for detection of antibodies in serum, showed a sensitivity of 92.00\% and 95.00\% respectively. The specificity of the ELISA using Eg-rCW12, Eg-rCW24 antigens for detection of antibodies in serum was observed to be 96.66\% and 95.55\% respectively. The sensitivity and specificity of the ELISA using \textit{E. granulosus} recombinant antigen-B/2 (Rec Eg-AgB8/2) for detection of antibodies in serum was observed to be 94.00\% and 93.33\% respectively. In comparison, the efficiency of the ELISA using Eg-rCW24 (95.26\%) for detection of IgG antibodies in serum was observed to be higher than the ELISA using both Eg-rCW12 (94.21\%) and Rec Eg-AgB8/2 (93.68\%).

The Dot-ELISA using \textit{E. granulosus} recombinant antigens, Eg-rCW12, Eg-rCW24, for detection of antibodies in serum, showed a sensitivity of 88.00\% and 91.00\% respectively.
SUMMARY AND CONCLUSION

The specificity of the Dot-ELISA using either Eg-rCW12 or Eg-rCW24 antigens was observed to be 94.44%. The sensitivity and specificity of the Dot-ELISA using *E. granulosus* recombinant antigen-B/2 (Rec Eg-AgB8/2) for detection of antibodies in serum was observed to be 90.00% and 93.33% respectively. In comparison, the efficiency of the Dot-ELISA using Eg-rCW24 (92.63%) for detection of IgG antibodies in serum was observed to be higher than the Dot-ELISA using both Eg-rCW12 (91.05%) and Rec Eg-AgB8/2 (91.57%).

The Dot-ELISA using Eg-rCW24 showed the highest efficiency in comparison to other two antigens (Eg-rCW12 and Rec Eg-AgB8/2) for demonstration of hydatid antibodies in serum. The ELISA using Eg-rCW24 however, showed a sensitivity similar to that of the ELISA using other antigens (Eg-rCW12 and Rec Eg-AgB8/2). The Dot-ELISA, therefore, using Eg-rCW24 showed to be a simple and rapid diagnostic test for diagnosis of CE.

**Evaluation of ELISA and Dot-ELISA to detect hydatid antigens in serum by using affinity purified antibodies raised against *E. granulosus* recombinant antigens for diagnosis of cystic echinococcosis**

The Ag-ELISA using affinity purified anti-Eg-rCW12 and anti-Eg-rCW24 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 80.00% and 83.00% respectively with a specificity of 97.77%. The Ag-ELISA using affinity purified anti-Rec Eg-AgB8/2 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 83.00% and a specificity of 98.88%. In comparison, the efficiency of the Ag-ELISA using anti-Eg-rCW24 (90.00%) and anti-Rec Eg-AgB8/2 (90.52%) antibodies for detection of hydatid antigen in serum was observed to be higher than the ELISA using anti-Eg-rCW12 antibody (88.42%).

The Dot-ELISA using affinity purified anti-Eg-rCW12 and anti-Eg-rCW24 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 76.00% and 83.00% respectively and a specificity of 96.66% and 98.88% respectively. The Dot-ELISA using affinity purified anti-Rec Eg-AgB8/2 monospecific antibodies for detection of hydatid antigens in serum, showed a sensitivity of 82.00% and a specificity of 96.66%. In comparison, the efficiency of the Dot-ELISA using anti-Eg-rCW24 antibodies (90.52%) for detection of hydatid antigens in serum was observed to be higher than the Dot-ELISA using both anti-Eg-rCW12 (85.78%) and anti-Rec Eg-AgB8/2 (88.94%) antibodies.
The Dot-ELISA using affinity purified anti-Eg-rCW24 antibody showed the highest efficiency in comparison to other two antibodies (anti-Eg-rCW12 and anti-Rec Eg-AgB8/2) for detection of hydatid antigens in serum. The Ag-ELISA using anti-Eg-rCW24 antibody showed the efficiency similar to that of the Ag-ELISA using other antibodies (anti-Eg-rCW12 and anti-Rec Eg-AgB8/2). The Dot-ELISA, therefore, using anti-Eg-rCW24 antibody showed to be a simple and rapid diagnostic test for diagnosis of CE.

