Isolation of *Listeria monocytogenes* from gallbladder of sheep and cattle in slaughterhouse of Najaf

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Abstract:

Listeria monocytogenes is the etiologic agent of listeriosis, a severe food-borne disease. The presence of *L. monocytogenes* in gallbladder explained that ability of the organism to survive and resistance the bile salt effect. The aim of this study was undertaken to explore the occurrence of *L. monocytogenes* in gallbladder of cattle and sheep. Three hundred gallbladder samples were collected randomly from sheep and cattle and screened for the presence of *L. monocytogenes* by using International Dairy Federation (IDF) protocol. The isolates were confirmed by API- *Listeria* system and the presence of haemolysin (*hly*) gene. A total of 8 (2.7%) *Listeria* spp were recovered in 6 (4.0%) samples of sheep and 2 (1.3%) samples of cattle. The isolates were identified to the level of species and it was found that all isolates belonged to *L. monocytogenes*. The isolates were obtained separately during the study period, the frequency of *L. monocytogenes* positive gallbladder samples tend to occur during cold months of the year. All isolates gave positive results with Hly specific primers. The present study concluded that the gallbladders of cattle and sheep may play a role in meat contamination and establishment of human infections.

الكشف الجزئي للأنماط المصلية لبكتريا *Listeria monocytogenes* المعزولة من كيس الصفراء للأبقار والأغنام

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الخلاصة:

تعد اللستريا وحيدة النواة من أهم الممرضات الغذاء التي تسبب داء اللستريا. إن وجود البكتريا في كيس الصفراء يدل على مقاومتها إلى أملاح الصفراء. اجري الغرض من هذه الدراسة للتحقق من وجود اللستريا وحيدة النواة في المرارة للأبقار والأغنام.

حيث تم جمع (300) عينة من المرارة عشوائيا للأبقار و للأغنام. تم الكشف والتحري عن اللستريا بواسطة استخدام طريقة International Dairy Federation (IDF) وتم استخدم فحص التحلل ألدمي وفحص (API test) لتحديد وتأكيد العزلات.

أظهرت النتائج ان نسبة عزل اللستريا وحيدة النواة كانت 8 (2.7. %) موزعة إلى 6 (4.0%) من عينات كيس الصفراء للأغنام و2 (1.3 %) في عينات كيس الصفراء للأبقار كما وجد إن جميع العزلات تعود إلى اللستريا وحيدة النواة عن طريق التحري عن جين التحلل ألدمي (*hly*) ، كما أظهرت النتائج تباين نسب العزل حسب أشهر السنة وان النسبة العالية لعزل الجرثومة كانت في الأشهر الباردة من السنة. يمكن ان نستنتج من هذه الدراسة إن كيس الصفراء في الأغنام والأبقار ربما تكون مصدر لتلوث اللحوم والتسبب بإصابات الإنسان.

Introduction:

Listeria monocytogenes is a Gram-positive, facultative, intracellular bacterial pathogen that causes morbidity and mortality in humans and livestock (1). It is a significant food-borne pathogen due its widespread distribution in nature, its ability to survive in a wide range of environmental conditions, and its ability to grow at refrigeration temperatures (2,3). The primary source of infection for both sporadic and epidemic listeriosis is almost invariably contaminated food and hence the gastrointestinal tract is the major portal of entry of *L. monocytogenes* into the host (1,4) Other possible infection routes include, direct transmission via the skin, characterized by a pyogranulomatous rash-generally on hands and arms-that is sporadically seen among farmers and veterinarians exposed to genital secretions or aborted fetuses from cases of listerial miscarriage in ruminants (5). Isolation of *L. monocytogenes* from the gallbladder has already been described in humans by (6). *L. monocytogenes* has important feature of virulent by it is ability to colonize in the gallbladder together with extracellular multiplication, which revealed to presence of an *L. monocytogenes*-specific gene, termed *bsh*, encoding a bile salt hydrolase (7). In Iraq there is a little information regarding the dissemination of *L. monocytogenes* in animals. Therefore, there is an increase demand to investigate the spreading of *L. monocytogenes* isolates, hence the aims of this study is to identify the occurrence of this species in gallbladder of cattle and sheep.

Meterials and Methods:

Culture enrichment of gallbladder samples:

During the period from November 2010 to April 2011, a total of 300 samples of bile slat were collected from gallbladder of the cattle (n=150) and sheep (n=150) from slaughterhouse of Najaf province (50 samples per month). The International Dairy Federation (IDF) method was used in isolation and differentiation of *Listeria*

isolates (8). All samples were collected into a sterile container and transported by ice box to the laboratory of Microbiology Department/ Collage of Medicine, Kufa University, then incubated at 4^{o}C for 1 week and centrifuge at 6000 rpm for 20 min. The supernatant discarded and the pellets used for isolation of *Listeria* spp. A 1ml of bile salt pellets were added into 9 ml of Listeria enrichment broth (Himedia, India). Then, the broth was incubated at 30°C for 48 hours. After that, 0.1 ml of the enrichment broth culture was spread on HiCrome Listeria agar, modified (Himedia). All plates were incubated at 37°C for 24-48 h.

