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Isolation and molecular characterization of *Listeria* species from animals, food and environmental samples

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Abstract
Aim: The aim of the current study was to isolate and characterizes *Listeria* species in different animal and environmental samples.
Material and Methods: The current study was undertaken with the objective of studying *Listeria* species from different eco-epidemiological units of animal interface in the rural and peri-urban areas of Shivamogga district. A total of 310 samples were collected, analysed, characterized by biochemical, sugar fermentation and molecular methods.
Results: Culturally, identified *Listeria* species were subjected to PCR targeting of *16SrRNA*, *Lmo1030*, *namA*, *scrA* and *Oxidoreductase* genes. Further, the isolates were subjected to PCR targeting the virulence associated genes *viz.*, *plcA*, *hlyA*, *iap* and *prfA*. Then, the isolates were subjected to ERIC PCR to known the genetic diversity.
Conclusion: Out of 310 samples eight isolates were identified as *L. monocytogenes* (0.6%), *L. ivanovii* (0.6%), *L. welshimeri* (0.6%) and *L. grayi* (0.6%). Genetic diversity of the isolates were performed by ERIC PCR where six ERIC types/cluster (C1to C6) were found with the Shannon weiver index of 0.752 and Simpson index of 0.928.

Keywords: *Listeria*, *16SrRNA* sequencing, ERIC-PCR

Introduction
Listeria is an important food borne pathogen having world- wide occurrence, associated with the foods of animal origin, vegetables and even in aquatic foods (Nayak *et al.*, 2015, Oyinloye *et al.*, 2018 and Basha *et al.*, 2019) [13, 14, 16, 2]. Animal do suffer from clinical/subclinical cases of listeriosis which may be the chief source of contamination of food. Since, *Listeria* is a cold loving and can withstand the food processing substance which results in the food borne listeriosis in ready to eat foods and it has been estimated that 99% of all human listeriosis cases are caused by consumption of contaminated food products (Mead *et al.*, 1999) [12]. *Listeria monocytogenes* is associated with septicemia, meningo-encephalitis and abortion in human and animals, pregnant, new born, and immune-comprised individual (Choi and Hong, 2003; and Rossmanith *et al.*, 2006) [6, 19]. While, *Listeria ivanovii* is exclusively linked to infecting sheep and cattle, and few sporadic occurance of *Listeria ivanovii* associated with human infection has occurred recently (Snapir *et al.*, 2006, Guillet *et al.*, 2010) [20, 11].
Species of *Listeria* are non-sporulating, intracellular pathogen, Gram positive short rods, occurring in chains and filamentous structure which are ubiquitously present in environment. They are also found in soil, manure/sewage, farm slurry, sludge, silage, animal feed, water, and excreta /faeces of mammals and birds (Fenlon, 1985) [8]. Advances in the detection and characterization methods of the organisms especially molecular tools have paved the way for better understanding of virulotype of organism, circulation of molecular subtypes and to differentiate different species, genetic diversity studies that is molecular fingerprinting and molecular diagnostic technique including ERIC PCR used as a valuable tool for epidemiological investigation of organisms/ epidemics (Basha *et al.*, 2019) [2]

Material and Methods
Collection of samples
The collection and analysis of the samples from different eco-epidemiological units of animal interface were carried out during the period of March 2021 to December 2021. The samples consisted of both environmental and animal samples.

Animal sample consisted of fecal sample from rectum, milk, vaginal swabs and clocal swab. Environmental sample *viz.*, soil, water, floor swab and sediment were collected from the respective units/farms from the rural areas such as Kodamaggi, Massur, Bhadravathi and peri-urban/urban areas of L.F.C, Anupinakatte and Abbalgere of Shivamogga district

Isolation of *Listeria* spp.

Isolation and identification of *Listeria* species was done according to standard protocol prescribsed by USDA- FSIS 2013, with slight modification.

Isolation and Identification of *Listeria* species as per USDA-FSIS 2013 involved four stages, that is 1) Primary selective enrichment with UVM I broth, 2) Secondary selective enrichment with UVM II broth, 3) Selective plating onto PALCAM agar and 4) Selection and subculture of suspect /putative *Listeria* isolates on BHI agar. The method under each stages is briefly described below.

1) Primary selective enrichment (UVM I)

Samples approximately 5 ml/5 g soild/semi-solid (soil, faeces, floor swab) or liquid (water and milk) were inoculated into 45 ml of University of Vermont medium I and supplements such as acriflavin and nalidixic acid were added to the broth and mixed thoroughly with vortex shaker for 2-5 sec. The homogenized sample/ media was incubated at 30 °C for 18±2 hours. Floor swabs and vaginal swab were directly inoculated into 10 ml of UVM I broth.

2) Secondary selective enrichment (UVM-II)

100 µl (0.1 ml) of primary enriched medium was taken and inoculated into 10 ml of UVM II broth, supplements such as acriflavin and nalidixic acid were added and vortexed for 2-5 sec to obtain a homogenized mixture and further it was incubated for 30 °C for 18±2 hours.

