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Cystic Echinococcosis in Buffaloes from Northern Region of India: Prevalence and Molecular Characterization

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A B S T R A C T

The present investigation was undertaken to study the prevalence of cystic echinococcosis (CE) in buffaloes slaughtered at slaughter houses in parts of northern India for a period of one year from January, 2017 to December, 2017. Of the 322 animals screened, the overall prevalence rate of 12.11% of CE was observed [Udham Singh Nagar district (n=150) - 10% (lungs=53.33%, liver=26.67% and liver + lung=20%); Nainital district (n=172) - 13.95% (lungs=50%, liver=33.33% and liver + lung=16.66%)]. Overall, the average prevalence of 30.76%, 51.28% and 17.94% was recorded in liver, lung and liver + lung, respectively with significantly (P<0.05) higher prevalence of cystic echinococcosis in lungs as against liver. Seasonal prevalence of CE revealed significantly (P<0.05) higher prevalence of CE in winter (15.38%) than summer (11.49%) and rains (10%). Different types of cysts were recovered from various infected organs with significantly (P<0.05) different rates of infection [single cysts (lungs=80%, liver=66.66%), multiple cysts (lungs=20%, liver=33.33%)]. The fertility rate of 39 hydatid cysts examined from total number of slaughtered buffaloes was found to be 61.53% [sterile=25.65%, calcified=12.82%} and significantly higher fertility rate of cysts from lungs (65%) was observed as against liver (58.33%) and lung + liver (57.14%). The overall viability rate of protoscolices that were recovered from cysts of all slaughtered animals was observed to be 83.33% [lungs (76.92%), liver (71.42%) and liver+lungs (100%)]. A significant (P<0.05) difference of the viability of protoscolices of different sizes of the cysts was also observed [<3cm (0%), 3-6cm (75%) and >6cm (93.33%)]. DNA was extracted from overall 28 samples (protoscolices and laminated layer). The DNA and cox1 gene amplification fragment length of all the isolates was found to be 18kb and 493bp, respectively. The cox1 gene sequence obtained from Udham Singh Nagar and Nainital isolate showed 100% and 99.9% identity with India, Sudan and Brazil isolates (G5 genotype), respectively which confirmed the prevalence of G5 genotype of *Echinococcus granulosus* in buffaloes in the study area. The nucleotide variation in the cox1 gene sequence as compared to Brazil isolate resulted in change in the translated amino acid sequence at only 1 site for Udham Singh Nagar isolate (at position 7, Serine was replaced by Alanine) and at 2 sites for Nainital isolate (at position 98, Serine was replaced with Leucine and at position 112, Cysteine was replaced by Serine). When Udham Singh Nagar isolate was compared with the Nainital isolate, change in the amino acid sequence was observed at 2 sites (at position 98, Leucine was replaced with Serine and at position 112, Serine was replaced by Cysteine). Phylogenetic analysis of cox1 gene obtained from Udham Singh Nagar and Nainital districts of Uttarakhand revealed that the Udham Singh Nagar and Nainital isolates originated from genotype of *Echinococcus granulosus* in buffaloes in the study area. The nucleotide same ancestor and both had maximum relevance with G5 genotype from Brazil isolates. This is the first report of G5 genotype of CE in buffaloes from this part of northern India and thus is even more significant in understanding its zoonotic potential.

Introduction

Cystic echinococcosis (CE) is a cestode disease which has been accounted as predominant zoonoses around the world (Deplazes *et al.,* 2017; Petrone *et al.,* 2015 and Thompson and Jenkins, 2014). Its place is second in helminthic disease of significance (Sangaran *et al.,* 2014). While hydatidosis alludes to contamination with the larval stage i.e. metacestode, echinococcosis implies disease with both adult and larval contaminations (NICD, 2005). Out of the six types of *Echinococcus* spp. that have been perceived till date, only four (*Echinococcus granulosus, E. multilocularis, E. vogeli and E. oligarthrus*) have got public health significance and also both of their adult and larval stages are distinct. *E. felidis* (African lions) and *E. shiquicus* (small animals) are the two species of genus *Echinococcus* that have also been recognized (Moro and Schantz, 2009). CE is a neglected cyclozoonoses related with an expected 3 billion US $ yearly misfortune to animals industry (WHO, 2017). In India, the financial misfortunes because of CE have been assessed at roughly 212 million US $ (Singh *et al.,* 2014). Financial misfortunes incorporate misfortunes to beef industry and are chiefly because of diminished production of milk, brought down nature of meat or fleece, impeded development, brought down fertility and condemnation of tainted organs.

Cystic echinococcosis (CE) and alveolar echinococcosis (AE) are two most essential types of echinococcosis which are of restorative and general well being significance in people (WHO, 2017). Occurrence of hydatid cysts in the intermediate hosts (sheep, goats, cows and buffaloes) in various areas leads to the condemnation of the influenced organs. The vital organs including liver, brain and lungs are most severely affected in case of CE. Herbivorous animals are the main target for CE while humans may contract this disease coincidentally by food stuffs contaminated with fecal material of dogs, foxes and wolves containing the causative agent of this disease *i.e. Echinococcus granulosus* (Brunetti *et al.,* 2010).

