

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.805.233>

Virulence Profiling of *Listeria monocytogenes* Isolated from Different Sources

S.R. Thorat¹, D.B. Rawool⁴, P.M. Sonkusale¹, S.R. Warke²,
S.P. Choudhari³ and N.V. Kurkure^{1*}

¹Department of Veterinary Pathology, ²Department of Veterinary Microbiology,
³Veterinary Public Health, Nagpur Veterinary College, Nagpur-440006 and
⁴Division of Veterinary Public Health, Indian Veterinary Research Institute,
Izatnagar 243 122, India

*Corresponding author

ABSTRACT

Keywords

Listeria monocytogenes,
Virulence profiling,
Multiplex-PCR

Article Info

Accepted:
17 April 2019
Available Online:
10 May 2019

A total of 23 putative *Listeria* isolates obtained from different sources, viz. food, animal, human, caterpillar and mosquito were screened for presence of the virulence factors by multiplex polymerase chain reaction (mPCR). Multiplex polymerase chain reaction for the amplification of *isp* and *prs* genes was employed for genus and species identification, while virulence profiling was employed by amplification of *plcA*, *hlyA*, *actA*, *prfA*, *inlC*, *inlJ*, *luxS* and *fla* genes. All strains harbours virulence genes *plcA*, *hlyA*, *actA*, *prfA*, *inlC*, *inlJ*, *luxS* and *fla*. Finally this study validated mPCR in the analysis and rapid detection and virulence profiling of *L. monocytogenes*. Irrespective of species of origin all the virulence genes are expressed by all isolates coequally.

Introduction

Listeria monocytogenes is a gram-positive bacterial pathogen that causes septicaemia, encephalitis, meningitis and gastroenteritis, particularly in children, immunosuppressed individuals and elder's; it also causes miscarriage in pregnant women (Radoshevich and Cossart, 2017) and in animals (Eruteya *et al.*, 2014; Pournajaf *et al.*, 2016). The bacterium is considered as a ubiquitous in nature and can be isolated from the

environmental sources, including surface water, soil, sewage, vegetables, milk, milk product and food-processing plants (Pournajaf *et al.*, 2016), even from fish and fishery products (Jallewar *et al.*, 2008).

The genus *Listeria* contains, 17 species and two subspecies among those, two species, *Listeria monocytogenes* and *Listeria ivanovii* are pathogenic (Liu *et al.*, 2007; Radoshevich and Cossart 2017, Doijad *et al.*, 2018). All *Listeria spp.* are rod-shaped facultative

anaerobes that can grow at low temperatures and are quite resistant to environmental stresses, such as low pH and high salt concentrations, that features make *L. monocytogenes* a major concern for the food industry (Liu *et al.*, 2007 and Bucur *et al.*, 2018).

Pathogenesis of *Listeria monocytogenes* is facilitated by the action of a set of virulence genes including hemolysin gene (*hlyA*), regulatory gene (*prfA*), Phosphatidylinositol Phospholipase C gene (*plcA*), Actin gene (*actA*) and *luxS*, *fla* located in a *Listeria* pathogenicity island-1 (LIPI-1) and other virulence factors located outside LIPI-1 such as internalins, cell-wall-associated proteins *internalin A (InlA)* and *internalin B (InlB)*, encoded by genes located within the *inlAB (internalin)* operon (Gregory *et al.*, 1996). Liu *et al.*, (2007) mentioned that internalin A (*InlA*) and internalin B (*InlB*) are species-specific surface proteins that play essential roles in *Listerial* entry into host cells, while *InlJ* (or *lmo2821*) gene is responsible for passage of *L. monocytogenes* through the intestinal barrier and can be used for evaluating virulence of *L. monocytogenes* (Pournajaf *et al.*, 2016).

