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Original Article

First substantiation of clinico-molecular investigation of pathogenic *Listeria monocytogenes* in Nili-Ravi buffaloes

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Abstract

Background: Listeriosis is a zoonotic disease of humans, animals, birds, fish, and crustaceans worldwide. Domestic animals, especially ruminants, are more susceptible to listeriosis. This infectious disease is caused by *Listeria monocytogenes*, an intracellular bacterium that can cross blood-brain, placental and intestinal barriers. In Pakistan, the incidence and reliable diagnostic tools for the *L. monocytogenes* are unidentified in Nili-Ravi buffaloes. **Aims:** This study was designed to inspect listeriosis in buffaloes through molecular techniques and haemato-biochemical analyses. **Methods:** A total of 230 samples (115 milk and 115 faecal samples) were collected from symptomatic listeriosis cases in Nili-Ravi buffaloes of 3 geographical districts (Rawalpindi, Faisalabad, and Muzaffargarh) Punjab, Pakistan. These samples were processed for DNA extraction using commercialized kits, and *L. monocytogenes* was confirmed by conventional PCR. **Results:** The results revealed that 6.08% and 4.34% of the isolates from milk and faecal samples were found positive for *L. monocytogenes*, respectively. The phylogenetic analysis of these isolates showed 97-100% similarity to isolates from the USA, Switzerland, Japan, and India. The accession numbers on NCBI GenBank appeared as HF558398 (Switzerland), KP965732 (India), EU372032 (USA), and LC259850 (Japan). Haemato-biochemical examinations showed that the values of WBCs, plasma fibrinogen, ALT, and AST significantly increased ($P<0.05$) in diseased buffaloes compared to healthy ones. **Conclusion:** The occurrence of listeriosis in buffaloes urges continuous monitoring and surveillance to prevent this emerging disease in Pakistan.

Key words: Buffaloes, *Listeria monocytogenes*, Listeriosis, Phylogeny, Polymerase chain reaction

Introduction

The genus *Listeria* has 20 species comprised of two main groups; (i) *Listeria sensu stricto*, includes the species *L. monocytogenes*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, *L. marthii*, and *L. seeligeri*, and (ii) *Listeria sensu lato*, includes the species *L. grayi*, *L. newyorkensis*, *L. booriae*, *L. riparia*, *L. grandensis*, *L. floridensis*, *L. cornellensis*, *L. aquatica*, *L. weihenstephanensis*, *L. fleischmannii*, *L. rocourtiae*, *L. goaensis*, *L. thailandensis*, and *L. costaricensis*. Among these species, *L. monocytogenes* is a highly pathogenic bacterium that causes listeriosis in ruminants and humans (Orsi and Wiedmann, 2016; Quereda *et al*., 2020). The ability of *L. monocytogenes* to cross the blood-brain and intestinal and placental barriers is conferred by the interaction between the internalin surface protein with the E-cadherin receptor (Lecuit, 2005). *L. monocytogenes* can survive in an extensive range of temperatures, pH and salt concentrations (Sleator *et al*., 2003); therefore, they are found in diverse environments such as water, sewage, silage, effluents, and dairy products. Raw milk is considered one of the main sources of infection for several pathogenic microorganisms (Meyer-Broseta *et al*., 2003). In animals, *L. monocytogenes* causes encephalitis, meningitis, abortion, and mastitis. Buffaloes (*Bubalus bubalis*) have been reported to be vulnerable to listeriosis (Shakuntala *et al*., 2006). Clinical signs in ruminants include fever, circling, head pressing, head tilting, cranial nerve paralysis, septicemia, keratoconjunctivitis, and various reproductive disorders such as stillbirth, late-trimester abortion and placentitis (Hunt *et al*., 2012). The clinical signs depend on the site of injuries in the nervous system (Walland *et al*., 2015). Various virulence factors of *L. monocytogenes* have been described, among which the *hly*A gene (encodes for hemolysin A) is an important virulence factor and pathogenic marker (Vázquez-Boland *et al*., 2001; Liu *et al*., 2007). To distinguish between virulent and avirulent strains of *L. monocytogenes*, the *hly*A gene has been targeted in previous studies. In addition, pursuing the *hly*A gene to detect *L. monocytogenes* is more reliable due to its pathogen-specific DNA sequences (Soni and Dubey, 2014). The