The prevalence of CE has been reported from different parts of the world *viz.* Iran (Ghasemian *et al.,* 2018), Egypt (Omar *et al.,* 2013), Pakistan (Latif *et al.,* 2010) and Turkey (Beyhan and Umur, 2011). In India, the prevalence of CE has been reported from Tamil Nadu, India (Raman and John, 2003), Mumbai, Maharashtra (Pednekar *et al.,* 2009), Punjab (Arif *et al.,* 2015), North India (Singh and Dhar, 1998, Rialch *et al.,* 2018) and Jammu (Godara *et al.,* 2014). The Indian subcontinent provides perfect conditions for the perpetuation, spread and dispersal of CE in animals (Gupta *et al.,* 2011) and human population as well (Samra *et al.,* 2000). Customarily, contingent upon the host, the size and predilection of cysts, hydatid cysts have altogether unique rates of occurrence. Several studies have been conducted in India to evaluate the fertility and viabilty rates of protoscolices in an assortment of slaughtered animals (Tashani *et al.,* 2002 and Elmajdoub *et al.,* 2007).

The various species of genus *Echinococcus* with genotypes causing CE includes the *E. granulosus sensu stricto* (s.s.) (G1/G2/G3), *E. granulosus sensu lato* (s.l.) complex groups, *E. canadensis* (G6/G7/G8/G10), *E. ortleppi* (G5), *E. equines* (G4) and *E. felidis* (lion strain) (Cucher *et al.,* 2016). At least 10 strains (G1–10) of *E. granulosus* s.l. have been recognized (McManus and Thompson, 2003) forming 4 major clades (G1–G3, G4, G5 and G6 to G10) (Nakao *et al.,* 2007, 2013) all of which have variable range of hosts, ability to infect host and genetic characteristics (Eckert *et al.,* 2001).

Taxonomic correction of G1 to G5 as *E. granulosus sensu stricto* (G1 to G3), *E. equinus* (G4) and *E. ortleppi* (G5) has been proposed through the ongoing re-assessments of *Echinococcus* species (Ito *et al.*, 2007).

Solid proof exists for species status of genotypes G6 to G10 (*E. canadensis*) and the lion strain (*E. felidis*). Natural varieties in *E. granulosus* impact its life cycle designs, pathogenesis caused in host, immunological result in host, capacity to transmit the disease and the affectability to various drugs. For instance, *E. equinus*, *E. granulosus s.s*, *E. canadensis* and *E. ortleppi* are transmitted essentially through domestic life cycles (Carmena and Cardona, 2014). This reinstates the fact that *Echinococcus* species identification is important as it may affect the designated developing and evaluation of prevention and control measures, diagnostic assays and therapeutics (Thompson and McManus, 2002).

In the area under study, only human reports of CE has been put forward from sporadic areas *viz.* Srinagar, Pauri Garhwal (Singh *et al.*, 2016), Haldwani, Nainital (Shahi *et al.*, 2015) and Dehradhun (Khare *et al.*, 2014). In several of these reports, there has been the history of the patient's association with domestic animals like goats/sheep and dogs which is quite a practice followed by several people of some parts of northern region of India (Shahi *et al.*, 2015) indicating the role of domestic animals in the life cycle of *E. granulosus*.

Keeping in view the above facts, the present study was undertaken with the objective to study the prevalence of cystic echinococcosis in buffaloes in some parts of northern region of India and to carry out molecular characterization of cystic echinococcosis recovered from buffaloes.

Materials and Methods

Prevalence study

The present study was conducted in the Department of Veterinary Parasitology, College of Veterinary and Animal Sciences., Govind Ballabh Pant University of Agriculture and Technology, Pantnagar and in and around two districts of (U.S. Nagar and Nainital) of northern region of India. The prevalence of cystic echinococcosis was determined in buffaloes in northern region of India for a period of 12 months (January, 2017- December, 2017) by making regular visits to the slaughter houses.

Collection of cysts

Cysts were collected from buffaloes from the various slaughter houses of Udhamsingh Nagar and Nainital districts (Fig. 1). All the organs and tissues were examined for the presence of the cysts. The cysts were placed in sterile saline solution and transported to laboratory in ice box.

Organ wise distribution

The collected cysts were counted organ wise from which they were isolated in order to assess the frequency of CE in different organs. The different organs were also examined for the type (single/multiple), size, fertility (fertile/sterile/calcified) and viability of the cysts (live/dead).

Sizes of cysts

The cysts collected from various organs were measured individually using scale in centimetres and were categorized in different groups (< 3cm, 3-6cm and > 6cm) as per the criteria described by Dalimi *et al.*, (2002). The different sizes of the cysts were also correlated for fertility (fertile/sterile/calcified) and viability (live/dead).

Fertility of cysts

Cyst contents were aspirated aseptically and also the germinal layer of the cysts was isolated by scraping the inner layer of the cysts. The cyst fluid and the germinal layer were examined under light microscope for the presence of protoscolices. The cysts with no protoscolices were considered infertile while as the cysts with protoscolices were considered fertile. The calcified or suppurative cysts were also considered infertile. Protoscolices were collected from individual fertile cysts, whereas germinal layer was collected from both fertile and infertile cysts under aseptic conditions. The collected material was washed three times with phosphate buffer saline [PBS (pH7.4)] by centrifugation and then stored in 70% ethanol for further use.

Statistical analysis

The percent prevalence of CE and data were analyzed by analysis of variance (ANOVA) (Jones, 1994). A computer program (SPSS 11.5 for windows) and Primer Minitab software was used for data analysis. Student's t-test and Chi-square test were used for the analytic assessment. The results were considered to be significant when the p-value obtained was less than 0.05.