Listeria monocytogenes isolation on selective enrichment media followed by biochemical studies is strenuous and requires sample time for detection from any specimen. Detection of virulent genes by multiplex polymerase chain reaction (mPCR) will be useful to decrease the time and labour required for diagnosis and will be useful in a large-scale investigation for detecting virulent strain of *L. monocytogenes* species. It has been observed since long time that numerous death and major illness in animals and humans are reported due to naturally virulent strains of *L. monocytogenes* (Rawool *et al.*, 2016; Pournajaf *et al.*, 2016). Hence, the present study is aimed to standardize multiplex PCR for the

simultaneous detection of various virulent genes in the *L. monocytogenes* isolates from different sources.

Materials and Methods

Isolates

Total twenty-three putative isolates of *L. monocytogenes*, out of which 6 isolates from human clinical cases (viz., human abortion and human aborted foetus), 8 from animal clinical cases (viz., bovine mastitis, caprine abortion, caprine aborted foetus, ovine abortion and ovine aborted foetus) and 7 from food (viz., seafood, chevon, meat, poultry meat, paneer and milk) 1 from each mosquito and caterpillar previously isolated and maintained at the department were included in the study.

Isolation and identification of *Listeria*

Briefly, all the lyophilized vials were handled aseptically and mixed with the 10ml University of Vermont-1 (UVM-1, Himedia Labs, Mumbai, India) and incubated at 30°C for 18 hrs. The enriched UVM-1 inoculum (0.1 ml) was then transferred to University of Vermont-2 (UVM-2) (Himedia, Mumbai, India) and again incubated overnight at 30°C for 18 hrs. A loopful of inoculum from enriched UVM-2 was streaked directly on Dominguez–Rodriguez isolation agar [DRIA consisting of (g l⁻¹) proteose peptone 3 (Difco, Becton Dickinson, Meylan, France)]; tryptone 3 (Himedia Labs, Mumbai, India); peptone 3 (Himedia Labs, Mumbai, India); ferric ammonium citrate 1 (Himedia Labs, Mumbai, India); aesculin 1 (Sigma); Sodium chloride 5 (Sigma); di-sodium hydrogen phosphate 12 (Merck); nalidixic acid 0.04 (Sigma); acriflavine 0.006 (Sigma); agar 15 (Sisco Research Labs, Mumbai, India); defibrinated sheep blood (50 ml) and plates were incubated at 37°C for 48 hrs. The typical

greenish yellow glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis were presumptively identified as *Listeriae*. The presumed colonies of *Listeria* (at least 3/plate) were further confirmed by biochemical tests.

Extraction of genomic DNA

A single colony was inoculated in 5 ml (BHI) broth and incubated at 37°C for 12-18 hrs with aeration. This fresh overnight grown bacterial culture was used later for genomic DNA isolation by using commercially available Himedia Multi-sample DNA Purification Kit (MB554-50PR) and quantified using NANODROP-1000 (Thermo-scientific USA), by measuring absorbance at 260/280 nm wavelength.

Polymerase chain reaction for detection of virulence associated genes

All the putative *L. monocytogenes* were confirmed by amplification of *prs* and *isp* genes and assessed for their presence of associated genes viz., *plcA*, *hlyA*, *actA*, *prfA*, *inlC*, *inlJ*, *luxS* and *fla* by multiplex Polymerase chain reaction (mPCR) as per the protocol described by Liu *et al.*, (2007); Lotfollahi *et al.*, (2014); Rawool *et al.*, (2016) and Warke *et al.*, (2017) with certain suitable modification. Briefly, the mPCR was standardized employing the standard pathogenic strains of *L. monocytogenes* EGD-e and MTCC, the *Enterococcus fecalis* available in the department was used as negative control. Subsequently, the test isolates were screened by the standardized mPCR for the detection of aforesaid virulence associated genes.