In conclusion, in the present study, both Ag-ELISA and Dot-ELISA using affinity purified anti-Eg-rCW24 and anti-Eg-rCW12 monospecific antibodies for detection of hydatid antigens in serum showed high sensitivity and high specificity. Hence both the techniques could be used for diagnosis of CE. The Dot-ELISA, has the added advantage of being more convenient, simple, rapid, cheap and reliable, does not require costly equipment and requires only lesser amounts of specimens and reagents.

Identification of the strains of *E. granulosus* causing human cystic echinococcosis in South India by polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP)

The internal transcribed spacer 1 (ITS-1) of the rDNA repeat was amplified from a total of 45 human isolates of *E. granulosus* collected in South India Pondicherry (Pondicherry), Chennai (Tamilnadu), Hyderabad (Andhra Pradesh), Trivandrum (Kerala) and Bangalore and Manipal (Karnataka), endemic for CE. The PCR product was digested with Alu I and Cfo I endonucleases and characteristic RFLP patterns were produced when samples within various species and strain groups were analysed. The presence of a number of shared bands indicated some degree of relatedness and considerable intra-specific genomic variation among the *E. granulosus* isolates from this part of South India. All these isolates, in the present study, were identified as three genetically distinct strains (sheep, cattle and buffalo) of *E. granulosus*.

The level of diversity in the lengths of ITS-1 gene and in the various RFLP patterns suggested the possibility of more significant changes rather than just point mutations occurring in the ITS-1 component of *E. granulosus*. Results of the present study also showed the presence of two or more distinct ITS-1 types in a single isolate of *E. granulosus*. 

*SUMMARY AND CONCLUSION*
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Study of intra and inter specific genetic diversity and phylogeographic trends of the *E. granulosus* populations from different geoclimatic regions of South India by random amplified polymorphic DNA (RAPD)-PCR

In the present study, the whole genome of *E. granulosus* strains obtained from human patients’ residing in different parts of South India was subjected to random amplified polymorphic DNA (RAPD)-PCR analysis. The RAPD-PCR analysis that requires no prior knowledge of the target sequence, proved to be a useful tool in the present study, for identification and phylogeographic analysis of the strains of *E. granulosus* by identifying robust molecular markers in those strains of *E. granulosus*.

Among the six random primers used in this study, OPB10 primer only was found to amplify all the samples tested. The RAPD-PCR using OPB10 primer produced 8-12 scorable and reproducible DNA fragments of sizes ranging from 2710 base pairs to 157 base pairs and could differentiate clearly all the strains of *E. granulosus*. Moreover, the RAPD-PCR marker was also able to reveal variations between the *E. granulosus* parasite populations collected from different parts of South India. Most importantly, the primer OPB10 produced reproducible RAPD-PCR profiles, when tested in three different trials. Therefore, result of the present study shows that RAPD-PCR primer OPB10 can be a very useful molecular maker for DNA fingerprinting of the strains of *E. granulosus* populations in India at their different spatially hierarchical levels and their phylogeographical levels.

In the present study, the primer OPB10 was employed to generate the RAPD-PCR profiles of a total of 45 human isolates of *E. granulosus* collected in South India. The RAPD-PCR analysis showed that the phylogeography of *E. granulosus* strains is complex, with a high genetic divergence and gene flow between the strains of *E. granulosus*. The RAPD-PCR analysis also showed that the sheep and cattle strains of *E. granulosus* were highly heterogeneous in a single area as well as among different areas. However, the buffalo strains appeared to be homogenous. The route of entry of the parasite into the Indian sub-continent, as far as South India concerned, possibly appeared to be through Chennai (Tamilnadu) and spanning to the neighboring states.

The well balanced atmospheric conditions with sufficient moisture and wide spread agriculture land and forestry, which favour the prolonged survival of *E. granulosus* larvae in the environment after excreted from definitive host, appeared to be the major factors for the increasing prevalence of CE in India, as demonstrated in the present study.