*hly*A gene is the most common (100%) virulence gene among *L. monocytogenes* strains isolated from milk (Skowron *et al*., 2019). Various outbreaks of human listeriosis have been reported owing to the consumption of raw milk (Silk *et al*., 2013). Infected dairy animals may continuously shed *Listeria* in their milk and faeces (Dehkordi *et al*., 2013). Laboratory methods used to detect *Listeria* pathogens are bacterial culture, biochemical analysis and molecular assay. Due to its zoonotic nature and limited access to advanced biosafety measures, the handling of the bacterium can be dangerous to laboratory workers and researchers (El-Liethy *et al*., 2020). Identification of *L. monocytogenes* has been carried out through the cultural method and has shown lower sensitivity and specificity compared to the PCR assay (Haj Hosseini *et al*., 2014). Serological tests can also detect *L. monocytogenes* in milk samples, but less sensitively (Amagliani *et al*., 2006).

The case mortality rate in ruminants due to listeriosis is approximately 20-100% (Nightingale *et al*., 2005; Esteban *et al*., 2009). In Pakistan, the frequency of *L. monocytogenes* in raw milk was 26.66%, and caused high morbidity and mortality outbreaks. Listeriosis has been described as an emerging milk-borne disease (Zafar, 2020). The animal's recovery from listeriosis depends on the disease duration and the severity of clinical signs (Braun *et al.*, 2002). According to Pakistan's Economics Survey 2020-21, the buffalo population in Pakistan is 42.4 million with a gross milk production of 38363,000 tonnes.

There is limited data regarding the occurrence of *L. monocytogenes* in Nili-Ravi buffaloes in Punjab, Pakistan. Therefore, the present study was conducted for the molecular detection and haemato-biochemical analysis of *L. monocytogenes* in buffalo milk and faecal samples gathered from dairy farms of Punjab.

Materials and Methods

This study was approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences of Lahore (Number. DR/946 Dated 13-09-2019 Office of Research Innovation and Commercialization). The guidelines of animal care and handling were followed as per the University's instructions.

Case description and inclusion-exclusion criteria

Buffaloes with clinical signs of head tilt, ataxia, one-sided facial paralysis, circling, drooping of the ear, jaw muscles paresis, lip paralysis, abortion, septicemia, gastroenteritis, and mastitis were included. Those that did not show listeriosis signs were excluded from our study.

Sampling and design

Milk and faecal samples were collected from 115 Nili-Ravi buffaloes (out of a total of 230), from 3 different geographical districts (Rawalpindi, Faisalabad, and Muzaffargarh) in Punjab, Pakistan. Samples were collected from organized and unorganized dairy farms. Milk samples were collected aseptically in sterilized vials placed in an icebox. Using sterilized gloves, the faecal samples were collected directly from buffaloes' rectums or from freshly secreted buffalo pat and placed in sterile plastic bags. The collected milk and faecal samples were transferred to the Diagnostic Laboratory, University of Veterinary and Animal Sciences (UVAS), Lahore, and stored at 4°C until the tests. Before processing, they were thawed at room temperature.

Isolation and identification of *Listeria*

25 g/ml of each fecal/milk sample was added into 225 ml half-strength Fraser broth (Cat. 1183 Condalab, Spain) for primary enrichment of *Listeria*, and placed in an incubator at 30°C for 25 h. 0.1 ml of the primary enrichment was transferred to 10 ml of full-strength Fraser broth (Cat. 1182 Condalab) and incubated at 37°C for 24 h in aerobic conditions. These isolates were streaked onto PALCAM *Listeria* agar (Cat. 0955 Condalab) and incubated at 35°C for 48 h. After that, colony characteristics and biochemical tests were performed (Aygun and Pehlivanlar, 2006).

