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Antibiogram and Identification of the *hlyA* gene in *Listeria Monocytogenes* Isolated from *Labeo Rohita* Samples

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ABSTRACT

Listeria monocytogenes is a zoonotic food-borne disease with significant public health and economic consequences. In animals, L. monocytogenes can cause clinical listeriosis, which is characterized by symptoms such as abortion, encephalitis, and sepsis. In the current study, 100 random samples of fresh fish (Labeo rohita) were purchased from various fish forms and markets in Pakistan's Rawalpindi and Islamabad regions. L. monocytogenes was detected bacteriologically in the collected samples. Then virulence tests were applied to positive isolated L. monocytogenes samples, all of which showed positive Christie–Atkins–Munch-Peterson results, a narrow zone of hemolysis on sheep blood agar, and positive beta hemolytic activity. Five out of seven samples were detected positive for the presence of the virulence gene (hlyA) using the specific primers via conventional PCR technique. Labeo rohita had the highest prevalence of virulence genes. The results showed that 7.0% prevalence (7/100) of L. monocytogenes was obtained from fresh Labeo rohita samples identified by a biochemical test. All L. monocytogenes-positive isolates tested for antimicrobial susceptibility Some isolates showed resistance to ampicillin, tetracycline, ceftazidime, and azithromycin. They were, however, sensitive to chloramphenicol, gentamicin, levofloxacin, amikacin, and trimethoprim. Listeriosis zoonotic potential has been demonstrated in raw fish and open-air fish market samples, necessitating further monitoring and awareness of antibiotic resistance to identify contaminated foods and ensure effective treatment. Furthermore, the health authorities must establish more active food control and surveillance. Population groups at higher risk of listeriosis require preventive education programs.

Kew words: Labeo rohita, Listeria monocytogenes, hlyA gene, antibiotic resistance

1 introduction

Listeria monocytogenes is intracellular pathogenic bacteria. *Listeria monocytogenes* abundantly present in dairy products like cheese from raw milk also raw milk beef, mutton, and poultry from animals or slaughter houses, decaying or raw vegetables from soil fish from water and utensils and also present in a variety of seafood's like shellfishes and fin fishes it can also present in soil silage sewage and animal waste. It can be found growing in refrigerated food as well (Embarek 1994) cause important bacterial disease listeriosis. In 1940, Pirie named this genus *Listeria* after catalase-positive gram-positive rods that had been isolated in other cases from humans (Pirie et al., 1940). *Listeria monocytogenes* is one of the most hazardous bacterial food-borne pathogens in the world, causing serious human infections (Maurtens et al., 2014). Listeriosis is a zoonotic illness transmitted mostly by the eating of contaminated food by *L. monocytogenes* (Hilliard et al. 2018). Humans may also become contaminated by direct contact with diseased animals and settings (Vázquez-Boland et al. 2001). Between 2017 and 2018, South Africa witnessed the world's largest outbreak of human listeriosis, with "polony," a beef-based ready-to-eat (RTE), being implicated in 2018 (Allam et al., 2018; Boatemaa et al., 2019; Olanya et al., 2019).

The Listeria pathogenicity island 1 (LIPI-1) contains several virulence genes, including *PrfA, plcA, plcB, hlyA, mpl*, and *actA.* They allow *L. monocytogenes* to survive and spread in host cells (Sereno et al., 2019). These genes have been found in *L. monocytogenes* isolates from both imported and local meat and meat products in the country (Matle et al., 2019), as well as food processing facilities (Mafuna et al., 2021). *PrfA* and *hlyA* are the most conserved genes due to their low nucleotide diversity, and bacterial virulence is overly reliant on genes found in the major *prfA*-regulated pathogenicity, also known as *LIPI-1*, which enhances the bacterium's subcellular spreading in the host and is composed of monocistron *hlyA*, which plays a key role in locus *alacithinase* operons, which includes the *mpl*, *actA*, and *plcB* genes (Chakraborty et al., 1992). This study aimed to check the prevalence and hemolysis causing *hlyA* gene in *L. monocytogenes* bacteria isolated from fish.

2 Material and method

This study was carried out in National Veterinary Laboratory (NVL) Islamabad Pakistan from Oct 2022 to June 2023 followed the guideline of the ethics committee of NVL Islamabad Pakistan.