3) Selective plating (plating out) onto PALCAM agar

Loopfull of enriched culture from UVM II broth were streaked separately onto PALCAM (Polymxcinacriflavin Lithium chloride Ceftazidimie Esculin Mannitol) incubated at 37±1 °C for 24 hrs and observed for the growth of greyish green colourcolonies with esculin hydrolysis (Plate 1).

4) Subculture of suspect/ putative *Listeria* colonies on BHI agar

Further, putative *Listeria c*olonies from PALCAM agar was sub-cultured and grown on BHI agar and preserved in glyercol stock for further analysis *viz.*, bio-chemical, sugar fermentation and molecular characterization of the isolates.

All PCR reaction were done according to Mazza *et al*., 2015 [24] mention in the table 4 with slight modification.

DNA extraction of *Listeria* species by hot-cold lysis (snap-chill) method and PCR

The DNA from *Listeria* isolates were extracted as per Zeinali *et al.* (2015) [22]. About 200µl of overnight grown broth from BHI broth was taken into sterile 2 ml micro-centrifuge tube and centrifuged at 12,000 rpm for 10min. The supernatant was removed and the pellet was resuspended in 100 µl nuclease free water and centrifuged. After proper mixing tubes were kept in boiling water bath at 100 °C for 20 min, then immediately placed in the deep freezer (-20 °C) for 20 min followed by centrifugation @ 10,000 rpm for 5 min. The supernatant containing DNA was collected and used for molecular reaction

Results and Discussion

Confirmation and Characterization of Isolates by Molecular Methods

In the current study the identification of genus *Listeria* is based on *16SrRN*A gene which is amplified at 938 bp. (fig 1, Table 2)

The results of the current study is in agreement with molecular characterization of genus *Listeria* isolates according to Das *et al.* (2013) [7], Osman *et al.* (2016) [15], Garedew *et al.* (2015) [9], Oyinloye *et al.* (2018) [16]. Identification of *Listeria* species in the current study was carried out by targeting the genes *Lmo1030, namA, scrA and Oxidoreductasi, Lin0464* and *Lmo33.* (Fig 2, 3,4, and 5, Table 2)

In the current study only four species of *Listeria* were identified *viz., L. monocytogenes, L. ivanovii, L. welshimeri* and *L. grayi* which were amplified at 509bp, 463bp, 281bp and 201bp is in agreement with Mazza *et al.* (2015) [24] and Basha *et al.* (2019) [2], Amusan *et al.* (2018) [1] and Wai *et al.* (2020) [21].

In the current study all eight *Listeria* isolates were screened for the virulent associated genes such as*plcA, hlyA, prfA* and *iap* which were amplified at 1484 bp, 456 bp, 1060 bp and 131bp.All species of *Listeria viz., L. monocytogenes, L. ivanovii, L. welshimeri* and *L. grayi* was found to be positive for *iap*gene. *L. monocytogenes* was found to be positive for all virulent genes *plcA, hlyA, prfA* and *iap.* While, *L. ivanovii* isolates positive for *plcA* and *iap*gene. (fig 6,7,8 and 9 and Table 2)

In the current study *Listeria monocytogenes* and *L. ivanovii* exhibited the presence of virulent genes such as *hlyA* and *plcA* genes which were amplified at 456bp, 1484bp which is in accordance with Rawool *et al.* (2007) [18].

Characterization of *Listeria* species by colony morphology and Gram's staining help in presumptive identification of *Listeria* species, while further biochemical tests such as Indole, Methyl red, Voges-Proskauer, nitrate and citrate test help in knowing stability of organism, determing the traits which may be used in identification and classification of *Listeria* species. Characterization futher helps in determining the metabolic and nutritional capabilities of *Listeria* species in identification of genus and species of bacteria. PCR based methods are very specific, sensitive and quick and relay results within a day. Detection of virulent associated genes helps to differeniate the pathogenic and non pathogenic strains of *Listeria* since, all *Listeria* species *viz., L. monocytogenes, L. ivanovii, L. welshimeri and grayi* were found to have *invasive associated* protein they are pathogenic of animal/human.

Among many genes, *I6SrRNA*gene was selected and targeted, since it was most commonly and widely used gene for the identification of genus *Listeri*a (Osman *et al.*, 2016; Amusan *et al.*, 2018) [15, 1]. And simplex PCR was adopted since the assay was rapid, reliable and had highly ability for the identification of *Listeria* species.

