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# Identification and molecular characterization of *Echinococcus granulosus* from domestic goat in Chittagong, Bangladesh

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**Abstract.** The tapeworm *Echinococcus granulosus* causes cystic echinococcosis (CE) in human and many domestic animals worldwide including Bangladesh. The parasite has significant public health importance in the country and no in-depth study has been conducted to determine this cestode in either human or animals. The aim of present study was to evaluate genotype of *E. granulosus* isolated from domestic goats reared in Chittagong, Bangladesh using DNA based tools. Partial gene fragment of *12S rRNA* gene and *Cytochrome oxidase 1* gene were accomplished by PCR followed by sequencing and phylogenetic analyses. A total of 19 hydatid cyst samples were collected from 385 goats from several local slaughterhouses located in Chittagong. The rates of fertile hydatid cysts were found as high as 57.89% while remaining cysts were found non-viable and sterile. Genomic DNA was extracted from germinal membrane and/or protoscolices for PCR assay. Sequence similarity based on BLAST search revealed variable prevalence of *E. granulosus* genotypes such as G1 (68.42%) and G1/G3 complex (31.58%) which is reported for the first time in the country. This result indicates common sheep strain G1 is the dominant subtype of *E. granulosus* in this region. The study generated six sequences of which four were aligned with G1 common sheep strain and two were aligned with G3 strain (commonly referred as Buffalo strain). Phylogenetic analysis of *12S rRNA* gene and *Cytochrome oxidase 1* gene also indicated that common sheep strain (G1) and Buffalo strain (G3) are circulating among domestic goats in Chittagong region of Bangladesh.

## INTRODUCTION

*Echinococcus granulosus*, a dog tapeworm causes cystic echinococcosis (CE) in human and many domestic animals worldwide including Bangladesh. The cestode is responsible for significant economic losses in livestock industry as resulting from condemnation of infected organs along with overall production losses (Valieva *et al.*, 2014; Torgerson & Budke, 2003). In addition echinococcosis is significant zoonosis with enormous public health importance in developing countries like Bangladesh (Karim *et al.*, 2015). Although dogs remain as the final host, animals including sheep, goats, buffalo, cattle, pig, camel, horses etc. are some of the known intermediate hosts of this important zoonotic cestode. A number of investigators have reported the prevalence of echinococcosis in animals in Bangladesh (Islam *et al.*, 2003; Basak *et al.*, 2011). These studies were based on detecting hydatid cysts in various affected organs from cattle, sheep and goats through slaughterhouse surveillance. While conventional diagnostic tools are capable of identifying the parasite morphologically, the use of modern molecular tools has now enabled us to identify the different genotypes available in different host species. Earlier reports have indicated *E. granulosus* presents a high level

of intra-specific variation and numerous host-adapted genotypes have been described in different geographical areas (Thompson 2008). These genetic variations of *E. granulosus* may determine phenotypic characteristics, host specificity, antigenicity, transmission dynamics, infection route, pathology, control, sensitivity to chemotherapeutic agent and vaccine development strategies (Thompson and McManus 2001, 2002). Studying genetic variations within and between *E. granulosus* populations might have significant implications for epidemiology and disease control (Saarma *et al.*, 2009). To date, ten distinct genetic types (G1-G10) of *E. granulosus* have been identified which is widely accepted by the scientific community (Snabel *et al.*, 2000). In addition recent phylogenetic studies splitted the *E. granulosus* into following groups; *E. granulosus sensu stricto* (G1; common sheep strain, G2; Tasmanian sheep strain, G3; buffalo strain), *E. equinis* (G4; horse strain), *E. ortleppi* (G5; cattle strain) *and E. canadensis* (G6; camel strain, G7; pig strain, G8; cervid strain, G9; human strain, G10; Fennoscandian cervid strain) (Nakao *et al.*, 2007, 2010; Hüttner *et al.*, 2008). The common sheep strain (G1 genotype) of *E. granulosus sensu stricto* group has the most wide geographic distribution around the world and frequent cause of disease in humans and animals (Ergin *et al.*, 2010). In Bangladesh, few epidemiological studies have carried out on CE in animals, and most of them have investigated epidemiological pattern (Islam *et al.*, 1976; Islam *et al.*, 2003; Basak *et al.*, 2011). Understanding the actual genotypes are crucial to formulate effective prevention and control strategies and highly sensitive DNA based tools are capable of reliably identifying the different genotypes from any biological samples. The aim of the present work was to investigate through PCR amplification of *12S rRNA* gene and *Cytochrome oxidase 1*(CO1) gene, which genotypes of the *E. granulosus* complex are circulating in goat population in southern parts of Bangladesh. This is the first molecular study of any animal-originated samples of echinococcosis in Bangladesh and can complement earlier epidemiological reports towards better understanding the transmission pattern of this important zoonotic cestode.

