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Molecular and morphological characterisation of *Echinococcus* from food producing animals in India

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Abstract

In view of the medical, veterinary and economic importance of hydatid disease in India, our study aimed to determine the prevalence and genotypes of *Echinococcus* present in domestic livestock in India. Out of 21,861 animals examined, cattle were found with the highest prevalence of hydatid cysts (5.10%) followed by buffaloes (3.81%), pigs (0.87%) and sheep (0.075%). Phylogenetic analysis of the cytochrome oxidase -1 gene revealed that the buffalo strain or G3 genotype was the predominant genotype (29/46) in all species of livestock followed by the cattle strain or G5 genotype (9/46), the G1 genotype or the common sheep strain (6/46) and the G2 genotype or Tasmanian sheep strain (2/46). The ability of the G3 (buffalo) and G5 (cattle) genotypes of *E. granulosus* to infect and produce fertile hydatid cysts in pigs was also demonstrated for the first time. Both morphological and molecular results support earlier studies

suggesting that *Echinococcus* of buffalo origin is phenotypically and genetically similar to the sheep (G1) and Tasmanian Sheep (G2) strains of *Echinococcus*, which adds further evidence to support its recognition as one species *viz*, *Echinococcus granulosus* sensu stricto. Our molecular, morphological and biological characteristics also support earlier studies suggesting that *Echinococcus* of cattle origin, designated the G5 genotype, should be recognised as a separate species *viz E. ortleppi.* Finally, the study reveals that the prevalence of hydatidosis in urban centres in India has been showing a consistently declining trend over the past few decades, possibly owing to economic development and improved government legislation of abattoirs.

Keywords: *Echinococcus granulosus*, India, cattle, pigs, sheep, buffalo, PCR, cytochrome oxidase.

1. Introduction

Cystic echinococcosis, a common metacestode infection in food producing animals also poses a major public health problem, especially in developing countries. Humans are infected with hydatid cysts during natural transmission of the disease from carnivores to domestic animals, by accidentally consuming eggs of *E. granulosus* through contaminated food, water and soil, or through direct contact with dogs. Although the disease in domestic animals is usually asymptomatic and detected only at the time of post-mortem inspection at the abattoir, it causes great economic loss through condemnation of infected offal, in particular, liver. In 2005, the contribution of the Indian livestock industry to the GDP was 6.8% and in 2002 India exported US $320.4 million worth of meat and edible meat offal (FAO, 2005). Previous surveys of hydatid disease in food producing animals in India have revealed that the disease is endemic throughout the country (see Table 1). Measures to control hydatid diseased would not be beneficial the Nation's economy, but also human health. Over 500 cases of hydatid disease requiring surgery has been sporadically reported in the human medical literature of India within the last 50 years (Traub et al., 2005). In India, ideal conditions exist for the establishment, propagation and dissemination of cystic echinococcosis in both humans and livestock. A lack of education and knowledge about the life cycle of the parasite and the lack of veterinary meat inspection and offal disposal at illegally run abattoirs significantly contributes to domestic cycles of transmission. Moreover, home-slaughter, especially for religious events or in rural communities, is commonly practiced throughout the country, and stray and semi-domesticated dogs are given ample opportunity to be exposed to infection.

To date, nine genotypes ($G_1 – G_{10}$) of *E. granulosus* have been identified using molecular tools and the strain variation closely follow the parasite's biological and phenotypic

characteristics (McManus and Thompson, 2003; Nakao et al., 2007). Recently it has been proposed that *E. granulosus* may be a species complex which are likely to be maintained in distinct cycles of transmission comprising of *E. granulosus* sensus stricto (genotypes G1-G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5), G6/G7, *E. canadensis* (genotypes G8 and G10) and *E. felidis* ('lion strain') (Nakao et al., 2007; Huttner et al., 2008). Studies correlating morphological criteria based on the metacestode rostellar hook dimensions with genotype have provided further support for this hypothesis (Thompson et al., 2006). To date, no data on the morphological characteristics of the Indian Buffalo strain (G3) of *Echinococcus* exists to support its proposed placement within the G1/2/3 cluster.

