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Original Research Article

Virulence Gene Profiling and Serotyping of *Listeria Monocytogenes* from Infertility Cases of Women

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ABSTRACT

Listeria monocytogenes is food-borne bacterial pathogen and listeriosis, a public health important disease which can cause an abortion in pregnant women. *Listeriosis* is emerging as worldwide bacterial zoonotic infection caused by *Listeria monocytogenes* a facultative intracellular bacterium causative agent of listeriosis. In present investigation aim for study of prevalence, virulence gene profiling and serotyping of Listeria *monocytogenes* isolates by employing multiplex and serotyping polymerase chain reaction. Total 113 samples, comprising vaginal (n=66) and endocervical (n=47) swabs were collected aseptically for isolation recovered from infertility cases of women. These isolates were subjected to amplify virulence-associated gens namely *plcA*, *actA*, *hlyA* and *iap* by multiplex and *prf A* gene by singleplex PCR. Serotyping PCR was adopted for further genotypic characterization. Most of the isolates revealed amplification of actA, *hlyA and prf* A gene. Serotyping of recovered 11 Listeria *monocytogenes* isolates grouped in 1/2b and 4b serotype. All strains amplified the *prs* gene fragment (370 bp). Prevalence of Listeria *monocytogenes* was tune to be 9.73%, which was higher than earlier finding. Serotyping PCR study revealed that the serovar 1/2b is the most frequently isolated from food borne outbreaks, although the majority of epidemic listeriosis is caused by type 4b indicating zoonotic and genetic relationships.

Keywords: Listeria, Serotyping, Infertility, Virulence, Zoonotic.

Highlights:

- The isolates belonged to serotypes having epidemic potential.
- 1/2a and 1/2b is the most frequently isolated strain from contaminated foods, the majority of epidemic listeriosis was caused by the type 4b strain
- There is a possibility of transfer of this pathogen from animals to foods of animal origin and thereafter the food chain.
- There may be a link between serotype and virulence potential indicating zoonotic and genetic relationships.

INTRODUCTION

Listeriosis is most important worldwide emerging food borne bacterial zoonotic infection. [(1)] It primarily affects immunocompromised individuals, the elderly and young babies, foetuses in utero. The pathogen represents major challenge in food borne infection and widely distributed in environment. [(2,3)] There are 13 serotypes reported of *L. monocytogenes* only three serotypes (½a, ½b and 4b) have been reported for the

majority of human diseases while serotype 4b causes the majority of human epidemics. [4] Listeriosis accounted for only 7.4% of all reported food-borne infections, although the incidence of the disease is low, the case the fatality rate (17%) and hospitalization rates (92.6%) are high. [5] Mortality rates for invasive listeriosis typically vary from 20 to 40%. [6] It may primarily cause miscarriage in pregnant women. [7] Generally an industrially processed food such as raw meat, fish, milk, milk–related products has been associated with the listeriosis. [8]

The first report of listeria infection in man was confirmed by Nyfeldt in 1929. [9] *Listeria monocytogenes* is comprises in the list of food borne pathogens by World Health Organization (WHO). [10]

The diagnosis of pathogenic *Listeria* spp. and listeric infection should preferably based on virulence markers. [11] The Multiplex PCR analysis was employed for detection of virulence-associated genes. [12] And serotyping method for sub typing. Serogroup classifications are a useful component of strain characterization due to ecotype divisions appears largely congruent with serogroup distinctions. [13] This rapid and reproducible technique can be easily adapted to different laboratories. [14] Comprehensive review of reported sporadic cases of human and animal listeriosis suggested insufficiency of available epidemiological data to assess degree of true infection in humans and animals. The disease remains mostly undiagnosed and under–reported due to unavailability of a suitable diagnostic assay. [15] Hence considering the unavailability of suggestive data present study was aimed to investigate the prevalence, serogroup and genetic diversity among Listeria *monocytogenes* isolated from infertility cases and its characterization using a PCR for various virulence genes as well as to determine the serovar through serotyping and molecular typing using Multiplex PCR method.

MATERIALS AND METHODS

Collection of clinical samples: Total 113 samples, comprising from vaginal(n=66) and endocervical (n=47) swabs from infertility cases in women were collected aseptically and quickly transported to laboratory under chilled condition and further processed for isolation and identification.

