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Prevalence and Molecular Characterization of *Echinococcos granulosus* in Cattle in Oman

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Abstract: This study determined the prevalence and genotypes of *Echinococcus granulosus* (*E. granulosus*) in the cattle of Oman. 28,269 cattle were examined for the presence of Cystic echinococcosis (CE) at 4 slaughterhouses belonging to the governorates of Muscat, Al-Dakhiliyah, Al-Batinah and Dhofar during March 2012 to April 2013. 257 suspected samples were collected and 79% were found infected with CE and the prevalence rate was 0.72%. The higher percentage of positive to submitted samples was observed from Salalah (87%) followed by Nizwa (76.7%), Bousher (73.5%) and Sohar (60%). The highest prevalence/100 of CE was found in cattle of Nizwa (9.9%) followed by Bousher (0.89%), Salalah (0.62%) and Sohar (0.16%) slaughterhouse, $p<0.001$. Pulmonary CE infection was higher (82.9%) compared to hepatic infection (75%) and multiple organs infection (76.7%) $p=0.001$. Female cattle were found to be more infected (82.7%) than males (76.5%), $p=0.23$. The Cattle older than 5 years of age were found to be more infected (88.6%) than the age groups of 3-5 years (80.8%) and less than 3 years (54.8%), $p=0.001$. The fertility of hydatid cysts in lungs and livers were similar (15.5% and 16% respectively) and the overall viability of protoscolices was high (63.3%). Genotyping of *E. granulosus* by complex PCR system revealed that the common sheep strain (G1) and camel strain (G6) are predominant and cycling among cattle in Oman. Further studies based upon molecular characterizations of *E. granulosus* isolates in other intermediate hosts are strongly recommended to contemplate control measures.

Key words: Abattoirs · Cattle · *Echinococcos granulosus* · Molecular Characterization · Oman

INTRODUCTION

Cystic echinococcosis, caused by the metacestode *E. granulosus*, is a globally distributed zoonotic disease. This disease is considered to be one of the most important parasitic infectious diseases with a significant veterinary, economic and public health impact [1-4].

Cystic echinococcosis is one of the 17 neglected tropical diseases listed by the World Health Organization, has a cosmopolitan distribution and can be transmitted through a variety of domestic, synanthropic and sylvatic cycles [5]. Livestock infection maintains the life cycle of *E. granulosus* as offal is often consumed by dogs [6]. The infection also leads to economic losses due to the condemnation of livers and to the lowered meat and milk production [7, 8].

Globally, *E. granulosus* is the most common and widespread species with endemic foci present on every habited continent [9]. CE represents a serious human and animal health concern in many rural, grazing areas of South America [10], the Mediterranean basin [9,11], North and East Africa [2,12, 13], Western and Central Asia, China [8.14,15] and Europe and Italy [16]. However, only few studies on the epidemiological situation of animal and human echinococcosis have been carried out in the Middle East especially in Gulf countries. The surveillance studies conducted by various authors [17- 22] have shown that medium to high infection rates were recorded in most countries of the Middle East.

Echinococcus genus comprises nine valid species including *E. granulosus sensu lato* which branched into (*E. granulosus sensu stricto* (genotypes G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6–G10) and *E. felidis, E. multilocularis, E. oligarthrus, E. shiquicus and E. vogeli* [5, 23- 25]. However, *E. granulosus sensu lato* (*E. granulosus s.l.)* has a world-wide distribution and its transmission is primarily maintained in a synanthropic cycle through dogs as

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definitive hosts and livestock species as intermediate hosts [6]. Cattle are the common host of CE worldwide and can be infected by different types of *E. granulosus s.l.* strains including *E. granulosus senso stricto (E. granulosus s.s.)* or (G1/sheep strain), *E. ortleppi;* (G5/cattle strain) and *E. Canadensis; (*G6/camel strain) [8]. Sheep strain is more prevalent in cattle than cattle strain. Moreover, according to Bowles, Blair, McManus [26], when cattle are infected by the sheep strain, the cattle are considered as an accidental host and the resultant cyst is usually infertile.

In our previous study on slaughtered goats in Oman, the results indicated that CE in goats is existed sporadically in all studied locations [27]. To our knowledge, no reports on prevalence or molecular genotyping have previously been conducted on cattle of Oman. Knowledge of these data in all intermediate host species is essential to implement appropriate control measures. Hence, this study was carried out to determine the prevalence and genotypes of *E. granulosus* existed in slaughtered cattle in Oman.