Barring the report published by Bhattacharya et al. (2006) and more recently, Gudewar et al. (2008), who found isolates of *E. granulosus* belonging to genotypes G1, G2 and G3 from livestock in West Bengal, a detailed investigation on the genotypes of *E. granulosus* within a larger geographical area of India, has yet to be performed. In view of the medical, veterinary and economic importance of hydatid disease in India, our study aims to ascertain the prevalence and molecular epidemiology of hydatid disease in food-producing animals in India by genetically and morphologically characterising hydatid cysts recovered from a range of domestic livestock, namely cattle, buffalo, sheep and pigs. From a practical point of view, the recognition of strain variation is a major prerequisite for strategic control efforts aimed at limiting transmission in endemic area.

2. Materials and Methods

2.1. Sampling Design and collection of hydatid material

Between January 2007 and February 2008 a total of 21,861 animals, including 824 cattle, 1050 buffaloes, 16,099 sheep and 3,888 pigs were examined for the presence of hydatid cysts on post-mortem inspection at Deonar Abattoir, run by the Municipal Corporation of Mumbai. The abattoir, the largest in the country, sources its livestock from a vast region spanning western India including Maharashtra and adjoining states *viz*. Gujarat, Rajasthan, Madhya Pradesh, Karnataka and Andhra Pradesh. This project was approved by the University of Queensland Animal Ethics Committee.

The visceral organs of every animal included in the survey were examined visually, palpated and incised for the detection of hydatid cysts at post mortem inspection. The infected visceral organs were separated from the carcass to note the size and number of hydatid cysts present. Intact hydatid cysts recovered from the infected animals were placed separately in the polythene bags containing ice and brought to Bombay Veterinary College for further processing.

Hydatid fluid was aspirated after washing the cyst with distilled water. The fluid was further subjected to centrifugation at 5000 rpm for five minutes and the sediment was observed under the low power objective of a compound microscope for protoscoleces. Germinal layer (sterile cysts) or protoscoleces (fertile cysts) were randomly collected from 15 animals from each intermediated host species for molecular characterisation. Only one cyst from each infected animal was subjected to molecular characterisation and assigned the status of a single isolate. The material was frozen at -20^0 C until used.

2.2 Morphological analysis

The protoscoleces were placed on a glass slide to which a drop of lactophenol was added before applying a coverslip. The coverslip was slightly pressed, so as flatten but not to damage the hooks. The hook components were measured according to Hobbs et al. (1990). Measurements of the total length and blade length were made on six large and six small hooks per rostellum from each of the six protoscoleces for each isolate.

2.3. Molecular Methods

Thirty milligrams of protoscoleces or a piece of germinal layer (1" X 1") were washed with PBS (pH 7.2) and followed by three cycles of alternative freezing in liquid nitrogen followed by thawing in water held at 96ºC. DNA extraction was performed using the GeneiUltrapure™ Mammalian Genomic DNA Purification Tissue Kit (Bangalore Genei) according to the manufacturer's instructions. The eluted DNA was kept at -20° C till further use.

A 434 base pair fragment of the mitochondrial cytochrome oxidase – 1 gene was amplified from each isolate using the previously published primer pairs: forward primer RT_1_E.g.Cox1_F 5-GCCATCCTGAGGTTTATGTGTT-3', reverse primer RT_1_E.g.Cox1_R 5'- CGACATAACATAATGAAAATGAGC -3' (Barnes et al., 2007). The PCR was carried out in a 20µl reaction mixture containing 2.0µl of 10 × PCR buffer, 1.6µl of 25 mM $MgCl_2$, 0.4µl of 10 mM dNTP Mix (Bangalore Genei), 12.5 pmol of each primer, 0.2µl of 1 unit of Taq polymerase (Bangalore Genei) and 1µl of template DNA (10 - 200 ng DNA). PCR amplification was undertaken using the following protocol: step 1 – 94°C for 2 min, 50° C for 1 min, 72°C for 2 min – one thermal cycle, step 2 – 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec – 35 thermal cycles, step 3 – 72°C for 7 min, hold at 12°C.

