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Secondary bacterial infection of hydatid cysts infected livestock animals (In vitro study)

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Abstract

Isolation and identification of the bacteria-infected hydatid cysts of livestock animals were carried out. The study lasted for five months. A total of 302 slaughtered livestock were checked to find hydatid cyst naturally infected livers and lungs of sheep and goat at local Mosul abattoir/ Nineveh/ Iraq. Each cysts' aspirated fluid was cultured separately on blood or nutrient agar at 37C for 24 hours then detected secondary bacteria. Each bacterium was incubated independently with 1000 viable protoscolices in a culture tube containing tryptic soy broth at 37°C and examined every two hours for 6 hours, followed by 12, 24, 36, and 48 hours afterward. The degeneration rate of protoscolices resulting from treatment with bacteria isolated depending on viability was studied *in vitro*. The results were revealed that the liver was the preponderant affected organ in sheep and goats. About 57.1% of sheep and 50% of goats harbored fertile cysts. *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* bacteria were observed in the infected cysts. The most common bacteria infect the cyst was *Escherichia coli*. The protoscoleces treated with the bacterial isolates had completely deteriorated, whereas 97% of protoscoleces throughout the control groups were still alive and intact even after completing the incubation time. The experiments detect a significant time-dependent scolicidal effect on decreased viability of protoscolices *in vitro* study. This could pave the door for more research into the scolicidal validity of bacteria or their by-products as protoscolices both *in vitro* and *in vivo*.

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Introduction

Cystic Echinococcosis (CE) or hydatid disease is a remarkable silent zoonotic infection caused by the larval stage of *Echinococcus granulosus*, which affect any internal organs of infected humans and herbivores, especially liver and lung, causing severe monetary damage in livestock animals and a significant public health consideration in both cities and suburbs (1,2). Age, sex, health status, address, and socioeconomic condition affecting human infection (3). It may be diagnosed incidentally from its complication signs (4). The clinical features of hydatid cysts depend on the cyst's site, size, and the presence of complications as either perforation, pain, or discomfort due to the swelling cyst and secondary bacterial infection in most of the delay or untreated cases (5,6). CE is a widespread public health issue, particularly in developing regions including Iraq (7,8). Protoscolices were developed into adult worms after ingested by suitable definitive hosts e.g., dogs (9). A human can get the infection from ingested eggs of contaminated food or water passed from infected carnivores (10). This disease is endemic in surrounding areas where humans, livestock, and dogs are found together, as this configuration allows the parasite to replenish its life cycle. Clinically, such

a disease is often asymptomatic. However, it can be discovered accidentally by observing the clinical signs of increased size or the cyst's complications as compression pain or rupture respectively (11,12). Generally, the contained rupture may be subsisting silent clinically, whereas the communicating one might cause biliary infection and liver abscess. When laceration occurs, the contents spread into physiological channels (bronchial tree, biliary) or adjacent organs, which could result in significant clinical consequences (6).

Hydatid cyst is a fluid-filled vesicle-like structure containing protoscolices, an infective stage of disease, will grow to the adult stage after ingestion by a definitive host and repeat the cycle (13). Cystic fluid (hydatid fluid) is clear and bacteriologically accessible. Protoscolices released from the original cyst were developed into daughter cyst that may degenerate by bacterial invasion from the matrix (4). This infection may cause the death of protoscolices, resulting in clear fluid with a damaged germinal layer and cyst wall (9). Rupture and spread of cyst contents are more dangerous than the mass effect of hydatid cysts, due to their anaphylactic reaction, except in the brain where the mass effect by itself has severe consequences (13,14). Cyst's expansion aids the diffusion of bacteria into the cyst due to the difference in the physiology of the organs resulting in multiple secondary hydatidoses (4). There are few studies on secondary bacterial infections of hydatid cysts in humans and animals and its scolicidal effect on the protoscolices by its toxins or enzymes *in vitro* and *in vivo*. The present study aims to isolate and identify the bacteria-infected hydatid cysts of livestock animals in Mosul local abattoir *in vitro* study.