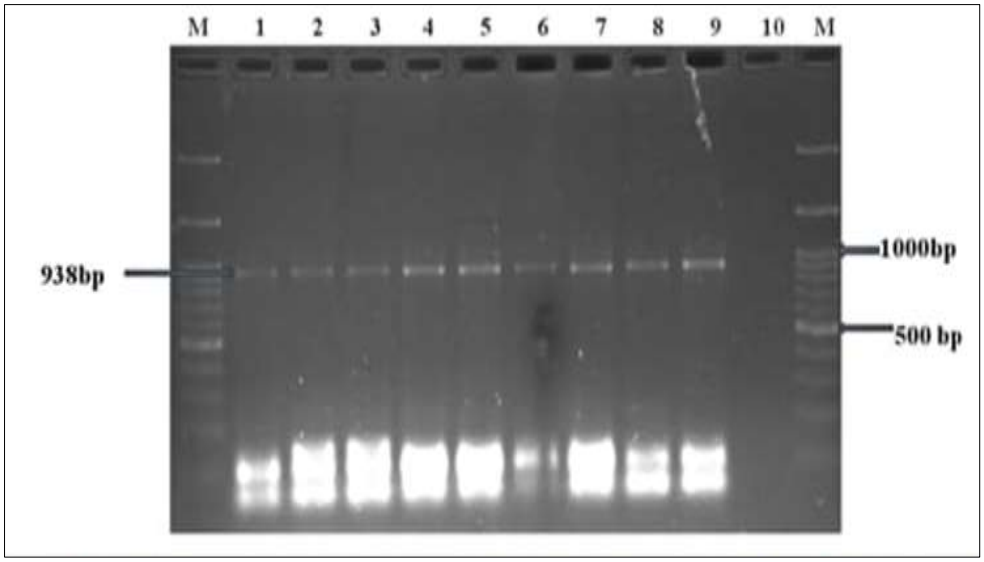
Genetic diversity of the isolates

In the current study genetic diviersity was performed by ERIC-PCR (Enterobacterial repetitive intergenic consenus). ERIC –PCR produced 1-5 bands ranging from 100-600bp and

the maximum bands were produced by all the isolates at 100 and 200 bp. At 70% cut off similarity, dendrogram showed six clusters (C1-C6) with the Simpson's genetic diversity index of (0.9286) and Shannon wiener index of (0.722). Among six clusters, C4 and C6 share two isolates each. One *L. monocytogenes* (M9) isolate and one *L. grayi* (S3) isolates were grouped into one cluster i.e., (C6) are circulating in rural eco-epidemiological unit of animal interface Massur (unit 3). Two *Listeria welshimeri* isolates (PF4 and F118) were grouped into one cluster i.e., (C4) are circulating in urban-peri urban eco-epidemiological units of animal interface L.F.C and Abbalgere (unit 1 and unit 6). This study indicated more genetic diversity was found among *Listeria* species. (fig 10 and Table 3).

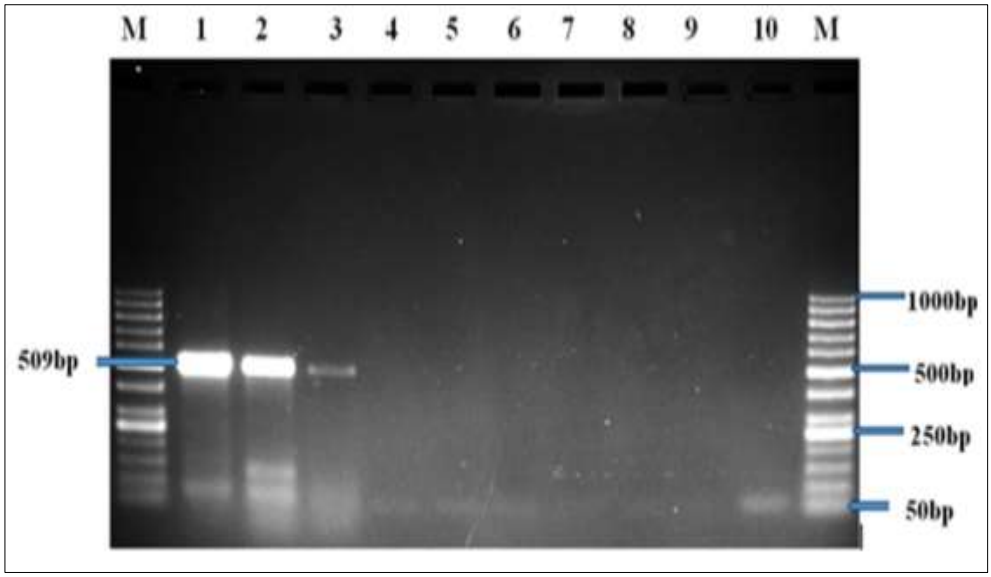
Soni *et al.* (2013) [23] performed ERIC –PCR for *Listeria monocytogenes* and they found that ERIC –PCR produced 3 to19 bands ranging from 140-5200 bp and maximum conserved fragements at 124 to 127 bp and showed 1% high discriminatory power which is higher than the present study. Another study conducted by Maurice-Bilung *et al.* (2018) reported that ERIC PCR produced 1-5 bands with the size ranging between 120-1450bp and showed high discriminatory power of 0.85 and Simpson index of 0.88 which is similar to the present study. mong species of *Listeria*.

ERIC PCR is a valuable tool for studying the genetic diversity of *Listeria* species. The present study revealed that all *Listeria* species isolated were diverse/heterogeneous.