PCR amplification products were cut from agarose gels and purified using GeneiPure™ Quick PCR Purification Kit (Bangalore Genei) according to manufacturer's recommendations. DNA sequencing was performed in both directions by Bangalore Genei. Sequence chromatograms were read and analysed using the software program Finch TV v 1.4.0 (Geospira Inc.©) . Clear sequences were obtained for a 312 base pair fragment. These were aligned and compared with previously published sequences of *E. granulosus* (GenBank accession numbers AJ508021, EF393619, DQ269942, M84663, M84662, M84664, M84665, M84666, M84667, DQ269944, AF525457, DQ144021) using Clustal W (GenomeNet, Japan) and Bioedit (Hall, 1999). Distance-based analyses were conducted using Kimura 2-parameter distance estimates and trees were constructed using the Neighbour Joining algorithm using Mega 4 software. *T. solium* (AB086256) was used as an outgroup. Bootstrap analyses were conducted using 1000 replicates.

3. Results

Out of a total of 21,861 animals examined, 126 were positive for hydatid cysts (prevalence 0.58%). The prevalence of hydatid cysts was highest in cattle (5.10%) followed by buffaloes (3.81%), pigs (0.87%) and sheep (0.075%). The highest percentage of fertile cysts was found in sheep (97.14%) followed by pigs (52.78%), cattle (25.0%) and buffaloes (22.37%).

Table 2 displays the organ-wise prevalence of sterile and fertile hydatid cysts recovered from animals in this study. With the exception of sheep, the majority of individual animals harboured hydatid cysts within a single organ. Irrespective of host species, lungs (0.35%) and liver (0.26 %) were found to be most common predilection sites for the parasites followed by

spleen (0.032%), heart in sheep (0.0046%) and kidney in pigs (0.0091%). In contrast, the percentage of multiple and single organ involvement in individual sheep were equal.

The average intensity of hydatid cysts per infected carcase was found to be highest in sheep (2.92) followed by pigs (2.24), cattle (2.09) and buffaloes (1.9). However, the average size of hydatid cysts was found to be highest in buffaloes (20.15 cm) followed by cattle (16.4 cm), pigs (8.1 cm) and sheep (5.62 cm).

Phylogenetic analysis

The neighbour-joining tree based on the alignment of partial cytochrome oxidase-1 sequences is displayed in Figure 1. Clear and readable sequences were obtained and phylogenetic analysis was performed for 14 cattle, 13 buffalo, 11 pig and 8 sheep isolates. Table 3 summarises the genotypes of *Echinococcus* obtained according to host, and cyst fertility. In total, 29 (63%) of isolates, including 8 cattle, 7 pig, 8 buffalo and 6 sheep clustered within the Indian Buffalo (G3) strain of *E. granulosus,* while 9 (20%) isolates, including 4 pigs, 3 cattle and 2 buffalo clustered within the cattle strain (G5) of *E. granulosus*. Six isolates (13%), including 3 buffalo, 2 sheep and 1 cattle isolate clustered within the sheep strain (G1) of *E. granulosus* and 2 (4%) isolates, both fertile cysts belonging to cattle clustered within the Tasmanian Sheep (G2) strain. Analysis of the cytochrome oxidase -1 gene provided strong bootstrap support (99%) for the separation of the G1/3 cluster from G2 and separation of G5 from the G6/7/8/10 cluster of *Echinococcus*.

Morphology

Figure 2. Displays a scatterplot of blade length and total length of: (A) large rostellar hooks, and (B) small rostellar hooks, measured in micrometres. The means for all isolates from each host species within G1 and G3 and means of individual isolates from each host species within G2 and

G5 are displayed along with data from previous studies according to Thompson et al. (2006). As can be seen, regardless of host species, the isolates belonging to G1, G2 and G3 group together for both large and small hook morphology. Although isolates from pigs and cattle belonging to G5 group in a distinct cluster, two isolates from buffalo belonging to G5, grouped within isolates belonging to G1/2/3. Isolate C7 sits as an outlier for small hook morphology but is clearly placed within isolates belonging to G5 for large hook morphology.

