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Isolation and Molecular Identification with Resistant Profile Determination of *Listeria monocytogenes* from Imported Chicken Carcasses in Duhok, Kurdistan Region, Iraq

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This study was carried out to determine the contamination rate of *L. monocytogenes* from imported chickens in Duhok, Kurdistan region of Iraq and to determine their susceptibility profiling to different antibiotics. A total chickens (100 samples) were swab sampled, examined by conventional cultural methods and confirmed by PCR technique. Out of 100 only 3 (3%) chickens were found to be contaminated with *L. monocytogenes.* (100%) of isolates were resistant to Ceftazidime, and Ceftriaxone, (66.6%) to Ciprofloxacin and Clindamycin, while (100%) were susceptible to each of Imipenem, Levofloxacin, Ticarcillin-calvulanic acid, Clarithromycin and Gentamicin. The presence of this pathogen with an MDR profile on chicken carcasses found in this study creates a major risk for the consumer and need good hygienic strategies.

Key words: *L. monocytogenes*; chicken carcass; isolation, molecular identification; resistant profiling.

Listeria monocytogenes is a Gram-positive, non-spore forming rod, facultative anaerobic bacteria, motile with peritrichous flagella[1]. The organism grows over a wide temperature range from 1-45 °C, with an optimal growth temperature between 30 °C and 37 °C, *L. monocytogenes* can grow at pH values between 4.4 and 9.4, and at water activities ≥ 0.92 with sodium chloride (NaCl) as the solute[1, 2]. There are ten species within the genus *Listeria; L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayii, L. marthii, L. rocourtiae, L. weihenstephanensis and L. fleischmannii*[3]. Only two species are pathogenic; *L. monocytogenes* that cause disease in both humans and animals and *L. ivanovii*, which cause illness only in an animal[1, 4]. *Listeria* spp., particularly *L. monocytogenes*, identification is important because a severe infectious disease caused by this pathogen, known as listeriosis, in humans that has been recognized as a significant health problem worldwide[5]. Two forms of listeriosis, have been identified; an invasive form which causes infection mainly between at risk population and causes meningitis and/or septicaemia, and non-invasive variant self-limiting form, febrile gastroenteritis form, which affects healthy people and characterized by aches, self-limiting fever, diarrhoea, nausea and fatigue[6]. In addition, *L. monocytogenes* is danger for consumer safety, because this organism having the capability for growth at refrigeration temperatures in both raw and cooked meat[7]. Poultry usually contaminated with *L. monocytogenes* during their production, in the processing plant, in storage or cross-contamination during preparation, cooking, and serving foods[8, 9]. The excessive use of antimicrobial agents in poultry for prophylactic purposes to decrease the risk of infectious diseases, allow the emergence and dissemination of resistant bacteria

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particularly *Listeria* spp., in the environment, which may have a public health problem[10].

There is limited data on the presence of *L. monocytogenes* on imported chicken carcasses in our area.

Therefore, this study was aimed to estimate the contamination rate of *L. monocytogenes* from chicken carcass surfaces, by using cultural and molecular techniques, and to find out their resistance to different antibiotics in Duhok city, Kurdistan region of Iraq.

MATERIALS AND METHODS

Sample collection

A total 100 samples were collected from randomly nominated chicken carcasses at the food control unit in the Duhok city / Ministry of Health, Iraq, from May to September, 2014. The process of sampling was done by taking the swab over the whole chicken carcass started from neck to leg. All swab samples were inserted into sterile tubes that contain 10 ml of buffered peptone water (BPW) (Lab M, UK). After that transported to the microbiology laboratory, Faculty of Veterinary Medicine University of Duhok.

Culture methods for isolation and identification

Isolation protocol was carried out according to[7, 11, 12] with some modifications in which each swab in a test tube with (BPW) at the delivery to the laboratory was vortex mixed and incubated at 37ºC for 24-48 hours. Then 1-2 loopful of BPW broth culture inoculated by surface streaking on to Harlequin Listeria Chromogenic (HLC) Agar (Lab m, UK) and incubated at 37°C for 24 hours. After incubation, three suspected blue-green colonies that surrounded by a white halo, presumptively *L. monocytogenes*, were selected from HLC agar to sub-culture on blood agar (Lab m, UK) containing 6% sheep blood and incubated at 37°C for 24-48 hours. The *L. monocytogenes* isolates were identified by using catalase production, oxidase activity, motility test in semi-solid motility media with nutrient agar at 25ºC, and CAMP test with *Staplylococcus aureus* previously isolated in the same laboratory[13, 14]. Finally, the isolates were subjected to a PCR assay for the final confirmation of the *L. monocytogenes* using the commercially available specific kit (VetPCR™ *L. monocytogenes* Detection Kit) from (Bioingentech [Ltd], Ref. VET-A022-48D).