Ethical approval: The necessary ethical clearance was obtained from institutional ethics committee. The ethical permission was taken for collection and processing of human clinical samples.

Isolation and identification of *Listeria monocytogenes*: Isolation of listeriae from the endocervical and vaginal swabs followed the method of the US Department of Agriculture (USDA) described by McClain & Lee (1998) [16] with some modifications. All Samples were processed for selective isolation of *Listeria monocytogenes* by two-step enrichment in University of Vermont (UVM) broth -I and II. Vaginal and endocervical swabs were aseptically enriched into 50 ml of University of Vermont Medium (UVM)–1 supplemented with acriflavin and nalidixic acid and incubated at 37°C for 18–24 h. Further enrichment of the samples was carried out by inoculating 0.1 ml of UVM–1 to 10 ml of UVM–2 broth. Inoculated UVM–2 broth was incubated further for 24 h at 37°C. A loopful of enriched UVM–2 broth was streaked directly on Dominguez-Rodriguez isolation agar (DRIA) agar for selective isolation of listerial colonies. The inoculated agar plates were incubated 35°C for 48-72 hrs. The isolated pinpoint grayish–green colonies surrounded by black zone of esculin hydrolysis were presumed as *Listeria monocytogenes* and further characterised as per the method describe. [17]

Confirmation of the isolates: Morphologically typical colonies were verified by Gram's staining and were characterized biochemically. [18] Genomic DNA was prepared as per standard Phenol: Chloroform method described by Donnelly (2002). [19] The DNA was quantified using spectrophotometer.

Genotypic Characterization: Standardized multiplex PCR assay for all the four primer sets namely *plcA, hly A, actA* and *iap* and singleplex PCR was employed for detection of individual virulence cluster gene namely *prf* A by following the methodology described by Notermans *et al.* [11], with suitable modification by Warke *et al.* [17]

The reaction volume was optimized as: Master mix 12.5µl, 10µM of forward and reverse primer of each set final concentration (0.1 µM each) 1 unit Taq DNA polymerase, 2.5 µl cell lysate and sterilized milliq water to make up the reaction volume.

The cycling condition including an initial denaturation at 95°C for 10 min. followed by 35 cycles each of 15 second denaturation at 95°C, 30 second annealing at 60° C and 1 min. 30 second extension at 72° C. It was followed by final extension of 10 min. at 72° C and 10 min. hold at 4°C after the reaction, PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

The primers for detection of phosphatidylinositol Phospholipase C gene (*plc* A), actin gene (*act* A) , Haemolysin gene (*hly*), p60 gene (*iap*) *and prf A* Master Regulator of virulence gene of *L. monocytogenes* used in this study were synthesized by Sigma Aldrich (Banglore). The details of the primer sequences are shown in Table.

Serotyping: The primers for detection of *L. monocytogenes* 0737 gene (*lmo0737*), transcriptional regulator gene (*ORF2819*), secreted protein gene (*ORF2110*) and phosphoribosyl pyrophosphate synthetase gene (*prs*) of *L. monocytogenes* used in this study were synthesized from Sigma Aldrich. [20]

The PCR was set for 50µl reaction volume for the detection of *L. monocytogenes* serotype by PCR, conditions were optimized by using varying concentrations of molecular biologicals (Sigma Aldrich), gradient annealing temperature and number of cycles for amplification of target genes.

PCR was optimized and cycling conditions included an initial denaturation at 94°C for 5 min. followed by 35 cycles each of denaturation at 94° C for 30 seconds, annealing at 54° C for 1 min. 15 seconds and extension at 72° C for 1 min. 15 seconds. It was followed by final extension at 72° C for 10 min. and hold at 4° C for 30 min. After the reaction, PCR products were kept at –20°C until further analysis by agarose gel electrophoresis.