Following formula was used to calculate the prevalence of parasites;

$$\text{Prevalence (P)} = \frac{\text{Total number of hosts infected}}{\text{Total number of hosts examined}} \times 100$$

Molecular characterization

Processing of cyst elements

Cyst contents already preserved in 70% ethanol were washed three times with PBS (pH 7.4) by centrifugation at 447 x g for 15 minutes. The pellet obtained was used for DNA extraction.

DNA extraction

Total genomic DNA was isolated from laminated membrane/protoscolices and hydatid fluid (for purification of DNA from any existed protoscolices suspended in hydatid fluid of fertile cysts). The DNA extraction was carried out using Uniflex^{TM} DNA isolation kit [Bangalore Genei^{TM} (India) Private Ltd., Bangalore] as per the protocol described by Zugin and Hartley (1985). The method included the removal of cellular proteins from the sample by adding salt (phenol: chloroform 1:1) that resulted into biphasic solution with upper aqueous phase (nucleic acids) and lower organic phase (proteins). DNA was recovered from aqueous phase by precipitation with 70% ethanol followed by centrifugation to make DNA pellets to be suspended in buffer and stored at -20^{0}c till further use.

PCR assay

Mitochondrial gene was amplified using specific primers for cytochrome C oxidase 1 (*cox1*) (Shahzad *et al.,* 2014) (Table 1). All PCR amplification reactions, including negative control samples were carried out in a final volume of 25 µl with 12.5µl of commercially available PCR master mix (GeNei^{TM} PCR-Master Mix-2X) [Bangalore Genei^{TM} (India) Private Ltd., Bangalore].

Forward primer

To the available lyophilized primer (324.9µg), 440µl sterile water was added to make it 100µM/L as per manufacturer's instructions. Twenty microliter of it was taken and diluted in 80µl of sterile water to make 20µM/L or 20pM/µl as working solution.

Reverse primer

To the available lyophilized primer (374.6µg), 502µl sterile water was added to make it 100µM/L as per manufacturer's instructions. Twenty microliter of it was diluted in 80µl of sterile water to make 20µM/L or 20pM/µl working solution.

The final reaction mixture was prepared as per the protocol of Shahzad *et al.,* (2014). Briefly, the master mix was thawed and mixed gently. 12.5µl of the master mix was taken into PCR tube to which 5µl DNA lysate, 1µl each forward and reverse primer and 5.5µl sterile water was added to make the final reaction volume of 25µl. The PCR program was carried out in temperature gradient thermocycler as per the protocol described by Shahzad *et al.,* (2014). Initial denaturation at 94^{o}C for 30 seconds followed by 40 cycles of each denaturation at 94^{o}C and annealing at 57^{o}C for 60 seconds. Amplification was done at 72^{o}C for 40 seconds followed by final extension at 72^{o}C for 5 minutes. PCR product was analyzed using 1.5% agarose gel. Before genotyping, the PCR product was subjected to purification as per the protocol described by Sambrook *et al.,* (1989).

Nucleotide and amino acid variability of cox1 gene

The sequenced partial *cox1* gene from Udham Singh Nagar and Nainital district isolates were compared and analysed with that of already published gene sequence available in the Gene Bank. After alignment of this gene, its nucleotide variability and amino acid variability were studied.

Phylogenetic analysis

The nucleotide sequences were compared and phylogenetic analysis was done using sequence data available in Gene Bank and the sequence data obtained from the present study. The sequence data was aligned using the software Mega 7 with Clustal *W* multiple sequence alignment. Percent similarity/percent divergence and phylogenetic tree was obtained by using BioEdit programme available in Mega 7 software.

Results and Discussion

Overall prevalence and organ wise distribution of cystic echinococcosis

Of a total of 322 buffaloes screened, an overall prevalence rate of 12.11% of CE was observed (Table 2). Overall 172 buffaloes slaughtered at slaughter houses in district Nainital were screened with a prevalence rate of 13.95%. From Udham Singh Nagar district, 150 slaughtered animals were examined with a prevalence rate of 10%. Overall, the average prevalence of 30.76%, 51.28% and 17.94% was recorded in liver, lung and liver +lung, respectively. The lungs had significantly ($P<0.05$) higher proportions of prevalence of cystic echinococcosis rather than liver. In Nainital district, liver of 8 animals, 12 lungs and 4 both liver and lungs were observed to be harbouring hydatid cyst with a prevalence rate of 33.33%, 50% and 16.66%, respectively. In Udham Singh Nagar district, the prevalence of CE was found to be 26.67%, 53.33% and 20% in liver, lungs and both liver and lungs, respectively. A significant association between hydatidosis and organ wise location of hydatid cysts ($P<0.05$) was observed. The proportional prevalence of hydatidosis was found to be homogenous in different districts. Organ wise, the % prevalence of CE was significant ($P<0.05$).

In the present study, the prevalence of CE can be correlated with the places from where these buffaloes were brought to the slaughter

houses which may have been frequented by several stray dogs. Wide variations have been recorded by several workers across India on prevalence of hydatidosis (Ghourai and Sahai, 1989; Irshadullah *et al.,* 1989).The observation made in the present study can be correlated with Nadery *et al.,* (2011) and Pour *et al.,* (2012), who reported higher rate of infection in lungs in comparison to liver. Nadery *et al.,* (2011) determined higher rate of infection in lungs (60%) followed by liver (32%), spleen (4%), kidney (2%), heart (0.9%) and brain (0.1%) (Table 3).

