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Molecular characterization and antibiotic resistance profile of *listeria monocytogenes* isolated from fish and broiler meat

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Listeriosis is a foodborne disease with low incidence and high fatality rate. It occurs by the intake of food-soiled with *Listeria monocytogenes*. A study was conducted to discern the presence of *L. monocytogenes* from fish and broiler meat. A total of 160 samples, fish (n=80) and broiler (n=80) were collected from the markets of Lahore and Rawalpindi. These samples were enriched in Listeria enrichment broth (LEB) at 37°C for 48hours. A loop ful of the LEB was streaked on PALCAM agar plates and these plates were incubated at 37°C for 48hours. Isolated gray-green colonies on the agar were identified by grams staining, catalase and oxidase tests. The results indicated that 21% (34) of total samples were positive for *Listeria monocytogenes.* These isolates were further subjected to PCR targeting its prfA gene of 479bp, and this molecular technique confirmed only 15% (24 of 34) isolates as *Listeria monocytogenes*. The prevalence of the bacterium was 17.50% more in fish (23.75%) than in broiler (6.25%). Ampicillin showed highest sensitivity 91.6% followed by trimethoprim 83.3% and tetracycline 75% to the isolates. Our study conclude that *Listeria monocytogenes* is prevalent in both fish meat and broiler meat however it is present more in fish meat than in broiler meat. The polymerase chain reaction is a specific tool for its detection. Our study demonstrate that this problem can easily be treated as most of the isolates were sensitive to the commonly used antibiotic.

Keywords: *Listeria monocytogenes*, PCR, Fish, Broiler, Antibiotic resistance.

INTRODUCTION

Pakistan is an agro-based country with an estimate of 199.71 million people (Pakistan, 2017) Cattle, sheep and goats are raised to meet its population's protein demand. Moreover, poultry and fish meat also contribute a huge share to the protein sources. Improper handling and under cooked meat may lead to food-borne poisoning. Different bacteria such as *Salmonella, Clostridium, Campylobacter*, *Staphylococcus* and *Listeria* cause the contamination of food and result in food poisoning (Le Loir, Baron, and

Gautier, 2003; Mead et al. 1999). Ingestion of food contaminated with *Listeria monocytogenes* results in a high mortality rate that may go up to 35% (Farber and Peterkin, 1991; Vázquez-Boland et al. 2001). *Listeria* comprises seven species with two well-defined groups that are based on their pathogenesis and hemolysis patterns. The two groups include pathogenic species such as *Listeria monocytogenes* and *Listeria ivanovii* while the rest are non-pathogenic group and the members include *L*. *murrayi*, *L*. *grayi*, *L*. *seeligeri*, *L*. *innocua*, and *L*. *welshimeri*.