Materials and methods

Collection of hydatid cysts

A total of 302 slaughtered livestock of both sexes (110 goats and 192 sheep) were explored to detect the presence of hydatid cyst in local Mosul abattoir, Nineveh, Iraq for five months from May to September 2021. Infected livers and lungs were inspected and examined cautiously to exclude cyst rupture at the local abattoir. Organs with cysts were carefully packed in sterile plastic containers and were transported to the microbiology laboratory at Mosul University's Department of Clinical Laboratory Science, where fluids were collected and studied.

Assessment of *E. granulosus* protoscolices viability

The uprooted cysts were investigated by incision and keeping fluid for subsequent investigation. Protoscolices (psc.) were aspirated according to Smyth (15). Collection and assessment of *E. granulosus* protoscolices from hydatid fluids were obtained under sterilized conditions. Briefly, the organ's surface was cleaned with normal saline and sterilized with 70% ethanol before the fluid sac was removed using a 50 ml syringe. Cyst fluid was collected from an individual cyst, washed by phosphate buffer saline (PBS), and checked for the presence of protoscolices. The sediment was checked microscopically for fertility and detection their bacterial infection. Cyst containing viable protoscolices were considered fertile. Others had dead protoscolices were considered as sterile cysts but if containing bacterium was assumed as "infected" according to their manifested by Fallah *et al.* (9) and Al-Juwary (16). Gravity sediment was spotted for protoscolices viability under the light microscope consummated by body contraction, peristaltic movement, and impermeability using vital stain (0.1% aqueous eosin). Twenty microliters of fluid were obtained from the cyst under sterile conditions. Apply 20 ul of stain on a slide using a light microscope and examine it. Because protoscolices membranes excluded eosin, bright green protoscolices were regarded as living, whereas red protoscolices were judged dead due to pigment acceptance (17).

Isolation and identification of secondary bacterial infection

Hydatid cyst replete with clear fluid, had transparent white membrane with one or more separate chamber, was a mainly non-infected one, otherwise infected cyst was overstuffed with turbid fluid and calcification may notice. The aspirated fluid from all cysts was incubated individually to detect secondary bacterial infection. The remaining fluids were centrifuged, pick the precipitate having protoscolices and/or bacteria used for further examination.

The isolated fluid from each hydatid cyst was inoculated directly onto BA (blood agar) (Merck, Germany) in a standard sterile condition, and incubated for 24-48 h at 37 °C, then observed colony appearance. To identify bacteria at the genus or species level whenever possible, additional assays were done by culturing on differential medium, and some basic biochemical tests were performed according to (18,19).

Protoscolices viability and isolated bacteria

The effect of bacteria isolated from cystic fluids on the viability of the protocols was examined in vitro. Each bacteria were cultured separately in 10 mL of Brain Heart Infusion (BHI) broth medium for 24 h at 37°C. Approximately 1 ml of bacteria were incubated independently in a culture tube containing tryptic soy broth and a total of 1000 live protoscoleces. As a control, another tube without bacteria was created. These tubes were incubated at 37 °C. Each culture tube was observed and examined microscopically to assess the viability of the *E. granulosus* protoscolices as well as each treated sample was cultured on blood or nutrient medium to assess bacterial growth every two hours for six hours, followed by 12, 24, 36 and 48 hours afterward (9).

Statistical analysis

All of the experiments in this study were carried out in triplicate. A statistical package for society SPSS program (version 19) was used to analyze the data, which was applied to analyze it by computer. Percentages, means, SDs, and One-Way ANOVA (Duncan) were used to analyze results. All results were significant with $P \leq 0.05$.

Results

In this work, we educated cystic echinococcosis of the livestock animals in a Mosul local abattoir for five months from May to September 2021. Of 302 livestock (192 sheep and 110 goats), 6.62% were infected with *E. granulosus*, representing 7.3% and 5.5%, respectively. A higher proportion of sheep were infected with hydatid cysts than goats. Table 1 reveals that the liver was the preponderant affected organ in sheep and goats. Concurrent infection of both liver and lung were estimated in sheep and goats 50% and 66.7%, respectively.