Seasonal prevalence of hydatidosis

Data revealed significant seasonal patterns for infection in buffaloes ($P<0.05$) and highest prevalence values were observed during winter (15.38%) then summer (11.49%) followed by the rains (10%) (Table 4). This is contrary to the findings of Arif *et al.,* (2015) who found that infection rate was low during winter (24.51%) as compared to spring (30%). However, the findings of present study are in agreement with that of (Mohamadin and Abdelgadir, 2011) who also reported that the prevalence rate of CE was higher in winter as compared to other seasons.

Moreover, Jithendran *et al.,* (1996) observed a high rate of CE in sheep (28.3%) and goat (19.45%), respectively in winter. However, this disease can occur in any season. One of the reasons for a higher number of infections recorded during winter may be attributed to the fact that in winter, there was an influx of buffaloes for slaughter in comparison to other seasons.

Only in some slaughter houses in the present study, slaughtering of buffaloes was done under the supervision of veterinarians. One potential purpose behind the varied rates of infection in the animals slaughtered during the study could be attributed to the changes in ecological factors. In addition, these variations could be related to the diverse strains of *E. granulosus* (McManus, 2010).

Infection rates of organs

Out of 20 infected lungs, 16 had single cyst (80%) and 04 multiple cysts (20%). From 12 infected livers, 08 were found to be harbouring single form of cysts (66.66%) and 04 had multiple cysts (33.33) (Table 5; Figures 2 and 3). The reason for harbouring of hydatid cyst in these organs can be related to factors such as physiological and anatomical characteristics of organ, host and strain of parasite (Polat and Atamanalp, 2009). Statistical analysis between the infected organs revealed a significant difference in infection rates of different organs ($P<0.05$). In the present investigation, the lungs of buffaloes were observed to be more usually tainted with hydatid cysts as compared to other organs.

The fertility rate of hydatid cysts from slaughtered buffaloes was found to be 61.53%. A total of 25.65% of the cysts were found to be sterile and 12.82% cysts were calcified. It was observed that there was a consequential difference in fertility rates of cysts recuperated from lungs and livers of slaughtered animals ($P<0.05$). In terms of the fertility rate for hydatid cysts from each study area, it was observed that in pulmonary cysts, it was 65%, which was higher than that for liver (58.33%) and commixed organs i.e., liver and lung (57.14%). However, most of the calcified cysts were found in liver (16.66%) followed by lungs (10%) and commixed organs i.e. liver and lungs (14.28%). The cysts recovered from the lungs of slaughtered buffaloes (65%) were found to be more fertile as compared to other organs. The statistical difference was found to be significant ($P < 0.05$) for the fertile cysts that were recovered from organs of slaughtered

buffaloes. The viability of protoscolices in fertile cysts was 76.92% in lungs, 100% in liver+lungs as compared to cysts recovered from liver which was 71.42% (Table 6). The prevalence of highly fertile cysts as encountered in present study has got public health significance.

Viability of hydatid cysts

In the present study, the viability rate of protoscolices that were recuperated from all slaughtered animals was observed to be 83.33%. In terms of the different cyst size of slaughtered animals, the viability rate of protoscolices was 0%, 75% and 93.33% for cyst size <3cm, 3-6cm and >6cm, respectively (Table 8; Figures 4). A significant statistical distinction between the viability rate of protoscolices recuperated as different sizes of cysts ($P < 0.05$) was observed. Information acquired on the fertility and viability of hydatid cysts in animals plays a significant role in giving sound designators of the centrality of every domesticated animals as a conceivable source of disease to final hosts, particularly canines (Elmajdoub *et al.,* 2007). Customarily, contingent upon the host, the size and predilection of cysts, hydatid cysts have altogether unique rates of occurrence. In such manner, vast number of studies has been directed in India to evaluate the fertility and viabilty rates of protoscolices in an assortment of slaughtered animals (Tashani *et al.,* 2002 and Elmajdoub *et al.,* 2007).

Molecular characterization

Electrophoretic analysis of DNA and cox1 gene o*f Echinococcus granulosus*

DNA was extracted from samples isolated from both Udham Singh Nagar and Nainital districts of northern part of India. The DNA length of all the isolates was found to be 18kb (Figure 5) and the amplification of*cox1* gene fragment from DNA of *E. granulosus* was of 493bp (Figure 6).

Cystic echinococcosis is a crucial zoonotic disease that constitutes a prime public health risk in many countries throughout the world and India in particular. Buffaloes have been found to have the highest prevalence of hydatid cysts. This is most possibly due to the older age at which the animals were slaughtered (Latif *et al.,* 2010). In order to develop preventive and control strategies for echinococcosis, a better knowledge of transmission cycle of *E. granulosus* complex is the need of hour. From the various intermediate hosts, various intraspecific variants of *E. granulosus* from different parts of the world have been isolated by molecular characterization of various isolates (Thompson, 2008).