Sample collection and processing

A total of 100 samples were taken at random from various retail fish markets and fish forms in Rawalpindi and Islamabad. Twenty-five grams of intestinal tissues from fish were collected and sent to the National Veterinary Laboratory (NVL) in a sterile plastic bag. For further processing, where they were grown on specialized conditions for microbiological testing, antibiotic resistance testing, and molecular characterization by using Fraser broth (OXOID CM0895) media and *Listeria* selective media (*Listeria* oxford agar base ISO 11290). Sample (25g) of fish intestinal tissue was homogenized in 225 ml Fraser broth, incubated at 37 °C for 24 hours (in a sterile sample bag stomached for 30 seconds). The material from the Fraser liquid media was then streaked onto *Listeria* selective media. *Listeria* produced small grey greenish or olive-colored colonies with a central black zone that resembled a halo. Morphological and biochemical characteristic of the bacteria were analyzed using gram staining, catalase test, sugar fermentation test and motility test according to FDA Bacteriological and Analytical Method (BAM) and International Organization for Standardization (ISO 11290) method (Hitchins, 2001; ISO, 1996). Beta hemolysis and CAMP tests performed. To confirm *Listeria* beta-hemolysis, colonies were streaked on blood agar plates and incubated for 24 hours at 37°C.Following incubation, bacterial growth exhibiting complete lysis of red blood cells was referred to as positive β –hemolytic. The CAMP test was used to distinguish between β- hemolytic *Listeria* species. Indole TSI Methylene red and Esculine test were performed for the biochemical identification.

Antimicrobial sensitivity testing of *L. monocytogenes* via disc diffusion method

Using the Kirby-Bauer method and according to the Clinical Laboratory Standards Institute recommends agar (CLSI) antibiotic resistance was performed for *Listeria* isolates strains grown on Mueller Hinton agar with a variety of antibiotics. Using the CLSI antibiotics chloramphenicol (30 μg/disc), azithromycin (15 μg/disc), gentamicin (120 μg/disc), levofloxacin (5 μg/disc), rifampin (5 μg/disc), ampicillin (10 μg/disc), tetracycline (30 μg/disc), trimethoprim (1.25 μg/disc) and ceftazidime (30 μg/disc) were used. Zone of inhibitions/break points were interpreted according to CLSI (Volume 40, 30$^{th\ edition}$) guidelines.

Polymerase chain reaction (PCR)

DNA was extracted with DNeasy Qiagen kit for gram positive bacteria (07/2020, hb-2061- 003@2020-qiagen) PCR mastermix recipe was prepared using Dreamtaq Green master mix

Table 1: Master mix composition and quantity

S. No	Ingredient	Quantity (µl)
1	Dreamtaq PCR mastermix	13
2	Nuclease free water	5
3	Forward primer	1
4	Reverse primer	1
5	DNA	5
Total		25 µl

Molecular characterization of *Listerial hly*A gene

Listeria *hlyA* gene of size 450bp in the selected isolates was amplified via following primer sequences,

F- 5'-AAATCATCGACGGCAACCT-3' & R-5'-ATTTCGGATAAAGCGTGGTG-3'. The following conditions were used for the successful completion of polymerase chain reaction.

Table 2. PCR thermal profile for amplification of *hly*A gene

Stage	Step	Time	Temperature	No. of cycles
1	Initial Denaturation	5 min	95°C	01
2	Final denaturation	30 sec	95°C	35
3	Primer annealing	1 min	60°C	
4	Initial extension	1 min	72°C	
5	Final extension	5 min	72°C	01

Gel electrophoresis used for the detection of PCR products after final extension were analyzed by agarose gel (1.5%) visualized under UV light in gel documentation system. All the data was interpreted using latest version of MS Excel (Microsoft 365).

3 Results

Among 100 meat samples (fish intestine) via cultural identification of *Listeria*, only 25 (25%) samples showed small transparent olive green colour colonies with black zone. The suspected *Listeria* colonies were subjected to gram staining among displayed as gram positive rods through microscopy (table 3).

Table 3. District wise prevalence of *Listeria* isolates

S.No	Locality	Type of sample	No. of (+ve) samples	Percentage (%)
1	Rawalpindi	Fish intestine	17	68
2	Islamabad	Fish intestine	8	32

Biochemical detection of Listeria

The 25 cultures positive suspected *Listeria* colonies were identified further by their biochemical characteristics upon performing different biochemical test. It was found that only 7 (28%) were *Listeria. Listeria* growth showing complete lysis of red blood cells was referred to as positive β –hemolytic.

Antibiotics Susceptibility Testing

Using the *Listeria* breakpoints specified in CLSI (Vol 40, 30th edition), almost all *Listeria* isolates were found sensitive to chloramphenicol (100%) and resistant (10%) to gentamycin and levofloxacin. Figures 1 shows that maximum Listeria isolates were sensitive to gentamycin, chloramphenicol, levofloxacin, and amikacin.