4. Discussion

The analysis of data generated during the present study in context to the findings of the studies conducted over time by different workers in Western India (Deshpande, 1977; Kulkarni et al., 1984; Dhote et al., 1992; Munde et al., 1999; Gatne, 2001), reveals that the prevalence of hydatidosis in urban centres has been consistently declining over the past few decades. This can be attributed to the increase in the number government-controlled abattoirs, where veterinary inspection of carcases and proper disposal of offal is routinely practiced. These large urban-based abattoirs, such as the one sampled from in the present study, are more likely to attract livestock from large-scale livestock production facilities that are intensively managed rather than the poorly resourced rural farmer. This study is therefore unlikely to represent the prevalence of hydatid disease of food producing animals in poorly resourced rural communities, which is expected to be significantly higher.

This study is in agreement with previous studies (Bowles et al., 1992; Bowles and McManus, 1993a, b & c; Bhattacharya et al., 2006; Gudewar et al., 2008) demonstrating that four genotypes, namely the Sheep strain (G1), Tasmanian Sheep strain (G2), Indian Buffalo (G3) strain and Cattle strain (G5) of *E. granulosus* are present in livestock in India. The Indian

Buffalo (G3) strain was the most commonly encountered genotype in all species of hosts in India. Barring cattle, which possessed a majority of sterile cysts, the G3 genotype appears to be well adapted to producing fertile cysts in other hosts such as sheep and pigs within India. The cattle strain, the second most common genotype of *E. granulosus* present in India, is capable of producing fertile hydatid cysts in both buffalo and pigs. All cysts characterised as the G5 genotype were fertile and localized in the pulmonary tissue, which was also observation by Eckert and Thompson (1998) and Worbes (1992). To date, only two genotypes of *E. granulosus* has been reported in pigs, namely the G7 (pig) and G1 (common sheep) genotypes. This is therefore the first study to demonstrate the ability of the G3 (buffalo) and G5 (cattle) genotypes of *E. granulosus* to infect and produce fertile hydatid cysts in pigs.

India has one of the largest populations of cattle and buffalo in the world and ranks first in milk production. The majority of the milk is sourced from buffalo and cattle and the industry is growing at 5% per annum (Kembhavi, 2003). It is therefore not surprising that the prevalence of hydatid disease is highest in large ruminants, which is in support of previous surveys (Abraham et al., 1980a; Abraham et al., 1980b; Vijayasmitha et al., 1993; Das & Das, 1998; Sharma et al., 2000). This is most likely due to the older age at which the animals are slaughtered and the presence of well established host-adapted strains. Interestingly, an inverse trend of higher cyst fertility rates in sheep and pigs compared to large ruminants appears consistently throughout the literature of surveys performed in India. Similar trends were recorded by Soulsby (1982), Kulkarni et al. (1986), Singh and Dhar (1988), Biswas et al. (1989), Sharma et al. (2000) and Gatne (2001). This epidemiological pattern may be a reflection of the younger age at which sheep and pigs are slaughtered coupled with the presence of host adapted G1 and G3 genotypes in sheep and G5 genotypes of *E. granulosus* in pigs. The age at which the animals

213 are slaughtered may also account for the relative sizes and intensities of the cysts isolated from
214 each host. Buffaloes were shown to harbour the largest cysts followed by cattle, pigs and sheep,
215 whereas the intensity of infection were reversed in each species probably owing to the lowered
216 immune status of younger animals.

217 In the present study only two isolates revealed their identity as the G2 genotype of *E.*
218 *granulosus*. Both these isolates were derived from fertile hydatid cyst sourced from cattle. In
219 West Bengal, in addition to cattle, the G2 genotype was also isolated from buffalo and sheep
220 (Bhattacharya et al., 2006), which was not observed in this study. Bhattacharya *et al* (2006) and
221 Gudewar et al. (2008) also proposed that the predominant genotypes occurring in the eastern
222 regions of India were G2 and microvariants of the G1 genotype of *E. granulosus* respectively,
223 which was in opposition to what was discovered in more western parts of India.