Listeria monocytogenes is a non-spore forming, Gram positive, rod shaped, facultative anaerobic bacteria (Black, 2012). They move through flagella at 20°C but they are non-motile at 37°C (Peel, Donachie, and Shaw, 1988). This bacterium is ubiquitous in nature and is found in soil, water, milk, sewage and dust. It can easily get into human food chain and can multiply there (Farber and Peterkin, 1991). The pathogen is catalase positive, oxidase negative and causes complete destruction of red blood cells through listeriolysin, a kind of hemolysin. Almost 16 serovars are described for *Listeria monocytogenes* (Quinn et al. 2011). It can grow at a temperature ranging from 0°C to 45°C (Annous, Becker, Bayles, Labeda, and Wilkinson, 1997). The zoonotic *Listeria monocytogenes* is acid-tolerant, able to grow at relatively high NaCl concentrations and temperature (Liu, Lawrence, Ainsworth, and Austin, 2005).. The bacterium is a biofilm producer (Borucki, Peppin, White, Loge, and Call, 2003). *Listeria monocytogenes* emerges as a main cause of Listeriosis (Vázquez-Boland et al. 2001). Annually in United States approximately 76 million cases of food-related illnesses occur. Percentages of food borne mortality with Listeria is 27.6% (499 deaths out of 2518) whereas with *Salmonella non-typhoidal* 30.6%; *Campylobacter* species 5.5%; and *Vibrio* species 0.71% (Mead et al. 1999). USA has zero tolerance policy for *Listeria monocytogenes* in food while Canada allows only 100cfu/g (MacPhee, 2005). The *Listeria monocytogenes* contains different virulence genes such as a transcriptional regulator or positive regulatory factor A (*prfA*), a hemolysins (*hly*), two phospholipases (*plcA* and *plcB*), a metalloprotease (*mpl*) and actin polymer (*actA*). Due to the presence of these genes, the bacterium replicates inside host cell and spreads to neighbor cells (Schmid et al. 2005). The virulence genes of the bacterium are expressed at 37°C and become silent at 30°C, as these virulence genes are regulated by a temperature-dependent prfA gene (Johansson et al. 2002). *Listeria monocytogenes* can be diagnosed using conventional (culturing, staining) and advanced (PCR) techniques. Polymerase chain reaction is an easy tool to diagnose *Listeria monocytogenes* (Jung et al. 2009). Currently, different antibiotics such as ampicillin, gentamycin, trimethoprim–sulfamethoxazoleate, erythromycin and vancomycin are used to control Listeriosis. Ampicillin (beta lactam) is the drug of choice but sometime combination of ampicillin with gentamycin is given to the patient (Temple and Nahata, 2000).

MATERIALS AND METHODS

Sample collections

A total of 160 swab samples (fish and broiler) were collected randomly, from various meat markets of Lahore and Rawalpindi. The distribution of sample is given in **Table 1.** The samples were transported to Post-Graduate Laboratory, Department of Epidemiology and Public Health, University of Animal and Veterinary Sciences, Lahore in test tubes containing transport medium.

Table 1: Fish and Broiler meat samples distribution

Type of samples	Lahore Sample(n)	Rawalpindi Sample(n)
Fish	40	40
Broiler	40	40

Isolation of Listeria monocytogenes

1ml of each samples was transferred into the tubes having 9ml Listeria enrichment broth (LEB) and the tubes were incubated at 37°C for 48hours (Kalender, 2012). A loopful of each broth sample was streaked on PALCAM agar (Oxoid) surface and kept the plates at 37°C for 48hours.

Identification

Isolated gray-green colonies with black zone were fished out and restreaked on PALCAM agar. The restreaked petri plates were kept in an incubator for 24 hours at 37°C. The purified colonies on PALCAM agar were further subjected to Gram staining, catalase test and oxidase test for organism's identification (Mena et al. 2004).

Molecular Confirmation

Pure colonies of *Listeria monocytogenes* were confirmed by Polymerase chain reaction targeting

prfA gene. The DNA from the positive isolates was extracted using Chelax (10%) method and subjected it to PCR targeting *Listeria monocytogenes prfA* gene of 479bp. Already reported primers by (Jung et al. 2009). shown in table 2 were used for DNA amplification.

Amplification conditions were optimized to thermal cycler and were follows: Initial denaturation 95°C for 10 min, 35 cycles of denaturation at 95°C for 45 sec, annealing at 50°C for 45 sec, and extension at 72°C for 45 sec, then a final extension at 72°C for 10 min. The amplified DNA was analyzed by Gel electrophoresis. PCR amplification was performed by mixing the template DNA, primers, nuclease free water and econoTaq 2X Master Mix by Lucigen company (Wisconsin USA). The composition of the reaction mixture is given in Table 2.

Table 2: Primers used in the study

Forward Primer	5´ AACCAATGGGATCCACAAG3´
Reverse Primer	5´ATTCTGCTAACAGCTGAGC 3´

2.5 Antibiotic susceptibility testing

Kirby-Bauer standard disk diffusion method, on Mueller Hinton agar plates, was used for the determination of antibiotic sensitivity (Bayer, Kirby, Sherris, and Turck, 1966). The currently used antibiotics were chosen for the study. They belonged to different groups: penicillin (ampicillin 25µg), aminoglycosides (gentamicin 10µg), macrolides (erythromycin 15µg), tetracycline (tetracycline 30µg), phenicol (chloramphenicol 30µg) and Folate pathway inhibitors (trimethoprim 5µg). The results were interpreted according to Clinical Laboratory Standards Institute (Wayne, 2011).