DNA extraction

After the enrichment step, DNA extractions from the *Listeria* broth were carried out using a Vivantis Nucleic Acid Extraction kit (Cat No. GF-BA-100, Vivantis Technologies Sdn Bhd, Malaysia) and GeneAll Exgene Stool DNA (Cat No. 115-150, GeneAll Biotechnology, Co. Ltd., Seoul Korea), respectively, following the instructions given in the kits. Following the Vivantis kit protocol: 2 ml bacterial pellet was prepared by centrifugation at 6,000 × g for 2 min. The supernatant was poured out and 100 µL of the R1 buffer was added to the pellet to resuspend the cells. These were treated with 20 µL of lysozyme, centrifuged, and the supernatant was later discarded. Then, 180 µL of buffer R2 and 20 µL of proteinase K were added to the pellet. 20 µL of RNase A was used for the removal of RNA. Buffer BG (2 volume) was added for homogenization. These samples were mixed with 200 µL of ethanol and centrifuged. The mixed samples (650 µL) were loaded on the collection tube and centrifuged, and the flow through was discarded. The column was washed with wash buffer (650 µL) and centrifuged, and the flow through was discarded. The elution of genomic DNA was done through elution buffer (50 µL) and centrifuged at 10,000 × g for 1 min. The extracted DNA was stored at -20°C.

As the GeneAll kit protocol instructed, 1 ml of buffer PBS was added into 2 ml bacterial culture in a microcentrifuge tube and homogenized. The supernatant was collected in a clean microcentrifuge tube and centrifuged, and the supernatant was discarded. Then 1.3 ml of buffer FL was added into the pellet and centrifuged. The supernatant was poured to an EzPass filter, centrifuged and shifted to a clean tube. This step was repeated 8 times. 100 µL of buffer EB was added into a filter and centrifuged. 500 µL of buffer PB was added and the solution was collected in mini-columns

and centrifuged. 500 µL of buffer NW was added into the mini-column and centrifuged. Then 50 µL of buffer EB was added into mini-column and centrifuged at ≥10,000 × g for 1 min. The DNA was stored at -20°C. The DNA purity was checked by Nanodrop 1000 spectrophotometer (Thermo Scientific, USA).

Designing the primers

The primers used for the detection of *L. monocytogenes* were synthesized from GeneLink, USA. The sequences of primers were *hly*A-Forward 5´-GCA GTT GCA AGC GCT TGG AGT GAA-3´ and *hly*A-Reverse 5´-GCA ACG TAT CCT CCA GAG TGA TCG-3´. The product size of *hly*A was 456 base pairs (Paziak-Domańska *et al*., 1999). The primers for the *hly*A gene were optimized before use.

Conventional PCR

To find out the virulence factor (*hly*A), the PCR reaction was uniformed as documented (Paziak-Domańska *et al*., 1999; Laximan *et al*., 2016) with slight modification. PCR reaction steps (concentration of primers, volume of the master mix, annealing temperature and cycling conditions for *hly*A gene) were optimized. The total volume of 50 µL of PCR reaction mixture contained 25 µL Master mix (DreamTaq DNA polymerase, 2X DreamTaq green buffer, dNTPs, 4 mM $MgCl_2$ and 4 ×1.25 ml nuclease free water, Catalog. No. K1081, ThermoFisher Scientifics, USA), 2 µL forward primer (10 pmol/µL), 2 µL reverse primer (10 pmol/µL), 3 µL DNA template (50 ng/µL), and 18 µL sterilized nuclease free water (Catalog. No. 129114, Qiagen, Germantown). The PCR tube was then micro-centrifuged so that the blend settled down at the bottom. PCR reaction was carried out in a Thermal Cycler (Catalog. No. A24811, ThermoFisher Scientific, USA). The cycling conditions comprised of initial denaturation at 95°C for 5 min followed by 35 cycles each of denaturation at 95°C for 45 s, annealing 60°C for 45 s, extension 72°C for 2 min and final extension at 72°C for 7 min. The PCR product was analyzed through agarose gel electrophoresis.