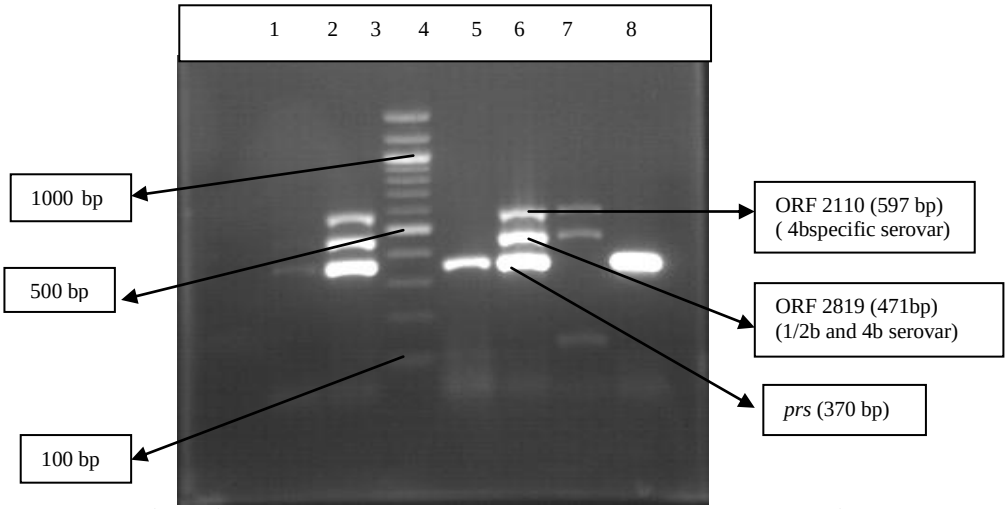
Table 1: Detail of Primer amplification of virulence associate genes of *L.monocytogenes*.

Sr.No.	Primers	Primer sequence		Amplicon size	Reference
1	*plcA*	F	5`-CTG CTT GAG CGT TCA TGT CTC ATC CCC C-3`	1484 bp	Notermans *et.al.*,(1991)
		R	5`-CAT GGG TTT CAC TCT CCT TCT AC-3`		
2	*act A*	F	5`-CGC CGC GGA AAT TAA AAA AAG A -3`	839 bp	Suarez and Vazquez Boland (2001)
		R	5`-ACG AAG GAA CCG GGC TGC TAG -3		
3	*hly A*	F	5`-GCA GTT GCA AGC GCT TGG AGT GAA-3`	456bp	Paziak-Domanska *et.al(1999)*
		R	5`-GCA ACG TAT CCT CCA GAG TGA TCG-3`		
4	*Iap*	F	5`-ACA AGC TGC ACC TGT TGC AG-3`	131 bp	Furrer *et al.*,(1991)
		R	5`-TGA CAG CGT GTG TAG TAG CA-3`		
5	*prf A*	F	5`-CTG TTG GAG CTC TTC TTG GGT GAAGCA ATC G -3`	1060 bp	Notermans *et.al.*,(1991)
		R	5`-AGC AAC CTC GGT ACC ATA TAC TAA CTC -3`		

Table 2: Details of primers for amplification of target genes of *Listeria monocytogenes* employed in serotyping PCR

Sr.No	Primers	Primer sequence	Amplicon size	Serovar specificity	Protein encoded by the target gene
1	*Imo0737*	F-5'-AGG GCT TCA AGG ACT TAC CC-3' R-5'-ACG ATT TCT GCT TGC CAT TC -3'	691bp	1/2a	Unknown, no similarity
2	*ORF2819*	F-5'-AGC AAA ATG CCA AAA CTC GT -3' R-5'-CAT CAC TAA AGC CTC CCA TTG -3'	471bp	1/2b and 4b	Putative transcriptional regulator
3	*ORF2110*	F-5'-AGT GGA CAA TTG ATT GGT GAA -3' R-5'-CAT CCA TCC CTT ACT TTG GAC -3'	597bp	4b	Putative secreted protein
4	*Prs*	F-5'-GCT GAA GAG ATT GCG AAA GAA G -3' R-5'CAA AGA AAC CTT GGA TTT GCG G -3'	370bp	All *Listeria spp.*	Putative phosphoribosyl pyrophosphate synthetase

Serotyping PCR



Title: Agarose gel electrophoresis of DNA fragment generated by multiplex PCR assay with the serotyping isolates from Human.
Lane 1 :- Negative control.
Lane 2 :- *L. monocytogenes* strain isolate of serovar 1/2b.
Lane 3 :- *L. monocytogenes* strain isolate of serovar 1/2b and 4b and *prs*
Lane 4 :- 100bp DNA ladder.
Lane 5 :- *L. monocytogenes* strain isolate of *prs*.
Lane 6 :- *L. monocytogenes* strain isolate from human of serovar 1/2b, 4b and *prs*
Lane 7 :- *L. monocytogenes* strain isolate of serovar 1/2b and 4b.
Lane 8 :- *L. monocytogenes* strain isolate of *prs*.