Table 3: Reaction mixture for PCR amplification

Components	For 25µl Reaction
EconoTaq PLUS 2X Master Mix	12.5µl
Forward primer (nm)	1.0µl
Reverse primer (nm)	1.0µl
DNA template	2.0µl
Nuclease free water	8.5µl

Nm=Nano-mole, µl=microliter

RESULTS

Swab samples of fish (n=80) and broiler meat (n= 80) were collected from Lahore and Rawalpindi markets. Only 34 of 160 isolates showed gray-green colonies characteristic for *L. monocytogenes* on PALCAM agar. **(Figure 1)**

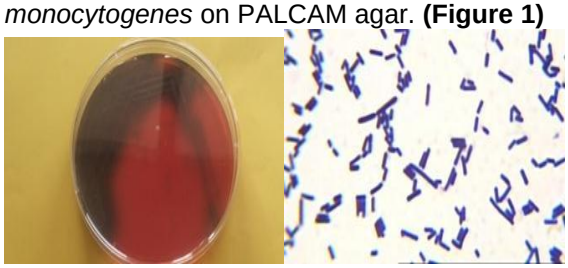


Figure 1: Bacterial growth (a), gram staining (b)

In Lahore samples, only 7 fish and 5 broiler isolates exhibited typically gray-green colonies on the agar. Most of isolates appeared as purple rods, two isolates looked like coccobacilli while one isolate had a circular shape when gram staining was done. All isolates were catalase-positive except one isolate, but these isolates were negative for the oxidase test. Similarly, 18 fish and 4 broiler isolates of Rawalpindi were identified as *L. monocytogenes*. Microscopic details after grams staining showed that all the isolates were, positive rod except two isolates, as they appeared coccobacilli. (Table 4) Bubble formation was observed in catalase test by the isolates except two samples.These isolates displayed no reaction against the oxidase test.figure 2.

Figure 2: Biochemical test (a) catalase test and (b) oxidase test

Table 4: Prevalence of *L. monocytogenes* in meat of Lahore and Rawalpindi samples

Area	Type of samples	No. of samples	cultural positive	
			No	Percentage
Lahore	Fish meat	40	7	17.50%
	Broiler meat	40	5	12.50%
Rawalpindi	Fish meat	40	18	45%
	Broiler meat	40	4	10%

Molecular confirmation of *Listeria monocytogenes*

Culturally positive 34 isolates (Figure 4) were confirmed by PCR targeting the organism's thermoregulatory prfA gene of 479bp. Specific primers and a positive control of *Listeria monocytogenes* (ATCC 13932) were used for the bacterium confirmation (Table 2).

Culturing and biochemically testing indicated that 31.25% fish and 11.25% broiler samples were positive for the organism. These positive samples were further confirmed by PCR. The PCR results revealed that out of total, only 15% samples (fish and broiler) were positive for the test organism. (Figure 3)

It was further found that more fish samples were positive for *L. monocytogenes* when compared to broiler samples. The fish sample percentage was 23.75 and broiler sample percentage was 6.25 (Table 5). The bar graph shows that 76.35% of the fish samples were without that bacterium.Figure 5

Similarly *Listeria monocytogenes* was not detected in 93.75% of broiler meat sample. The gel picture shows *Listeria monocytogenes* specific amplicons (size 479bp) of fish and broiler meat isolates. Lane m shows 100bp DNA ladder, Lane +ive indicates positive control (ATCC 13932). Lane 1 to 6 shows fish amplicons and lane 7 to 11 are broiler bands (a) while lane 1 to 13 shows fish amplicons(b).