Nucleotide sequencing of cox1gene of *Echinococcus granulosus*

The *cox1*gene sequences received were compared with the available sequences of cox1 gene in the GeneBank using BLAST analysis available in the NCBI website (www.ncbi.nlm.nih.gov). The *cox1* gene sequence obtained from Udham Singh Nagar isolate showed 100% identity with India, Sudan and Brazil isolates (G5 genotype). Nainital isolate exhibited 99.9% identity with India, Sudan and Brazil isolates (G5 genotype). On the basis of this analysis, it can be concluded that there is prevalence of G5 genotype of *E. granulosus* in buffaloes in the study area.

For genetic variability analysis, the sequences of both the isolates were aligned with the sequence from eight field isolates using Clustal*W* programme available in Mega 7 computer software. The changes in the gene squences were thus identified. The nucleotide sequences of both the isolates of *cox1* gene of

present study and other strain sequences were translated to amino acid sequences for comparison. The amino acid sequences were translated using Mega 7 computer software.

In Udham Singh Nagar isolate (Accession number MH428013), the nucleotide variation in the *cox1* gene sequence as compared to Brazil (Accession number KT337323.1) isolate resulted in change in the translated amino acid sequence at only 1 site. This change was in correspondence with the change in nucleotide sequence. At position 7, Serine was replaced by Alanine. In Nainital isolate (Accesion number MH428014), the nucleotide variation in the *cox1* gene sequence as compared to Brazil (Accession number KT337323.1) isolate resulted in change in the translated amino acid sequence at 2 sites. These changes were in correspondence with the change in nucleotide sequence. At position 98, Serine was replaced with Leucine and at position 112, Cysteine was replaced by Serine. When Udham Singh Nagar isolate was compared with the Nainital isolate, change in the amino acid sequence was observed at 2 sites also. At position 98, Leucine was replaced with Serine and at position 112, Serine was replaced by Cysteine. The genotypes are also important regarding the host specificity and life cycle of the *E. granulosus* (Bowles and McManus, 1993 and Dinkel *et al.,* 2004). In the current study, a mitochondrial marker (*cox1*) was used for phylogenetic studies and population differentiation because of its relatively rapid rate of evolution, importance in differentiation and discrimination of closely related genotypes. It is maternally inherited and does not undergo any recombination (McManus and Thompson, 2003; Ahmadi and Dalimi, 2006; Vural *et al.,* 2008; Amin-Pour *et al.,* 2011 and Sharifiyazdi *et al.,* 2011).

Recently, Balbinotti *et al.,* 2012 reported G5 genotype from 277 isolates collected from lung, liver and kidney of cattle which have 100% identity with the isolates of Udham Singh Nagar and Nainital in the present study. In India, Pednekar *et al.,* (2009) carried out the molecular characterization of isolates of *Echinococcus* in animals slaughtered in Mumbai (Maharashtra). Mitochondrial cytochrome c oxidase -1 (*cox1*) gene was amplified and sequenced. Based on the phylogenetic analysis, buffalo strain (G3) was found to be predominant followed by cattle strain (G5), sheep strain (G1) and then Tasmanian sheep strain (G2).This study is in partial agreement with the present study in which G5 genotype was found to be present in buffaloes in Uttarakhand.

Sharma *et al.,* (2013) examined 32 patients infected with CE in North India. Mitochondrial cytochrome-c oxidase subunit1 (*cox1*) gene was amplified and sequenced for molecular identification of the isolates. From all the isolates, G3 genotype was isolated from Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir and G5 genotypes was isolated from the patients of our study area. This study fully supports the zoonotic importance of G5 genotype that was also isolated during present study from buffaloes.

Phylogenetic analysis of *cox1* gene

The *cox1* gene sequences of both isolates (Udham Singh Nagar and Nainital) in this study were subjected to phylogenetic analysis with 8 GeneBank sequences. The transformative history was induced utilizing the neighbor-joining method (Saitou and Nei, 1987). The ideal tree with the aggregate of branch length = 8.37919662 is displayed. Evolutionary study was directed in MEGA7 (Kumar *et al.,* 2016). The phylogenetic tree revealed that the Udham Singh Nagar and Nainital isolates originated from same ancestor and both had maximum relevance with G5 genotype from Brazil isolates (Accession number KT337323.1) (Fig. 7).

Estimation of evolutionary divergence and percent identity

Analysis of evolutionary divergence and percent identity was conducted using the maximum composite likelihood model (Tamura *et al.*, 2004). The analysis involved 10 nucleotide sequences including two sequences of present study and eight published sequences of *cox1* gene. There were a total of 341 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). Udham Singh Nagar isolate showed 100% identity with India, Sudan and Brazil isolates (G5 genotype). Nainital isolate exhibited 99.9% identity with India, Sudan and Brazil isolates (G5 genotype) (Fig. 8). This is the first report of G5 genotype of CE in buffaloes from this part of India and thus is even more significant in understanding its zoonotic potential.

Prior examinations about the strains of *E.granulosus* in animals of Eastern India exhibited the prevalence of four genotypes G1, G2, G3 and G5 (Bhattacharya *et al.*, 2008). G1, G2 and G3 genotypes have been secluded from domesticated animals of West Bengal (Craig *et al.*, 2007). Four unique genotypes i.e. G1, G2, G3 and G5 genotype have been secluded from food producing animals in Maharashtra and bordering region of Western India (Pednekar *et al.*, 2009) while as from North India, G1 and G3 genotypes have been found in domesticated animals (Singh *et al.*, 2012). G1 and G3 genotypes have zoonotic potential and are additionally prevalent genotypes affecting people in India. G3 genotype has been isolated from Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir and G5 genotypes have been isolated from the patients of the study area (Sharma *et al.*, 2013).

