Molecular Based Detection and Antibiotic Sensitivity Profiling of *Listeria monocytogenes* Isolated from Different Meat Sources

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Abstract:

Objective: Food borne infections are the major and growing public health concern. *Listeria monocytogenes* is one of the emerging pathogens responsible for major food borne outbreaks round the globe. In the following study, the prevalence and antimicrobial susceptibility pattern of *L. monocytogenes* in fresh and frozen meat samples was detected.

Method: A total of 120 meat samples (60 fresh and 60 frozen) were collected from various sources including 10 samples each from chicken meat, chicken boneless meat, mutton, minced mutton, beef and minced beef. Identification of *Listeria species* was done using selective and differential medium (PALCAM agar and blood agar). The confirmation of *L. monocytogenes* was done by Polymerase chain reaction (PCR) using species specific primers for the amplification of *hly* A and *iap* genes. The confirmed isolates were subjected to antimicrobial susceptibility profiling.

Results: Of 120 meat samples 19 (15.83%) were found positive for *L. monocytogenes. L. monocytogenes* was more prevalent in frozen samples18.33% (11/60) in comparison to fresh meat samples 13.33% (08/60). Highest resistance was observed against Penicillin (63.15%) followed by Clindamycin (57.89%), Ciprofloxacin (36.84%) and Rifampicin (26.31%). All the 19 (100%) isolates were found susceptible to Cephalothin, chloramphenicol and vancomycin. Nine of the 19 isolates were found resistant to more than three classes of antibiotics hence, were declared as multidrug resistant (MDR). The most common pattern found was PEN-CLN-CIP among MDR phenotypes.

Conclusion: *L. monocytogenes* could be a source of food born disease outbreak due to its simultaneous presence in fresh and frozen meat with a special focus to MDR strains. PCR based detection is a good tool for its detection in meat samples but there is a need to develop rapid and isothermal methods which may be directly applied on food samples for quick detection of food pathogens.
Key Words: *Listeria monocytogenes*. PCR. Fresh meat. Antibiotic sensitivity. MDR

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1. Introduction

Food borne diseases are one of the growing threats to human beings round the globe. Incidence of such illnesses has increased in both industrialized as well as non-industrialized countries [1]. Several bacterial pathogens are associated with food borne diseases which may include *Salmonella species, Campylobacter species, Listeria species, Yersinia species* etc. [2]. Among all other bacterial pathogens, *L. monocytogenes* is one of the major causative agents of food borne illness, *Listeria monocytogenes* has recently been recognized as an important foodborne pathogen in human diseases [3]. *Listeria monocytogenes* is a zoonotic pathogen which can cause disease to both human and animals and widely distributed in the environment [4]. Meat and meat products are one of the major source of *L. monocytogenes* [5][3]. This is gram positive, rod shaped, facultative anaerobic bacterium which enter in the body through food contaminated with *L. monocytogenes* and results into a disease known as listeriosis with variable morbidity and mortality. According to World health organization, In United States, 76 million people were affected due to food borne diseases, out of which 325,000 people were hospitalized while 5000 people were died in a year. Moreover, the prevalence of food borne diseases due to contaminated food in United States is estimates 48 million illnesses and 1000 disease outbreaks [6]. *L. monocytogenes* poses a serious threat to public health due to its presence in different fermented foods and ability to grow at refrigeration temperatures and its pathogenicity among pregnant women and individuals with weaken immune system [7]. In European member, the prevalence of Listeriosis varies between population and it is significantly correlated with the incidence of *L. innocua*. The incidence of human Listeriosis is greatly increased over the year in some countries. Different species of *Listeria* affects about 2 to 3 million persons and mortality rate is about 500 to 2000 deaths each year [8].

Several studies proved PCR a promising methodology for detection of food-borne pathogens. Diagnosis of *Listeria species* is widely based on molecular assays like PCR instead of conventional methods of identification [9]. *hlyA* and *iap* genes are one of the most popularly selected targets for PCR detection of *L. monocytogenes*. *hlyA* gene encodes a well-recognized virulence factor Listeriolysin O while *iap* encodes protein p60 [10]. Primers are very specific to the targeted regions on these genes in *L. monocytogenes*. Non-*Listeria* bacterial species are screened out in the PCR products of *Iap* gene while the middle section of the gene is highly

variable for *Listeria species* but constant for the given species. So, the species-specific primers are more helpful in detection of *L. monocytogenes* [11].