Antibiotic Susceptibility Assay

Confirmed *Listeria monocytogenes* isolates were tested against different antibiotics on Mueller Hinton agar plates (Figure 6). The plates were incubated at 37°C for 24 hours. All antibiotics used in the study inhibited the bacterial growth to some extent. Ampicillin proved sensitiveand effectively stopped the growth of 91.6% isolates. Chloramphenicol and trimethoprim were sensitive to 83.3% of isolates whereas tetracycline blocked the growth of 75% isolates. Moreover, least susceptibility was recorded when gentamicin 45.3% and erythromycin 25% were used against the isolates.Table 6.

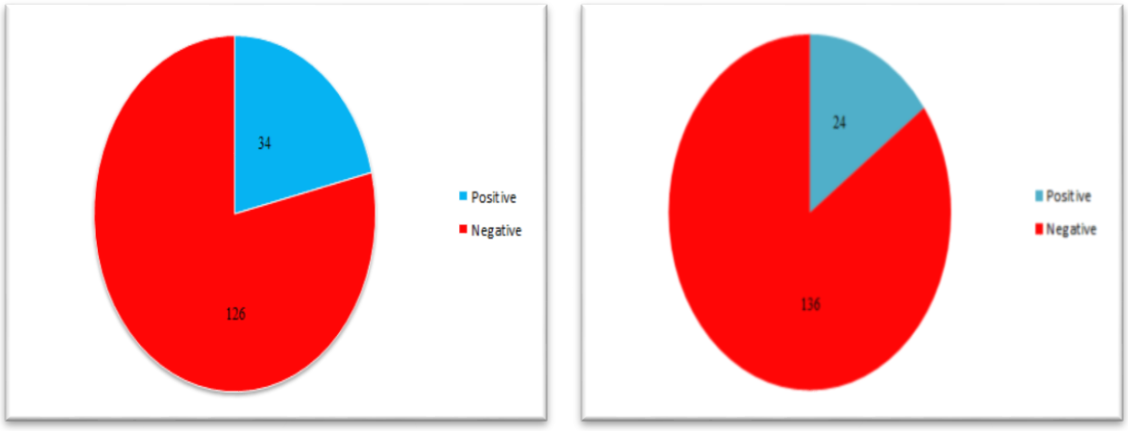
Table 5: PCR confirmation of *Listeria monocytogenes* in test samples

Sample type	Sample number(n)	Positive sample			
		Cultural		PCR	
		(n)	Percentage (%)	(n)	Percentage (%)
Fish meat	80	25	31.25	19	23.75
Broiler meat	80	9	11.25	5	6.25
Total	160	34	21.25	24	15

Table 6: Antibiotic susceptibility pattern of *Listeria monocytogenes*

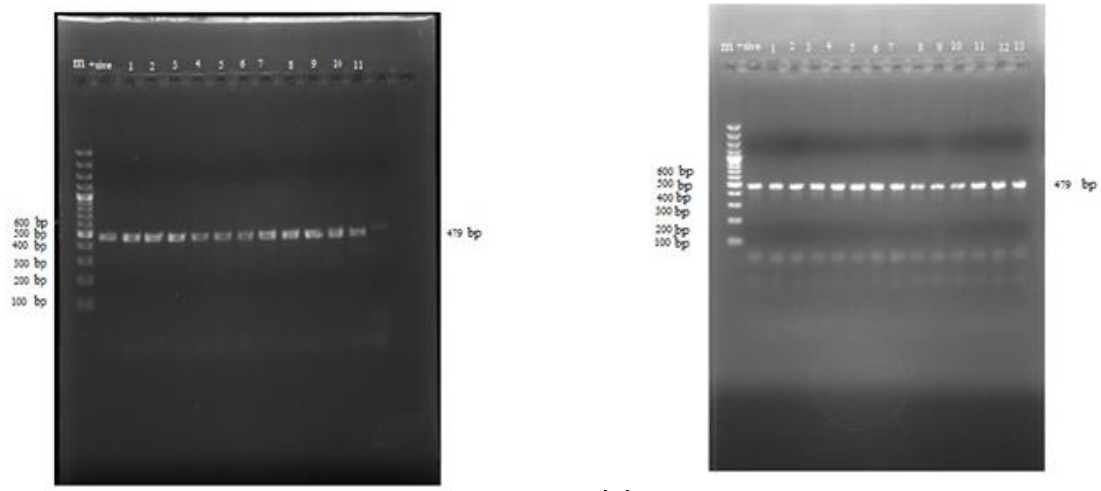
Antibiotic	Sensitive		Intermediate		Resistance	
	n	%	n	%	n	%
Ampicillin	22	91.7	-	-	2	8.3
Gentamicin	9	37.5	4	16.6	11	45.8
Tetracycline	18	75	2	8.3	4	16.6
Erythromycin	7	29.1	11	45.8	6	25
Chloramphenicol	20	83.3	4	16.6	0	0
Trimethoprim	20	83.3	0	0	4	16.6

