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Isolation and Detection of *Listeria monocytogenes* in Minced Meat, Frozen Chicken and Cheese in Duhok Province, Kurdistan Region by using RT-PCR

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Abstract

Listeria monocytogenes (L. monocytogenes) is the food borne pathogen responsible for listeriosis, which is considered a serious public health risk and are the most important pathogens which can be spread through food products consumption. The disease that can be serious and fatal to human and animals. The objective of present study was carried out to detect, isolate and identify *L-monocytogenes* from frozen chicken, minced meat and cheese in duhok province. Within a period of six months from march to October 2015, a total of 150 samples were collected including 50 samples of minced meet, 50 samples of frozen chickens and 50 samples of cheese. Biochemical and microbiological test with Real time PCR technique by using specific primer was performed to determine the prevalence of the *L-monocytogenes* in the samples. Detection of such bacteria in different kinds of food product is crucial to safeguard public health due to it is potential hazard in human and animals. Out of 150 samples 20 samples displayed garish and black colonies with black halos on Oxford and Palcam agar. From the total of 20 suspected Listeria 12 isolate of *L. monocytogenes* identified by PCR. *L. Monocytogenes* were detected in 1(2%), 7(14%), and 4(8%) isolates from cheese, minced meat and frozen chicken respectively. The PCR technique has demonstrated to be a rapid and reliable method appropriate for the routine analysis of different types of food.

Keywords: *Listeria monocytogenes*; Real time PCR; Agar

Introduction

Listeria spp. is broadly distributed in environment. It can be isolated from soil and water because the organisms of the Listeria are omnipresent in nature [1]. A wide range of animal species can be contaminated by *L. monocytogenes*, including mammals, domesticated animals, pets, fish, birds and crustaceans [2].

In mammals, *L. monocytogenes* might cause premature births and it is the reason for developing of a sign of meningoencephalitis [3]. In addition, *L. monocytogenes* considered as a vital reason for zoonoses that causes listeriosis [4,5].

L. monocytogenes has a great concern to general well-being and the food economy because of its extensive occurrence in the environment and capability to survive or even grow under very harsh conditions [3]. The expanding rate of *L. monocytogenes* in food born outbreaks, in nowadays eating style, has led to the immediate action for a fast discovery method for testing food products. Almost all cases of listeriosis tend to be foodborn, and a number of food items could be contaminated by *L. monocytogenes* including raw chicken meat, raw minced meat, soft cheese and fish. Most of these items are widely consumed in Kurdistan and Duhok province, especially [6].

A number of molecular biology methods and techniques have been described for detection of *L. monocytogenes* including DNA probes and PCR techniques [7-10]. In addition, direct detection of *L. monocytogenes* in food products by PCR has been reported in several cases [11].

To our knowledge, there is no published data covering variety sources of food samples for isolation and detection of *L. monocytogenes* in Duhok province. In fact, there is no incidence and no official data of food born listeriosis recorded in Duhok province as *L. monocytogenes* is tested in the food samples. The objective of present research was to assess the use of PCR in the detection of *L. monocytogenes* in food products and to find out the contamination level with Listeria pathogen in food products in Duhok province.

Materials and Methods

One hundred fifty raw samples of different food product including 50 samples of chicken, 50 samples of minced meat, and 50 samples of cheese were obtained from different supermarket; restaurant and veterinary quarantine of Duhok province were tested. All samples had been properly stored were placed in separate sterile plastic bags to prevent spilling and cross contamination and the samples were brought to the laboratory on crush ice and were kept in a refrigerator at 4°C until testing within 4 hours.

Food samples were analyzed for the presence of Listeria spp. using selective enrichment procedure and isolation protocol, recommended by United States Department of Agriculture (USDA) [12].

Microbiological investigation

A food sample of 25 g was added to 225 ml of half strength Fraser broth which use as primary enrichment media (Conda, Spain), to obtain a 1:10 sample dilution. All samples were homogenized 30-60 seconds and incubated at 30°C for 24 hrs. From this primary enrichment, 0.1 ml was then inoculated into 10 mL of Fraser Broth

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which use as secondary enrichment medium, and incubated for 48 hrs at 37°C in shaking incubator. A loopful of the Fraser Broth enrichment culture was streaked on the surface of different selective agar PALCAM listeria agar (Conda, Spain) with supplement and OXFORD agar (Lab UK) with X122 supplement. These selective agars were then incubated for up to 48 h at 37°C. Selective agars were observed for suspected colonies at 24 to 48 hrs of incubation. Suspected colonies were those that appeared greyish colonies surrounded by black halos and sunken centers with possible greenish sheen on Oxford agar or black colonies on PALCAM listeria agar.