The results showed that the intensity of the disease ranges from moderate to severe depending on the animal's posture and the number of cysts. The consistency was relatively high in investigated animals. Mainly 57.1% and 50% had more than ten cysts in sheep and goats, respectively, as manifested in table 2.

The study's findings revealed that hydatid cysts in the liver or lung were scarce compared to the presence of a significant number in both organs as shown graphically in figure 1.

Table 1: Number and percentage of hydatid cyst infections among slaughtered livestock considering habitat

Animal	Examined number	Infected number (%)	Location and number(%) of cysts in infected animals organ		
			liver only	Lung only	Both
Sheep	192	14 (7.29)	5 (35.7)	2 (14.3)	7 (50)
Goat	110	6 (5.45)	2 (33.3)	0 (0)	4 (66.7)
Total	302	20 (6.62)	9 (45)	2 (10)	9 (45)

Table 2: Number and percentage of hydatid cysts in infected livestock animals

Animal	Number of cysts (%)				Total infected cysts
	one –three	four –six	seven-nine	> ten	
Sheep	2 (14.3)	0 (0)	4 (28.6)	8 (57.1)	14
Goat	0 (0)	2 (33.3)	1 (16.7)	3 (50)	6
Total	2 (10)	2 (10)	5 (25)	11 (55)	20

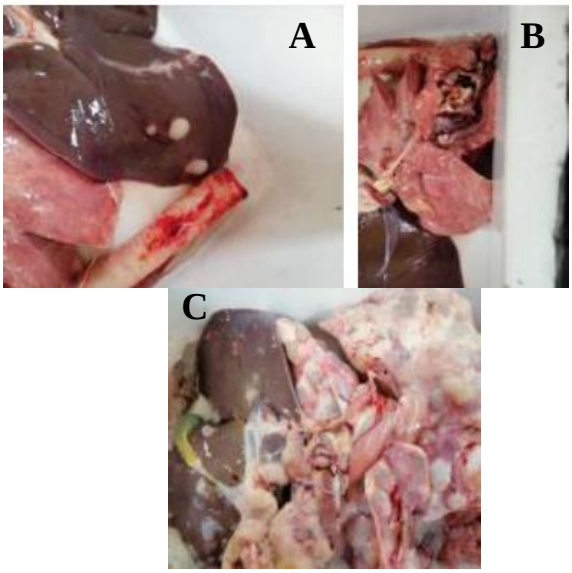

Figure 1: Prevalence of hydatid cysts in (A): liver only, (B): lung only, and (C): both liver and lung.

Considering that cysts had fertile protoscolices, others implicate that dead protoscolices were considered sterile cysts. Live protoscolices excluded eosin give a bright color, whereas dead protoscolices accepted eosin pigment give red color as shown in figure 2.

Our finding manifested that 57.1% sheep and 50% goat harbored fertile cysts, while sterile cysts were present in 35.7% sheep and 33.3% goats. A lower incidence of calcified cysts was found in sheep (7.14%) and goats (16.67%), as seen in figure 3.

Infected cysts were discovered in 55% of animals, where infected cyst rates were 71.43% in sheep and only 33.33% in goats. Secondary bacterial infection as a complication of delayed treatment may lead to sepsis and death. *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Proteus mirabilis*, and *Pseudomonas aeruginosa* were the bacteria secluded from the infected cysts. The growth of bacterial colonies was observed after culturing them on different media, which helps discover their types, as shown in figure 4.