Standardization of PCR protocol

In brief, the multiplex PCR assay was standardized in two sets., In first set

identification of genus and species of *L. monocytogenes* was carried out by amplification of *prs* and *isp* gene, wherein a 25µl reaction volume was prepared; containing 2.5 µl of 10X PCR buffer, 2µl of 10mM dNTP mix, 2µl of 50mM MgCl₂ and 1µl of each primer sets (*prs* and *isp*) at 10µM for each primer set, 1 unit of Taq DNA polymerase, 20ng of DNA and sterilized nuclease free water to make up the final reaction volume 25µl. The DNA amplification reaction was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid. The cycling conditions for PCR included initial step of denaturation of DNA at 95°C for 5 min. followed by 40 cycles each of 30 sec denaturation at 95°C, 1 min annealing at 53°C and 2 min extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The PCR products were stored at -20°C for future analysis by agarose gel electrophoresis.

Second set was standardize for the detection of virulence associated genes sub-grouped into 3 subsets; subset-1: consisted of *hlyA*, *actA* and *plcA* primers; subset-2: *prfA*, *inlC*, *inlJ* primers and subset-3: *luxS* and *fla*. A total of 25µl reaction volume was prepared for each set, which comprised of 2.5µl of 10X PCR buffer, 2µl of 10mM dNTP mix, 3µl of 50mM MgCl₂ and 1µl of each primer sets at 10µM for each primer set, 1 unit of Taq DNA polymerase, 20ng of DNA and sterilized nuclease free water to make up the final reaction volume 25µl. The DNA amplification reaction was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 5 min. followed by 35 cycles each of 15 sec denaturation at 94°C, 30 sec annealing at 57.7°C and 30 sec extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The resultant PCR

products were further analysed by agarose gel electrophoresis (1.5% low melting temperature agarose L), stained with ethidium bromide (0.5 µg/ml) and visualized by UV transilluminator and photographed in gel documentation system (Syngene, USA).

Results and Discussion

In total, 23 putative *L. monocytogenes* isolates were used in the present study, out of which 6 isolates from human clinical cases (viz., human abortion and human aborted foetus), 7 from food (viz., seafood, chevon, meat, poultry meat, paneer and milk) and 8 from animal clinical cases (viz., bovine mastitis, caprine abortion, caprine aborted foetus, ovine abortion and ovine aborted foetus). These results highlight the role of *L. monocytogenes* in spontaneous abortions, the results of animal and human abortion are in agreement with previous reports by Rocha *et al.*, (2017); Pournajaf *et al.*, (2016) while, 1 samples from each invertebrate host like mosquito and caterpillar have been investigated for the first time.

The molecular detection has facilitated the identification and characterization of major virulence associated genes and proteins in *L. monocytogenes*. The mPCR is most frequently employed for screening of samples, because it not only saves time and labor but also it is economical and have the advantage of screening large number of samples. As many of the pathogenic genes are commonly shared by the different pathogens. Detection of *L. monocytogenes*, targeting single virulence-associated gene by PCR is neither sufficient to identify the isolate nor to reveal its true pathogenic potential (Rawool *et al.*, 2016; Pournajaf *et al.*, (2016).

The genus and species nature of *L. monocytogenes* was demonstrated by *prs* and *is p* primers. It was particularly noteworthy

that the genus and species identity of 23 *L. monocytogenes* strains was validated through the formation of 844bp and 713bp band size by all the *L. monocytogenes* isolates (Fig. 1), these results were in correspondence with the Rawool *et al.*, (2016); Chen *et al.*, (2017).

Moreover, detection of *hlyA* and *plcA* gene by mPCR in *L. monocytogenes* isolates is not sufficient to elucidate the true pathogenic potential, because both the genes are regulated by a key regulatory gene i.e., *prfA* (Shakuntala *et al.*, 2006; Kaur *et al.*, 2007; Rawool *et al.*, 2007; Aurora *et al.*, 2008). In addition, other genes such as *actA*, *internalins* and *luxS*, *fla* virulent associated genes do play an essential role in pathogenesis of this bug. Therefore, mPCR targeting eight virulence-related genes was employed through three subsets PCR tube reactions to assess the virulence potential of *L. monocytogenes*.

