DOI: 10.7860/JCDR/2026/77953.23346

Research Protocol

Microbiology Section

Isolation and Identification of *Listeria monocytogenes* from Spontaneous Abortion Cases in Humans and its Association with Household Cattle: A Research Protocol

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ABSTRACT

Introduction: *Listeria monocytogenes (L. monocytogenes)* is a Gram-positive bacterium responsible for listeriosis, a foodborne disease causing significant health problems, especially for pregnant women. During pregnancy, immune modulation increases the vulnerability to infections, resulting in severe complications such as spontaneous abortions. Rural populations are disproportionately affected due to poor sanitation, limited healthcare access, and close human-animal interactions.

Need of the study: Studying the association between humans and household cattle is important, as household cattle can carry and transmit the *L. monocytogenes*, a zoonotic pathogen that may spread through contaminated food, water and contribute to spontaneous abortions in humans.

Aim: To investigate the association between *L. monocytogenes* in women with spontaneous abortions and their household cattle.

Materials and Methods: This cross-sectional study will be conducted at Datta Meghe Medical College, Nagpur, Maharashtra, India. The study for human samples will be conducted from January 2025 to December 2026, and this study will recruit 385 women aged 18-45 years with confirmed spontaneous abortions and their association with household cattle from rural settings. The study on animal samples collected from rural settings will be conducted after receiving approval from the animal ethics committee. Vaginal swabs and faecal samples will be collected and analysed for *L. monocytogenes* using selective culture methods and Real-Time Polymerase Chain Reaction (RT-PCR). Data will be statistically analysed using the chi-square test to identify correlations, and p-value <0.05 will be considered statistically significant.

Keywords: Microbiological diagnostic techniques, Pregnancy complications, Zoonoses transmission, Zoonotic bacterial infections

INTRODUCTION

A rare but serious foodborne infection, listeriosis, is caused by the facultative intracellular Gram-positive bacterium *Listeria monocytogenes* (*L. monocytogenes*). The organism contaminates various foods, including processed items in refrigerators, and is present in natural habitats [1]. It is a facultative intracellular Gram-positive bacterium. It affects population with weakened immune systems, newborns, old people, and pregnant women. High levels of progesterone and estradiol during pregnancy significantly impact the immune system, decreasing the number of T-lymphocytes, boosting or preserving innate immunity, and raising the risk of listeriosis [2]. Literature has documented the seasonal distribution of listeriosis, with summertime being the most common time for cases to arise. Listeriosis can result in preterm labour and frequently manifests as flu-like symptoms along with other symptoms like inflammation and respiratory distress. Meningitis, encephalitis, and severe sepsis are among its side-effects in patients with severe cellular immune deficiencies [3].

L. monocytogenes can develop a tolerance to sanitisers, create difficult-to-remove biofilms in food processing facilities, and multiply even at refrigerated temperatures. These characteristics make it more difficult to effectively avoid *L. monocytogenes* contamination in a variety of ready-to-eat foods, such as fruits, vegetables, dairy, meat, and fish, which have all been linked to listeriosis outbreaks in the past [4]. Genetically diverse, *L. monocytogenes* strains can be categorised using 7-gene Multilocus Sequence Typing (MLST) into phylogenetic lineages, Clonal Complexes (CCs), and Sequence Types (STs); alternatively, strains can be classified using whole-genome sequencing data, which expands the MLST concept to a larger number of genes in the core genome, into lineages, Sub-Lineages (SLs), and cgMLST types (CTs). All strains of *L. monocytogenes* carry the essential virulence genes found on Listeria Pathogenicity Island 1 (LIPI-1) that allow the bacteria to invade and multiply in host cells. Phenotypes connected to hypervirulence have been connected to internal genes and accessory genome components, such as LIPI-3 and LIPI-4 [5,6].

Pregnant women are more likely to get listeriosis, a serious infection caused by *L. monocytogenes*, which can lead to miscarriage or stillbirth. While relatively rare, the impact of listeriosis can be devastating for both mother and child [7]. The incidence of pregnancy-associated listeriosis in India is 14.8% [8]. The source of *L. monocytogenes* infection in pregnant women is often unknown, with contaminated food being a common mode of transmission [9]. Domestic cattle have been identified as potential carriers of *L. monocytogenes*, spreading the bacteria through their faeces and contaminating the environment. Introducing *L. monocytogenes* into the diet or water supply of these animals can lead to transmission to humans through tainted meat or dairy products [10].