Gel electrophoresis

The agarose gel 1.5% was prepared. The gel was stained with ethidium bromide, and Tris-borate EDTA buffer was added to the electrophoretic apparatus. PCR products along with positive, negative controls and molecular weight markers (Catalog. No. DM001-R500, GeneDirex, USA) were loaded on separate wells of the gel. The gel was run at 100 V for 45 min. The product size was observed under the Gel documentation system (Omega fluor plus, USA).

Sequencing and phylogenetic analysis

The sequence results obtained from the commercial sequence provider (PT. Genetika Science, Indonesia) were analysed by the NCBI Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA X software (https://www.megasoftware.net/) was used to determine the relatedness among the isolates of *L. monocytogenes*. The phylogenetic examination was derived by the Neighbor-Joining strategy. The evolutionary distances were processed utilizing the p-distance method. All ambiguous positions were eliminated for each grouping pair (pairwise deletion option). There was an aggregate of 408 positions in the last dataset.

Haematological and biochemical analysis

Blood samples

Blood samples (3 ml) were collected aseptically from the jugular vein of 12 PCR positive and 12 healthy buffaloes using disposable syringes. The samples were then transferred to vacuum tubes with and without $EDTA.K_3$ (BIO-VAC, China) to collect blood and serum, respectively. For serum collection, the blood samples were allowed to clot, after which the clotted blood was centrifuged at 3000 × g for 5 min. After centrifugation, the supernatant, clear straw-colored fluid (serum) was aspirated into Eppendorf tubes with the help of a pasture pipette. The serum samples were stored at -20°C for later examinations. The haematological and biochemical parameters were checked by a haematological analyzer (Abacus Junior Vet, Diatron, Austria) and a biochemical analyzer (BioSystems BTS-350, Spain), respectively. The values of the haemato-biochemical profile of buffaloes suffering from listeriosis were checked and compared with the healthy buffaloes' values.

Statistical analysis

The gathered data was compiled on a Microsoft excel worksheet 2013. The haemato-biochemical parameters were analysed by independent samples t-test on MedCalc® Statistical Software version 20.009 (Belgium). The value of $P<0.05$ was considered significant.

Results

Clinical signs/symptoms

Based on the clinical signs/symptoms, a total of 115 buffaloes were suspected of listeriosis. Each susceptible buffalo showed a different pattern of clinical signs. Seven buffaloes showed signs of head tilted and 4 displayed signs of heads pressing. Nineteen buffaloes had shown abnormal gait. Circling on one side was observed in only 3 buffaloes, whereas ataxia was noted in 24. Pricking of different body regions with a hypodermal syringe showed hyposensitivity of the head region in 23 buffaloes. Physical examinations revealed that swallowing reflexes were weak in 31 and absent in 18 buffaloes. Pupil light reflex was weak in 17 buffaloes. Other signs included mastitis in 23 and abortion (in the last trimester) in 3 buffaloes (Table 1).

Bacteriological analysis of *L. monocytogenes*

A total of 230 samples (115 milk and 115 fecal samples) from 115 buffaloes were subjected to culture and biochemical tests.

L. monocytogenes appeared coccobacilli or rod-

shaped on Gram-staining. *L. monocytogenes* was motile at ≤30°C and showed tumbling motility. The colony indicated a small zone of blurry haemolysis on blood agar media. Typical grey-green colony growth with blacking of adjacent media on *Listeria* selective agar (PALCAM) was observed.