MATERIALS AND METHODS

Study Areas: The suspected organs for CE (liver, lungs, kidney, spleen and heart) from slaughtered cattle were obtained from four slaughterhouses located at various governorates; Boushar slaughterhouse in Muscat governorate, Sohar slaughterhouse in Al-Batinah North governorate, Nizwa slaughterhouse in Al-Dakhiliyah governorate and Salalah slaughterhouse in Dhofar governorate (Fig 4).

Study Design and Data Collection: An epidemiological surveillance for CE in slaughtered cattle was conducted from March 2012 to April 2013. During the study period, 28,269 slaughtered cattle including males and females of various ages were examined for CE. Among this population, 257 cattle were found suspected for CE.

Ante and Post-Mortem Inspection of Slaughtered Cattle: The slaughtered cattle were examined by veterinarians through visual inspection, palpation and symmetric incision of different visceral organs including liver, lungs, kidney, heart, spleen and mesenteric fats as recommended by [28]. Organs harboring hydatid cysts were removed separately and collected in sterile bags in a cool box and sent to the laboratory for morphological and microscopic examination.

Examination of Fertility, Viability and Size of Cysts: Individual cysts were grossly examined for any evidence of degeneration and calcification. For the determination of the proportion of fertile cysts, the cyst wall was penetrated with a sterile needle and opened up with a scalpel and scissors. The contents were transferred into a sterile petri dish and examined under a stereo microscope (x20, Olympus, SZ40, Japan) with aid of a lump (Olympus, LG-PS2, Japan) for the presence of protoscolices by adding glycerin and placing between two microscopic slides [29]. The morphology and ultra-structure of the protoscolices were determined under a light microscope (x10, Olympus, BX41TF, Japan).Cysts that contained no protoscolices as well as heavily surpurative or calcified were considered unfertile.

The viability of fertile protoscolices was assessed by the motility of flame cells together with staining of 0.1% aqueous eosin solution [30]. The viable protoscolices were completely or partially excluded from the eosin stain while the unviable fertile protoscolices had taken up the stain [29, 30].

The diameter of the hydatid cysts from the affected organs was measured and classified as small (less than 4 cm), medium (between 4 and 8 cm) and large (greater than 8 cm) [31].

PCR Reaction for Conformation of *E. granulosus*: The hydatid cystic fluid containing protoscolices and germinal membranes were collected in 10 ml sterile tubes by inspiration with a 5 ml sterile pipette and rinsed three times with 10 ml of phosphate buffer saline (PBS) solution by centrifugation at 1000 rpm (SIGMA centrifuge 2-16 PK) for 15-20 minutes. The collected protoscolices and

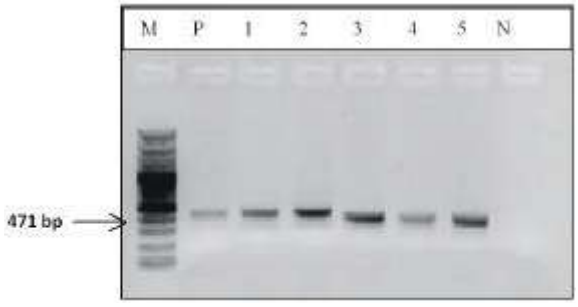

Fig. 1: Gel electrophoresis (1.5% agarose gel); of PCR products from *E. granulosus* showing bands of mitochondrial *nad 1,* 471 bp. (M): DNA molecular marker of (100bp), (P): positive control, (1-5): DNA amplified samples extracted from cattle; (N): negative control.

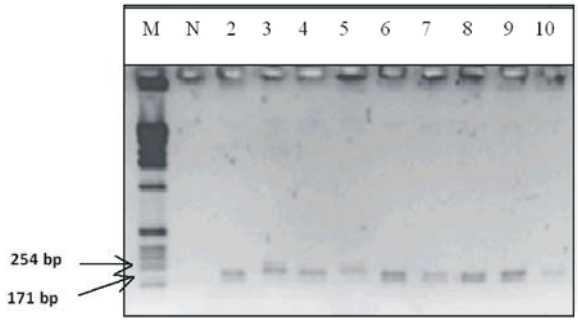

Fig. 2: Semi-nested g5PCR of the 171 bp specific PCR product of *E. Canadensis* (G6/7). 254 bp: product of the preceding the G5/6/7 PCR. (M): 100bp Molecular marker, (N): negative control, (2-10): negative samples for *E. Ortilepi* (G5)