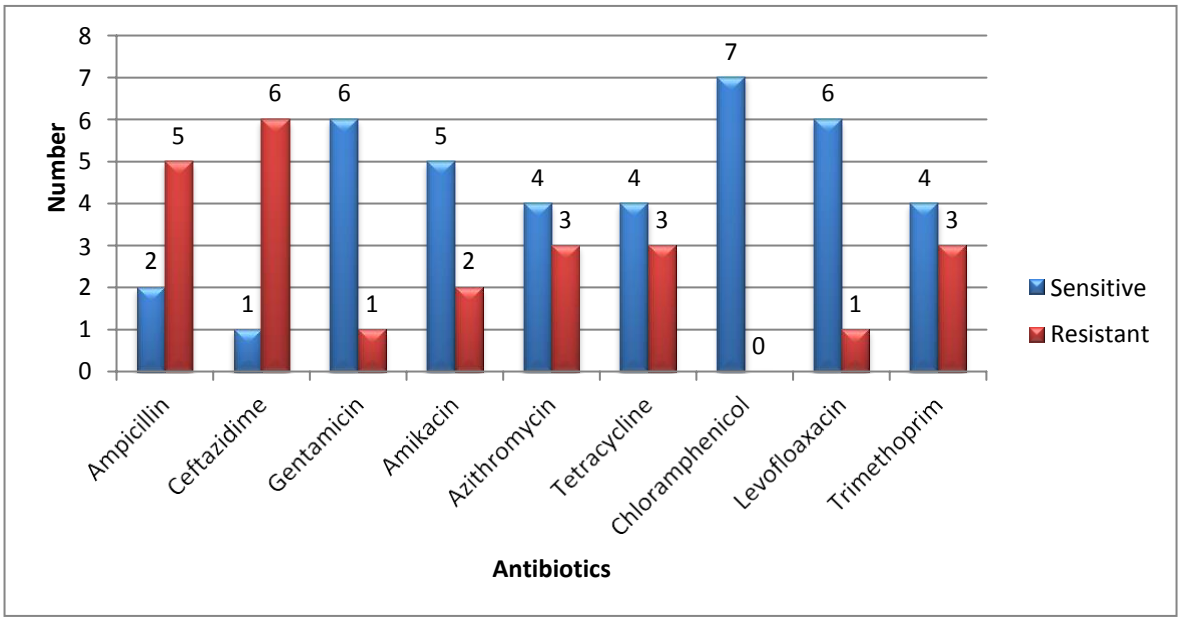

Figure 1. Antibiogram of *Listeria monocytogenes*

PCR Amplification

Listeria *hly*A gene was subjected to PCR amplification using conventional pcr. PCR results show that out of 7 samples, 5 (71%) isolates carried *hly*A gene as shown in figure 2.

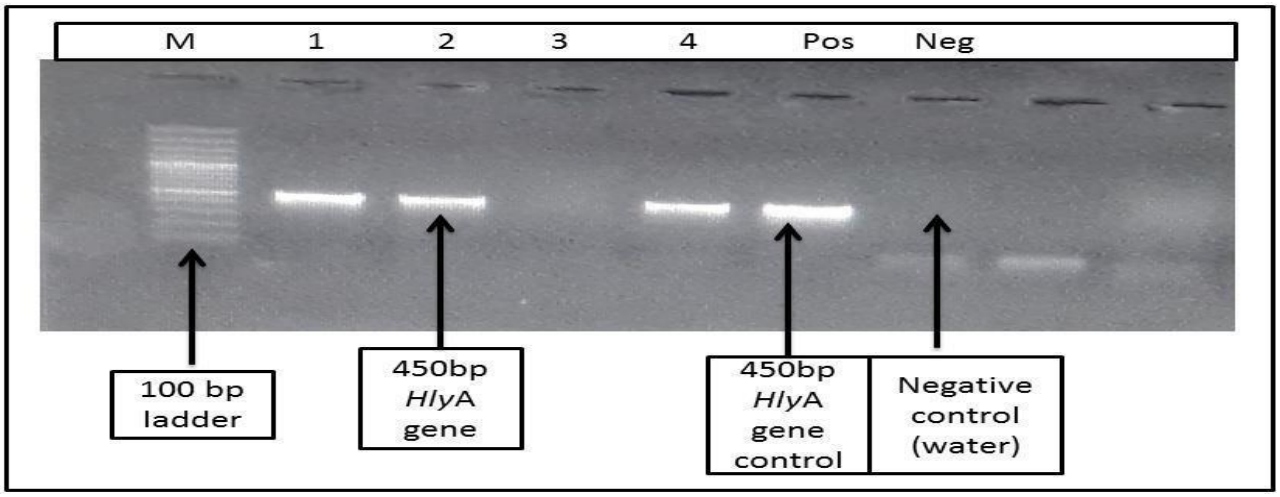

Figure 2. Amplification of hlyA gene

4 Discussion

Infection with *L. monocytogenes* in humans has also been linked to fish and fishery products. According to the most recent EFSA (European Food Safety Authority) data, nearly half (41.7 percent) of *L. monocytogenes* reports in the Rapid Alert System for Food and Feed from 2008 to 2016 involved fish and their products. Among the members of the genus