DNA extraction

The bacterial DNA was extracted by boiling (heat-freeze) technique according to Chai study[15] with minor modifications. Briefly, 2-3 colonies from each biochemically identified isolates, was suspended in 500 µL sterile nuclease free ddH2O and boiled for 10 minutes. Then, the suspended sample was chilled for 5 minutes. Finally, the supernatant was stored for PCR after centrifugation at 15,000 ×g for 10 minutes.

PCR Amplification

All three isolates were tested by PCR technique to confirm the detection of *L. monocytogenes*. VetPCR™ *L. monocytogenes* Detection Kit (Bioingentech [Ltd], Ref. VET-A022-48D) was used. The reaction of PCR was achieved in a volume of 13.5 µl, according to Bioingentech [Ltd] company manual procedure, containing 2 µl of the extracted DNA template, 5.5 µl of premixture solution, 6 µl DNase/RNase free water and 11 µl mineral oil solution. The PCR conditions were set according to the company instruction (Bioingentech [Ltd], Ref. VET-A022-48D) as detailed in table1. After that, the PCR products were subjected to 1.5% agarose gel electrophoresis containing ethidium bromide. Finally, the resulted PCR products were identified by ultra-violet transilluminator. The positive control, Negative control and BrigTM molecular weight marker (Bioingentech [Ltd]) were used in this experiment.

Antimicrobial susceptibility testing

The susceptibility testing of *L. monocytogenes* isolates for antibiotics was performed as applied by[1, 16], using disc diffusion method on Mueller-Hinton agar (Lab m, UK) supplemented with 5-7% sterile sheep blood. The results of susceptibility testing were interpreted in accordance with the Clinical and Laboratory Standards Institute[16]. The antimicrobial agents that tested and their corresponding concentrations from Bioanalyze Turkey were as follows: Ciprofloxacin (CIP) 5 µg, Imipenem (IM) 10 µg, Ticarcillin-calvulanic acid (TCC) 75/10 µg, Ceftriaxone (CRO) 30 µg, Clarithromycin (CLR) 15 µg, Clindamycin (DA) 2 µg, Gentamicin (CN) 10 µg, Levofloxacin (LEV) 5 µg, Ceftazidime (CAZ) 30 µg[17, 18, 14, 19].

RESULTS AND DISCUSSION

In this study from total 100 raw chicken carcasses examined for the presence of *L. monocytogenes* by using the conventional methods, Harlequin Listeria Chromogenic (HLC) agar (fig. 1), observing the umbrella shaped sub-surface growth in semi-solid motility media (fig. 2), type of haemolysis on blood agar (fig. 3) and enhancement the beta-haemolysis of *Staphylococcus aureus* in CAMP test (fig. 4), only 3 chicken samples were found to be contaminated with a percentage rate of about (3%). Finally, all contaminated samples were confirmed by PCR technique using VetPCR™ *L. monocytogenes* Detection Kit (fig.5). These results were greater than the research outcome (1.3%) that conducted in Morocco by Ennaji *et al*.,[13], (1.92%) studied in Iran by Sohrabi *et al.*,[11], and also greater than the results of Abd El-Malek *et al*.,[7] in Egypt, which did not record any contamination from frozen chicken fillets. In addition, the study that completed in Alberta, Canada by Bohaychuk *et al*.,[20], showed the same result, (3%) of *L. monocytogenes* contamination rate, from Chicken wieners. In other hand, these results were lower than the study of Alzubaidy *et al*.,[21] (7.3%) in Erbil, Iraq, (23.3%) in Mexico by Castaneda-Ruelas *et al*.,[17], (20%) in Malaysia by Goh *et al*.,[22], and (17.6%) from raw chicken meat in Iran by Fallah *et al*.,[18]. Also the contamination rate, (13.6%) of Alsheikh *et al*.,[23] in Sudan, (10.4%) in Thailand by Indrawattana *et al*.,[19], (30%) in Serbia by Dimic *et al*.,[2], and (16 and 34%) from broiler meat and skin samples respectively in Egypt by Ahmed and El-Atti,[24], were greater than the results of this research.