## MATERIALS AND METHODS

*Sample collection*

A total of 19 hydatid cysts were collected from the goat liver and lung samples from local slaughterhouses in Chittagong, a division located in the southern part of Bangladesh. The animals originated from various locations of Chittagong division that were brought to a municipal slaughterhouse. The cysts were removed from the parasitized organs (liver and lungs) aseptically and kept in separate clean Petri-dishes. Hydatid fluid was aspirated from cyst by 50 ml sterile plastic syringe and protoscolices were scraped from sides of germinal layer to store in sterile test tubes. The collected fluid with protoscolices was centrifuged at 2500 rpm for 5 minute at room temperature. The supernatant was removed and sediments were used for measuring viability of protoscolices by staining with commercially available Eosin stain (BDH Chemicals Ltd, Poole, England) through examining under light microscope at 40x magnification (where the red protoscolices considered dead and green ones were regarded as alive). The protoscolices or larval tissue materials were then frozen, refrigerated or preserved in 90% ethanol for future use.

*DNA extraction*

Samples from each individual cyst were processed as an isolate for subsequent characterization. The protoscolices and germinal layers were rinsed several times with PBS to remove the ethanol prior to DNA extraction. DNA was extracted from protoscolices and from the germinal layers using the commercial DNA extraction kit (G-spin™ total DNA extraction kit, Intron, Korea) according to the manufacturer's protocol. DNA concentration was measured through Qubit® 2.0 Fluorometer (Invitrogen).

*Polymerase chain reaction (PCR)*
During this study, we followed a previously described PCR protocol for species/strain discrimination through amplifying *12S rRNA* gene (Dinkel *et al.*, 2004) and *Cytochrome oxidase 1*(CO1) gene (Singh *et al.*, 2012).

**i. PCR assay for amplification of 12S rRNA gene region**

**PCR assay specific for *E. granulosus* G1:**
The mitochondrial *12S rRNA* gene was amplified using specific primers: E.g.ss1for. 5-GTATTTTGTAAAGTTGTTCTA-3 and E.g.ss1 rev.5-CTAAATCACATCATCTTA CAAT-3. The PCR was performed with a reaction volume of 25µl containing 12.5µl of 2X GoTaq® Green Mastermix (Promega Cat no. M7121) [containing GoTaq® DNA Polymerase supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$], 2.5 µl template gDNA (100ng), and 1.5 µl (10 pmol) of each forward and reverse primers. The PCR conditions were 3 min at 94°C (initial denaturation), 40 cycles of amplification round (30s at 94°C, 1 min at 57°C and 40s at 72°C) and 5 min of final extension at 72°C. The PCR products were separated on 1.2% agarose gel in presence of ethidium bromide (0.5µg/µl of stock solution) at a concentration of 3µl/50ml of agarose solution.

**PCR assay specific for *E. canadensis* G6/7 and *E. ortleppi*:**
In first PCR, Primer E.g.cs1 for. 5'-ATTTTT AAAATGTTCGTCCTG-3' and E.g.cs1 rev. 5'-CTAAATAATATCATATTACAAC-3' were used for amplification of *E. ortleppi* (G5) and *E. granulosus* G6/G7. Similar reaction compositions (25 µl volume) were followed as described earlier with some modifications. The PCR conditions were 5 min at 95°C (initial denaturation), 40 cycles of amplification round (30s at 94°C, 1 min at 53°C and 40s at 72°C) and 5 min of final extension at 72°C. The amplicons were stored at -20ºC for further use.