All the isolates of *Listeria* were further characterized by mPCR for subset-1 virulence associated genes *hly*, *actA* and *plcA*. The PCR amplification lead to product size of 456bp and 839bp and 954bp respectively (Fig. 2). Second subset of virulence primer consisted of *inlC*, *inlJ* and *prfA* genes, the result of this investigation showed the genomic DNA of isolated *L. monocytogenes* to form the expected band of 517bp, 238bp and 1060bp (Fig. 3). Further, the third subset of primers i.e; *luxS*, *fla* were characterized by mPCR, which obtain PCR product of 208bp, 363bp (Fig. 4). All the *L. monocytogenes* isolates amplified all the targeted virulence associated genes respectively. The results of the present investigation revealed that all the 23 *L. monocytogenes* isolates amplified all the targeted virulence associated genes indicating that the *L. monocytogenes* isolates are pathogenic in nature irrespective of their source of origin. Similar findings have also been reported in several studies which suggest that *L. monocytogenes* isolates harbouring

crucial associated genes such as *prfA*, *plcA* and *hlyA* are pathogenic (Shakuntala *et al.*, 2006; Rawool *et al.*, 2007; Shouk *et al.*, 2013) (Table 1).

Table.1 Primer sequences for amplification of virulence genes of *Listeria monocytogenes*

Name of Primer	Sequence 5'---3'	Product Size (bp)	References
<i>inlC</i>	F 5'- AATTCCCACAGGACACAACC-3' R 5'- CGGGAATGCAATTTTTCTACTA-3'	517	Liu <i>et al.</i> , 2007
<i>inlJ</i>	F 5'- TGTAACCCCGCTTACACAGTT-3' R 5'- AGCGGCTTGGCAGTCTAATA-3'	238	
<i>hlyA</i>	F 5'- GCAGTTGCAAGCGCTTGGAGTGAA -3' R 5'- GCAACGTATCCTCCAGAGTGATCG -3'	456	Rawool <i>et al.</i> , (2007)
<i>prfA</i>	F 5'- CTGTTGGAGCTCTTCTTGGTGAAGCAATCG -3' R 5'- AGCAACCTCGGTACCATATACTAACTC -3'	1060	
<i>actA</i>	F 5'- CGCCGCGGAAATTAATAAAAGA -3' R 5'- ACGAAGGAACCGGGCTGCTAG -3'	839	
<i>plcA</i>	F 5'- CTGCTTGAGCGTTCATGTCTCATCCCC -3' R 5'- CATGGGTTTCACTCTCCTTCTAC -3'	954	Lotfollahi <i>et al.</i> , (2014)
<i>luxS</i> (Imo1288)	F 5'- GGA AAT GCC AGC GCT ACA CTC TTT-3' R 5'- ATT GCA TGC AGG AACTTC TGT CGC-3'	208	Warke <i>et al.</i> , (2017)
<i>fla</i> (Imo0689)	F 5'- GCG CAA GAA CGT TTA GCA TCT GGT-3' R 5'- TTG AGT AGC AGC ACC TGT AGC AGT-3'	363	
<i>Isp</i>	F 5'- TGCAGCGAATGCTCTTAGTG-3' R 5'- AGCCAAGCACGGCTACTTTA-3'	713	Rawool <i>et al.</i> , (2016)
<i>Prs</i>	F 5'- AGCTGAAGATTCCGAAAGA-3' R 5'- TTCACCAAGAAGAGCTGCAA-3'	844	

Fig.1 Multiplex PCR for *isp*(713 bp) and *prs* (844 bp) gene, revealing detection of genus *Listeria* and species *L. monocytogenes* in the recovered *L. monocytogenes* isolates. (M- Ladder, 1-8 are number of samples, P-Positive control and N- negative control)