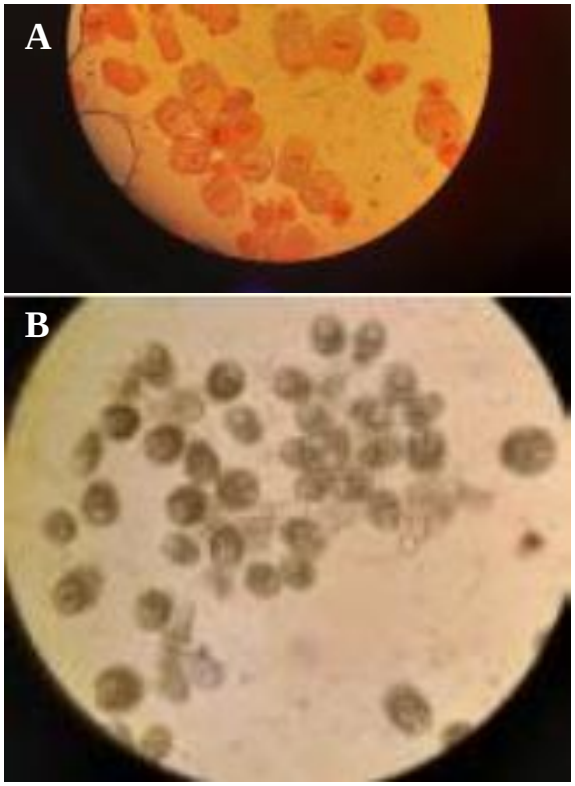

Figure 2: Hydatid cyst protoscolices treated with 0.1% aqueous vital eosin stain; (A): red color of dead protoscolices, and (B): green color of vital protoscolices (40X).

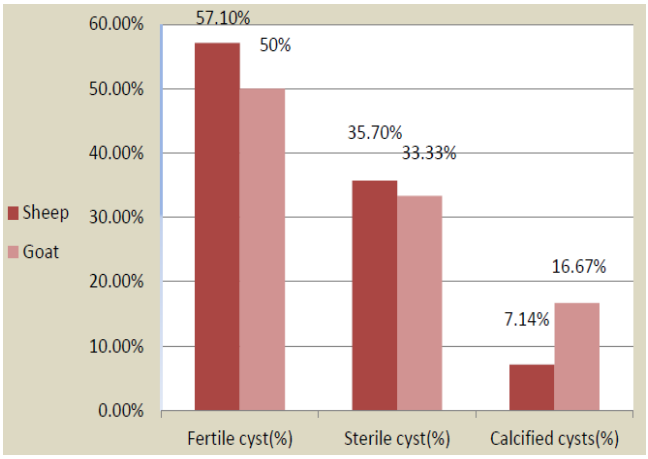

Figure 3: Distribution of uprooted hydatid cysts concerning the viability of protoscolices and clinical manifestation.

A metallic green sheen with a dark center growth on Eosin Methylene Blue (EMB) agar indicated *Escherichia coli*. At the same time, *Klebsiella pneumonia* showed a pink mucoid lactose fermenter on MacConkey agar. Mannitol Salt Agar (MSA) is used as a selective and differential medium in which *Staphylococcus aureus* fermented mannitol and grew as yellow colonies surrounded by yellow areas. The swarming colony-forming concentric circles motif indicated Proteus mirabilis, while green colonies were a feature of *Pseudomonas aeruginosa* when grown in Mueller Hinton Agar. Some biochemical reactions were further investigated, as represented in tables 3 and 4.