The following tests were used for confirmation; Gram's staining, motility test, catalase reaction, and oxidase reaction and RT-Polymerase Chain Reaction (PCR).

DNA extraction

The DNA from Listeria was extracted from the sweep of few colonies grown on PALCAM listeria Agar plates by boiling method. One loop of Listeria from agar plates was suspended in 100 µl of sterile de-ionized water in a 1.5 ml microcentrifuge tube and a bacterial suspension was vortexed. The bacterial suspension was boiled at 95-100°C for 10 min and centrifuged at 10,000x g for 10 min. The supernatant was used as a DNA template for PCR [13]. Purification of DNA was achieved by using a genomic DNA purification kit (Qiagen, Germany) according to the manufacturer's instruction. The DNA was measured by using Nanodrop Spectrophotometer QIAxpert (QIAGEN) and stored at 4°C.

Confirmation of samples with Real time PCR technique

Screening of suspected Listeria that isolated microbiologically were conducted according to Food proof® Listeria detection kit (Biotecon Diagnostics, Catalog) which is PCR kit for the qualitative detection of Listeria Spp., using real-time PCR instruments. In this method a fluorescent dye is used to follow the PCR amplification in real-time and can be used to detect the amplified products from a number of genes at the same time [14]. The real-time PCR was done with a final volume of 25 µl of reaction mixture in each well of a 96-well plate, in which containing of 20 µl of PCR Master Mix (provided) and 5 µl of DNA template, beside positive and negative control were run. The DNA from samples was amplified in the standard mode running (2 hours) on a 7500 fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The reactions were run according to the amplification conditions were optimized to the thermalcycler as defined in Table 1. The amplification results were visualized and analyzed during the last 50 cycles of the amplifications using the 7500 fast software V.1.4.0 (Applied Biosystems) with the thermocycler AB 7500 fast (Applied Biosystem, Foster City, CA, USA).

Results

A total of 150 samples tested, 13.3% presumptively positive by culturing method while 8% were confirmed to be positive for *L. Monocytogenes* by means of PCR.

Microbiologically all sample streaked on Palcam and Oxford agar. On Palcam plate which is selective media that recommended for the isolation of *L. monocytogenes* from foods, Listeria colonies appeared grey-green with black–sunken centers and a black halo, the selectivity of the medium comes from its content of polymyxin, acriflavin, ceftacidim, and lithium chloride. While on Oxford plate which has the same properties of Palcam as well as it is preferred by adding various antimicrobial agents to the base, and both of them inhibit gram-negative organisms and most gram-positive organisms during 24 hrs. Twenty samples were suspected by culture as *L. monocytogenes*. according to catalase and motility test were positive and oxidase negative as show in Table 2, the isolation sample was distributed between three type of food in Duhok city (50 sample from each type), from cheese 3 (6%), chicken 6 (12%) and from meat 11 (22%) founded as such bacteria.

Stage	Temperature	Time	Cycles
Holding stage	37°C	4 minute	1
Holding stage	95°C	5 minute	1
Denaturation	95°C	5 second	50
Annealing	60°C	60 second	50

Table 1: RT-PCR conditions and cycling for *L. monocytogenes* detection.

Sample	Sample positive for culture	Catalase	Oxidase	Motility
Cheese	3	+ve	-ve	Motile
Meat	11	+ve	-ve	Motile
Chicken	6	+ve	-ve	Motile
Total	20			

Table 2: Suspected of *L. monocytogenes* by means of microbiology and biochemical test.

Sample	No. Of Sample	Suspected Listeria M. By culture (Oxford and Palcam)	Amplifying by PCR
Cheese	50	3	1
Meat	50	11	7
Chicken	50	6	4
Total	150	20	12

Table 3: Number of positive samples according to Real time PCR.

Confirmation of *L. Monocytogenes* by real time PCR

A total of 20 samples tested were identified as presumptively positive for Listeria species using Oxford and Palcam agar, which does not differentiate between Listeria species, non-pathogenic species of Listeria, therefore, cannot be eliminated when selecting suspect colonies for confirmation.

All 20 isolated samples are detected with *L. Monocytogenes* underwent to the DNA extraction from bacterial colony by boiling to achieve real time PCR using food proof® *Listeria monocytogenes* Detection Kit Hybridization Probes (LC 1.x, 2.0) as shown in Figure 1. The majority of the suspected samples (12) were detected as positive as in Table 3.