Identification of *L. monocytogenes* isolates

The isolates were identified according to (9) using Gram staining, haemolysis of blood, oxidase, catalase and motility tests. The *L. monocytogenes* isolates were confirmed by API-Listeria test (BioMerieux, France). The colonial morphology and biochemical tests of the *Listeria* isolates were compared with *L. monocytogenes* wild type strain 10403s.

DNA extraction and PCR conditions for detection of haemolysin *(hly)* gene

Genomic DNA of *L. monocytogenes* isolate was extracted by using Genomic DNA Mini Kit (Geneaid, USA). DNA templates were subjected to PCR using set (F and R) of primers targeting haemolysin (*hly*) gene (Bioneer, Korea), F; CGGAGGTTCCGCAAAAGATG and R; CCTCCAGAGTGATCGATGTT as described by (10). The reaction mixture contained AccuPower^{TM} PCR PreMix (Bioneer, Korea), which is premixed ready-to-use solution containing *Tag* DNA polymerase, dNTP, $MgCl_2$ and according to Bioneer procedure. The reaction mixture was prepared in 0.2 ml eppendorf tube with 20 µl reaction volumes, and done under the following thermocycling conditions in a GeneAmp PCR system (Geneamp, Singapore); 95°C for 3 min for 1 cycle. Then 94°C for 1 min, 60°C for 1 min, 72°C for 1 min for a total of 30 cycles, and 72°C for 10 min. The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide using gel documentation system (BioDocAnalyze Live; Biometra biomedizinische Analytic GmbH, Germany).

Results and Discussion:

The presence of *L. monocytogenes* in gallbladder explained that organism has ability for survive and resistance the bile salt effect. Confirmed that the bacteria multiply within the gallbladder for some carriers without showing clear clinical signs that is distinguish the bacterial infection(11). However, *L. monocytogenes* is known to cause listeriosis in humans and animals. Information on the occurrence and

distribution of *L. monocytogenes* and other *Listeria* species is very limited in both the veterinary and public health sectors in Najaf.

Bacterial isolation results found that 8 (2.7%) of *L. monocytogenes* isolates were recovered from subclinical sheep (n=6, 4.0%) and cattle (n=2, 1.3%). While, all other samples turned out to be negative in this respect (Table 1).

Table (1): Distribution of *L. monocytogenes* in sheep and cattle

Type Animal	Number of collected samples	Number of positive samples	Percentage (%)
Sheep	**150**	**6**	**4.0**
Cattle	**150**	**2**	**1.3**
Total	**300**	**8**	**2.7**

Present data show that the occurrence of *L. monocytogenes* in cattle and sheep gallbladder in Najaf is reasonable. This was comparable with results of surveys undertaken in Al-Muthena province reported by (12) who revealed that the isolation rates of *L. monocytogenes* from sheep gallbladder were 20%, and from human was (4%) but no isolate appeared in cattle. In previous study, in Faculty of Veterinary Medicine, Ghent University, Belgium, by (13) documented that only one *L. monocytogenes* isolate was recovered from the gallbladder of a dog. No other studies were available on dissemination of this species in gallbladder of the animals. However, hazard linked to the consumption of meat contaminated with *L. monocytogenes*. It is generally assumed that raw meat products cannot be free from *L. monocytogenes* because of slaughter methods evisceration and food processing that allows greater chance for contamination in Najaf. Furthermore, *Listeria* species are ubiquitous in the environment (14). People handling food at different levels can also be sources of contamination (15,16). According to (17), *L. monocytogenes* transmission to the carcasses does not occur primarily through the animal, but is mainly linked to the slaughterhouse environment. As *L. monocytogenes* may persist in cattle and sheep processing environments, such as slaughterhouses, chilling rooms and cutting rooms, the efficiency of cleaning and disinfection procedures are of the utmost importance (18). Though there are, no reports of *L. monocytogenes* infection in Najaf environment, in view of the high fatality rate attention should be focused on correct and early diagnosis of the etiological agent and disease.

Bile is mainly composed of bile salts, cholesterol, and phospholipids. The detergent activity of bile is primarily attributed to the bile salt component (19). *L. monocytogenes* is highly resistant to bile salts (20,21). In present demonstration, the isolation rate of *L. monocytogenes* from gallbladder of

sheep was more than the isolation rate from cattle (Table 1). This result agreed with (12) who found that the isolation rate (0%) of *L. monocytogene* from gallbladder samples of cattle was less than in sheep (20%). This may be due the reason why so many things first are that the cows inherently less susceptible to this disease than other animals such as sheep and goats (22). This may be because the components of bile in the cows have a number of additional bile acids more than human and sheep bile, the bile in human and sheep contain mainly cholic acid and genodeoxy cholic (23).