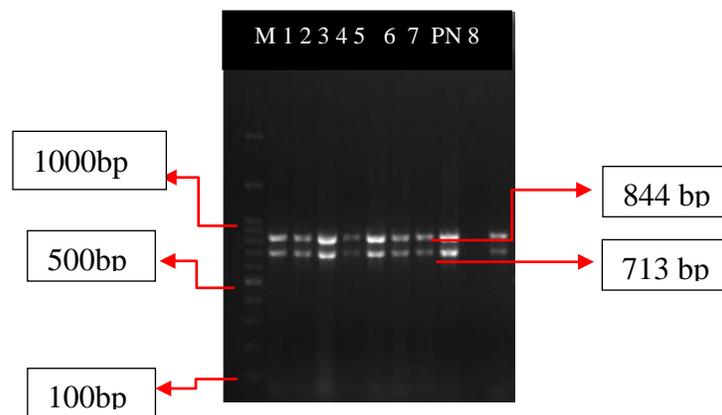


Fig.2 Multiplex PCR revealing detection of virulence associated genes *actA* (839 bp), *hlyA* (456 bp) and *plcA* (954 bp) in *L. monocytogenes* isolates. (M- Ladder, 1-8 are number of samples, P- Positive control and N- negative control)

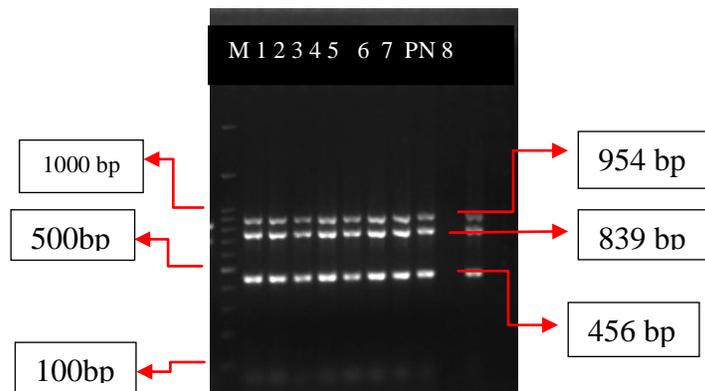


Fig.3 Multiplex PCR revealing detection of virulence associated genes *prfA* (1060 bp), *inlC* (517 bp) and *inlJ* (238 bp) in *L. monocytogenes* isolates. (1-8 are number of samples, N- negative control, P-Positive control and M- Ladder)

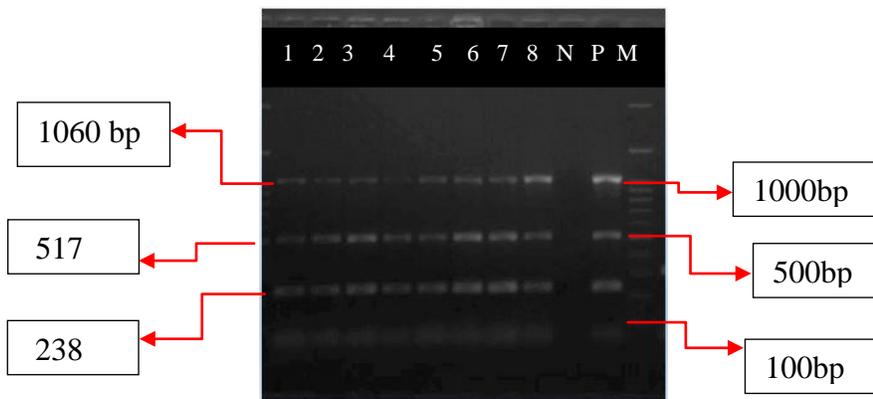
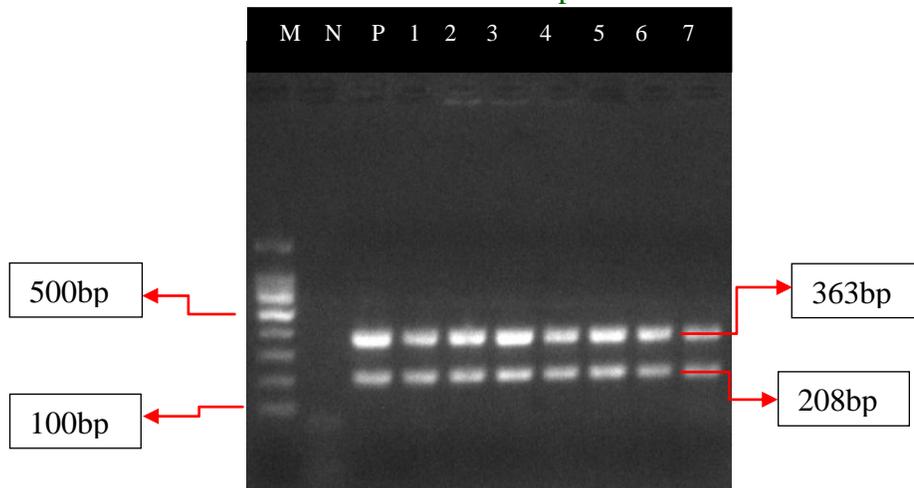