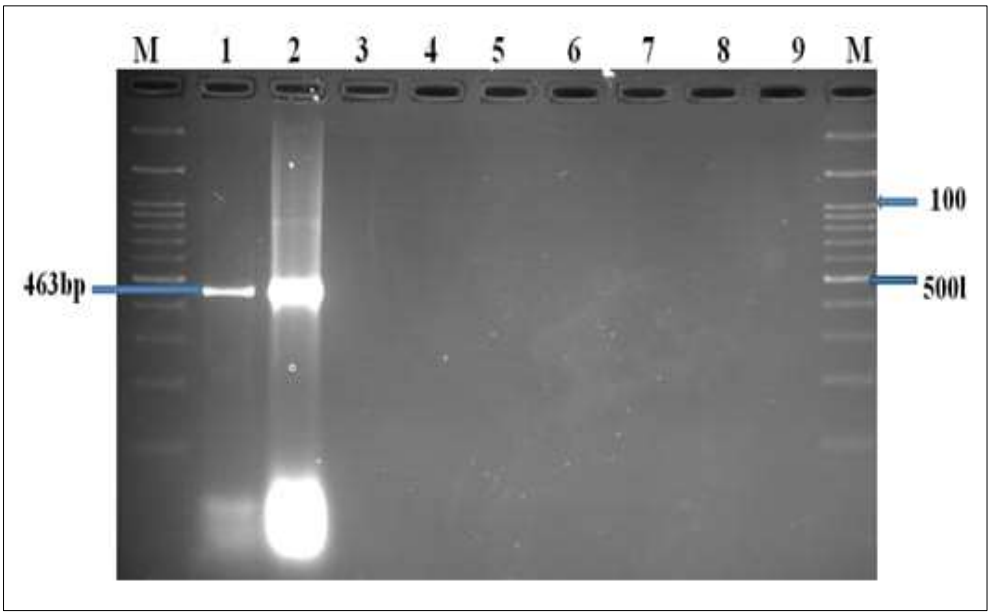
Lane M: 1001 bp ladder
Lane I: Positive control L. *monocytogenes*
Lane 2: 2 to 9 Listeria isolates showing amplicon of 938 bp (PF4, F82, VS6, F113, F118, M9, SDI and S3)
Lane 10: No template control

Fig I: Agarose gel picture showing genus specific PCR result for 16SrRNA gene of Listeria Species



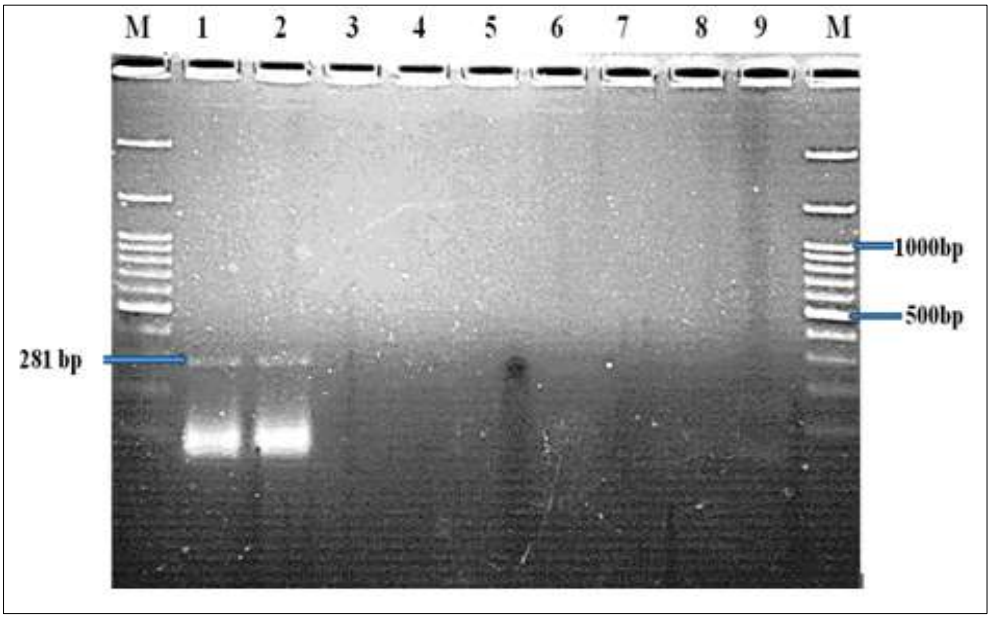
Lane M: 50 bp ladder
Lane I: Positive control L. *monocytogenes*
Lane 2 to 3: *Listeria monocytogenes* showing amplicon of 509 bp (F113 and M9)
Lane 4 to 9: other *Listeria* isolate showing negative result (PF4, SD1, S3, VS6, F82 and F118)
Lane 10: No template control

Fig 2: Agarose gel picture showing species specific PCR result for gene *Lmo1030* of *L. monocytogenes*