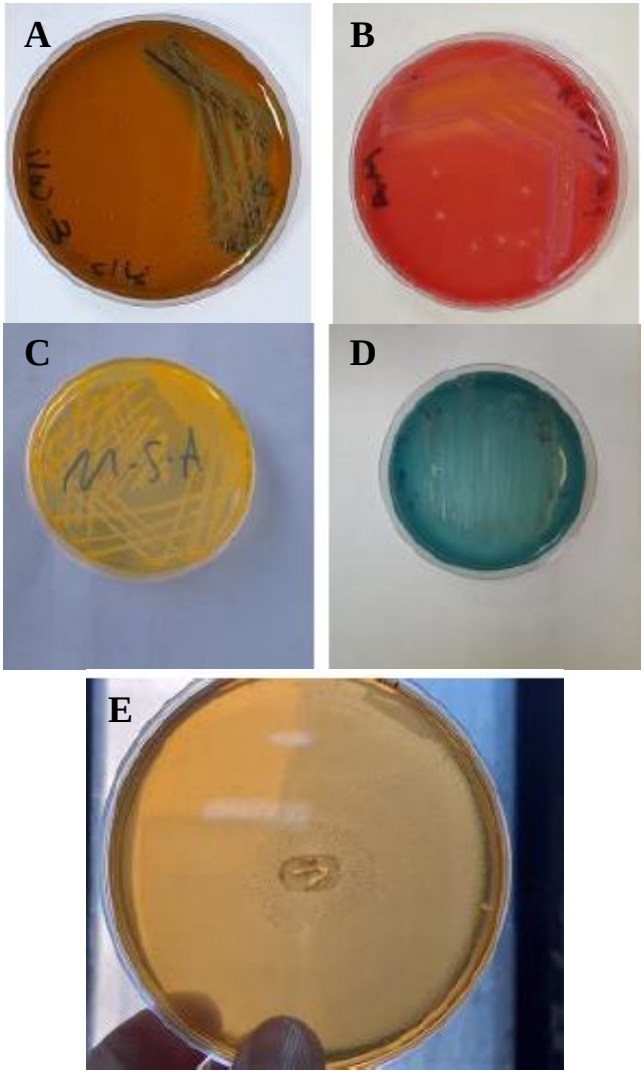

Figure 4: Growth of bacteria isolated from hydatid cyst fluid; (A) *Escherichia coli* on EMB, (B) *Klebsiella pneumonia* on MacConkey agar, (C) *Staphylococcus aureus* on MSA, (D) *Pseudomonas aeruginosa* and (E) *Proteus mirabilis* on MHA on MHA.

Table 3: Biochemical tests used to diagnose bacterial species (gram-negative)

Biochemical Tests		Bacteria			
		E. coli	*K. pneumoniae*	*Prot. mirabilis*	*Pseud. aerogenosa*
Catalase		+	+	+	+
Oxidase		-	-	+	-
IMVC	Indole	+	-	-	-
	Methyl red	+	+	-	V
	Voges Proskauer	-	V	-	+
	Citrate	-	V	+	+
Urase		-	+	V	+
Gelatin Hydrolysis		-	+	-	-
Motility		+	+	+	-
Nitrate Reduction		+	+	+	+
Triple Sugar Iron		A/A	K/A	K/K	A/A
Fermentation	Lactose	+	-	-	+
	Glucose	+	+	-	+
	Sorbitol	+	-	-	+

+: positive result, -: negative result, V: variance result, A: acid, K: alkaline.

Table 4: Results of biochemical tests used to diagnose bacterial species *Staphylococcus aureus* (gram-positive)

Biochemical Tests		*S. aureus*
Catalase		+
Oxidase		-
Hemolysis		+
Coagulase		+
Nitrate Reduction		+
Gelatin Hydrolysis		+
Motility		-
Mannitol Salt Agar		+
DNAase		+
Fermentation	Glucose	+
	Lactose	+
	Mannitol	+

+: positive result, -: negative result.

Multiple infections were diagnosed in some infected cysts being examined Concerning the organs, *E.coli* was the bacteria that were most frequently isolated 54.55% in the liver, while 45.46% and 27.28% of *K. pneumonia* and *E.coli* respectively isolated from the lung. The results showed a dense growth of bacteria for all the treated culture tubes in a different period, which means that the five bacteria grew on the medium and were not affected by the presence of protoscolices. Most infected hydatid cysts had dead protoscoleces, which could be due to the bacteria's effect on them, leading them to lose their viability. To find out, we used bacteria to treat protoscolices, and the results revealed that the bacteria had a significant impact on the vitality of protoscolices *in vitro* but at varied rates and timeframes. Incubation of protoscoleces with all isolated bacteria resulted in complete degeneration, which began after four hours. After 48 hours, all protoscoleces in control groups were intact and viable; however, after one week, half of the protoscoleces in control groups remained intact and alive, as represented in table 5.