RESULT

Prevalence of *Listeria monocytogenes:* A total of 113 samples from vaginal and endocervical swabs of human origin were subjected to screen for listeriae out of them 11 (9.73%) were found to be positive for *Listeria monocytogenes.* Details of recovered isolates of *Listeria monocytogenes* is presented in Table.

Table3: Frequency of Listeria monocytogenes recovered cases of infertility in women.

Sample	No. Samples Investigated	No.and % of *L.monocytogenes* isolated
a)Vaginal swab	66	8(12.12)
b)Endocervical swab	47	3(6.38)
Total	113	11(9.73)

The genomic DNA samples of recovered isolates were analysed by multiplex PCR assay with virulence-associated genes primer sets namely, *plc*A, *act*A, *hly*A and *iap* and singleplex with *prfA*. Each primer set was found to be specific to the corresponding gene amplifying the DNA fragments of the expected size.

PCR analysis of all recovered isolates revealed the variable genotypic patterns for virulence-associated genes namely *plc A*, *act A*, *hly A iap*. and *prf A*. PCR analysis confirmed that *act* A, *hly* A and *prf A* genes amplified by four *Listeria monocytogenes* isolates. Two virulence-associated genes namely *act* A, and hly A *were* detected in six and hly gene in a isolate.

Table 4: Frequency of occurrence of virulence-associated genes in *Listeria monocytogenes* isolates recovered.

Source	No. of isolates	Virulence-associated genes detected				
		*plc*A(1484bp)	*act*A(839bp)	*hly*A(456bp)	*iap*(131bp)	*prfA*(1060bp)
Human	4	-	+	+	-	+
	6	-	+	+	-	-
	1	-	-	+	-	-

+ = Positive, - = Negative

Serotyping:

Serotyping PCR study revealed that 1/2b and 4b serovar detected in most of the isolates while 1/2b sereovar in one isolate. All strains amplified the *prs* gene fragment (370 bp).

Table 5: Frequency of occurance of serotypes in *Listeria monocytogenes* isolates recovered

Sr.No.	Source	Isolates	Serotype
1	Vaginal	G7a	*prs,1/2b,4b*
2	Vaginal	G20a	*prs,1/2b,4b*
3	Vaginal	G27a	*prs,1/2b,4b*
4	Vaginal	G36a	*prs,1/2b,4b*
5	Vaginal	G40a	*Prs*
6	Vaginal	G41a	*prs,1/2b*
7	Endocervical	G41b	*prs,1/2b,4b*
8	Vaginal	G42a	*prs,1/2b,4b*
9	Vaginal	G43a	*prs,1/2b,4b*
10	Vaginal	G44a	*prs,1/2b,4b*
11	Endocervical	G44b	*prs,1/2b,4b*

DISCUSSION

Globally, listeriosis has been reported to occur either in sporadic and epidemic form; however there are certain Asian countries where the disease has been under reported due to lack of active surveillance systems. [21] In India there was no case report of Listeria *monocytogenes* until 1973. A study was undertaken in that same year. [22] Due to lack of awareness, burden of other traditional diseases, rare expertise and poor reporting, the incidence of listeriosis is unknown. [23] Systematic and coordinated studies needed to understand the listeriosis to estimate the prevalence of *Listeria. monocytogenes* in different habitats, occurrence of Listeriosis in humans and animals, their concordance of occurrence and actual disease.