Rural communities may face an increased risk of foodborne infections due to limited access to clean water, poor sanitation, and inadequate healthcare services [11]. There is a lack of information on the incidence of *L. monocytogenes* infections in pregnant women and household cattle in rural areas, highlighting the need for further research in this area. Detection of *L. monocytogenes*

in pregnant women during the first trimester and domestic cattle in rural communities using RT-PCR is crucial for understanding transmission and incidence in rural populations. RT-PCR is a rapid and sensitive diagnostic tool that can detect *L. monocytogenes* in various samples, aiding in risk mitigation strategies [12].

REVIEW OF LITERATURE

Listeriosis, caused by *Listeria monocytogenes*, is a severe infectious disease that poses significant risks during pregnancy, potentially leading to adverse foetal and neonatal outcomes. Pregnant women must avoid consuming unpasteurised dairy products, meats, and other high-risk foods that may be contaminated with *L. monocytogenes* [13,14]. Ohadi E et al., conducted a comprehensive study in Tehran, Iran, on the serotyping of *L. monocytogenes* isolates from women with spontaneous abortion using the PCR method. The results revealed a notable discrepancy between the detection rates of microbial culture and PCR, with microbial culture identifying *L. monocytogenes* in 4.2% of specimens and PCR detecting the bacterium in 16.7% of specimens. This significant difference highlighted the superior sensitivity of PCR over traditional culture methods [15].

El-Naenaeey ES et al., revealed that *Listeria* spp. was present at a 16% isolation rate among the 350 samples analysed, including raw milk, mastitis milk, faeces of dairy cows, and stool samples from pregnant women. The prevalence of *Listeria* spp. varied across the sample types, ranging from 8% in faeces of dairy cows to 4% in stool samples from pregnant women. Additionally, the study showed that *L. ivanovii* and *L. welshimeri* were present in 6% and 4% of milk samples from dairy cows, respectively. Internalin A (*inlA*) and internalin B (*inlB*) genes were detected in 80% and 40% of *L. monocytogenes* isolates, respectively. Two *L. monocytogenes* isolates from normal raw milk and faeces of dairy cows lacked both *inlA* and *inlB* genes [16].

The findings indicate that *Listeria* spp. in milk, faeces, mastitis milk, and stool samples of pregnant women pose a potential health hazard and can lead to infection and abortion in pregnant women. Internalin A and B genes are useful markers for evaluating the virulence of *L. monocytogenes* isolates from diverse sources.

Yousefi A et al., investigated the relationship between extraintestinal *Listeria monocytogenes* infections and the incidence of spontaneous abortions during pregnancy. The study follows rigorous methodological guidelines, conducting a comprehensive literature search across multiple databases and employing strict inclusion criteria to ensure high-quality case-control studies. Key findings of the review indicate that *L. monocytogenes* infection increases the likelihood of spontaneous abortion, particularly when associated with the presence of the *hlyA* gene. Additionally, the meta-analysis provides pooled estimates of the prevalence of *L. monocytogenes* in pregnant individuals experiencing spontaneous abortions compared to healthy controls. The study employs a thorough approach to evaluating the included studies, utilising the Joanna Briggs Institute Evaluation Checklist to appraise the quality of individual studies. Furthermore, the authors acknowledge potential limitations and controversies in the field, suggesting future directions for research [17].

Ahmadi A et al., conducted a study to assess the prevalence of *L. monocytogenes* infection in women with different reproductive outcomes. The research included 417 women aged 19-40 years, categorised into groups based on pregnancy status. The study revealed infection rates of 3.66% in women with spontaneous abortion, 1.83% in those with normal delivery, 3% in fertile women, and 0% in infertile women. Notably, higher unpasteurised dairy consumption was linked to normal delivery and fertility, while a higher smoking rate was associated with infertility. Despite no significant correlation between *L. monocytogenes* infection and pregnancy outcomes, the study stresses the importance of educating pregnant women and the public about the risks of consuming non pasteurised dairy products. It also advocates for further research to understand the factors contributing to *Listeria* prevalence and develop effective intervention strategies to mitigate infections, emphasising the need for continued monitoring and control measures to safeguard women of reproductive age from *Listeria*-related infections [18].

Ke Y et al., conducted a retrospective cohort study that investigated the clinical characteristics and outcomes of pregnancy-associated listeriosis in China and found that, among 14 pregnancy-associated listeriosis cases with an incidence of 16.69/100,000 births, all maternal patients recovered completely without sequelae, while neonatal outcomes showed 11 survivors and three deaths (13 early-onset, 1 late-onset) [14]. Outborn neonates experienced significantly higher fatality rates compared to those born in the hospital.