Antibiotic resistance is increasing day by day in food associated pathogens and the occurrence of such pathogenic bacteria in food chain plays a unique role in spreading the resistance and ultimately leads to huge economic losses [12]. Meat and dairy products are heavily affected by pathogens like *E. coli, Campylobacter jejuni, Yersinia enterocolitica, L. monocytogenes, Staphylococcus aureus* but *Listeria monocytogenes* has its own importance due to the ability to grow at low temperatures and a major public health concern [9]. The virulent genes along with emergence of antibiotic resistance in *L. monocytogenes* can increase the virulence of bacteria up to many folds which may be a big trouble if associated with food-borne disease outbreaks [13].

Detection of *L. monocytogenes* in milk and different food items using conventional methods is studied intermittently in Pakistan [14]. Among these, to our knowledge raw meat has never been the part of these studies in Pakistan. The objective of this study was to determine the prevalence of *Listeria monocytogenes* from different meat sources targeting *hlyA* and *iap* genes using PCR and phenotypic detection of antibiotic resistance in the isolates.

2. Material and Method

2.1. Sample collection

A total of 120 meat samples (60 fresh and 60 frozen) were collected in sterile plastic bags from different retails shops and super markets comprising of 10 samples each from fresh and frozen meat sources including chicken meat, chicken boneless, mutton, minced mutton, beef and minced beef. The samples were transferred to research laboratory.

2.2. Isolation and Identification of Listeria

Samples were pre-enriched in 25 ml of the Fraser broth at 4°C for 24 hours for Listeria culturing. After the incubation for 24 hours, 100 µl of enriched culture was spread on PALCAM agar (Oxoid-Themo Fisher Scientific™) and blood agar and incubated for 24 hours at 37°C [15]. Further confirmation was done through gram staining and standard biochemical tests like catalase, oxidase, indole, methyl red, Voges-Proskauer, TSI, H2S production, motility at 25 °C and 37 °C, acid production from various sugars, nitrate reduction, hemolysis on blood agar, and CAMP test [16].

2.3. Molecular identification of *hlyA* and *iap* genes of Listeria monocytogenes

2.3.1. DNA extraction and amplification

The isolated Listeria colonies were subjected to DNA extraction with the help of commercially available kit, Gene JET Genomic DNA Purification Kit (Thermo Scientific™, USA).

Amplification of hemolysin *(hly A)* gene and *(iap)* gene was done by using specific primers with polymerase chain reaction (PCR) as previously described by [17]. We used pair of primers targeting both genes *hylA* and *iap*, 01 (F: 5'-CGGAGGTTCCGCAAAAGATG-3'; R:5'-CCTCCAGAGTGATCGATGTT-3' with product size of 234 bp), 02 (F: 5'-

ACAAGCTGCACCTGTTGCAG-3'; R: 5'-TGACAGCGTGTGTAGTAGCA-3' with product size of 131 bp) respectively.

2.3.2. Detection of PCR products

The amplified PCR products were further confirmed using gel electrophoresis system. The agarose gel was prepared by adding agarose gel in TBE buffer making final concentration 0.8 %. To facilitate visualization of DNA after electrophoresis, ethidium bromide was added to the gel after cooling it down a bit. DNA fragments were visualized by placing on an ultraviolet transilluminator gel documentation system. DNA bands were compared with known loaded DNA ladder of 1000 bp [10].

2.4. Antimicrobial Susceptibility Testing

Kirby-Bauer disc diffusion method was used for the detection of antimicrobial susceptibility pattern and zone of inhibition against each antibiotic was measured using Clinical & Laboratory Standard Institute (CLSI) guidelines [18]. The plates were incubated aerobically at 37 °C for 18-20 hours. After incubation plates were examined and interpreted according to CLSI guidelines. The implanted antibiotics were Penicillin G (10 µg), Cephalothin (30µg), Ciprofloxacin (5µg), Clindamycin (2µg), Chloramphenicol (30µg), Gentamicin (10µg), Rifampicin (30µg), Trimethoprimsulfamethoxazol (1.5/23.5µg), Tetracycline (30µg) and Vancomycin (30µg).

3. Results

L. monocytogenes colonies appeared greyish-green having black sunken centers along with a black halo on PALCAM agar. Under the microscope, the isolated microorganism showed Gram positive reaction. A non-spore forming short rods having positive motility test were observed. The isolates were catalase and methyl red test as positive while indole, oxidase and Voges–Proskauer were negative. On TSI agar after 24 hours of incubation revealed fermentation of sugar with alkaline slant and H_2S production. Results of CAMP test showed 19 isolates making a characteristic enhancement of the zone of hemolysis with *S. aureus* on blood agar.