**Semi-nested PCR (171 bp fragment):**
To discriminate *E. ortleppi* (G5) from *E. canadensis* G6/7, a semi-nested PCR specific for *E. ortleppi* (G5) or for G6/7 was used in the second step, each amplifying different fragment of 171 bp. For *E. ortleppi* (G5 PCR), the primers E.g.cattle.for. 5' ATG GTC CAC CTA TTA TTT TG 3' and E.g.cs1rev and for G6/7 PCR, the primers E.g.camel.for. 5' ATG GTC CAC CTA TTA TTT CA 3' and E.g.cs1 rev (as above) were used. Similar reaction compositions (25 µl volume) with 2.5 µl amplicons product were followed as described earlier with some modifications. The PCR conditions were 30 cycles of amplification round (30s at 94°C, 1 min at 60°C and 40s at 72°C) and 5 min of final extension at 72°C. The PCR products were separated on 1.2% agarose gel.

**ii. PCR assay for amplification of Cytochrome oxidase 1 gene region**
The fragments of CO1 gene was amplified using primers as reported by Barnes *et al.* (2007) using the RT 1 E.g.Cox1 F 5'-GCCATCCTGAGGTTTATGTGTT-3' and RT 1 E.g.Cox1 R 5'-CGACATAACATAAT GAAAATGAGC-3' forward and reverse primer respectively. Similar reaction compositions (25 µl volume) were followed as described earlier with *12SrRNA* gene. The following PCR conditions were performed as reported by Singh *et al.* (2012): First thermal cycle of 2 min at 94°C, 1 min at 57.6°C and 2 min at 72°C, then 35 cycles of amplification round (30 sec at 94°C, 30sec at 55°C and 30sec at 72°C) and final extension for 7 min at 72°C. The PCR products were separated on 1.4% agarose gels in presence of ethidium bromide (0.5 µg/µl) at a concentration of 3 µl/50ml of agarose solution.

*DNA sequencing and phylogenetic analysis*
Six PCR products were purified by PCR purification kit (FavorPrep™ PCR Clean-Up Mini kit, Favorgen, Taiwan) according to the manufacturer's instructions before sending for Sanger sequencing by commercial suppliers (Bioneer Corporation, Korea).

Sequence chromatograms were analyzed using the ChromasPro 2 (South Brisbane, Australia) and Mega 6.0 (Tamura *et al.*, 2013) software programs. Sequences were compared with each other and available reference sequences in GenBank using Chromas and NCBIBLASTn program. Reference sequences of *E. granulosus* genotypes (G1-G3) and *Echinococcus vogeli* (as outgroup) were inferred from previous publications (Oskouei *et al.*, 2016) and NCBI (http://www.ncbi.nlm.nih.gov/). After multiple alignments by ClustalW, phylogenetic analyses were performed using *12S rRNA* and *CO1* sequences and phylogenetic tree was originated through MEGA6 software tool.

## RESULTS

A total of 19 hydatid cyst samples collected from 385 slaughtered goats were used during this study. The types of cysts and their distribution are mentioned below (Table 1). Eleven cysts were found fertile (57.89%) as evident by the presence of protoscolices viewed by microscopy. Amplification of *12S rRNA* gene fragment with G1 specific primer yielded an amplicon sized 254 bp in 13 samples independently (Fig. 1). These samples were identified as common sheep strain G1 of *E. granulosus sensu stricto* based on the previous reports (Dinkel *et al.*, 2004). Four sequences derived from these *12S rRNA* gene amplified products were submitted to the GenBank under following accession numbers- KU599929, KU599930, KU961545 and KX150849. Samples that were not amplified by 12S rRNA gene (G1 specific primers), were further assessed to amplify with *E. canadensis* G6/7 and *E. ortleppi* specific primers and was not successful. Furthermore all 6 negative samples were amplified with *CO1* gene and were amplified