The rural communities have a practice of rearing domestic animals like sheep, goat, cattle and buffaloes and dogs together. Sometimes people also go for home slaughter/open slaughter of animals on specific occasions and the offal is fed to dogs in the vicinity.

Table.1 Primers specific to *cox 1* gene

Name	Type	Sequence
Primer (*cox 1*)	Forward	5-TTT TTT GGG CAT CCT GAG GTT TAT-3
	Reverse	5- TAA AGA AAG AAC ATA ATG AAA ATG-3

Table.2 Overall prevalence and organ wise distribution of hydatidosis in buffaloes from January, 2017 to December, 2017

Districts	Animals examined	Animals Infected	Prevalence (%)	Infected Organs					
				Liver		Lungs		Liver +Lung	
				No.	%	No.	%	No.	%
Nanital	172	24	13.95*	08	33.33	12	50*	04	16.66
Udham Singh Nagar	150	15	10*	04	26.67	08	53.33*	03	20
Overall	**322**	**39**	**12.11**	**12**	**30.76**	**20**	**51.28**	**07**	**17.94**
***P < 0.05**									

Table.3 Month wise prevalence of hydatidosis in buffaloes

Month	Buffalo		
	Examined	Infected	%
January, 2017	45	04	8.88
February	40	04	10
March	22	02	9.09
April	20	03**	15
May	15	03*	20
June	25	02	08
July	15	01	6.66
August	20	04*	20
September	30	06*	20
October	20	02	10
November	40	04	10
December, 2017	30	04**	13.33
Overall	**322**	**39**	**12.11**
***(P<0.01) **(P<0.05)**			

Table.4 Seasonal prevalence of hydatidosis in buffaloes

Season	Buffalo		
	Examined	Infected	% Prevalence
Winter	95	16*	15.38
Summer	112	10	11.49
Rains	115	13**	10
Overall	**322**	**39**	**12.11**
*** (P<0.01) **(P<0.05)**			

Table.5 Frequency of the forms of hydatid cysts from different organs

Animal	Number Examined	Number Positive	Forms of Cyst			
			Single		Multiple	
			No.	%	No.	%
Buffalo	Lungs	20	16*	80	04	20
	Liver	12	08	66.66	04	33.33
	Liver+ Lung	07	05	71.42	02*	40
Overall		**39**	**29**	**74.35**	**10**	**25.65**
***(P<0.05)**						

Table.6 Fertility rate of hydatid cysts and viability of protoscolices recovered from different organs

Animal	Infected Organs Examined	No. of Infected Organs	Type of Cyst						Viability of Protoscolicies in Fertile Cysts	
			Sterile		Suppurative or Calcified		Fertile			
			No.	%	No.	%	No.	%	No.	%
Buffalo	Lungs	20	05	25	02	10	13**	65	10	76.92
	Liver	12	03	25	02	16.66	07**	58.33	05	71.42
	Liver+ Lung	07	02	28.57	01	14.28	04*	57.14	05	100
Overall		**39**	**10**	**25.65**	**05**	**12.82**	**24**	**61.53**	**20**	**83.33**
***P<0.05; T-Value = 5.07P-Value = 0.015 **P<0.01; T-Value = 6.99P-Value = 0.006**										

Table.7 Fertility rate of different sizes of the hydatid cysts

Animal Species	Cyst Size (Cm)	No. of Cysts	Type of Cyst					
			Sterile		Suppurative or Calcified		Fertile	
			No.	%	No.	%	No.	%
Buffalo	<3	10	06*	60	03	30	01	10
	3-6	12	03	25	01	8.33	08**	66.66
	>6	17	01	5.88	01	5.88	15**	88.23
Total		**39**	**10**	**25.65**	**05**	**12.82**	**24**	**61.53**
***P<0.05; T-Value = 5.07,P-Value = 0.015 **P<0.01; T-Value = 6.99,P-Value = 0.006**								

Table.8 Viability of different sizes of the fertile hydatid cysts

Animal Species	Cyst Size (Cm)	No. of Cysts	Fertile Cysts		Viability of Protoscolices	
			No.	%	No.	%
Buffalo	<3	10	01	10	0	0
	3-6	12	08	66.66	06	75%
	>6	17	15	88.23	14*	93.33
Total		**39**	**24**	**61.53**	**20**	**83.33**
***(P<0.05)**						

Fig.1 Maps showing collection sites of hydatid cysts in Udham Singh Nagar and Nainital districts