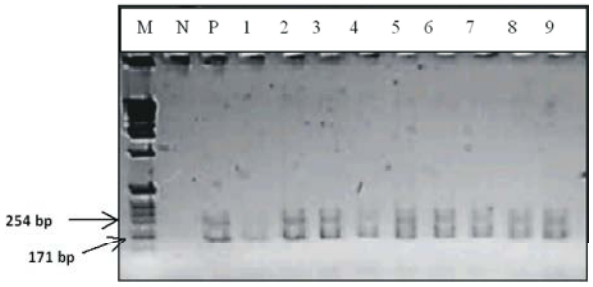

Fig. 3: Semi-nested gG6/7 PCR of the 171 bp specific PCR product of *E. canadensis* (G6/7). 254 bp: product of the preceding the G5/6/7 PCR. (M): 100bp Molecular marker (N): negative control, (P): Positive control and 1-9 are positive samples for *E. Canadensis*

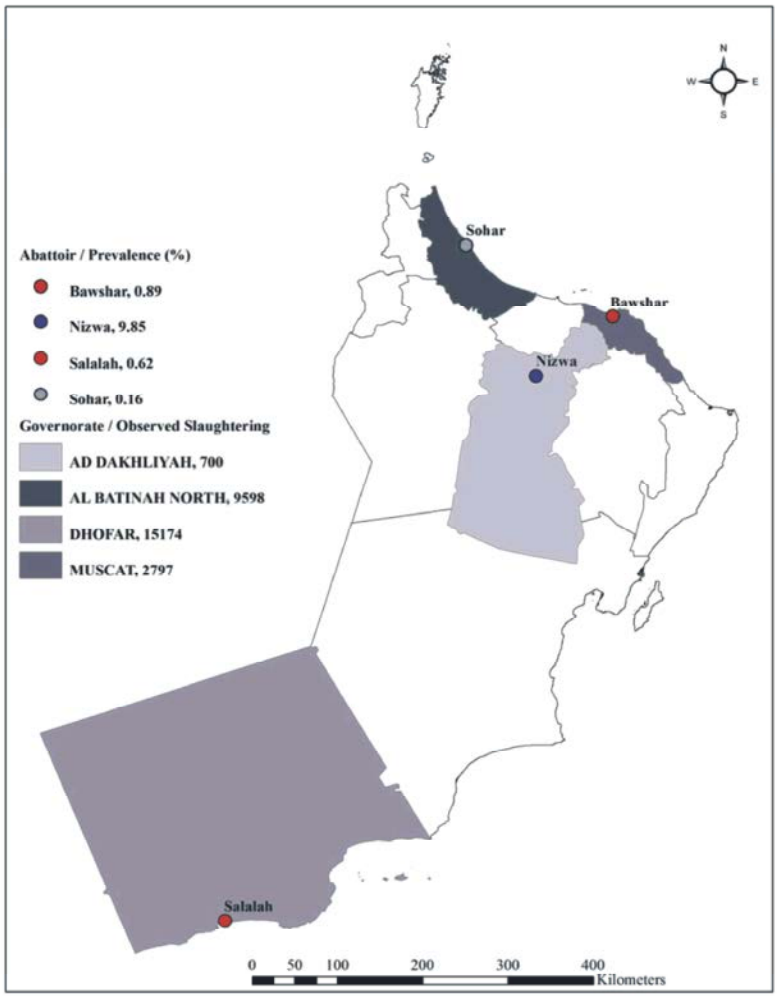

Fig. 4: A map of Oman showing the locations of the four slaughter houses included in this study from different governorates, the number of cattle slaughtered during the study period and the prevalence percentage of each slaughter house

germinal layers were fixed with 70% ethanol (v/v) and preserved in an ultra-low temperature freezer at -80°C for molecular analysis.

DNA Extraction and PCR Amplification: The protoscolices and germinal membrane were thawed and washed 3 times with PBS to remove the ethanol. The DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol: 250mg of a sample was suspended in 180µl of ATL tissue lysis buffer and incubated in a water bath at 56 °C with 40 µl of Proteinase K for 18 hours. The DNA was eluted to a final volume of 30µl in AE buffer. A 471bp fragment of the mitochondrial NADH dehydrogenase subunit 1 (*nad-1)* gene was amplified in a thermal cycler using the following primer pairs:

Forward primer; MS1 (5' CGT AGG TAT GTT GGT TTG TTT GGT 3') and reverse primer; MS2 (5' CCA TAA TCA AAT GGC GTA CGA T 3'). The primers and PCR conditions were set up as described previously by Sharbatkhori *et al*. [32].