There were large differences between the results of *L. monocytogenes* contamination rate on chicken meat with different studies. This may result from differences in the country that supply the chickens or differences in the slaughtering process and hygienic status during slaughtering. However, this may lead to contaminate the chicken carcasses, because the poultry is considered as a main carrier of *L. monocytogenes* in their intestinal tract, which in turn act as a major source of carcass contamination[25]. However the low prevalence of *L. monocytogenes* from chicken carcasses found in this study could not reveal that the absence of this pathogen, but actually indicate that imported

Table 1. PCR protocol for detection of *L.monocytogenes*

Step	Number of cycles (x)	Temperature (C°)	Time
Initial Denaturation	1X	94	2.0 min
Denaturation		94	0:30 min
Annealing	30X	55	0:30 min
Extension		72	0:30 min
Final extension	1X	72	5:00 min

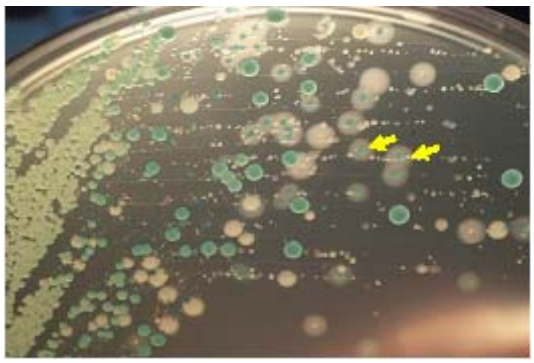

Fig. 1. Characteristic green colonies surrounded by a white halo (arrows) of *L. monocytogenes* on (HLC) agar from a primary isolation.

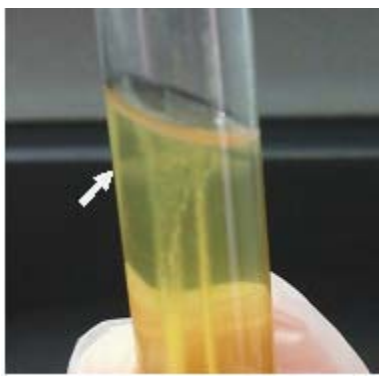

Fig. 2. Characteristic umbrella shaped sub-surface growth of *Listeria* spp., in semi-solid motility media.

chicken meat could be a reservoir of *L. monocytogenes*. As it is usually not possible to hold food products for 7 days prior to distribution, thus the food industries need faster methods for the detection of *L. monocytogenes*[26].

To overcome this problem, varieties of chromogenic media have been introduced to provide convenient management and faster identification protocol[26]. Therefore, a specific chromogenic agar media, HLC Agar was chosen in this study for the detection of the β-D-glucosidase enzyme, produced by all *Listeria* species. The blue-green colonies resulted from cleaving the chromogenic substrate (β-D-glucopyranoside) in the agar media for all *Listerial* species. While the specific differential activity of this agar is obtained from a lecithin substrate for the detection of phospholipase enzyme that will only be present in *L. monocytogenes* resulting in a white halo of precipitation surrounding the blue-green colonies (Lab M, UK).

Regarding to resistant profiling, (100%) of isolates were resistant to Ceftazidime and Ceftriaxone, (66.6%) to Ciprofloxacin and Clindamycin, while none of the isolates were resistant (100%, susceptible) to each of Imipenem, Levofloxacin, Ticarcillin-calvulanic acid, Clarithromycin and Gentamicin. All isolates were found to be resistant to three or more antibiotics and were considered as a multidrug resistant (MDR)

Table 2. Level of resistant profile of *L. monocytogenes* to different antibiotics.

Sample No.Antibiotics	1	23	88
LEV 5 µg	S	S	S
CRO 30 µg	R	R	R
CAZ 30 µg	R	R	R
DA 2 µg	S	R	R
CLR 15 µg	S	S	S
CIP 5 µg	R	R	S
TIM 75/10 µg	S	S	S
CN 10 µg	S	S	S
IMP 10 µg	S	S	S

LEV: Levofloxacin, CRO: Ceftriaxone, CAZ: Ceftazidime, DA: Clindamycin, CLR: Clarithromycin, CIP: Ciprofloxacin, TIM: Ticarcillin-Clavulanic acid, CN: Gentamicin, IMP: Imipenem, R:resistant, S: susceptible.