Although the species Listeria *monocytogenes* has long been known as genetically diverse with strain showing differences in their virulence potential. Detailed knowledge of strain diversity and evolution is still lacking. Genotypic characterization of *Listeria monocytogenes* isolates from human clinical cases and reveals present of *hly* A, *act*A and *iap* genes while none of isolate was positive for *plc* gene24 [24] In our study, genes in three different combinations were reported. Most of isolates were positive for *actA, hly A* and *prf A*. Whereas the *actA* gene was detected in all of 12 (100%) positive samples. [25]

There are few reports on the incidence of *L. monocytogenes* in clinical samples in India. Therefore present study was performed to determine the prevalence of *Listeria monocytogenes* .The recovery of *L. monocytogenes* isolated from vaginal and endocervical swabs among infertility cases in women was found to be 9.73%, which was higher than the earlier reporting on the isolation of *L. monocytogenes* from cervical swab in which 100 cases presenting with bad obstetric history only three cases were found to be positive. [21] The earlier studies from India indicated that the incidence of *L. monocytogenes* in pregnant women with bad obstetric history ranging between 1.34 and 4 % [26] and one of 958 listeriosis cases studied. [27] *L. monocytogenes* isolated from the cervix from 150 patients with bad obstetric history i.e. past history of abortions, miscarriage, stillbirth or neonatal deaths only 14 % were found to be positive. [28] The occurrence of *L. monocytogenes* in women with bad obstetrics cases was found to be 1.94 %. [29] *Listeria monocytogenes* was isolated from cerebrospinal fluid (CSF) and blood of the baby, and also from the genital tract of the mother. [30] A total of 5 (7.6%) *L. monocytogenes* isolates were identified by PCR in different clinical samples, [31] while in present study prevalence was found to be 9.37 % which was higher than

earlier finding. This might be due to difference in geographic location, nature and time of sample collection. Considering the above described result difference reported among the studies due to differences in the population under study including race, culture, geographical region, nutrition and laboratorial diagnosis methods.

Listeria monocytogenes isolated from 96 vaginal swabs from patient with spontaneous abortion reveal 7(7.2%) isolates by microbiological and biochemical analysis which was quite similar with our results. [(25)] About one-third of reported human listeriosis cases happen during pregnancy, which may result in spontaneous abortion. The finding are in agreement with the earlier reports on isolation of *L. monocytogenes* from 9 of 100 [(32)] and 22 of 428 patients with bad obstetric history. [(33)]

Silk *et al.* [(3)] reported an incidence rate of listeriosis of 0.27 cases per 100,000 general US populations Risk groups of 100 samples collected from women with spontaneous abortions, 9 isolates were identified as *L. monocytogenes.* Contamination rate of samples in women with spontaneous abortion was 2, 3, 3, 1 and 0% for rectal swab, vaginal swab, placental bit, urine and blood respectively. [(32)]

Highlighting the role of L. *monocytogenes* as a causative agent of human abortions. Three isolates of *L. monocytogenes* was recovered from placental tissues of women with spontaneous abortion. Two of these three women had previous bad obstetric history. Infection with *L. monocytogenes* in pregnant women sometimes giving rise to urinary tract infection symptoms. [(34)] 311 specimens were collected from 107 pregnant women including urine, blood, and placenta and cervix swab. In total, from 107 pregnant women, in 11 specimens (10.28%) *hly A* genome were isolated. Among positive specimens, 63.6% of the specimen belonged to cervix, 36.4% to urine and 18% to urine and cervix respectively. [(34)] Listeriosis cases reported through the U.S. Listeria Initiative during 2004–2007. Cases were classified as pregnancy-associated if illness occurred in a pregnant woman or an infant aged <28 days. Out of 758 reported *Listeria* cases, 128 (16·9%) were pregnancy-associated. Maternal infection resulted in four neonatal deaths and 26 (20·3%) fetal losses. Invasive illnesses in newborns (*n*=85) were meningitis (32·9%) and sepsis (36·5%). [(35)] Out of 118 isolates from non pregnancy associated cases, 114 (96.6%) were isolated from blood and/or CSF. Cases having no underlying medical conditions were 12 (11.6%) out of 103 with a known clinical history. The all-cause fatality rate of 83 cases with a known survival outcome was 25.3%. [(36)] Indeed, invasive listeriosis cases notified to the local health authorities increased from 35 in 2006 (incidence rate 0.37 per 100,000 population) to 70 in 2010 (incidence rate 0.74 per 100,000 population). [(37)] This increase mainly affected non-pregnant subjects, in accordance with previous reports from other European countries using PCR, 12.5% isolates of *L. monocytogenes* were recovered from 96 human abortion cases; but in culture method 7.2% isolates of *L. monocytogenes* were detecte. [(38,39)]