Table 1. Types of cysts used during this study and the outcome of PCR assay of two different gene fragments. Out of nineteen cyst samples only two samples were successfully amplified by both *12S rRNA* and *CO1* gene through PCR assay

| Type of cyst | No. of Samples (n) | Positive cases in PCR assay of *12S rRNA* gene | Positive cases in PCR assay of *CO1* gene | Positive cases found by PCR assay of both genes |
|---|---|---|---|---|
| Fertile | 11 | 9 | 1 | 1 |
| Non-viable | 4 | 1 | 2 | 1 |
| Sterile | 4 | 3 | 1 | 0 |
| Total | 19 | 13 | 4 | 2 |

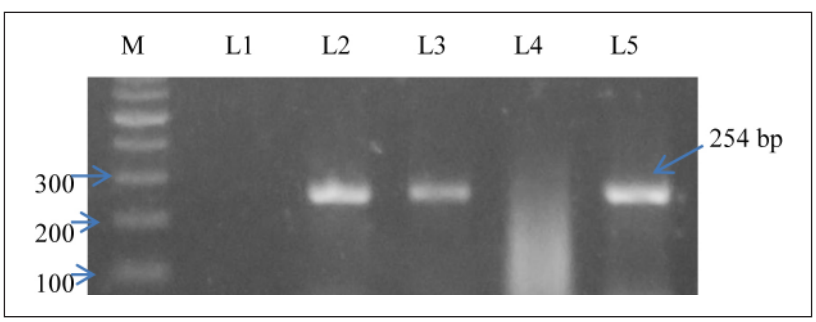


Figure 1. Agarose electrophoresis of PCR amplified 254 bp amplicons of partial mitochondrial 12S rRNA gene. The lanes M indicate 100bp DNA ladder; Lane 1 Negative control (no template); Lane 2, 3, and 5 positive goat isolate; Lane 4 negative goat isolates.

successfully (Fig. 2). Among the six amplicons, two were sequenced and submitted to the NCBI Genbank (Accession numbers KU961547 and KX150850). Further bioinformatics analyses of the derived sequence was attempted to identify homologous sequences based on previously submitted NCBI GenBank entries. Based on phylogenetic tree construction these samples were identified as buffalo strain G3 which needs further validation by sequence analyses or restriction digestion patterns. Overall, during this study *E. granulosus sensu stricto* genotypes- G1 (68.42%) and G1/G3 complexes (31.58%) were identified based on the previously published sequence at GenBank.

Sequencing BLAST and phylogenetic revealed specific genotypes based on sequence similarity search. Common sheep strain (G1 genotype) found during this study have almost complete identity with the *12S rRNA* gene fragment previously described by Dinkel *et al.* (2004) except one nucleotide position. The difference was a transversion substitutional mutation of a thymine (T) to guanine (G) in position 200 (Fig. 3). Phylogenetic tree constructed through using the sequence data indicates all the isolates from the present study were grouped into a distinct cluster with the most closely relation to G1 genotype (Fig. 4). However this cluster contains one recorded isolate from human

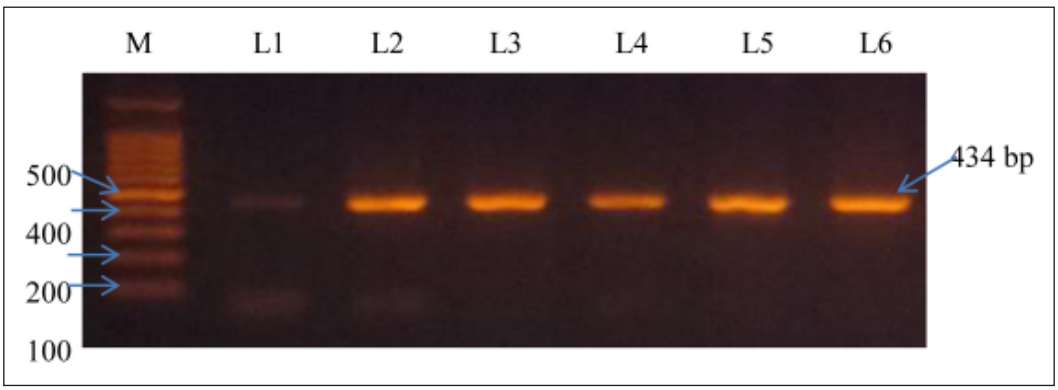


Figure 2. Agarose gel electrophoresis of the PCR-derived 434 bp amplicons of the hydatid cyst of *E. granulosus* mitochondrial Cytochrome oxidase-1 gene, separated on 1.4% agarose gel and stained with ethidium bromide Lanes: (M) 100 bp DNA ladder, lane 1 Negative control (No template), lane 2-6 goat isolate.