3.1. Prevalence of *L. monocytogenes*

Out of 120 samples 19 (15.83%) were positive for *L. monocytogenes* in meat samples collected from different sources both (fresh and frozen meat), while frozen meat samples were found to have higher percentage 18.33% (11/60) as compared to fresh meat samples 13.33% (08/60).

3.2. Confirmation of *Listeria monocytogenes* by PCR

The suspected *Listeria monocytogenes* were further confirmed by PCR amplification of DNA fragment of *hly* A and *iap* genes. The results of the current study revealed that all the 19 isolates showed positive amplification of *hlyA* gene **Fig. 1** for Listeria species with 234bp band on agarose gel and *iap* gene **Fig. 2** for *Listeria monocytogenes* with 131bp band.

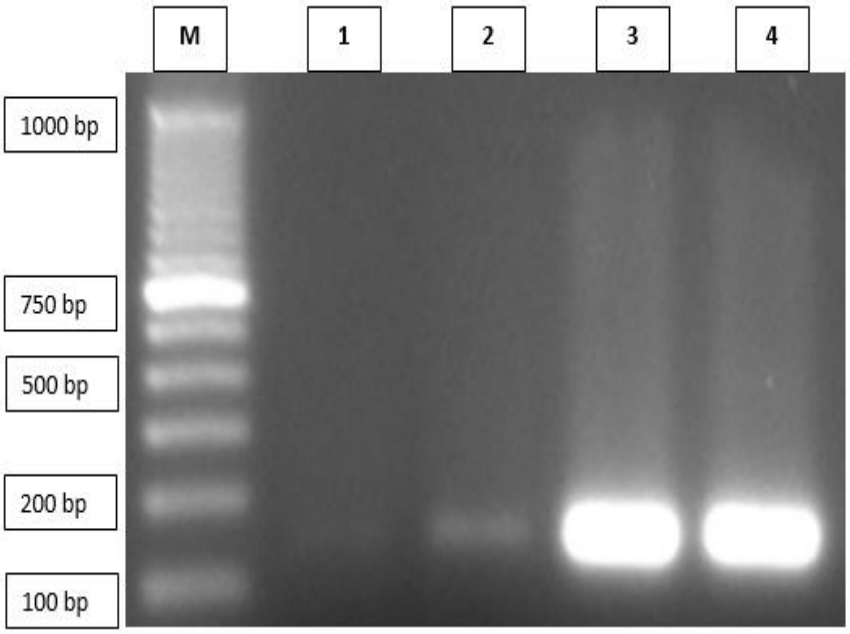

Fig.1 DNA marker in lane M and amplified product of *iap gene of 131bp from lane 1-4*

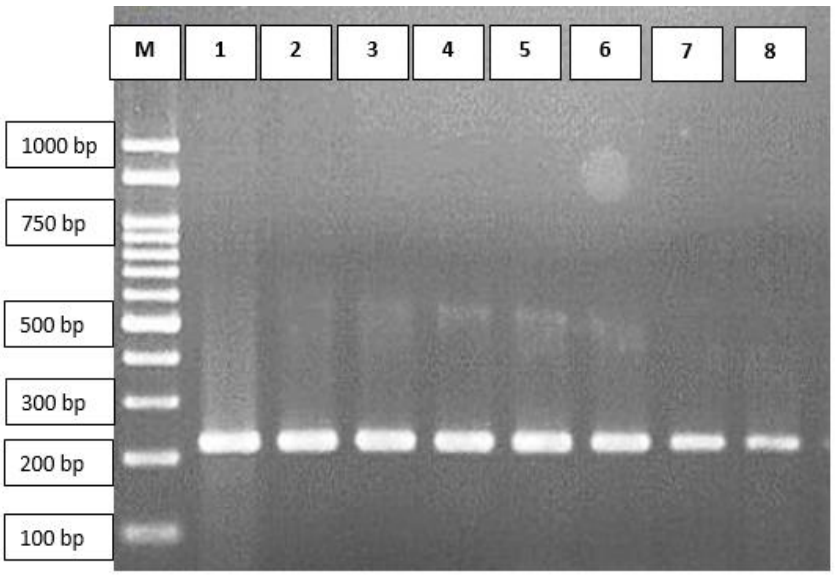

Fig.2 DNA marker in lane M and amplified product of hlyA gene of 234bp from lane 1-8