n= number of isolates %=percentag



(a) (b)

Figure 3: Over all cultural (a) and molecular (b) confirmed isolates



(a) (b)

Figure 4: A representative gel image for positive *L. monocytogenes* isolates from fish and broiler meat. The gel picture shows *Listeria monocytogenes* specific amplicons (size 479bp) of fish and broiler meat isolates. Lane m shows 100bp DNA ladder, Lane +ive indicates positive control (ATCC 13932). Lane 1 to 6 shows fish amplicons and lane 7 to 11 are broiler bands **(a)** while lane 1 to 13 shows fish amplicons **(b).**

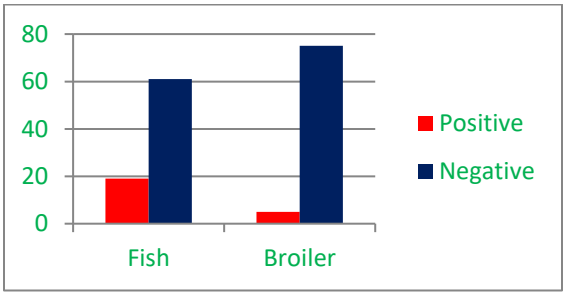


Figure 5: Prevalence of *Listeria monocytogenes* in fish and broiler meat

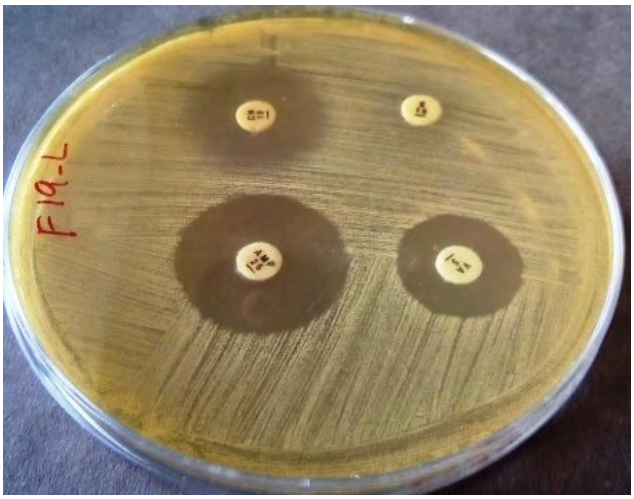


Figure 6: MHA plate displaying the outcome of antibiotics against *L. monocytogenes* isolate.

DISCUSSION

In both animals and human *L. monocytogenes* causes serious problems. It is mainly a foodborne pathogen found in different sources, like water, soil and many kinds of food products as well as in humans and animals (Rabiey, Hosseini, and Rezaei, 2013). In this study a high prevalence (23.75%) of *Listeria monocytogenes* was recorded in fish meat, while a low prevalence (6.25%) was detected in broiler meat. This indicated that 17.50% more prevalence was found in fish meat than in the broiler meat. These results were in similarity with (Latorre et al. 2007), who examined 5,788 food samples, and found only 121 (2.1%) food sample contaminated with *Listeria monocytogenes.* Furthermore, they recorded higher prevalence of the organism in smoked salmon 10.6% than in the poultry meat (8.5%). (Molla, Yilma, and Alemayehu, 2004) also found that fish meat had a higher prevalence (2.3%) of *Listeria monocytogenes* than in the chicken meat (1.9%) in their study. (Cabedo, Picart i Barrot, and Teixido i Canelles, 2008) also detected *Listeria monocytogenes* in 7.9% smoked salmon samples while in chicken, the bacterium was 6.2%.