The degeneration rate of protoscolices treated with isolated bacteria differed according to bacteria type and incubation time. *E. coli* and *K. pneumonia* were the bacteria that degraded the protoscoleces faster than the others. The experiments detect a significant ($P \leq 0.05$) time-dependent scolicidal effect on decreased viability of protoscolices in vitro study as shown in figure 5.

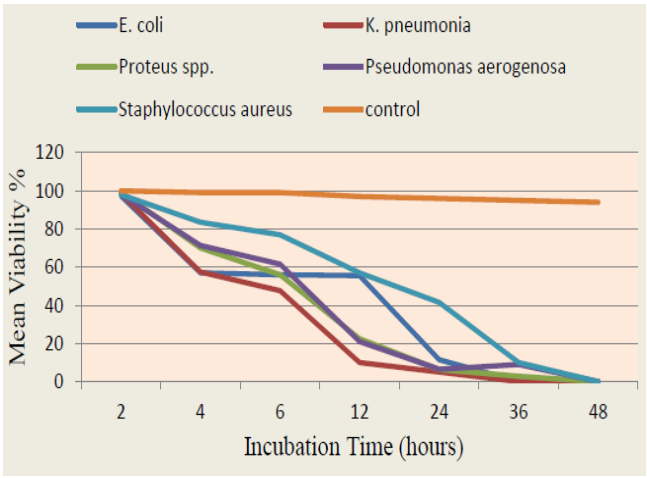

Figure 5: *E.granulosus* protoscolices viability after being treated with the isolated bacteria.

Table 5: Protoscolices Viability after Being Treated with Different Bacterial Species at different time intervals.

Bacteria	mean viability % of protoscolices ± SD after:							P-value
	2 hrs.	4 hrs.	6 hrs.	12 hrs.	24 hrs.	36 hrs.	48 hrs.	
E. coli	97.33±0.57 E	57±0.86^{a} D	56±0.5^{b} C	55.5±1.5^{b} C	11.4±1.73^{c} B	0.0±0.33^{a} A	-----	0.010
K. pneumonia	98.67±0.58 F	57.5±1.5^{a} E	47.6±0.36^{a} D	20±4.5^{a} C	5±0.62^{a} B	0.0±0.00^{a} A	-----	0.000
Prot. mirabilis	99.33±0.57 G	70.07±0.9^{b} F	56±0.86^{b} E	22.5±1.32^{a} D	6.3±0.34^{b} C	2.85±0.5^{b} B	0.0±0.00^{a} A	0.000
Pseud. aerogenosa	98.33±0.57 G	71.4±0.85^{b} F	61.5±0.81^{c} E	21±1.5^{a} D	6.5±0.00^{b} C	9.0±0.00^{c} B	0.0±0.00^{a} A	0.000
S. aureus	98±1.00 G	83.5±2.17^{c} F	77±1.47^{d} E	57±0.00^{b} D	41.5±0.5^{d} C	10±1.00^{c} B	0.0±0.00^{a} A	0.000
Control	100±0.98 E	99±0.07^{d} E	99±0.00^{e} E	97±0.86^{c} D	96±0.00^{e} C	95±0.86^{d} B	94±0.00^{b} A	0.000

Significant at P≤0.05. Each treatment had three duplicates. At zero time, the viability % was regarded to be 100%. At zero time, each test tube had around 15000 protoscolices. The number of protoscolices utilized in each treatment was 1500 protoscolices /20l at the start. Different letters vertically (a), (b), (c), (d), (e) indicate that the mean is significantly different at P≤0.05 between different groups at a time interval. Different letters horizontally (A), (B), (C), (D), (E), (F), (G) indicate that the mean is significantly different at P≤0.05 between each group at different times interval.