Table 1: Clinical manifestations of listeriosis in 115 Nili-Ravi buffaloes

Clinical signs/symptoms observed	Number of Nili-Ravi buffaloes
Posture	
Head tilted	07
Head pressing against inanimate objects	04*
Gait	
Abnormal gait	19
Circling on one side	03
Ataxia	24
Sensitivity of superficial body regions	
Hyposensitivity of the head region	23
Swallowing reflex	
Weak	13
Absent	18**
Pupil light reflex	
Weak	17***
Other signs	
Mastitis	23
Abortion (in the last trimester)	03

* 04 buffaloes showed combined signs of head pressing and weak swallowing reflex, ** 18 buffaloes exhibited shared signs of absent swallowing reflex and head region hyposensitivity, and *** 17 buffaloes displayed combined signs of weak pupil light reflex and ataxia

Biochemical tests revealed catalase, coagulase, and methyl red positive. Indole and urease tests were negative. Glucose and maltose fermentation tests were positive with the production of acid. Citrate utilization and nitrate were negative. The Christie, Atkins, and Munch Peterson (CAMP) test showed the enhanced zone of haemolysis adjacent to *Staphylococcus aureus* streak.

Out of the 230 samples that were bacteriologically examined, 17 samples (9 milk and 8 fecal) were phenotypically positive for *L. monocytogenes.*

Conventional PCR for detection of *L. monocytogenes*

The isolates phenotypically positive for *L. monocytogenes* were processed for PCR reaction by amplification of the *hly*A gene. Out of these, 12 isolates included 7 milk (6.08%) and 5 fecal (4.35%) isolates were found positive for the *hly*A gene of *L. monocytogenes* (Fig. 1). The 7 PCR positive milk isolates were originated from head tilted (4 No.) and mastitic (3 No.) buffaloes. The 5 PCR positive fecal isolates were detected from those that showed combined signs of absent swallowing reflex and head region hyposensitivity (3 No.), abnormal gait (01 No.), and circling on one side (01 No.). The results revealed that shedding of *L. monocytogenes* in milk and fecal samples varied from one buffalo to another.

Phenotypic and biochemical properties of bacteria may be altered by external factors, i.e. growth phases and mutations of specific genes. The molecular technique for the detection of a specific gene of bacteria is more accurate and not influenced by external factors (Shamloo *et al*., 2019). Hence, the recognition of *L. monocytogenes* by PCR assay is more sensitive and specific as compared to conventional cultural methods.

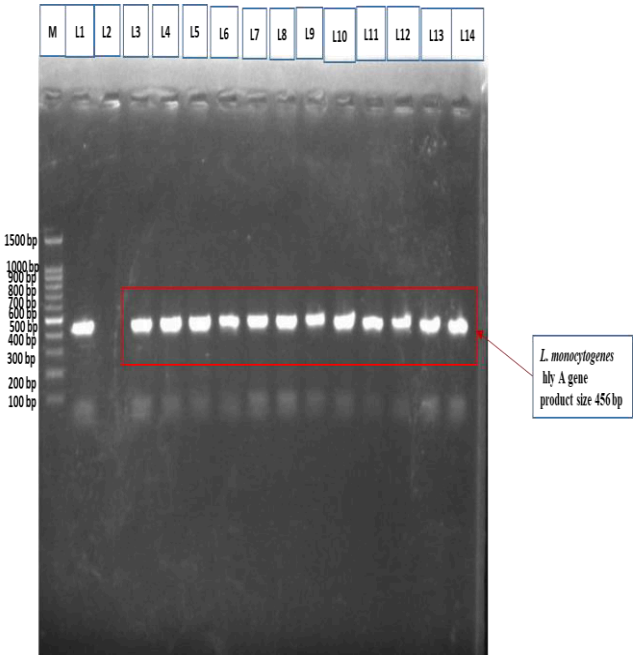

Fig. 1: Agarose gel electrophoresis indicated PCR product of *L. monocytogenes* isolates. M: DNA molecular weight marker (100 bp). Lane 1: Positive control, Lane 2: Negative control, Lanes 3 to 14: Buffaloes isolates were positive for *hly*A gene of *L. monocytogenes*

Haemato-biochemical profile

Haematological analyses indicated increased values of WBCs and plasma fibrinogen in diseased buffaloes compared to healthy ones. A significant relationship ($P<0.05$) was found between increased WBCs and listeriosis in buffaloes. The other haematological variables recorded were not significantly linked ($P>0.05$) to listeriosis (Table 2). Biochemical parameters showed that values of ALT and AST were significantly related ($P<0.05$) to listeriosis (Table 3).