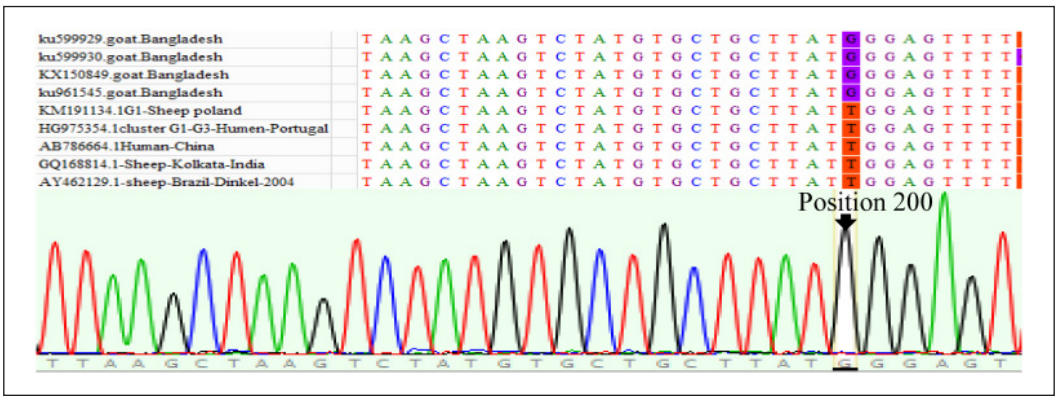


Figure 3. Partial chromatogram of the mitochondrial 12S rRNA sequence with studied and reference sequence alignment. Dark arrow shows a transversion mutation of a thymine to guanine in position 200.