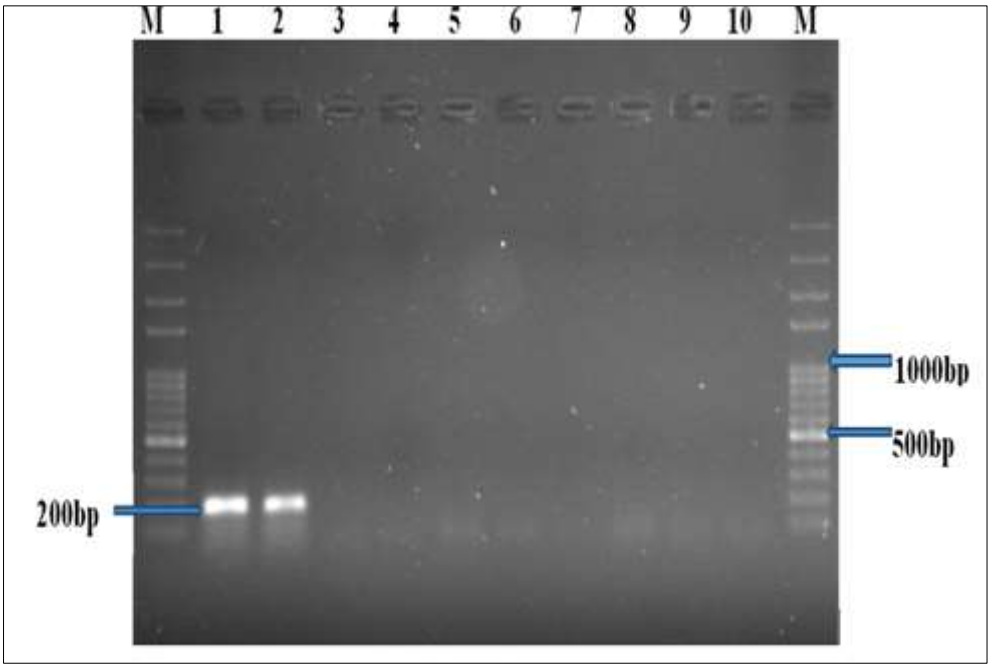
Lane M: 100 bp ladder
Lane 2 and 3: *L. ivanovii* showing amplicon of 463 bp (F82, VS6)
Lane 3 to 8: other *Listeria* isolate showing negative result (PF4, F113, F118, M9, SD1 and S3)
Lane 9: No template control

Fig 3: Agarose gel picture showing species specific PCR result for *namA* gene of *Listeria*



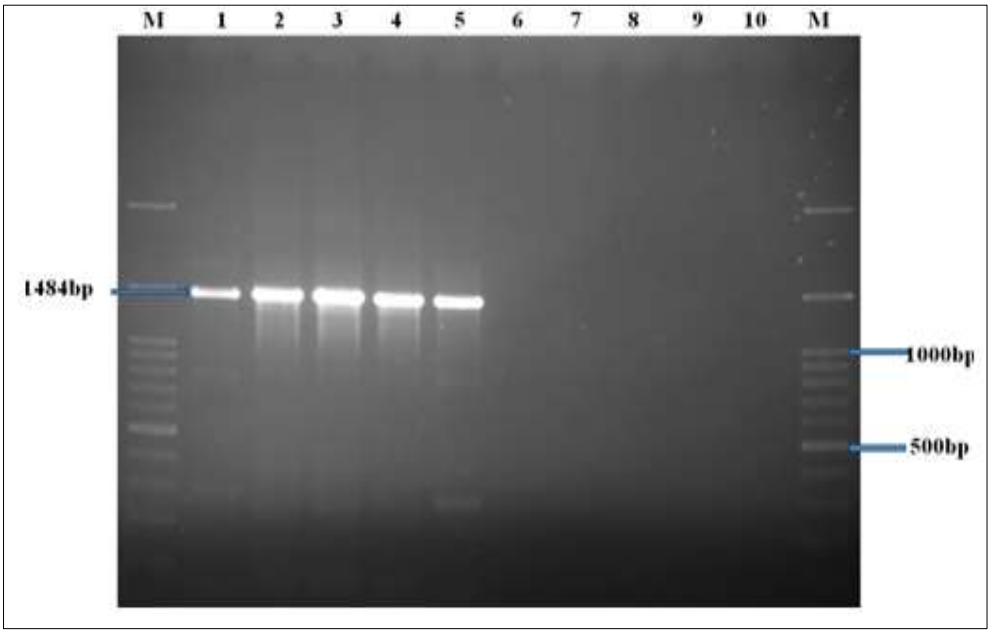
Lane M: 100 bp ladder
Lane 2 and 3: *L. welshimeri* showing amplicon of 281 bp (F118, PF4)
Lane 3 to 8: other *Listeria* species showing negative result (F82, F113, VS6, M9, SD1 AND S3)
Lane 9: No template control

Fig 4: Agarose gel picture showing species specific PCR result for *scrA* gene of *Listeria welshimeri*