Phylogenetic exploration

The phylogenetic examination was derived from the Neighbor-Joining strategy. Evolutionary distances were processed using the p-distance method. All ambiguous positions were eliminated for each grouping pair (pairwise deletion option). There was an aggregate of 408 positions in the last dataset. Evolutionary analyses were conducted in MEGA X software which revealed 97-100% similarity of *L. monocytogenes* Pakistani isolates with the USA, Switzerland, Japan, and India isolates. On NCBI, GenBank, the accession numbers appeared as HF558398 (Switzerland), KP965732, KJ883238 (India), EU372032, DQ812456 (USA), and LC259850 (Japan). Accession numbers of the Pakistani isolates obtained from NCBI were GenBank OL442087-OL442098 (Fig. 2).

Table 2: Comparison of haematological profile between healthy and diseased (listeriosis) buffaloes

Haematological profile	SI Units	Diseased ± StDev[1]	Healthy ± StDev	Md[2]	95% CI[3]	P-value
Hemoglobin	g/L	111.666 ± 14.992	107.833 ± 11.582	3.8333	-7.5087-15.1753	0.490
PCV	L/L	0.292 ± 0.044	0.291 ± 0.0425	0.0008	-0.03565-0.03732	0.962
RBCs	$\times10^{12}$/L	6.242 ± 1.362	6.300 ± 0.8873	-0.0583	-1.0316-0.9149	0.902
MCV	fL	43.500 ± 4.796	42.8333 ± 4.7832	0.6667	-3.3884-4.7217	0.736
MCH	pg	16.367 ± 1.906	15.7917 ± 1.5588	0.5750	-0.8989-2.048	0.427
MCHC	g/L	375.425 ± 8.213	372.50 ± 5.8387	2.9250	-3.1081-8.958	0.325
Thrombocytes	$\times10^{9}$/µL	458.917 ± 181.795	448.75 ± 168.982	10.1667	-138.426-158.759	0.888
MPV	fL	5.0417 ± 1.2325	5.0583 ± 1.2117	-0.01667	-1.0514-1.0180	0.973
WBCs	$\times10^{9}$/L	16.800 ± 5.8022	9.9083 ± 2.5224	6.8917	3.1040-10.679	0.001*
Neutrophils	$\times10^{9}$/L	3.9167 ± 2.0369	3.8500 ± 1.6676	0.06667	-1.5093-1.6426	0.930
Lymphocytes	$\times10^{9}$/L	4.0667 ± 1.455	3.7500 ± 1.3860	0.3167	-0.8863-1.5197	0.590
Monocytes	$\times10^{9}$/L	0.4583 ± 0.2539	0.4250 ± 0.2491	0.03333	-0.1796-0.2463	0.748
Eosinophils	$\times10^{9}$/L	0.4583 ± 0.2109	0.4667 ± 0.3025	-0.0083	-0.2291-0.2124	0.938
Plasma proteins	g/L	70.8500 ± 10.6255	70.333 ± 6.760	0.5167	-7.0228-8.0561	0.888
Plasma fibrinogen	g/L	7.6417 ± 2.8637	5.3167 ± 1.792	2.3250	0.3026-4.3474	0.026*

* Statistically significant (P<0.05), [1] Standard deviation, [2] Means difference, and [3] Confidence interval

Table 3: Comparison of biochemical parameters (LFT and RFT) between healthy and diseased (listeriosis) buffaloes

Biochemical parameters		SI Units	Diseased ± StDev[1]	Healthy ± StDev	Md[2]	95% CI[3]	P-value
LFT	ALT	Units/L	40.5167 ± 13.1011	25.7083 ± 9.7292	14.8083	5.0388-24.5779	0.0047*
	AST	Units/L	146.9167 ± 31.1228	105.0000 ± 21.3371	41.9167	19.3259-64.5075	0.0009*
	ALP	Units/L	184.1667 ± 75.6137	145.4167 ± 46.5373	38.7500	-14.4047-91.9047	0.1448
	Bilirubin	µmol/L	6.9833 ± 2.0876	6.2500 ± 2.4763	0.7333	-1.2056-2.6723	0.4412
RFT	Creatinine	µmol/L	153.0000 ± 35.1102	148.3333 ± 25.8785	4.6667	-21.4456-30.7790	0.7145
	BUN	mmol/L	9.2583 ± 5.1502	7.3333 ± 2.0628	1.9250	-1.3964-5.2464	0.2422