Discussion

Listeria monocytogenes considered as a highly pathogenic bacterium that contaminate a wide range of food products with a high mortality rate in the world. From a total of 150 samples of various type of food products, soft cheese 1(2%), raw chicken 4(8%) and red minced meat 7(14%), were positive for *L. Monocytogenes* by PCR. The findings of these results indicated that the meat products were considerably more likely to be contaminated with *L. monocytogenes* than other food products. This could be due to the fact that preparation and processing of minced meat, like kebab, might be done in a poor hygienic condition [15]. Although other factors like equipments and food additives can also have a role in contamination, Kebab is well cooked before eating and thus reduces the chance of getting Listeriosis in this kind of food [15].

There are variances in outcome of our results when compared to other research papers. For example, a research conducted in Isfehan, Iran on various food products including the dairy products, meat,

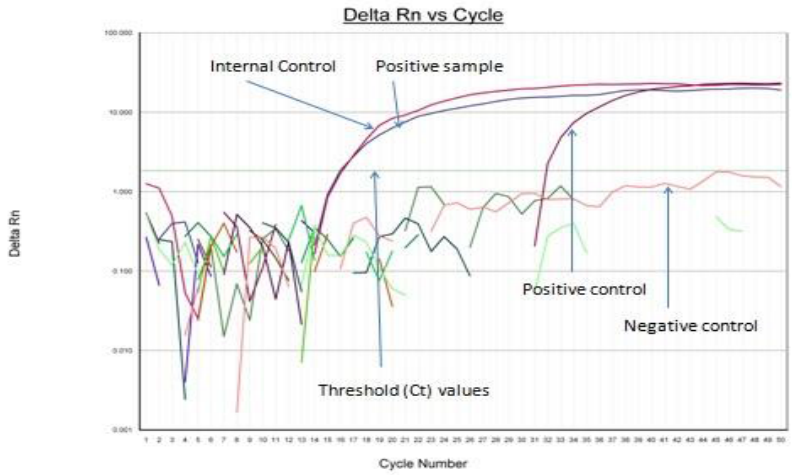

Figure 1: Amplication plot of Listeria monocytogenes flourescence versus cycle number. The figure shows that suspected Listeria M. sample amplifying. No amplification in negative control and positive control with internal control are worked.

Ready to eat food had found a 4.7% contamination rate with Listeria spp., which is lower than our result (16%) [16]. Interestingly, Zhou and Jiao [17] have confirmed that the highest contamination rate was seen in meat products. These results are in favour with the findings of the current study.

With regards to the chicken meat, our results have shown the lower incidences (4%) samples of listeria spp. These values are in agreement with a study done by Mahmood and his colleagues [18]. However, the occurrence of *L. monocytogenes* in the current research was seen to be lower than that carried out by Goh et al. [19].

Turning into soft cheese samples, current study showed that the prevalence of Listeria was not significant. In soft cheese about 1 (2%) out of 50 samples were found positive for *L. Monocytogenes* by PCR. Our results were contradicted to other study conducted in Jordan by Osaili et al. [20] where they were reported about 30% of pathogenic *L. monocytogenes* in soft cheese. It should be noted that the pathogenic *L. monocytogenes* in our research for the contamination rate of Listeria in dairy products, cheese are consistent with the results indicated by Jalali and his team [16]. A low level (1.1%) of *L. monocytogenes* contamination was found in soft cheese by Akya et al. [15]. These results are not going with the current study. This might be due to the contamination during the processing of making cheese as well as the pasturalization of the cheese product. The acceptable contamination rate of *L. monocytogenes* was revealed to be ranged between 10% and 15.3% for cheese and raw milk [21,22].

Conclusion

In conclusion, the contamination level of *L. monocytogenes* was comparatively low in food samples in Duhok governorate. While it was clear that the meat products have shown the highest percentage of contamination level, cheese recorded the lowest contamination rate and the chicken meat come into between these two food products.

In addition PCR technique estimated to be more dependable than conventional identification sine is based on constant genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable (Lawrence and Gilmour). Incidence of Listeria Spp. and *L. Monocytogenes* in poultry products and their rapid confirmation by multiplex PCR (1994).

Further researches on *L. monocytogenes* in Duhok are needed in order to provide a better background of contamination rate and the routes of transmission for this bacterium.

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