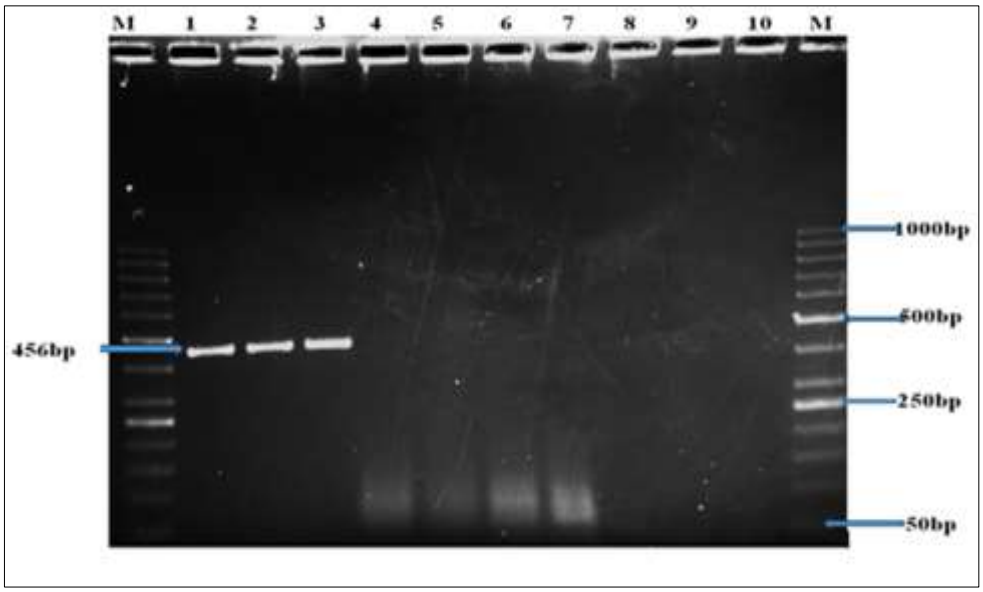
Lane M: 100 bp ladder
Lane 2 and 3: *L. grayi* showing amplicon of 201 bp (SD1, S3)
Lane 3 to 8: Other *Listeria* isolate showing negative result (F82, F113, VS6, M9, PF4 and F118)
Lane 9: No template control

Fig 5: Agarose gel picture showing species specific PCR result for *Oxidoreductasi* gene of *Listeria grayi*



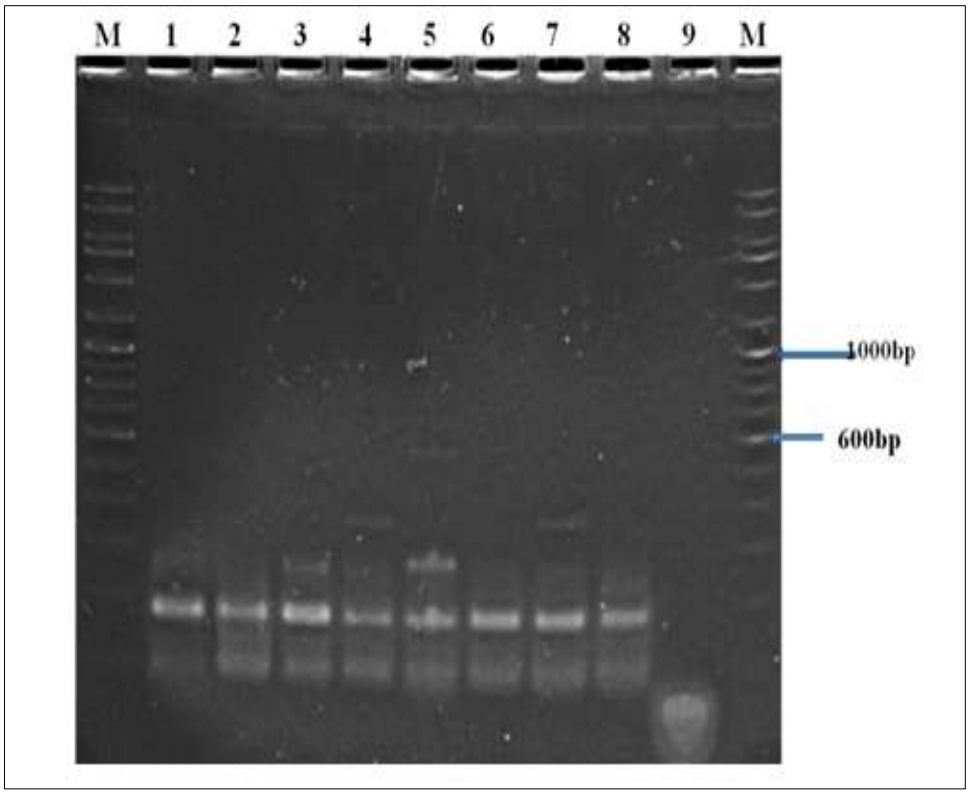
Lane M: 100 bp ladder
Lane 1: Positive Control *L. monocytogenes*
Lane 2 and 3: *L. monocytogenes* showing amplicon of 1484 bp (F113, M9)
Lane 4 and 5: *Listeria ivanovii* showing amplicon of 1484 bp (F82, VS6)
Lane 6 to 9: Other *Listeria* isolate showing negative result (PF4, F118, SD1, S3)
Lane 10: No template control

Fig 6: Agarose gel picture showing PCR amplification of virulent gene *plcA* of *Listeria* Species



Lane M: 100 bp ladder
Lane 1: Positive Control *L. monocytogenes*
Lane 2 and 3: *Listeria monocytogenes* showing amplicon of 456 bp (F113, M9)
Lane 4 and 9: Other *Listeria* isolate showing negative result (F824, VS6, F118, SD1 and S3)
Lane 10: No template control