Listeria, *L. monocytogenes* is considered the most dangerous to humans, while there have been rare cases of infection with other species (Andrews et al., 2018). In this work, out 100 fresh fish samples (*Labeo rohita*), 25% were found positive for listeria and 7.0%. *L. monocytogenes* was detected molecularly that is related to the previous studies of Autio et al. (1999) and Handa et al. (2005) that estimated 1.5 and 2.0 % *L. monocytogenes* presence. *L monocytogenes* and *L. innocua* were detected in 1.9 % and 5.7 % of frozen and fresh sea samples, respectively; according to Fallah et al. (2012) which is consistent with our findings. The rate of *L. monocytogenes* strain isolation in this investigation was comparable to that reported in other countries, including 6.25 % in Ethiopia, 9.5% in Italy, and 6.2 % in Spain (Meloni et al., 2009). The CAMP tests, as well as *in vitro* pathogenicity assays like beta-hemolysis on sheep or horse blood agar, have been used to evaluate the pathogenic potential of *Listeria monocytogenes* isolates according to study of Schonberg et al. (1989) and Mckeller et al. (1994) which corelated our findings.

In this work, the presence or absence of the Listeriolysin O (*hly*A) gene was used to determine virulence and non-virulence in *L. monocytogenes* isolates. A number of virulence-related genes have been sequenced and utilized as PCR targets to identify *L. monocytogenes* but listeriolysin O encoded by the *hly*A gene, is thought to be the most important virulence factor as this assessment line with the study of Border et al. (1990). Several virulence genes in *L. monocytogenes* have been identified and targeted for PCR detection, including the (*hly*A) gene, which codes for listeriolysin O (LLO), which is relevant to our research. In this assessment, isolates were found to have *hly*A genes, which is strongly supported by all of the preceding studies. Positive isolates in our study were also tested for antibiotic susceptibility. The number of reports has increased since the identification of *L. monocytogenes* strains resistant to one or more antibiotics in food products as reported by Gomez et al. (2014). All *L. monocytogenes* isolates from sea foods in the present study were resistant to β-lactam antibiotics, which is essential in the treatment of human listeriosis. The same study was conducted by Ruiz-Bolivar et al. (2011), in which the resistance to ampicillin and clavulanic acid was 71.0 % which was greater than the value given. The high rate of ceftazidime resistance (85%) seen in this investigation matched the findings of another study of Khen et al. (2015). Tetracycline (43%) and azithromycin (43%) resistance was found in *L. monocytogenes*, which was much lower than the rate reported by Garedew et al. (2015).

L. monocytogenes was found sensitive to chloramphenicol (100%), gentamicin (85%), and levofloxacin (85%) in this study that resembled to the findings of Osaili et al. (2011). In our study, *L. monocytogenes* isolates were susceptible to trimethoprim (57%) which corresponded to the findings of Ennaji et al. (2008). In this assessment, amikacin susceptibility was found to be (71%), which is higher than the figure reported in previous studies of Kocaman and Serimehmetoglu (2017). Our findings add to the growing body of data that multi-resistant strains are surfacing in nature, posing a concern to human health.

5 Conclusion

The presence of *L. monocytogenes* in fish (*Labeo rohita*) samples cannot be ignored, and the risk of listeriosis from eating sea foods should not be underestimated. According to our findings, strict controls on the hygienic quality of Pakistani sea foods should be put in place. As a result of these developments, the level of contamination with *L. monocytogenes* has increased. Contact with intestinal contents, cross contamination from diseased workers, use of contaminated equipment, fish manipulation, and incorrect shipping are the main sources of sea-food contamination. To restrict Listeria growth during the fishing, collection, transmission, distribution, and

storage phases, various food safety and quality standards may need to be enforced, and most Pakistani supermarkets and even fishing centers may need to implement them. Simultaneous recognition of virulence genes in assays is also desirable because it will facilitate large-scale investigations into pathogenic *Listeria* detection. The study's findings should enhance the development of guidelines for effective antibiotic treatment of listeriosis.

6 Declarations

Conflicts of interest/Competing interests
The authors have no conflicts of interest to declare.

Ethics approval
Not applicable

Consent to participate
All authors consent to participate in the manuscript publication

Consent for publication
All authors approved the manuscript to be published

Availability of data and material
The data supporting the conclusions of this article are included within the article. Any queries regarding these data may be directed to the corresponding author.

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