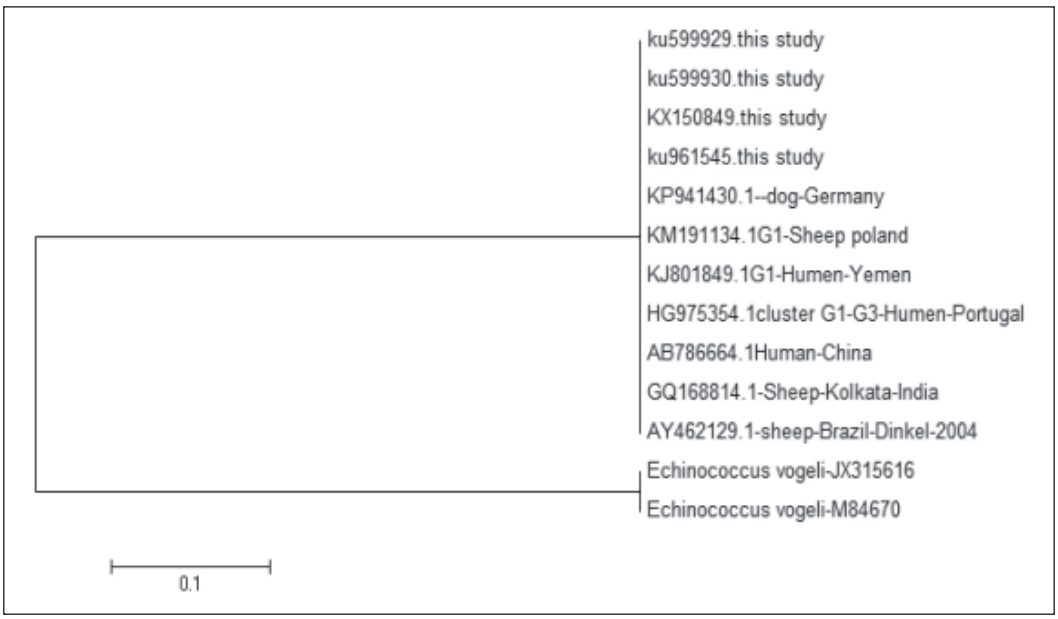


Figure 4. Phylogenic tree of *E. granulosus* goat isolates of Chittagong, Bangladesh and reference sequences for G1 and G1-G3 cluster genotypes of *E. granulosus* as well as *E. vogeli* as the outgroup. Isolates of this study were grouped into G1 and G1-G3 cluster.

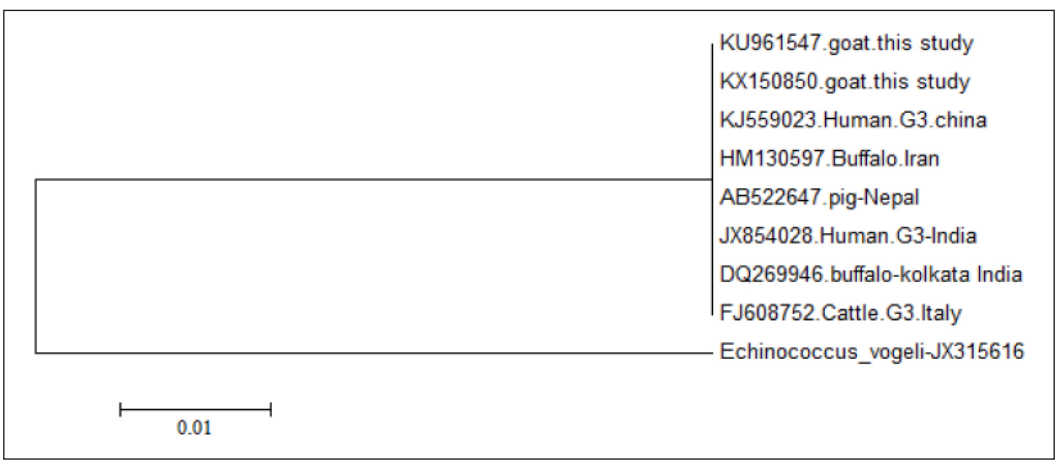


Figure 5. Phylogenic tree of *E. granulosus* goat isolates of Chittagong, Bangladesh and reference sequences for G3 genotypes of *E. granulosus* as well as *E. vogeli* as the outgroup. Goat isolates of this study were grouped into G3 (Buffalo strain) genotype of *E. granulosus*.

specimen from Portugal (Accession no. HG975354).

Phylogenetic tree constructed from two *CO1* gene sequences of *E. granulosus* is shown in Fig. 5. The clustering pattern was indicative of the different genotypes where G3 (Buffalo strain) clustered with the G3 reference sequences of human reported from other neighboring countries including India and China.

## DISCUSSION

The principal objective of the present study was to determine the existing genotypes or strains of *Echinococcus* sp. circulating in Bangladesh. While the previously reported studies in other countries were based on amplification of several genes such as *12S rRNA*, *16S rRNA*, *CO1*, *Cytochrome B (CYTB)*, *ITS1*DNA and *NAD1*. We have used only two genes (*12S rRNA* and *CO1* gene) during this study. In absence of the whole genome sequence information, partial gene amplification might help us unravel the specific genotypes. However, for a comprehensive study of each different isolates/ genotypes complete genome sequencing is always preferred and necessary.