Fig.4 Multiplex PCR revealing detection of virulence associated genes *LuxS* (208 bp), *fla* (363 bp) in *L.monocytogenes* isolates (M- Ladder, N- negative control, P-Positive control and 1-7 are number of samples)



However, several workers viz., Pournajaf *et al.*, (2016), Eruteya *et al.*, (2014), Warke *et al.*, (2017); reported that many of pathogenic genes were missing in *L. monocytogenes* isolated from different sources. Unique finding of the present study is that in spite of the different source of the bacteria all the isolates were positive for all the virulence associated genes tested with similar intensity.

Acknowledgements

Authors are thankful to Associate Dean, Nagpur Veterinary College, MAFSU, Nagpur, Maharashtra and ICAR-Indian Veterinary Research Institute, Izatnagar for providing necessary help and facilities for the successful completion of this work.

References

- Aurora, R., Prakash, A., Prakash, S., Rawool, D.B and Barbuddhe, S.B. 2008. Comparison of PI-PLC based assays and PCR along with in-vivo pathogenicity tests for rapid detection of pathogenic *Listeria monocytogenes*. *Food Control* 19:641–647.
- Bucur, F.I., Grigore-Gurgu, L., Crauwels, P., Riedel, C.U and Nicolau, A.I. 2018. Resistance of *Listeria monocytogenes* to Stress Conditions Encountered in Food and Food Processing Environments 9: 1-18.
- Chen, J.Q., Healey, S., Regan, P., Laksanalamai, P and Hu, Z. 2017. PCR-based methodologies for detection and characterization of *Listeria monocytogenes* and *Listeria ivanovii* in foods and environmental sources. *Food Science and Human Wellness* 6: 39–59.
- Doijad, S.P., Poharkar, K.V., Kale, S.B., Kerkar, S., Kalorey, D.R., Kurkure, N.V., Rawool, D.B., Malik, S.V.S., Ahmad, R.Y., Hudel, M., Chaudhari, S.P., Birte, A., Jorg, O., Weigel, M., Hain, T., Barbuddhe, S.B and Chakraborty, T. 2018. *Listeria goaensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 68: 3285-3291.
- Eruteya, O.C and Odunfa, S.A. 2014. Species and virulence determination of *Listeria monocytogenes* isolated from goat meat in Port Harcourt, Nigeria. *Int.J.Curr.Microbiol.App.Sci* 3(5): 32-39.
- Gregory, S.H, Sagnimeni, A.J and Wing, E.J. 1996. Expression of the inlAB operon by *Listeria monocytogenes* is not required for entry into hepatic cells in vivo. *Infect Immun* 64(10): 3983–3986.
- Jallewar, P.K., Kalorey, D.R., Kurkure, N.V., Pande, V.V and Barbuddhe, S.B. 2007. Genotypic characterization of *Listeria* spp. isolated from fresh water fish. *International Journal of Food Microbiology*, 114: 120–123.
- Kaur, S., Malik, S.V.S., Vaidya, V.M and Barbuddhe, S.B. 2007. *Listeria monocytogenes* in spontaneous abortions in humans and its detection by multiplex PCR. *Journal of Applied Microbiology* 103:1889-1896.
- Lotfollahi, L., Pournajaf, A., Irajian, G and Nowrouzi, J. (2014) Polymerase chain reaction (PCR4)-based detection of *hly* and *plc-A* genes in *Listeria monocytogenes* isolated from dairy and meat products in Iran. *African Journal of Microbiology Research* 8(10): 1098-1101.
- Liu, D., Lawrence, M.L., Austin, F.W and Ainsworth, A.J. 2007. A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *Journal of Microbiological Methods* 71: 133–140.
- Notermans, S.H.W., Dufrenne, J., Leimeister-Wachter, M., Domann, E and Chakraborty, T. 1991a. Phosphatidyl inositol-specific phospholipase C

- activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria species*. *Appl. Environ. Microbiol* 57: 2666–2670.
- Pournajaf, A., Rajabnia, R., Sedighi, M., Kassani, A., Moqarabzadeh, V., Lotfollahi, L., Ardebili, A., Emadi, B and Irajian, G. 2016. Prevalence, and virulence determination of *Listeria monocytogenes* strains isolated from clinical and non-clinical samples by multiplex polymerase chain reaction. *Rev Soc Bras Med Trop* 49(5): 624-627.
- Rawool, D.B., Malik, S.V.S., Shakuntala, I, Sahare, A.M and Barbuddhe, S.B. 2007. Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases. *International Journal of Food Microbiology* 113: 201–207.
- Rawool, D.B., Doijad, S.P., Poharkar, K.V., Negi, M., Kale, S.B., Malik, S.V.S., Kurkure, N.V., Chakraborty, T and Barbuddhe, S.B. 2016. A multiplex PCR for detection of *Listeria monocytogenes* and its lineages. *Journal of Microbiological Methods* 130: 144–147.
- Rocha, C.E., Mol, J.P.S., Garcia, L.N.N., Costa, L.F., Santos, R.L., Paixao, T.A. 2017. Comparative experimental infection of *Listeria monocytogenes* and *Listeria ivanovii* in bovine trophoblasts. *PLoS ONE* 12(5):1-13.
- Radoshevich, L and Cossart, P. 2017. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nature Reviews* 1-15.
- Suarez, M., Gonzalez-Zorn, B., Vega, Y., Chico-Calero, I and Vazquez-Boland, J.A. 2001. A role for *actA* in epithelial cell invasion by *Listeria monocytogenes*. *Cell Microbiol.* 3(12): 853–864.
- Shakuntala, I., Malik, S.V.S., Barbuddhe, S.B and Rawool, D.B. 2006. Isolation of *Listeria monocytogenes* from buffaloes with reproductive disorders and its confirmation by polymerase chain reaction. *Veterinary Microbiology* 117: 229– 234.
- Shoukat, S., Malik, S.V.S., Rawool, D.B., Kumar, A., Kumar, S., Shrivastava, S., Das, D.P., Das, S and Barbuddhe, S.B. 2013. Comparison of indirect based ELISA by employing purified LLO and its synthetic peptides and cultural method for diagnosis of ovine listeriosis. *Small Rumi. Res.* 113: 301-306.
- Warke, S.R., Ingle, V. C., Kurkure, N. V., Tembhurne, P. A., Prasad, M., Chaudhari, S.P and Barbuddhe, S. B. 2017. Biofilm Formation and Associated Genes in *Listeria monocytogenes*. *The Indian Journal of Veterinary Sciences & Biotechnology.* 12(3): 07-12.

How to cite this article:

Thorat, S.R., D.B. Rawool, P.M. Sonkusale, S.R. Warke, S.P. Choudhari and Kurkure, N.V. 2019. Virulence Profiling of *Listeria Monocytogenes* Isolated from Different Sources. *Int.J.Curr.Microbiol.App.Sci.* 8(05): 2010-2017. doi: <https://doi.org/10.20546/ijcmas.2019.805.233>