3.3. Antimicrobial Susceptibility Testing

The different isolates of *Listeria monocytogenes* from fresh and frozen meat showed variable susceptibility pattern against different antimicrobial agents. The results of the conducted study showed that highest resistance was observed for Penicillin (63.15%) in *L. monocytogenes* isolates followed by Clindamycin (57.89%), Ciprofloxacin (36.84%) and Rifampicin (26.31%) as shown in figure 1. All the nineteen isolates showed resistance to at least one antibiotic class except to Cephalothin, Chloramphenicol and Vancomycin. Against these three antibiotics all isolates were found susceptible. Of 19, nine isolates were found resistant against three or more than three

classes of antibiotics and regarded as multidrug resistant *L. monocytogenes*. The most common pattern observed in MDR *L. monocytogenes*. was PEN-CLN-CIP (Fig. 4)

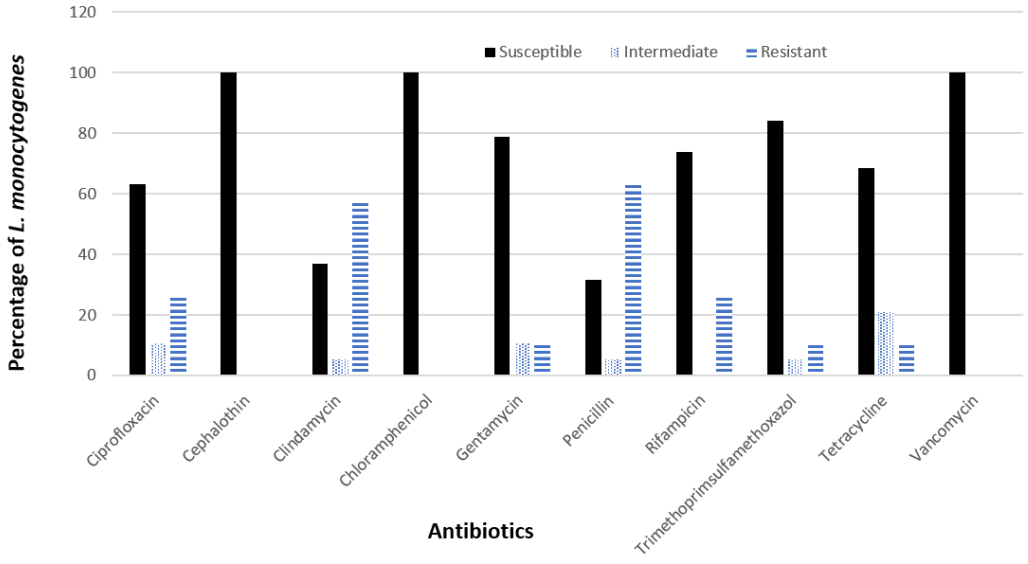

Figure 3: Resistance patterns of *Listeria monocytogenes* isolates to different antimicrobial agents

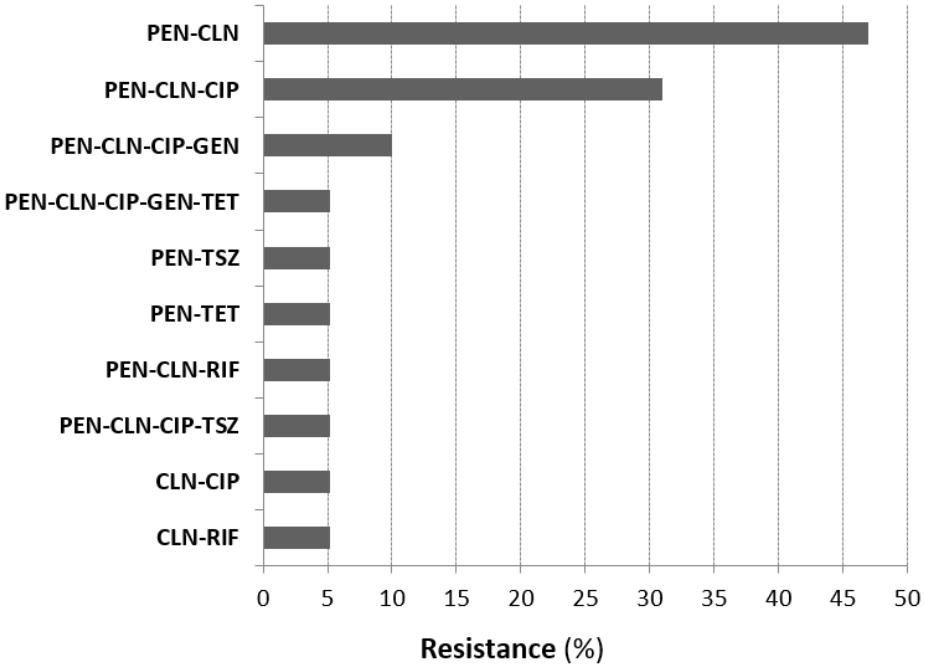

Fig.4 Phenotypes of drug resistance

PEN, Penicillin; CIP, Ciprofloxacin; CLN, Clindamycin; GEN, Gentamicin; RIF, Rifampicin; TSZ, Tri methoprimsulfamethoxazol; TET, Tetracycline.