224 Our morphological and molecular results support earlier studies suggesting that
225 *Echinococcus* of buffalo origin is phenotypically and genetically similar to the sheep (G1) and
226 Tasmanian Sheep (G2) strains of *Echinococcus*. All three strains occur sympatrically, which
227 adds further evidence to support its recognition as one species *viz*, *Echinococcus granulosus*
228 sensu stricto. Our molecular, morphological and biological characteristics also support earlier
229 studies suggesting that *Echinococcus* of cattle origin, designated G5, should be recognised as a
230 separate species *viz E. ortleppi* (reviewed by McManus & Thompson, 2003). Phenotypically, all
231 cysts were characterized by the nature of their pulmonary metacestode development with the
232 production of predominantly fertile cysts. Moreover, protoscoleces of all G5 isolates belonging
233 to cattle and pigs were morphologically distinct from isolates from the G1/2/3, G4, G6 and
234 G8/10 cluster for both large and small hook lengths. It is difficult however to interpret the

235 morphological data for the two buffalo isolates B4 and B9 that were phylogenetically
236 characterised G5 as both isolates clearly grouped within the G1-3 isolates of *E. granulosus*.
237
238 5. Conclusion
239
240 In conclusion, this study has demonstrated that in India the Buffalo (G3), Cattle
241 (G5), Sheep (G1) and Tasmanian Sheep (G2) strains of *E. granulosus* exist. Except for the
242 Buffalo strain (G3), all other strains present in India have been shown to infect humans. This has
243 important implications for hydatid control and public health. To date, no information about the
244 genotypes of *E. granulosus* infecting humans in India exist. Since human hydatidosis is very
245 common in India and the Indian Buffalo strain has emerged as the most prevalent strain in a wide
246 range of intermediate host, there is every possibility that the G3 genotype might well have
247 zoonotic potential. Therefore genotyping of human infections in India should be a research
248 priority.
249
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387

388 Figure 1. Phenogram construction of the cytochrome oxidase -1 gene of *Echinococcus* isolates
389 from food producing animals in India sourced in this study (each number represents one isolate,
390 "S" refers to a sterile cyst) together with GenBank reference strains, using the neighbour-joining
391 algorithm and maximum parsimony.
392
393 Figure 2. Displays a scatterplot of blade length and total length of: (A) large rostellar hooks, and
394 (B) small rostellar hooks, measured in micrometres from isolates of *Echinococcus* characterised
395 in this study as well as previously published and unpublished data.

Table 1

Region	Host	Prevalence (%)			References
		1980 -1990	1991-2000	2001 onwards	
North	Cattle	7.8	21.9	Information not available	Deka et al. (1983); Dhar and Singh (1995).
	Buffalo	11.3 – 48.1	18.39	Information not available	Singh and Dhar (1988); Deka and Guar (1990); Varma and Malviya (1992)
	Sheep	4.7 – 30.5	2.56 - 7.2	Information not available	Singh and Dhar (1988); Varma (1990); Jithendran (1996); Deka and Guar (1998).
	Pig	1 -11.25	0.73 -1.42	Information not available	Irshauallah et al. (1989); Singh et al. (1988); Varma and Malviya (1992); Deka and Guar (1998)
South	Cattle	1.7 – 42.12	6.37- 11.85	14.8	Prabhakaran et al. (1980); Reddy et al. (1983); Vijaysmitha et al. (1993); Hafeez et al. (1994); Balamurugan et al. (2003).
	Buffalo	4.0 - 22	7.24-9.8	7.3	Reddy et al. (1983); Kulkarni et al. (1986); Vijaysmitha et al. (1993); Shanmugan et al. (1994); Balamurugan et al.(2003).
	Sheep	2.5 – 9.7	3.7 -47.6	8.92	Abraham et al. (1990a & b); Kulkarni et al. (1986); Murlidharan & Sastry (1996); Das and Sreekrishnan (1998); Balamurugan et al.(2003).