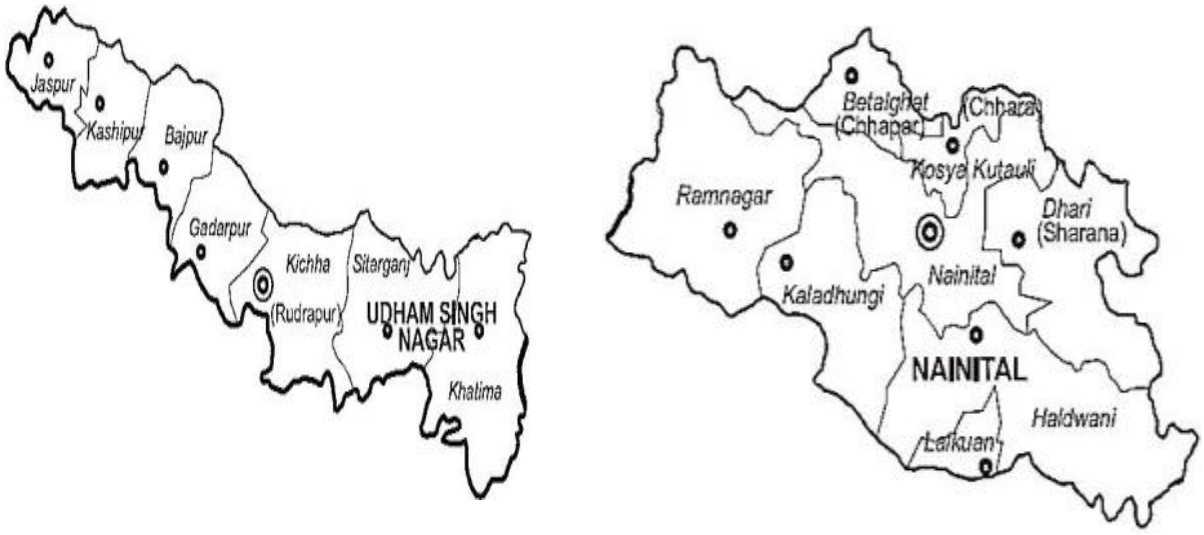
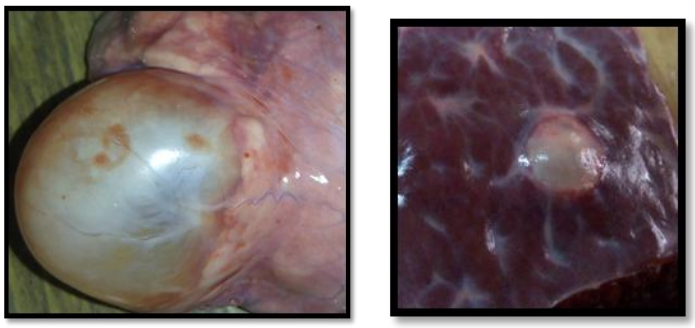


Fig.2 Photograph showing single cysts collected from lungs (a) and liver (b) of buffaloes

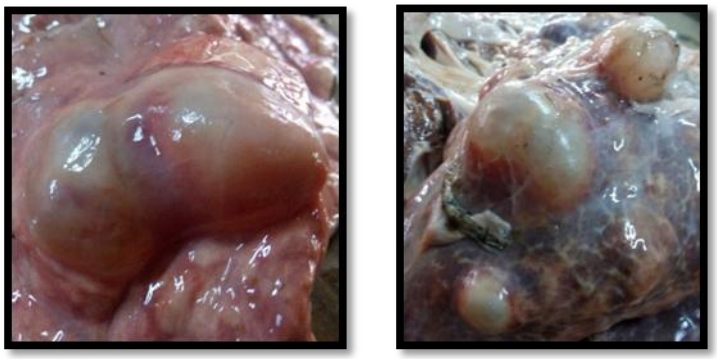


(a) **(b)**

NE=No. of infected organs examined NP= No. of infected organs

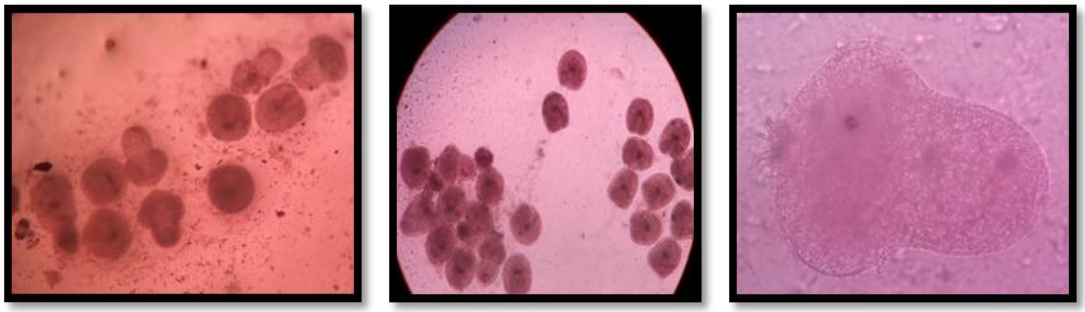
Fig.3 Photograph showing multiple cysts collected from lungs (a) and liver (b) of buffaloes

Fertility rate of hydatid cysts



(a) **(b)**

Fig.4 Photographs showing protoscolices stained with 0.1% aqueous eosin



Live protoscolices (10X) Dead protoscolices (10X) Dead protoscolices (40X)

Fig.5 Electrophoretic analysis of DNA of *Echinococcus granulosus*: M: 100bp DNA ladder; N1, N2, N3 and N4: Negative control; P1 and P2: Positive for Udham Singh Nagar isolates; P3 and P4: Positive for Nainital isolates