4. Discussion

Today, food safety is everybody's concern and it is very hard to find someone who has not encountered an unpleasant moment of food-borne illness at least once in a year [19]. The World Health Organization (WHO) claims that food associated problems affects many people every year in the world. Majority of the food borne diseases are self-limiting and mild in nature, but severe cases can occur in high risk groups including infants, young children, the elderly and the immunocompromised persons [20].

Meat and meat products are among the most important edible commodities of animal origin like cattle, sheep, poultry and fish. Contaminated raw and frozen meat is one of the vital sources of food borne illness due to the fact that it has favorable conditions and nutrients for the efficient growth of pathogenic microorganisms such as proteins, fermentable carbohydrates, minerals and growth factors [21]. These contaminated meats products play an important role in spreading food borne diseases across the world and poses a public health threat for millions of peoples in different communities around the globe.

The results of present study revealed noticeable frequency of *Listeria monocytogenes* in fresh and frozen meat samples. It was found 13.33% in variety of fresh meat samples and in frozen samples it was raised to 18.33% making the cumulative percentage of 15.83%. A similar type of result was observed by [22] with 16.6% and [23] with 13.3% prevalence of *Listeria monocytogenes* in meat and dairy products. The results of current study are much higher than findings of [24] showing 2.97% and with 3.5% occurrence of *L. monocytogenes* in different foods. The increased number of Listeria occurrence in current study may be due to unhygienic processing of meat. The utensils and equipment's used in these processes may also contribute towards the bacterial contamination. Method used for identification of *L. monocytogenes* in current study were by targeting *hylA* and *iap* genes which are part of Listeria genome, also used previously as a useful detection tool for Listeria in food. In a study by, [25] they used *iap* gene for the detection and differentiation between *L. monocytogenes* and other species. In another study used *hylA* and *iap* genes as a PCR targets for detection of *L. monocytogenes* and found *hylA* and *iap* based assay more sensitive, specific and accurate for detection and quantification of *L. monocytogenes* [26].

In the current study the frozen meat samples were found more prone to Listeria contamination than the fresh ones which is in agreement with the statements of [27]and It was also previously recorded that lower incidence of Listeria species found in fresh poultry meat by [28] and at the same time higher incidence of Listeria was observed in frozen meat samples by. This may be due to the ability of Listeria to grow and survive at cold temperatures followed by the fact that frozen meat and products are at more risk of contamination during their preparation and storage than the fresh meat where the possibilities of contamination are very low

The results of antimicrobial susceptibility conducted in present study showed that *Listeria monocytogenes* isolates were resistant to Penicillin in (63.15%) and Clindamycin (57.89%) which are supported by the results of previous studies indicating that Listeria showed resistance against Clindamycin. This finding is also in agreement with [1] and the fact behind this resistance is

excessive use of Clindamycin in veterinary practice. Additionally, the role of mutations in chromosomal genes in conferring antibiotic resistance to Listeria species cannot be neglected. In contrast, Cephalothin, Chloramphenicol and vancomycin found 100% effective against the isolated Listeria. The findings of [29] were same against Cephalothin and Chloramphenicol while very close results were obtained by.

During this research, four isolates were found resistant to 4 or more drugs which is a strange pattern leading towards an alarming situation. There should be controlled use of antimicrobials to limit the emergence of multi drug resistant Listeria. Furthermore, the discovery of new antimicrobials can help to prevent us from this situation.

5. Conclusion

The results of present study revealed that out of total 120 meat samples 19 (15.83%) were found positive for *Listeria monocytogenes*. It was found that *Listeria monocytogenes* was more prevalent in frozen samples18.33% in comparison to fresh meat samples 13.33%. It was found that highest resistance was observed for Penicillin (63.15%) in *L. monocytogenes* isolates followed by Clindamycin (57.89%), Ciprofloxacin (36.84%) and Rifampicin (26.31%). All the 19 (100%) isolates were found susceptible to, Cephalothin, chloramphenicol and Vancomycin. Two of the 19 isolates were found resistant to more than 4 antimicrobials including Ciprofloxacin, Clindamycin, Penicillin and Gentamycin.

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Conflict of Interest:

The following study have no conflict with any other.

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