	Pig	0.0	3.02 – 6.89	Information not available	Reddy et al. (1983); Vijaysmitha et al. (1993); Hafeez et al. (1994).
East	Cattle	17.8 - 31.9	13.3 – 45	16.76 – 21.43	Sanyal and Sinha (1983); Biswas et al. (1989); Das and Das (1998); Sharma et al. (2000); Deka et al. (2008).
	Buffalo	42.25	27.6 - 48	6.52	Biswas et al. (1989); Das and Das (1998); Sharma et al. (2000); Deka et al. (2008).
	Sheep	8.3 – 50	9.0	Information not available	Prasad and Prasad (1980); Katiyar and Sinha (1981)
	Pig	7.6	1.79 - 8.0	0.34 – 0.43	Prasad (1981); Sharma et al. (2000); Deka et al. (2008).
West	Cattle	4.2 – 21.6	4.16 -21.8	13.17	Kulkarni et al. (1984); Gatne et al. (1989); Dhote et al. (1992); Munde (1999); Gatne (2001)
	Buffalo	Information not available	4.6	34.5	Munde (1999); Khan and Purohit (2006)
	Sheep	Information not available	0.2	0.85	Munde (1999); Gatne (2001)
	Pig	Information not available	0.21	3.14 – 5.58	Gatne (2001); Gaurat and Gatne (2005).

Table 1. A summary of published literature on the prevalence of hydatid disease in livestock (expressed as a range) in different geographical locations in India.

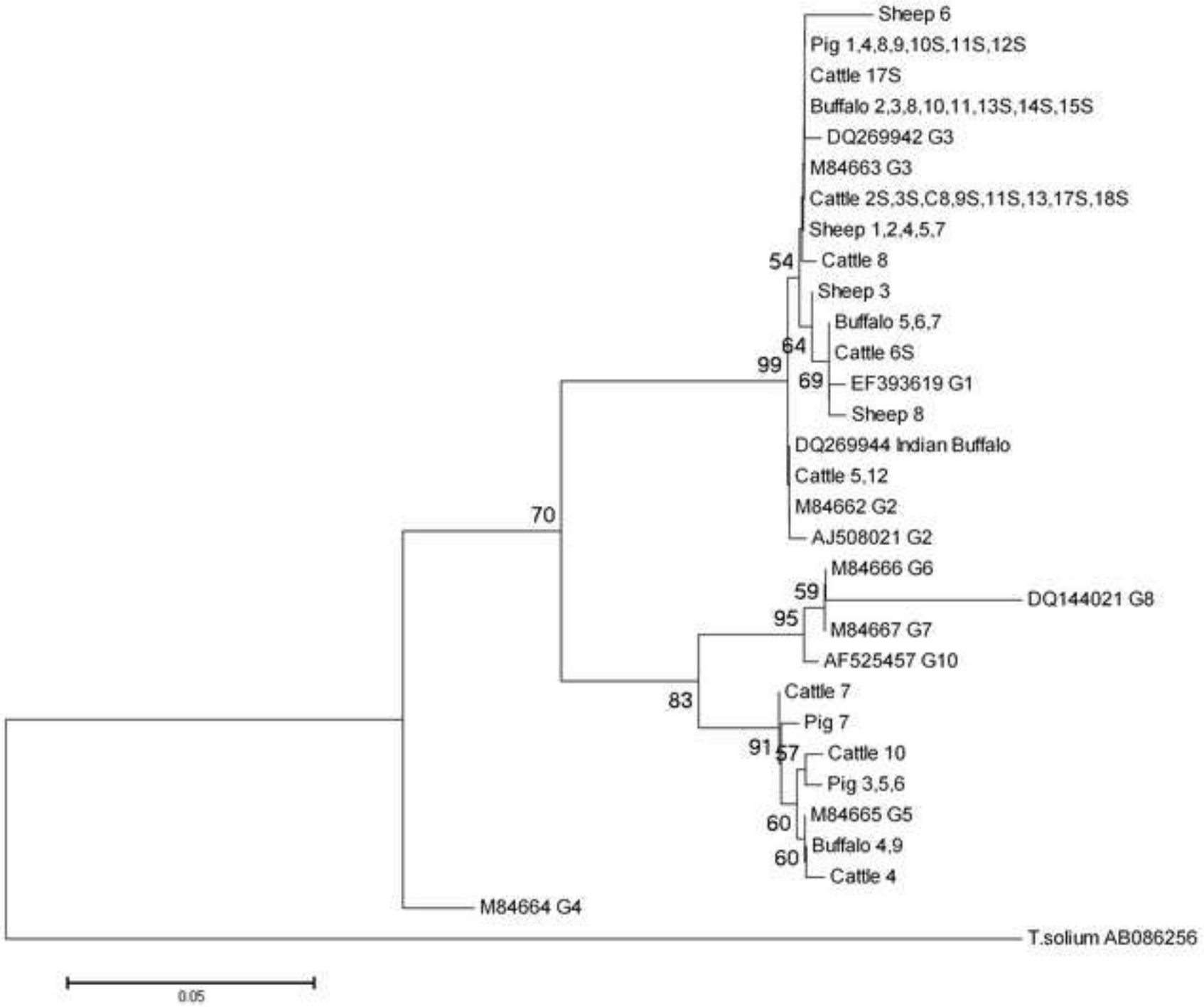
Table 2.Organwise number and prevalence (parentheses) of sterile and fertile hydatid cysts recovered from animals in this study