* Statistically significant (P<0.05), [1] Standard deviation, [2] Means difference, and [3] Confidence interval. LFT: Liver function test, and RFT: Renal function test

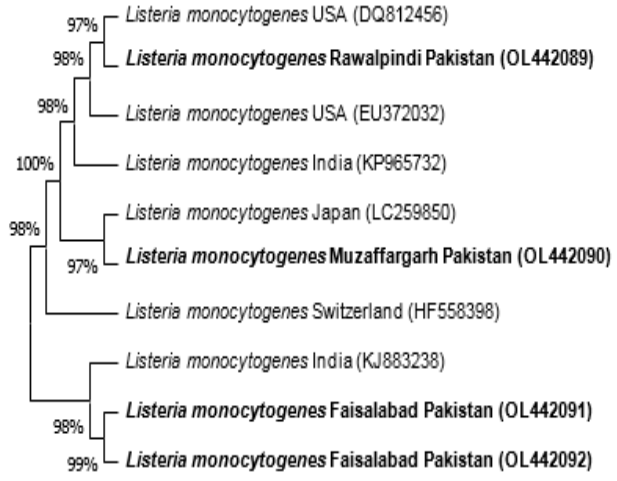

Fig. 2: Phylogenetic analysis of *L. monocytogenes hly*A gene partial cds by Neighbor-Joining method indicated the relatedness among the *L. monocytogenes* Pakistani isolates with other countries isolates. Accession numbers are in parentheses

Discussion

Clinical manifestations of listeriosis in buffaloes

In the present study, each buffalo suspected of listeriosis showed varied signs/symptoms depending on which cranial nerve was affected. Oculomotor, trochlear, and abducens nerve infections bring about visual anomalies i.e. unusual pupillary light reflex. The infection of the trigeminal nerve causes difficulties in chewing and loss of the corneal reflex. Infections of the vestibulocochlear nerve lead to nystagmus, circling, head slant and ataxia. The contagion of hypoglossal and vagal nerves diminish the strength of the tongue. These findings are in congruence with the previously reported clinical signs of bovine listeriosis (Schweizer *et al*., 2006; Hunt *et al*., 2012). On the contrary, it has been reported that buffaloes affected with listeriosis have manifested the signs of paralysis of limbs, loss of sensation of body parts and recumbency (Prado *et al*., 2019).

Occurrence of *L. monocytogenes* in buffaloes

In the present study, the *L. monocytogenes* occurrence in raw milk of buffaloes suspected of listeriosis was 6.08%, which is similar to the findings of Vilar *et al*., 2007, who reported 6.1% occurrence. On the contrary, previous studies have reported 8%, 1%, and 2.25% frequencies of *L. monocytogenes* in Hyderabad, Quetta, and Faisalabad, Pakistan (Chandio *et al*., 2007; Usman and Mukhtar, 2014; Samad *et al*., 2018), respectively. The occurrence of *L. monocytogenes* has been recorded as 5%, 1.9% and 3% in Iran and Turkey (Rahimi *et al*., 2014; Mansouri-Najand *et al*., 2015; Terzi Gulel *et al*., 2020). Lower and higher frequencies of *L. monocytogenes* 5.1% and 8% were identified in milk samples in India (Kalorey *et al*., 2008; Nayak *et al*., 2015). In Sudan and Egypt, 2.5% and 1.4% occurrence of *L. monocytogenes* were reported in milk samples (Osman *et al*., 2016; El Hag *et al*., 2021). Zero

occurrence of *L. monocytogenes* in milk samples was also recognized in India (Shantha and Gopal, 2014). Previous research maintains that low occurrence of *L. monocytogenes* indicates good farm management and hygienic standards adopted in dairy farms (Nightingale *et al*., 2005). Compared to this study, high a occurrence (11.11%) of *L. monocytogenes* in buffalo milk was reported in Iran (Dehkordi *et al*., 2013).