Based on the cultural characteristics on PALCAM agar, grams reaction, catalase positive, and oxidase negative colonies, *Listeria monocytogenes* were selected and thirty four of 160 samples were positive for the organism. Similarly (Kalender, 2012).

isolated the bacterium from ground beef and chicken meat through culturing and identified them by biochemical testing. He recognized *Listeria monocytogenes* to be 7.2% from beef and to be 17.8% from chicken meat. (Yücel, Cıtak, and Önder, 2005) also purified the bacterium from 11.5% chicken samples through culturing and confirmed them through biochemical tests. These conventional methods were found time consuming and these techniques gave false-positive results also because when the positive samples were subjected to PCR, only 24 of 34 isolates were confirmed as a targeted bacterium. A few samples on PALCAM agar showed morphologically characters like *Listeria monocytogenes* but molecularly they were not identified as the targeted bacterium. The data are consistent with the study of (Gouws and Liedemann, 2005) as they isolated 44% isolates culturally, but only 37% of isolates were confirmed as *Listeria monocytogenes* through PCR. Swetha, Madhava Rao, Krishnaiah, and Vijaya Kumar (2012) isolated the bacterium from fish samples through culturing and then confirmed them through PCR. Only 2 out of 25 samples (8%) were confirmed by PCR assay. Elmali, CAN, and Yaman (2015) confirmed *Listeria monocytogenes* in 57 (47.5%) out of 120 poultry meat samples through PCR assay. PCR is basically a specific and sensitive technique compared to the standard cultural method because it is based on constant and established genotypic characteristics. Antibiotics are the only choice to treat various bacterial infections but bacterial resistance against different antibiotics is an important issue. It is believed that *Listeria monocytogenes* growth can be inhibited by using any antibiotic that is effective against gram-positive organisms with the exception of its natural resistance towards some antibiotics like nalidixic acid, fosfomycin and the third-generation cephalosporins (Hof, 2004). The resistance of *Listeria monocytogenes* to different antibiotics, along with the scarcity of its data in

Pakistan creates a challenging situation for its control. One of the objectives of the study was to test the positive isolates for their sensitivity against antibiotics. The antibiotics used in the study were all effective against the organism. Ampicillin gave highest sensitivity up to 91.6%, chloramphenicol and trimethoprim sensitivity was 83.3%, tetracycline 75%, gentamicin 37.5%, and least sensitivity was recorded with erythromycin 29.1%. These results were matched with the findings of (Evrim Gunes et al. 2012) as they found that *Listeria monocytogenes* was absolutely susceptible (100%) to antibiotics like penicillin G, vancomycin, gentamicin, tetracycline, erythromycin, chloramphenicol and trimethoprim. They suggested that ampicillin, penicillin or rifampicin alone or in combination with gentamicin as drug of therapy for Listeriosis. If patient is allergic to penicillin, trimethoprim with sulfamethazole could be used. Moreover, (Ennaji, Timinouni, Ennaji, Hassar, and Cohen, 2008) reported that isolates of *Listeria monocytogenes* (from poultry and red meat) were sensitive to ampicillin, chloramphenicol and trimethoprim. (Çon, Gökalp, and Kaya, 2001) and (Pesavento, Ducci, Nieri, Comodo, and Nostro, 2010) also suggested that different combinations of antibiotics could be used for its treatment.

CONCLUSION

Our study concludes that *Listeria monocytogenes* are prevalent in both fish meat and broiler meat however it is present more in fish meat than in broiler meat. The polymerase chain reaction is a specific tool for its detection. Therefore we highlight that most of the isolates were sensitive to the antibiotics in our report and antibiotics like ampicillin and trimethoprim could be used for its treatment.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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