A total of 25 µl PCR mix of amplification for a single sample was prepared as following: 2x PCR Master Mix (12.5 µl), Deionize H2O (9 µl), *NAD 1*.F (1.25 µl), *NAD 1*.R (1.25 µl) and DNA template (1 µl). The thermal profile for amplification was set up as following: Initial denaturation (7 min/94°C), amplification (30 s/94°C; 30 s/55°C; 30 s/72°C), number of cycles (38) and final extension (5 min/72°C). The PCR products were electrophoresed at 80 Volt in 1.2 % agarose gel for 30 minutes containing 5 µl ethidium bromide. 4 µl of 100 bp DNA molecular size marker was mixed with 4 µl of 6x loading dye and loaded in first well. The rest of wells were loaded with 8 µl of the amplification product mixed with 4 µl loading dye. After 30 minutes, the gel was shifted on U.V. Transilluminator of gel documentation system to visualize the bands (Fig. 1).

Genotyping of *E. granulosus* Isolates from PCR Product: A total of 25 DNA samples that amplified *NAD* 1 were run using the 12S rRNA complex PCR method according to the published protocol for genotyping of *E. granulosus* [33, 26]. Genotyping of *E. granulosus* isolates was performed using a previously described PCR system and published primers by Dinkel *et al*. [34]. This method includes a PCR assay specific for G1 (g1 PCR) and PCR assays specific for G6/7 and (g5/6/7 PCR, g6/7 PCR and g5 PCR). G5/6/7 PCR was performed with all samples. However, samples which were positive to G5/6/7 PCR, underwent semi-nested PCR specific for G6/7 (g6/7 PCR) (Fig. 3) and for G5 (g5 PCR) in a second step (Fig. 2). In addition, G1 PCR was done with all samples that exhibited negative result with G5/6/7 PCR.

Statistical Analysis: A computer software IBM SPSS version 21 was used for analysis. Chi-square test ($x2$) with a p value of 0.05 was applied in this study.

RESULTS

Distribution of CE According to Abattoir Locations, Age, Sex and Breed of Slaughtered Cattle: The distribution of positive percentages related to the locations of slaughterhouses, age, sex and breed of slaughtered cattle is shown in table 1. The highest percentage of positive samples was from the Salalah slaughterhouse (87%) followed by Nizwa slaughterhouse (76.7%), Bousher slaughterhouse (73.5%) and Sohar slaughterhouse (60%), where $x2=10.5$, p=0.014.

The present study showed that, CE was significantly lower in the age group of less than 3 years as compared to the older group of > 3 and > 5 (88.6%, 80.8%) respectively, $x2=13.7$, p=0.001 (Table 1). Regarding to the sexwise distribution of the positive CE samples as shown in table 1; the percent positivity was found higher 82.7% in females as compared to 76.5% in males, $x2=1.44$, p=0.23. However, this difference was not significant.

Organs Localization, Fertility, Viability and Size of Hydatid Cysts: Table 2 shows organs related distribution of CE according to cyst localization, percentages of fertility, sterility and size of the hydatid cysts. The highest percentage of CE was found in single infection in lungs 97(82.9%) as compared to multiple organs infection (livers with lungs, lung with spleen and liver with spleen) organs 23 (76.7%) and single infection in livers 81 (75%), $x2=13.1$, p=0.001. A significant difference was associated with the size of the cysts, as 63.1% of the cysts were found small (<5cm), 36.0 % were medium (5-10cm) and 1% were large (>10cm), $x2=13.9$, p<0.01.

In this study, the majority of hydatid cysts (56.2%) were found sterile, while (14.8%) were fertile (Table 2). However, the fertility of liver and lungs (16% and 15.5% respectively) were higher as compared to other organs. In addition, the overall percentage of viability from the fertile cysts was high (63.3%) and the fertility rate of viable pulmonary cysts was higher (73.3%) as compared to that of other cysts (Table 3).