The variation in findings might be due to different geographical regions, variations in local weather, climate, unhygienic conditions, contamination during milking, transportation, storage, and environmental contaminations. The raw milk could have been contaminated with faecal material and/or environmental contagion during the processes of milking, storage and transportation (Konosonoka *et al*., 2012).

In this study, the occurrence (4.34%) of *L. monocytogenes* in faecal samples is similar to the 4.71% occurrence reported in buffalo faeces by Dehkordi *et al*., 2013. On the contrary, lower and higher-level (12% and 1.53%) occurrences of *L. monocytogenes* in faecal samples were recorded (KALENDER, 2003; Fox *et al*., 2009). In northwest Spain, the frequency of *L. monocytogenes* in faecal samples was 9.3% (Vilar *et al*., 2007), which is comparatively higher than our findings.

The dissimilarity in findings might be due to a high level of soil contamination and the presence of carrier animals in the dairy herds which ultimately led to listeriosis.

Virulence gene (*hly*A) identification of *L. monocytogenes*

In the present study, the virulence gene (*hly*A) was targeted through PCR for the detection of *L. monocytogenes* in buffaloes suspected of listeriosis, which is in accordance with the results reported in previous studies (Barbuddhe *et al*., 2002; Kalorey *et al.*, 2008). Comparable to this study, it has been described that identifying pathogenic and non-pathogenic strains of *L. monocytogenes* is essential to assess the importance of *L. monocytogenes* in general wellbeing hazards and food safety (Soni *et al*., 2013). An earlier study has also reported, the high occurrence of 98.4% of *hly*A gene among the isolates, whereas other virulence genes *iapA*, *plcA*, and *plcB* were found 85.7%, 73%, and 68.2% (Shakuntala *et al*., 2019). Contrary to this study, virulence cluster genes (*hly*A, *iap*, *plc*A, *act*A, and *prf*A) were targeted for the identification of *L. monocytogenes* from milk samples in Laximan *et al*., (2016) and Usman *et al*., (2016).

Haemato-biochemical analysis

In this study, the haemato-biochemical analysis indicated elevated levels of leucocytes, plasma fibrinogen, alanine transaminase (ALT), aspartate aminotransferase (AST) in listeriosis, while the values of blood urea nitrogen (BUN) and creatinine decreased. These findings are similar to those of the study that reported high levels of leucocytes, AST, Gamma-Glutamyl Transferase (GGT), and decreased values of BUN in bovine listeriosis (Schweizer *et al*., 2006). The slight changes in plasma fibrinogen might be due to inflammation (bacterial/traumatic/chemical) or cell injury. On the contrary, elevated values of WBCs, RBCs, neutrophils and eosinophils have been documented in listeriosis compared to healthy cattle in Turkey (Kennerman, 2014). The haemato-biochemical investigation was not vital for the identification of listeriosis but these can only guide for fluid infusion (Braun *et al*., 2002). In Brazil, no significant correlation was found between bovine listeriosis and hemato-biochemical parameters (Headley *et al*., 2014).

Such variations in findings might be due to different physiological conditions, stage of infection (encephalitis, septicemic, and reproductive) and severity of infection in buffaloes.

It is concluded from this study that *L. monocytogenes* has been recognized in milk and faecal samples of buffaloes in the province of Punjab in Pakistan. The occurrence of *L. monocytogenes* in milk (6.08%) and faecal (4.34%) samples highlighted the possible risk of transmission to humans and animals. The haemato-biochemical parameters specified increased values of WBCs, plasma fibrinogen, ALT and AST in buffaloes affected with listeriosis compared to healthy buffaloes. The information gathered from the current study are in the interest of public safety and may be helpful for dairy farmers and veterinarians.

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Conflict of interest

The authors declare that there is no conflict of interest.

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