Present investigation revealed that the frequency of *L. monocytogenes* positive gallbladder samples tend to occur during cold months, and exactly from November to April (Figure 1). Since the results obtained show, there are seasonal tendency for the isolation of *L. monocytogenes* were observed in this study. Therefore, present study suggested that the detection of a higher prevalence of *L. monocytogenes* carriers among cattle and sheep might be during cold months. Similar observation were detailed by (24) who found that *L. monocytogenes* positive fecal samples were collected during cold months, and exactly from January to April, and those examined in 1997 and found to be contaminated by *L. monocytogenes* were collected during the winter season too.

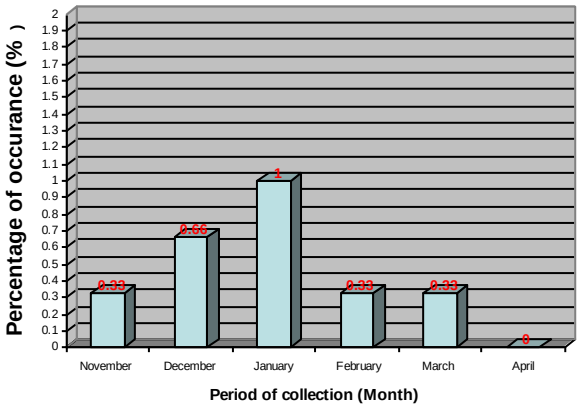

Figure (1): Histogram represented distribution of isolated *L. monocytogenes* from 300 bile salt samples of the sheep and cattle according to period (month of collection).

The present study used specific primers for hemolysin gene (*hly*) which encoded important virulence factor (listeriolysin O), a pore-forming exotoxin essential for invasion into the host cells and lysis of the phagosomes and that responsible for intracellular replication of *L. monocytogenes* (25). Our study revealed that all the eight isolates were carried the virulent *hly* gene indicating the isolates were *L. monocytogenes* (Figure 2).

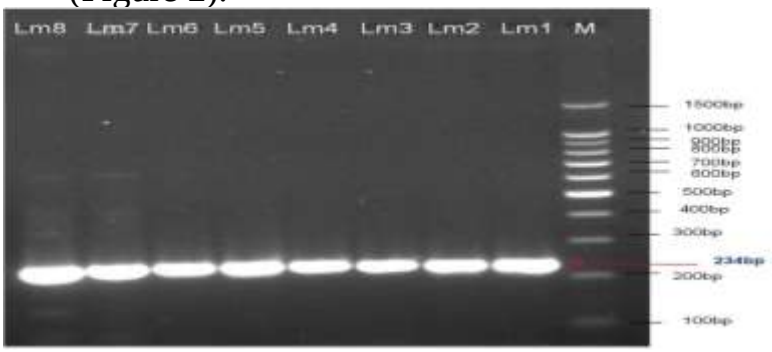

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted *L. monocytogenes* DNA amplified with

primers *hly* F and *hly* R. the electrophoresis was performed at 80 volt for 1.25 hours product.
Lane (M), DNA molecular size marker (l500-100bp ladder)
Lane (Lm1): *L. monocytogenes* isolate No. 1 show positive results with *hly* gene 234bp
Lane (Lm2): *L. monocytogenes* isolate No. 2 show positive results with *hly* gene 234bp
Lane (Lm3): *L. monocytogenes* isolate No. 3 show positive results with *hly* gene 234bp
Lane (Lm4): *L. monocytogenes* isolate No. 4 show positive results with *hly* gene 234bp
Lane (Lm5): *L. monocytogenes* isolate No. 5 show positive results with *hly* gene 234bp
Lane (Lm6): *L. monocytogenes* isolate No. 6 show positive results with *hly* gene 234bp

In this study, PCR was found to be a good confirmatory test. Moreover, PCR is simple, faster, cheaper, less difficult, and more reliable for confirmation and differentiation of *L. monocytogenes* dependent on amplification of specific gene. However, present result consistent with result reported by (26) who found that all *L. monocytogenes* isolates testing carried *hly* gene by using PCR technique as confirmation rapid screen test for classification into their respective phylogenetic divisions. The ability of *Listeria* species to produce haemolysis is closely correlated with their pathogenicity (1). Subsequent characterization of the *hly* locus led to discovery of the chromosomal virulence gene cluster in which most of the genetic determinants required for the intracellular life cycle of pathogenic *L. monocytogenes* (1). As a final point, present data concluded the hypothesis that gallbladders of cattle and sheep may be representing a reservoir for human *L. monocytogenes* infections in Najaf.

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