Fig 7: Agarose gel picture showing PCR amplification of virulent gene *hlyA* of *Listeria* Species



Lane M: 100 bp ladder
Lane 2 to 8: PF4, S3, M9, F82, F113, SD1, F118, and VS6 (Listeria Isolates showing multiple ERIC bands ranging from 100 to 600 bp
Lane 9: No template control

Fig 7: Agarose gel picture showing ERIC PCR Pattern of *Listeria* Isolate

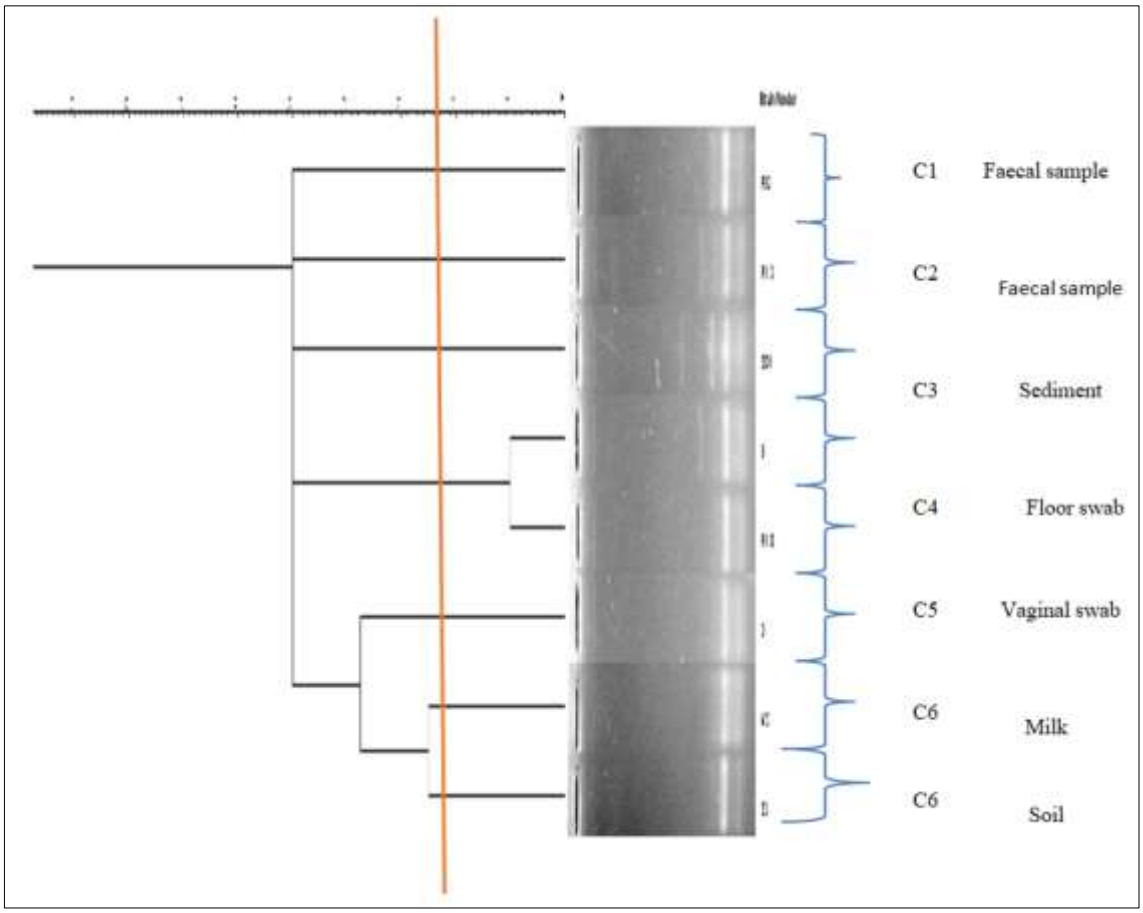


Fig 9: Dendrogram of *Listeria* isolate derived from analysis of ERIC PCR profile

Table 1: Colony morphology, staining, biochemical tests and sugar fermentation tests for *Listeria* species isolated from different eco-epidemiological units of animal interface.

Sl. No.	Isolates /Species	Grayish green colonies on PALCAM agar G	Esculin hydrolysis	Grams reaction	Motility at 25ºC	Motility at 37ºC	Biochemical tests							Sugar fermentation tests			
							Indole	Methyl red	Voges-Proskauer	Citrate	Nitrate	Catalase	Oxidase	Rhamnose	Xylose	Mannitol	Mannose
1.	*L.monocytogenes* (F113 and M9)	+	+	+	+	-	-	+	+	-	-	+	-	+	-	-	-
2.	*L.ivanovii* (F82 and VS6)	+	+	+	-	+	-	+	+	-	-	+	-	-	+	-	-
3.	*L.welshimeri* (PF4 and F118)	+	+	+	-	+	-	+	+	-	-	+	-	-	+		-
4.	*L.grayi* (SD1 and S3)	+	+	+	-	+	-	+	+	-	-	+	-	-	-	+	-