Globally, the common sheep strain (G1 genotypes) is the most widely distributed strain and has been found to be dominant strain both in human and animals (Thompson and McManus, 2001). During this study, previously described methods were followed where*12S rRNA* gene and CO1 gene was amplified to detect the common sheep strains (G1), cattle strain (G5), camel & pig strain (G6/7) and buffalo strains (G3) (Dinkel *et al.*, 2004; Singh *et al.*, 2012). The result of this study apparently indicate that the G1 genotype of *E. granulosus* (sheep strain) was the most commonly identified genotype from goat in Chittagong (with 68.42% prevalence). Considering the higher fertility rate (57.89%) of the cysts, in domestic goat, it appears that this species possibly is the most important host that maintains the transmission of disease in Chittagong region. However this hypothesis needs further validation by comprehensive studies with high sample numbers. About 82% of fertile cysts (out of 11 fertile cysts) were identified as G1 genotype indicating strong possibility of transmission to dog populations who are always roaming in the rural and urban areas of the country. With the absence of necessary slaughterhouse rules and regulations and indiscriminate killing of food animals in places accessible by stray dogs, one would assume the transmission cycle very easy and frequent between goat and dogs. The present study and its finding therefore indicate this hypothesis of goat-dog *E. granulosus* life cycle occurring in this part of Bangladesh, as revealed for the first time through molecular investigation.

Among all the different available genotypes, G1 is considered as the most common genotype in the world with a wide host range (Craig *et al.*, 2003; Moro and Schantz 2009). However, in some North African countries (such as Sudan) and in South America (such as Argentina, Peru), G6 is the dominant genotypes (Omer *et al.*, 2010; Soriano *et al.*, 2010; Moro *et al.*, 2009). Again, G7 (pig strain) genotypes was reported as dominant in goats in Greece (Varcasia *et al.*, 2007) and Spain (Mwambete *et al.*, 2004). Common sheep strain (G1) is reported as predominant genotype in different countries such as Iran (RostamiNejad *et al.*, 2008), India (Gudewar *et al.*, 2009), Kenya (Dinkel *et al.*, 2004), China (Yang *et al.*, 2005), Turkey (Utuk *et al.*, 2008), Italy (Busi *et al.*, 2007) and Tunisia (M'rad *et al.*, 2005) that are consistent with this study.

Based on the sequence data of amplified *12S rRNA* gene fragment, the nucleotide sequences from this study (KU599929, KU599930, KU961545, KX150849) varied from previously submitted reference sequences (GQ168814, AY462129) where a single nucleotide position at 200 involved a possible point mutation (Fig. 3). Further comprehensive bioinformatics analyses can identify genotype specific variation and their significance for zoonotic transmission among animals and human. Thereby molecular epidemiology can unravel significant biological information that will be useful for their effective prevention and control.

During this study two isolates were found as buffalo strain (based on *CO1* gene fragment sequence) which was earlier described as dominant genotype by other investigators in Italy and neighboring India (Calderini *et al.*, 2012; Singh *et al.*, 2012). This is consistent with the findings by other neighboring countries like Pakistan where the sheep strain (G1) and buffalo strain (G3) were detected among sheep, goats, cattle and buffalo (reviewed by Latif *et al.*, 2010).

Notable that only two isolates were found positive through using two genetic markers where G3 genotypes were identified using the *CO1* gene and G1 genotypes were identified using the *12S rRNA* gene fragments. This study provides evidence that the G1 and G3 genotypes were considered as *E. granulosus sensu stricto*.

The high prevalence or dominance of any specific genotype in an area indicates the transmission patterns among different hosts and key role of each intermediate hosts in the life cycle of parasites. The relative abundance of G1 strains and the presence of fertile cysts in goats likely to play the most important role in the transmission of G1 strain parasites to dogs which may lead to transmission of life cycle stages in between humans and livestock. This can be further validated by comparing the respective sequences of human and animal's samples from any selected areas which will shed light on novel life cycle stages of this important cestode. The present study documented the presence of G1, G3 strains of *E. granulosus* in domestic goats in Bangladesh. This represents the first molecular investigation of this kind in the country where hydatid cysts are frequently reported in human and animals.

## CONCLUSIONS

The analysis of goat isolates of hydatid cysts confirmed the existence of of *E. granulosus sensu stricto* (G1 and G3 genotypes) in this region. This study is the first of its type in Bangladesh using animal originated samples and their molecular characterization. Further study comparing both livestock and human specimen will identify the actual strains and/or species and their biology. Together this will increase our understanding about other possible sources of transmission between human and animals in developing countries like Bangladesh.

**Conflict of interest**

There is no conflict of interest to this research.

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