Table 1: Distribution of positive cases CE according to the locations of slaughterhouses, age groups and sex of slaughtered cattle

Categories	Details	Suspected	Positive	Percent (%)	p Value
Abattoirs	Boushar	34	25	73.5	p=0.014
	Nizwa	90	69	76.7	
	Salalah	108	94	87.0	
	Sohar	25	15	60.0	
Age (years)	<3	31	17	54.8	p=0.001
	3 - 5	182	147	80.8	
	>5	44	39	88.6	
Sex	Female	104	86	82.7	p=0.23
	Male	153	117	76.5	

Table 2: Distribution of CE in different organs with respect to the sizes and fertility status of the cysts

Positivity			Type of	Cysts				Size of	Cysts	
Organs localization	Total	Positive(%)	Sterile(%)	Fertile(%)	Calcified(%)	Degenerated(%)	Underdeveloped(%)	>8 cm(%)	4-8 cm(%)	< 4 cm(%)
Liver & lung	5	4 (80.0)	3 (75.0)	1 (25.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1 (25.0)	3 (75.0)
Heart	1	1 (100.0)	1 (100.0)	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1 (100.0)
Kidney	1	1 (100.0)	1 (100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1 (100.0)	0(0.0)
Liver	108	81 (75.0)	52 (64.2)	13 (16.0)	7 (8.6)	5 (6.2)	4 (4.9)	0(0.0)	23 (28.4)	58 (71.6)
Lung & spleen	24	18 (75.0)	15 (83.3)	1 (5.5)	2 (11.1)	0(0.0)	0(0.0)	0(0.0)	5 (27.8)	13 (72.2)
Liver & spleen	1	1 (100.0)	1 (100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1 (100.0)
Lung	117	97 (82.9)	41 (42.3)	15 (15.5)	14 (14.4)	25 (25.8)	2 (2.1)	2 (2.1)	43 (44.3)	52 (53.6)
Total	257	203 (79.0)	114 (56.2)	30 (14.8)	23 (11.3)	30 (14.8)	6 (3.0)	2 (1.0)	73 (36.0)	128 (63.1)

Table 3: Percentages of viability of protoscolices from fertile hydatid cysts in slaughtered cattle

Organs	Viable N (%)	Non-Viable N (%)	Total
Lungs	11(73.3)	4(26.7)	15
Livers	6(46.2)	7 (53.8)	13
Heart	1 (100.0)	0(0.0)	1
Livers with lungs	1 (100.0)	0 (0.0)	1
Total	19 (63.3)	11 (36.7)	30

Genotypes of *E. granulosus* in Cattle: The result of genotyping of *E. granulosus* (fig. 2 & fig. 3) has indicated that, G1 genotype (*E. granulosus sensu stricto*) or (sheep strain) was the most predominant genotype (68%) from the samples obtained from slaughtered cattle and the remaining samples were belonged to G6/7 (*E. canadensis*) or (camel strain) genotype.

DISCUSSION

This study was carried out in four slaughterhouses located at different governorates in Oman (fig. 4) to assess the situation of CE in slaughtered cattle. Our data indicated that CE infection exists in slaughtered cattle of Oman with statistically significant ($p<0.001$) differences in prevalence between all the studied locations. Although a previous study of CE in goats in Oman, reported that the prevalence of CE was low in goats [27], there have been no reports on the prevalence and intensity of this disease in other intermediate hosts such as cattle or sheep and data on cyst fertility and viability in these animals is lacking. Knowledge of these data in all intermediate host species is essential to implement effective strategies for disease control.

In this study, the total prevalence/100 of CE in slaughtered cattle (n=28269) in four slaughterhouses was found 0.72%. The low CE prevalence in cattle may reflect animal husbandry practices where cattle may have lower contact with stray dogs and cannot freely feed on pastures in most areas of Oman. The highest prevalence/100 of CE was found in the cattle slaughtered at Nizwa slaughterhouse followed by Bousher, Salalah and Sohar slaughterhouse respectively (fig. 4).

In general, this result has indicated that, the prevalence of CE in slaughtered cattle varies from low and sporadic in some studied areas (Boushar, Salalah and Sohar) and higher in other areas (Nizwa). The number of cattle slaughtered at Nizwa abattoir was lower (700) (fig. 4) as compared to other three abattoirs and the majority of the infected cattle with CE were found from local breeds. This could be indicated that CE is circulated among the local animals at Al Dakhiliyah governorate and more investigation with a plan of control measures which should include improvement of slaughter facilities and animal husbandry practices and control the number of stray dogs is required to eliminate this disease.