Serotyping PCR

The serovar 1/2a and 4b characterized by the specific markers *Imo0737* and *ORF2110*, respectively. The marker *Imo0737* produces 691bp fragment in 1/2a isolates and at least 95% of strains isolated from contaminated food and infected patients were of serotypes 1/2a, 1/2b, 1/2c and 4b. While serovar 1/2a is the frequently isolated strain from contaminated foods, the majority of epidemic listeriosis was caused by the type 4b strain. [(40)]

Parihar *et al.*, [(24)] have serotyped 20 isolates of *Listeria monocytogenes* from

India and all belong to serovars 4b. *Listeria monocytogenes* isolated from cerebrospinal fluid of premature newborn was identified as the 4b serovar after analysis using the Multiplex –PCR technique. [(41)] In agreement with our serotyping results 10 (90.90%) belong to serovar 1/2b and 9(81.81%) 1/2b and 4b group. Presence of such outbreak–associated serotype is of public health concern. In accordance with our result, the genotypic analysis of 17 *Listeria monocytogenes* isolates recovered during 2006-2009 have been studied from human in India. Multiplex PCR based serotyping assay showed 88.24 % (15/17) of the strain belonging to the serovar group 4b,4d,4e, and 11.76 % (2/17) to the serovar group 1/2b,3b. [(42)] In Andalusia (Southern Spain),154 isolates from human listeriosis cases in the period 2005-2009 were grouped into serotypes 4b (94 ,61%),1/2 b (30,19 %) 1/2a (27,18 %)and1/2 c(3,2%) by multiplex PCR serogrouping. [(43)] Among the 13 serovars of *Listeria monocytogenes, serovars* 1/2a and 4b accounts for most of the major listeriosis outbreaks and interestingly our study showed that all identified epidemic clones of *Listeria monocytogenes* belongs to these two serotypes. Human listeriosis cases, in Sweden, were subtyped as eighteen percent of the human isolates (2010) belonged to serotype 4b. [(44)] Pregnancy-associated cases were equally associated with serotype 4b (seven cases) and 1/2a (seven cases), with only one case caused by serotype 1/2b. Serotypes 1/2a and 4b comprised 52.2% and 38.8% of isolates, respectively. [(35)] One of the isolate was of *prs* serogroup. The *prs* gene, specific for strains of the genus *Listeria*, was targeted for an internal amplification control. [(45)]

CONCLUSION

The present study prevalence of serovar in clinical samples from infertility cases was confirmed by virulence gene profiling and serotyping. The prevalence of *Listeria monocytogenes* in cases of infertility in women was found to be 9.37% indicating important role in infertility. Genotypic characterization employing PCR confirmed amplification of multiple virulence associated genes viz. *act* A, *hly* A and *prf A*. Serotyping PCR revealed presence of 1/2b and 4b serotype in most of isolates. Serotype 1/2b is the most frequently isolated food borne pathogen, although the majority of epidemic listeriosis is caused by type 4b. The isolates belonged to serotypes having epidemic potential. *Listeria monocytogenes* have significant potential to transfer from animals to foods of animal origin and thereafter the food chain. It has been suggested that there may be a link between serotype and virulence potential. As each lineage is genetically heterogeneous a precise delineation of *Listeria monocytogenes* clones needful to determine which one mostly contributes to human or animal infection. Biological characteristics of the clones and evolution of molecular mechanism by which they cause disease and this knowledge would set a landmark for further studies. The epidemiological studies would help to understand the sources of infection and their risk assessment, routes of transmission, clinical forms and allowed them for better management of the listerial infection. In conclusion this study in terms of microbiological criteria reflects food safety requires the reliable detection clones of zoonotic pathogens such as *Listeria monocytogenes along* the entire food chain by appropriate methods to track the source of infection.

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