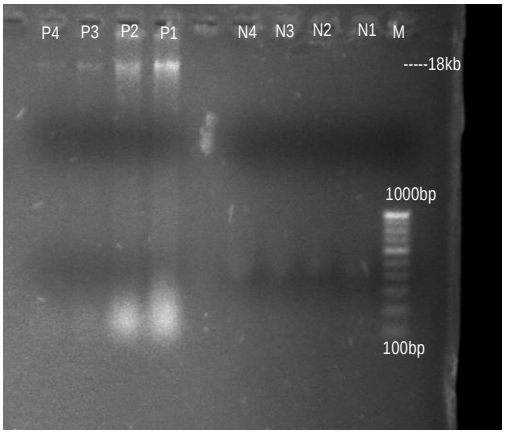


Fig.6 Electrophoretic analysis of *cox1* gene of *Echinococcus granulosus*: M: 100bp DNA ladder; P1 and P2: positive for Udham Singh Nagar isolates; P3 and P4: Positive for Nainital isolates

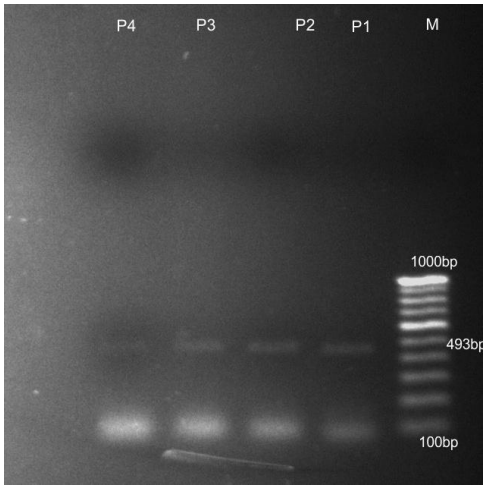


Fig.7 Phylogenetic tree based on comparison of *cox1* gene isolates obtained from Udham Singh nagar and Nainital districts of Uttarakhand to other genebank sequences. The lineages are shown on the tree

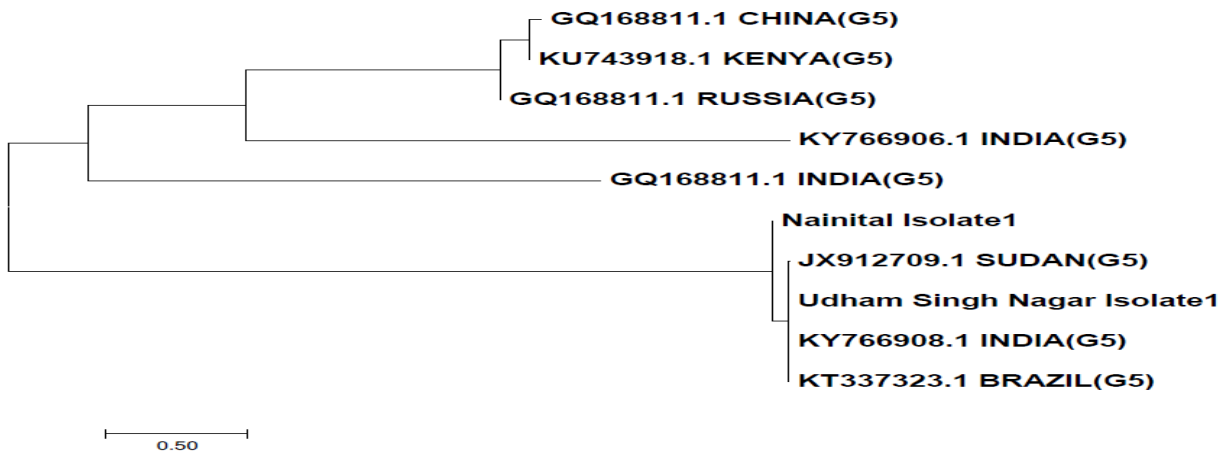


Fig.8 Percent identity and divergence between two isolates and eight published sequences of *cox1* gene

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10		
1		96.56	95.78	95.16	95.18	95.16	95.16	96.49	96.71	95.16	1	GQ168811.1_INDIA(G5)
2	3.44		97.15	95.32	95.50	95.32	95.32	99.96	99.9	95.32	2	GQ168811.1_CHINA(G5)
3	4.22	2.85		94.44	94.67	94.44	94.44	97.14	9.13	94.44	3	KY766906.1_INDIA(G5)
4	4.84	4.68	5.56		99.99	100	100	95.40	95.56	100	4	Udham_Singh_Nagar_Isolate1
5	4.82	4.50	5.33	0.01		99.99	99.99	95.58	95.73	99.99	5	Nainital_Isolate1
6	4.84	4.68	5.56	0.00	0.01		100	95.40	95.56	100	6	KY766908.1_INDIA(G5)
7	4.84	4.68	5.56	0.00	0.01	0.00		95.40	95.56	100	7	JX912709.1_SUDAN(G5)
8	3.51	0.04	2.86	4.60	4.42	4.60	4.60		99.93	95.40	8	KU743918.1_KENYA(G5)
9	3.29	0.10	2.87	4.44	4.27	4.44	4.44	0.07		95.56	9	GQ168811.1_RUSSIA(G5)
10	4.84	4.68	5.56	0.00	0.01	0.00	0.00	4.60	4.44		10	KT337323.1_BRAZIL(G5)
.	10	9	8	7	6	5	4	3	2	1		

Besides offals of animals from unmanaged slaughter houses is easily accessible to dogs frequenting those areas (Moon and Khemalapure, 2017). This clearly indicates the role of domestic animals in the life cycle of *E. granulosus*. However, such studies specifically in buffaloes in the study area are rare. This study will serve as the platform to the future scope of this disease in India in order to know the cyclic relation of CE between animals and humans and accordingly effective control strategies can be formulated.

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