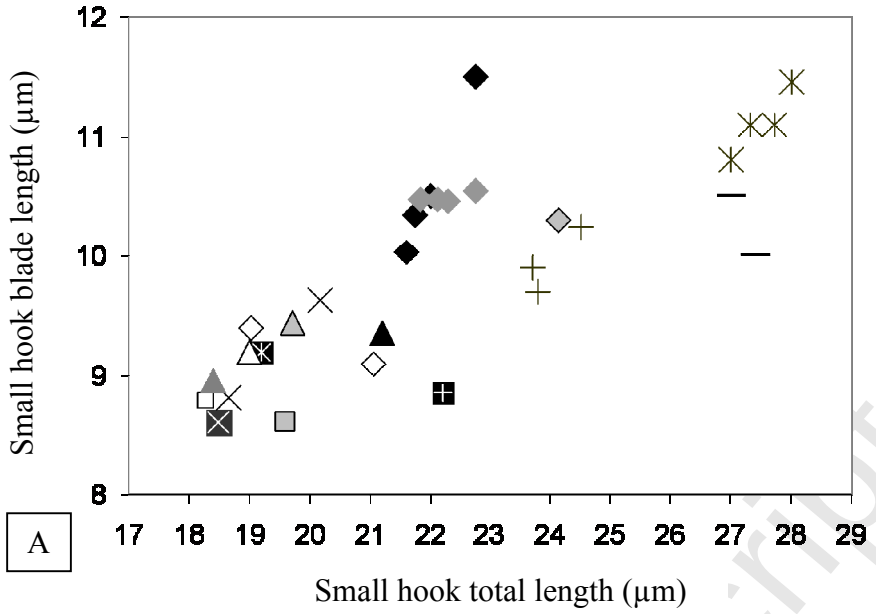
Species of animal	Lungs		Liver		Spleen		Other		Total	
	Sterile	Fertile	Sterile	Fertile	Sterile	Fertile	Sterile	Fertile	Sterile	fertile
Cattle	39 (81.25%)	9 (18.75%)	20 (74.07%)	7 (25.93%)	7 (53.85%)	6 (46.15%)	-	-	66 (75.0%)	22 (25.0%)
Buffalo	30 (78.95%)	8 21.05%)	29 (76.32%)	9 23.68%)	-	-	-	-	59 (77.63%)	17 22.37%)
Sheep	1 (5.56%)	17 (94.44%)	-	13 (100%)	-	1 (100%)	-	3 (100%)	1 (2.86%)	34 (97.14%)
Pigs	7 (25.93%)	20 (74.07%)	10 (35.17%)	18 (64.29%)	15 (100%)	-	2 (100%)	-	34 (47.22%)	38 (52.78%)