+ (*L. monocytogenes*), Xylose: + (*L. ivanovii* and *L. welshimeri*), Manitol:+(*L. grayi)*, Mannose: - (*L. monocytogenes, L. ivanovii, L. welshimeri* and *L. grayi*)

Table 2: Molecular characterization of *Listeria* species isolated from different eco-epidemiological units of animal interface

Isolates	*16SrRNA*	*Lmo1030*	*plcA*	*hlyA*	*Iap*	*prfA*	*namA*	*scrA*	*Oxidoreductasi*	*Lin0646*	*Lmo0333*
L. monocytogenes (F113 and M9)	+	+	+	+	+	+	-	-	-	-	-
L. ivanovii (F82 and VS6)	+	-	+	-	+	-	+	-	-	-	-
L .welshimeri (PF4 and F118)	+	-	-	-	+	-	-	+	-	-	-
L. grayi (S3 and SD1)	+	-	-	-	+	-	-	-	+	-	-

Note: +:positive , -:negative

Table 3: Cluster analysis and genetic diversity of different *Listeria* strain

Eco-epidemiological units	No of cluster	No of strain/isolates in cluster	Relative abdundanceRA	LNp(i) Log (RA)	Pi* LN(Pi) RAXLog (RA)
Anupinakatte	C1	1	0.125	-0.903	0.1128
Bhadravathi	C2	1	0.125	-0.903	0.1128
Bhadravathi	C3	1	0.125	-0.903	0.1128
L.F.C, Abbalgere	C4	2	0.25	-0.602	0.1505
Abbalgere	C5	1	0.125	-0.903	0.1128
Massur	C6	2	0.25	-0.602	0.1505
Total		8	Shannon Wiener index		0.7522
			Simpson's diversity index		0.952

Note**:** Relative abundance: No. of strain/isolates in cluster /Total no. of positive isolates, LNp(i) : log (RA), Pi* LN(Pi) : Relative abundance X log RA

Table 4: Primers for genus and *Listeria* species

Gene	Primer	Primer sequences	Amplicon Size	Reference
16SrRNA	U1 L11	5^1-CAGCAGCCGCGGTAATAC-3^1 5^1- CTC CATAAAGGTGACCCT-3^1	938 bp	Amusan *et al.*, 2018 [1]
Lmo1030	*Lmo1030F* *Lmo1030R*	5^1GCTTGTATTCACTTGGATTTGTCTGG-31 5^1ACCATCCGCATATCTCAGCCAACT- 3^1	509 bp	Mazza *et al.*, 2015 [24]
namA	*Liv22228* F Liv2228R	5^1-CGAATTCCTTATTCACTTGAGC-3^1 5^1 - GGTGCTGCGAACTTAACTCA 3^1	463 bp	
scrA	*Lwe1801*F *Lwe1801*F	5^1-CGTGGCACAATAGCAATCTG -3^1 5^1-GACATGCCTGCTGAACTAGA- 3^1,	281 bp	
oxidoreductasi	*Jograyi*F *Jograyi*R	$5^1$$5^1$-GCGGATAAAGGTGTTCGGGTCAA-3^1-5^1GCGGATAAAGGTGTTCGGGTCAA-3^1	201 bp	
Gene	Primer	Primer sequences	Amplicon Size	**Reference**
plcA	*plcAF* *plcAR*	51-CAGCAGCCGCGGTAATAC-31 5'-ATG GGT TTC ACT CTC CTT CTA C-3'	1484bp	Amusan *et al.*, 2018 [1]
hlyA	*hlyAF* *hlyAR*	5^1- GCA GTT GCA AGC GCT TGG AGT GAA-3^1 5^1- GCA ACG TAT CCT CCA GAG TGA TCG-3^1	456 bp	Mazza *et al.*, 2015 [24]
PrfA	*PrfA F* *PrfAR*	5^1-CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG-3^1 5^1-AGC AAC CTC GGT ACC ATA TAC TAA CTC-3^1	1060 bp	
	ERIC –IR *ERIC-2*	5^1-CATTAGGGTCCTCGAATGTA-3^1 5^1 –AGTAAGTGACTGGGTGAGCG-3^1.	Multiple band	

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Conclusion
The present study was taken up to known the dynamics of interaction in various eco-epidemiological units of animal interface. In fact no exact or similar studies were reported in relation-ship with eco-epidemiological units of animal interface having multiple species in particular eco-eystem. However, the present study showed some unique results suggesting that different dynamics may be taking place and may be a matter of futher investigation. The present study delt with single visit, cross sectional epidemiological investigation. The full pattern of interaction and dynamics may require multiple visits with much larger sample size.
Especially *Listeria*s species becomes an important in many angles as these are ubiquitous organism almost prevailing in all type of environmental condition, habitat and also important intracellular organism affecting many species of animals as well as human beings especially, *L. monocytogenes*, *L. ivanovii*, *L. welshimeri* and *L. grayi*. It is important to note that there are good number of interaction taking place in an eco-epidemiological units of animals interface like commensalism, antagonism and mutalism *etc.*, such that the dominance/presence of particular species may lead to microbial interaction.

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