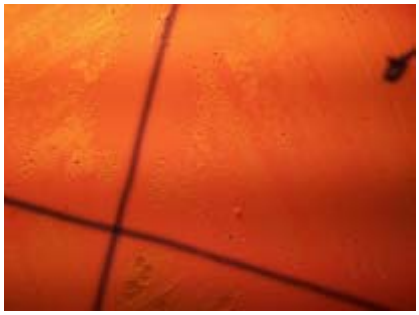

Fig. 3. Characteristic narrow zone of beta-hemolysis around small pinpoint colonies of *L. monocytogenes*.

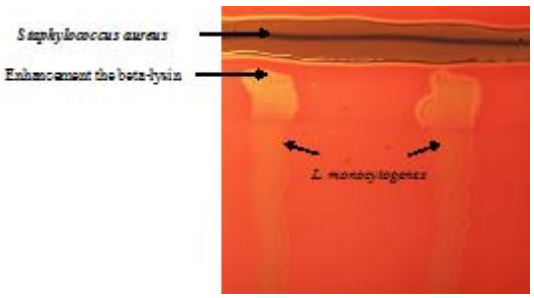

Fig. 4. CAMP test, enhancement the beta-lysin of *Staphylococcus aureus* by *L. monocytogenes*.

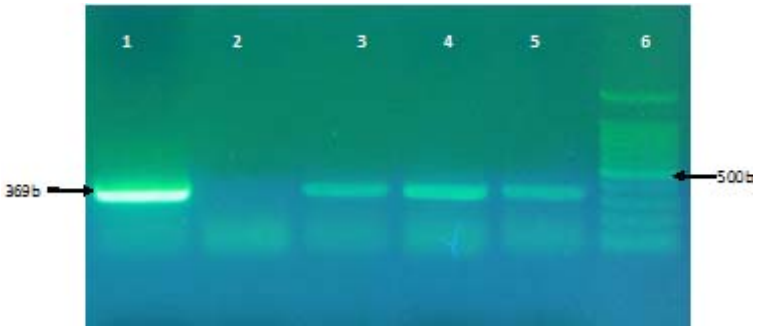

Fig. 5. PCR products confirm the detection of *L. monocytogenes* on 1.5 % Agarose gel showed under UV light. Line 1 Positive control, Line 2 Negative control, lines 3, 4 and 5 samples(1 , 23 and 88) and line 6 (1XSM1331) 1Kb Marker.

especially to Ceftriaxone, Ceftazidime, Clindamycin and Ciprofloxacin, Table (2).

These results agreed with Indrawattana *et al*.,[19] study in Bangkok, which found that (100%) of *L. monocytogenes* isolates were resistant to each of ceftriaxone and ceftazidime and (100%) susceptible to gentamicin and imipenem. Osaili *et al*.,[14], in Jordan which found that all isolates were susceptible to both imipenem and gentamicin and (62%) to clindamycin. Also Ennaji *et al*.,[13] found that all isolates (100%) were resistant to ceftazidime and (100%) susceptible to gentamicin, and Altuntas *et al*.,[27] that showed (100%) of isolates were susceptible to gentamicin. The results of Miranda *et al*.,[28] in Spain were nearly similar to this study results, which found that (60%) of isolates were susceptible to ciprofloxacin and (77.4%) to gentamicin. In other hand, these results disagreed with Fallah *et al*.,[18], in Iran, which found that (24.5%) of isolates were resistant to ciprofloxacin, (8.16%) clindamycin and (10.2%) gentamicin and with Castaneda-Ruelas *et al*.,[17] which found that (80.8%) of the isolates were resistant to ceftazidime. In this study all isolates (100%) have a MDR profile in which several studies were reported that *L. monocytogenes* was a MDR[13,14, 17, 18,19]. The high resistance of *L. monocytogenes* found in this study could be related to the usage of antibiotics in poultry farm as for prophylactic or therapeutic purposes.

CONCLUSIONS

To our knowledge this is the first try to isolate, *L. monocytogenes* from imported chicken in Duhok city, Kurdistan region. The presence of this pathogen with a MDR profile on chicken carcasses found in this study create a major risk for the consumer mainly in case of eating chicken meat when under cooked or cross contamination with other ready to eat food in a retail shop or in the kitchen. The potential health risk of chicken meat consumption for listeriosis is usually low after cooking, but the chance of cross-contamination with other food in the kitchen is possible, so strict sanitary plans are requested to prevent contamination. For this reason, this study is recommended for ensuring a good cooking quality with good hygienic strategies in the slaughterhouse and retail shops to prevent this risk and the administration of antibiotics should be at appropriate doses for the requested period after consideration the antimicrobial susceptibility test results.

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