The present study aimed to determine the association between *L. monocytogenes* isolated from spontaneous abortion cases in humans and those found in household cattle that are in close association.

Objectives

- To isolate *L. monocytogenes* from spontaneous abortion cases in women.
- To identify *L. monocytogenes* in spontaneous abortion cases in humans and its association with isolates found in household cattle in close contact.

Null hypothesis: There is no significant association between *L. monocytogenes* isolated from spontaneous abortion cases in humans and those found in household cattle.

Alternate hypothesis: There is a significant association between *L. monocytogenes* isolated from spontaneous abortion cases in humans and those found in household cattle.

MATERIALS AND METHODS

This cross-sectional study will be conducted in the Microbiology Department of Datta Meghe Medical College, Nagpur, Maharashtra, India, from January 2025 to December 2026. The study will involve the collection and analysis of clinical samples following standard microbiological procedures. Before participation, informed consent will be obtained from all individuals or their legal guardians. Ethical approval for the study has been granted by the Institutional Ethics Committee for human samples, with clearance number IEC/DMMC/2025/01-01 and from the Animal Ethics Committee with clearance: NVC/IAEC/02/2025.

Inclusion criteria: Females within the age range of 18 to 45 years who were diagnosed with spontaneous abortion recruited from rural areas, specifically those who have household cattle, including ruminants such as cattle, sheep, goats, and buffalo. Additionally, the study will involve samples of both human and ruminant origin that are accessible and suitable for testing *L. monocytogenes*.

Exclusion criteria: Females with spontaneous abortion not confirmed by medical records, those who are not in contact with ruminants, and human participants outside the specified age group. Furthermore, human or ruminant subjects who have received antibiotics or treatments known to affect *Listeria* detection will also be excluded from the study.

Sample size calculation:

$$n=(Z^2\times P\times(1-P))/e^2$$

Where,

'n' is the sample size; 'z' is selected critical value of the desired confidence level (CL 95%); 'p' is the proportion of abortion cases in humans associated with ruminants in the selected study area (50% =0.5); 'e' level of precision (5%)

Sample size: 385

This means 385 or more measurements/surveys are needed to have a confidence level of 95%, that the real value is within ±5% of the measured/surveyed value.

Study Procedure

Women with spontaneous abortions will be recruited from rural areas around the place of study during their routine antenatal visits. Recruited participants will be categorised based on the presence of ruminants and their close contact with ruminants. Vaginal swabs will be collected aseptically using sterile swabs. Each swab will be labelled with a unique identifier and immediately transported to the laboratory for processing to avoid sample contamination and maintain sample viability.

After females associated with ruminants test positive for *L. monocytogenes*, faecal samples from the associated ruminants will be collected. The samples will be securely transported to the laboratory for further analysis while maintaining appropriate temperature conditions. Samples will be cultured on selective culture media such as CHROM agar *Listeria* for isolation of *Listeria monocytogenes*.

Outcomes: Detection of *Listeria monocytogenes*

Nucleic acid extraction: Total nucleic acid extraction will be performed from the collected samples using a commercially available extraction kit following the manufacturer's instructions. This extraction method ensured efficient isolation of DNA from both human and animal samples, facilitating subsequent molecular analysis.

Real-Time Polymerase Chain Reaction (RT-PCR): The PCR amplification of the cDNA will be carried out using specific primers designed to target *L. monocytogenes* genes of interest. The RT-PCR conditions included an initial denaturation step, followed by a series of denaturation cycles, annealing of primers to the target sequences, and extension of the DNA strands. This amplification will increase the amount of *L. monocytogenes* DNA in the samples, making it detectable.

STATISTICAL ANALYSIS

Statistical analysis will be conducted using Statistical Package for the Social Sciences (SPSS) software (version 26.0). Descriptive statistics, including mean and standard deviation, will be used to summarise continuous variables, while categorical variables will be presented as frequencies and percentages. The association of *L. monocytogenes* in women with a history of spontaneous abortion associated with household cattle will be calculated by determining the proportion of samples positive for *L. monocytogenes* DNA among the total samples tested in each group and a comparative analysis, including the chi-square test, while the independent t-test will be used to compare means between two groups. Logistic regression analysis will be performed to evaluate potential risk factors associated with *L. monocytogenes* infection. A p-value of <0.05 will be considered statistically significant.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Jan 10, 2025
- Manual Googling: Dec 02, 2025
- iThenticate Software: Dec 04, 2025 (3%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jan 09, 2025**
Date of Peer Review: **Feb 17, 2025**
Date of Acceptance: **Dec 06, 2025**
Date of Publishing: **May 01, 2026**