In contrast, the presence of significantly higher prevalence of CE observed in slaughtered cattle from Salalah slaughterhouse in the present study was in agreement with a previous study by AlKitani et al. (2014) [27]. It is well established that, the grazing behavior and climatic conditions of Dhofar governorate is playing a great role in adaptation and transmission of this parasite. The majority of livestock at Salalah are free grazing on the green mountains with fresh pastures possibly contaminated with the eggs of *E. granulosus*. According to the studies of Daryani et al, (2007), high prevalence of hydatid cysts in domesticated farm animals, especially in sheep and cattle, was likely to be related to the presence of green pastures, differences in animal husbandry systems, unsupervised slaughter of animals, lack of proper removal of infectious carcass and the presence of more number of stray dogs. In addition, the population of stray dogs plays a great role in hydatid disease transmission and must be put in consideration in each studied location before planning any control measure.

The present study showed that, CE was significantly lower in the age group of less than 3 years as compared to the older group of > 3 and > 5.The significant linear increase of the prevalence with age has previously been reported in goats [27]. This is also supported by the statement suggested that aged animals have more chance of exposure to the eggs of *E. granulosus* [21, 22, 36]. Despite a sampling bias as the majority of cattle sent for slaughtering are from older age groups, this finding was in agreement with other studies on CE infection in different intermediate hosts, where the prevalence of hydatidosis was higher in older animals as compared to young animals [4, 37, 38].

More numbers of the female cattle were observed to harbor hydatid cysts as compared to male cattle. This finding has indicated that male cattle are probable either have a lower exposure to the parasite or greater resistance to infection following exposure [38]. However, it is difficult to compare these values due to the fact that, the majority of cattle in Oman are slaughtered at the age of post puberty especially the females that remain for dairy purposes.

Regarding to organ localization of CE in cattle, the result of this study has showed that the highest percent of CE was found in in lungs as compared to multiple organs infection (livers with lungs, lung with spleen and liver with spleen). This finding is in agreement with the previous studies of Adinehbeigi *et al.* and Daryani *et al.* and Azlaf and Dakkak and Kebede *et al.* [22, 35, 36, 39] in which they explained that the Echinococcus oncospheres migrate to the lungs that have greater capillaries sites than any of the other collected organs.

Fertility of cysts in various domestic herbivores provides reliable indicators of the importance of each type of animal as a potential source of infection to dogs [22]. In this study, the majority of hydatid cysts isolated from infected cattle were found sterile. However, the fertility of liver and lung cysts were found higher as compared to other cysts isolated from other organs. On the other hand, the overall percentage of viability from the fertile cysts was high (63.3%) and the fertility rate of viable pulmonary cysts was higher as compared to that of other organs. These findings suggest that, cattle of Oman could play a great in transmission of this to parasite to human.

This is a first study to highlight the genetic characterization of *E. granulosus* isolates from cattle in Oman. Our results indicate that, G1 genotype (*E. granulosus sensu stricto*) and G6/7 (*E. canadensis*) were the most commonly identified genotype from the samples obtained from slaughtered cattle. This finding suggests that sheep–dog and camel–dog cycles are circulating in Oman. On a global basis, G1 and G6 genotypes are the most predominant found in livestock [10, 40- 45]. Both genotypes (G1 and G6) were reported to cause the CE in human in different countries. Moreover, all intermediate hosts play a role in the maintenance of the *E. granulosus* cycle and act as reservoirs of human infection through the dominant G1 strain [38].

CONCLUSION

In this study, we demonstrated that CE is present and circulating among cattle of Oman and is widespread at some studied regions (Nizwa). The high cyst fertility rate (14.8%) in found in cattle in this study could contribute to the transmission of disease in Oman. We have also identified the genotypes G1 and G6/7 in cattle of Oman for

the first time. Both genotypes can infect human and efforts should be made to control the transmission of CE from slaughterhouses by safe disposal of infected offal and reducing dog population. This will reduce the transmission of this parasite from slaughterhouses to the final hosts. Moreover, a large scale molecular epidemiology of *E. granulosus* in all intermediate hosts is highly recommended since data on prevalence and molecular characterization of *E. granulosus* in other intermediate / final hosts in Oman is still lacking.

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Conflict Interest Statement: The authors declare that they have no conflict of interest with the contents of this paper in any respect.

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