Discussion

The present study investigated the most frequent secondary bacteria infected with the cyst in Mosul city/Nineveh governorate *in vitro* study. Three hundred and two livestock animals (192 sheep and 110 goats) have been explored. Only 6.62% (7.3% sheep and 5.5% goat) had hydatidosis. This result came to agree with Fallah *et al.* (9) and Fallah *et al.* (20). As regards the organ involvement, the majority of cysts in the selected region were ordinarily seen in the liver with the respective percentage of 35.7%, and concurrent infection of both liver and lung were estimated more in goats with the respective percentage of 66.7%, that conformed with the observations of Garcia *et al*. (4) and Sarkar *et al*. (13). Most hydatid cyst prospectors have demonstrated similar outcomes, for example, Jawad *et al.* (8) and Jarjees and Al-Bakri (21). These prospectors' recognition depends on the fact that the liver acts as the first barrier to the bloodstream by portal circulation then to all body sections. Most of *E. garnulosus* larvae pulled in the liver due to their large size and colonized forming a cyst. However, an asymptomatic disease that delays treatments indicates observation of hydatid cysts in both liver and lung of some investigated animals accorded with Fallah *et al.* (9).

In the present study, 55% of examined animals had more than ten cysts in each infected organ agrees with Kamhawi *et al*. (22). This consequence may be mentioned because each hydatid cyst primarily undergoes silent growth interval, which may engross several years before hydatidosis debut in respect of the response of the host's immune system and the slow growth and development of cysts. Sheep offered the highest recurrence of hydatidosis consistent with that observed by Nyero *et al*. (23). From a gross perspective, infected organs have pathological changes in both sizes, appearance, inflammatory signs existence, and their consistency. These changes depend on the cyst number involved. Undoubtedly, the danger of infection could be enhanced if diseased offal is not adequately removed using unsanitary slaughter procedures. Degenerative or necrosis processes, most often resulting from infection or immune processes, may cause the cysts to repair spontaneously, resulting in solidification or calcification (3). Cyst degeneration goes to partial or complete calcification lesions. Jarjees and Al-Bakri (21) observed that a sterile cyst might be referred to as the degenerative atrophy induced by body reaction against it, which goes to caseation and calcification. In this work, only two cysts 10% were obliterated furthermore a noticeable shrinkage wall. Muhaidi *et al*. (24) deduced that cyst fertility has an excellent significant value in epidemiological investigations due to the possibility of the fertile cyst to spread the disease in the body and act as a source for disseminating the infection to humans and other predisposing animals. Among the two species slaughtered for human exhaustion, the present study deduced undoubtedly that sheep play an essential role in the disease expansion because of their high prevalence of infection and presence of fertile cysts act as a source for dissemination of hydatidosis that agrees with Founta (25) and Santucciu (26).

After erosion, bacterial colonization in hepatic and lung cysts leads to suppuration, rupture, or abscess formation which raises the problem of differential diagnosis with hydatidosis (11). Black color and increased cyst fluid density often mean some microorganisms' impurities or inflammation. Also, bubbles inside the cyst indicate

secondary infection with gas-producing bacteria as observed by Sarkar *et al.* (13). The hydatid cyst infection always probably comes in this unnatural connection. Surgical treatment is typically challenging, and recuperation is dependent on the afflicted gastrointestinal organ (6). Early diagnosis and treatment of complications must be essential to prevent a life-threatening state.

Microscopical and biochemical examinations of cyst fluid show that such cysts were frequently infiltrated with bacterial pus or degenerated protoscolices debris. Different infective rates were established depending on animal species and the infected organs, other studies instituted that sheep have a higher infection rate than goats as mentioned with Grubor *et al.* (27). In the present investigation, bacterial infections were observed in the plurality of hydatid cysts 71% that came agree with Fallah *et al.* (9). Cyst infection, especially in animals, is a familiar complication. Although the cystic components have not spontaneously erupted into the bronchial tree or biliary ductal system, our research elucidates that this situation is exceptionally prevalent in animals, especially in this region, which is confirmed with the observations of García *et al.* (4) and Ziino *et al.* (28). However, hydatidosis with secondary bacterial infection usually had a systemic inflammatory response which may cause death by multiple organ dysfunction. This success shows that hydatid cyst superinfection can be the leading cause of mortality in cystic hydatidosis (4).