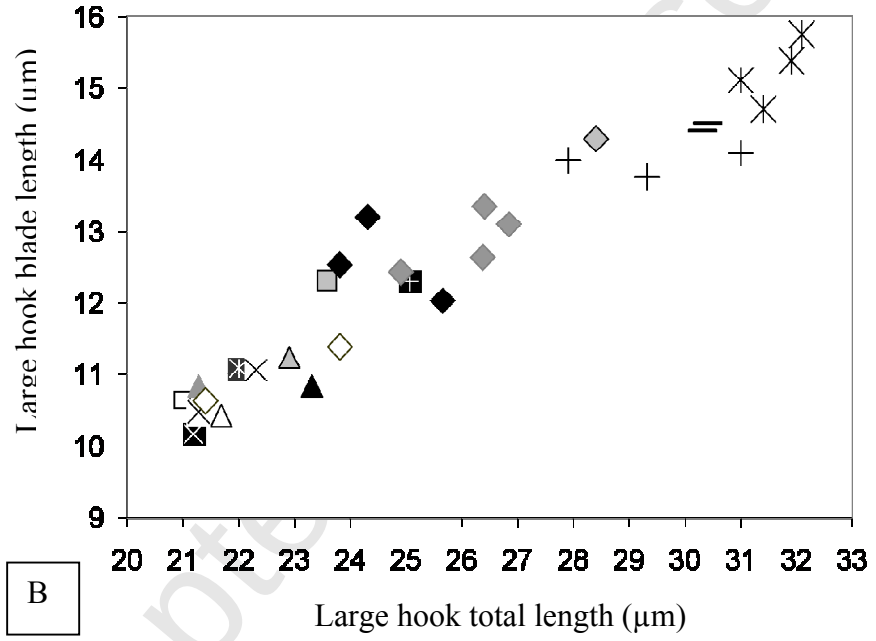
Table 3. Genotypes of *Echinococcus* obtained in this study according to host, and cyst fertility.

Host	Total number of isolates sampled	Number of isolates (number of fertile isolates)			
		G1	G2	G3	G5
Cattle	14	1 (0)	2 (2)	8 (2)	3 (3)
Buffalo	13	3 (3)	0	8 (5)	2 (2)
Pig	11	0	0	7 (4)	4 (4)
Sheep	8	2 (2)	0	6 (6)	0
Total	**46**	**6 (5)**	**2 (2)**	**29 (17)**	**9 (9)**

Figure 1


Sheep 6
Pig 1,4,8,9,10S,11S,12S
Cattle 17S
Buffalo 2,3,8,10,11,13S,14S,15S
DQ269942 G3
M84663 G3
Cattle 2S,3S,C8,9S,11S,13,17S,18S
Sheep 1,2,4,5,7
54
Cattle 8
Sheep 3
Buffalo 5,6,7
64
Cattle 6S
99
69
EF393619 G1
Sheep 8
DQ269944 Indian Buffalo
Cattle 5,12
M84662 G2
70
AJ508021 G2
M84666 G6
59
DQ144021 G8
95
M84667 G7
AF525457 G10
83
Cattle 7
Pig 7
91
57
Cattle 10
Pig 3,5,6
60
M84665 G5
Buffalo 4,9
60
Cattle 4
M84664 G4
T.solium AB086256
0.05





G1- Australian Sheep Isolate (Hobbs, unpublished)
G1- Mean Iranian Sheep Isolates (Harandi et al., 2002)
G1- Cattle Isolate India
G1- Mean Sheep Isolates India
G1- Mean Buffalo Isolates India
G2- Mean Cattle Isolates India
G3- Mean Buffalo Isolates India
G3- Mean Cattle Isolates India
G3- Mean Pig Isolates India
G3 - Mean Sheep Isolates India
G4- Horse Isolates (Kumaratilake et al., 1984)
G5- Mean Cattle Isolates (Thompson et al., 1984)
G5- Cattle Isolates India
G5- Pig Isolates India
G5- Buffalo Isolates India
G6- Camel Isolates (Hobbs, unpublished, Eckhert et al., 1989, Harandi et al., 2002)
G8/10 - Elk and Moose Isolates (Thompson et al., 2005)