A large percentage of hydatid cysts were bacteriologically contaminated in this investigation. The most frequent bacteria revealed were *E. coli, Klebsiella pneumoniae, Proteus spp., Pseudomonas aerogenosa, and staphylococcus aureus* that stratify with the observation of Ziino *et al.* (28) and Grubor *et al.* (29). It is worthy to say that in the present study, *E. coli* was the most common bacteria isolated from the investigated cysts that came agree with that of García *et al.* (4) and Pakala *et al.* (30). The degeneration rate of protoscolices treated with isolated bacteria differed according to the type of bacteria and incubation period. *In vitro*, the bacterial isolates degraded the protoscoleces during short-period incubation up to 48 hours, agreeing with Fallah *et al*. (9). Bacterial degeneration of protoscoleces could develop new protoscoleces and improve surgical safety. Bacteria degrade protoscoleces through unknown methods. On live protoscolices, these pathways might include impacts of bacterial toxins (endotoxin or exotoxin), specific enzymes, or other biological agents (9). Further research is required to investigate this effect within the body.

Conclusion

According to the findings, Bacteriological contamination was found in a high percentage of hydatid cysts. *E. coli* was among the most frequently isolated microorganisms. During *in vitro* incubation, the bacteria could deteriorate the protoscoleces. This research may be helpful for future research such as the efficacy studies concerning bacterial or their by-products as protoscolices both in vitro and in vivo.

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Conflict of interest

The authors have no competing interests in the publication of this paper.

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العدوى البكتيرية الثانوية للأكياس العدارية في حيوانات الماشية (دراسة في الزجاج)

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الخلاصة

اجري عزل وتشخيص الأنواع البكتيرية المختلفة للعدوى الجرثومية في الكيس العداري للماشية. استمرت الدراسة لمدة خمسة أشهر. تم فحص ما مجموعه ٣٠٢ من الماشية المذبوحة للعثور على أكباد ورئات الأغنام والماعز المصابة بشكل طبيعي والتي تم ذبحها في المسلخ المحلي لمدينة الموصل / نينوى / العراق. زرع كل سائل مأخوذ من الأكياس المائية بشكل منفصل على وسط الدم أو الأكار المغذي عند ٣٧ درجة مئوية لمدة ٢٤ ساعة ثم كشف عن العدوى البكتيرية الثانوية. حضنت كل بكتريا بشكل مستقل مع ١٠٠٠ من الرؤيسات الأولية الحية في انبوب استزراع يحتوي على وسط تريبتك مرق الصويا عند37 درجة مئوية وفحصت كل ساعتين لمدة ٦ ساعات، تليها ١٢ ، ٢٤ ، ٣٦ ، ٤٨ ساعة. درس معدل تحلل الرؤيسات الأولية نتيجة معاملتها بالبكتيريا المعزولة اعتمادا على قابليتها للحياة في المختبر. أظهرت النتائج أن الكبد هو العضو الأكثر إصابة في الأغنام والماعز. حوالي٥٧,١٪ من الأغنام و ٥٠٪ ماعز كانت تؤوي أكياسا خصبة. لوحظت بكتيريا الاشريكية القولونية و الكلبسيلة الرئوية و المكورات العنقودية الذهبية و المتقلبة الرائعة و الزائفة الزنجارية في الأكياس المصابة. كانت البكتيريا الأكثر شيوعا التي تصيب الكيس هي الإشريكية القولونية. تم تحلل جميع الرؤيسات الأولية المحضنة بالبكتريا المعزولة خلال فترة الحضانة، في حين أن ٩٧٪ في مجموعة السيطرة بقيت سليمة وحية حتى بعد الانتهاء من فترة الحضانة. كشفت التجارب عن تأثير معنوي يعتمد على الوقت على انخفاض قابلية بقاء الرؤيسات الأولية في الدراسة المختبرية والتي قد تفتح الطريق لمزيد من التجارب حول صلاحية البكتيريا أو منتجاتها الثانوية كمبيدات أولية في المختبر وفي الجسم الحي.