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Isolation and Identification of *Pseudomonas paralactis* in Cow Milk Samples Collected from "Andella" Area, Hambantota District, Sri Lanka

T.W.N.K. Perera

Department of Microbiology, Faculty of Science, University of Kelaniya, Sri Lanka

This study, conducted in the "Andella" area, Hambantota district, Sri Lanka, investigated the presence of bacteria in cow milk samples from semi-intensively managed nine dairy farms. The study aimed to identify bacterial contamination levels and to emphasise the importance of monitoring the contaminants to ensure milk quality and safety in the local dairy industry. Bacterial enumeration, isolation and identification were carried out using standard microbiological and molecular biological techniques, including 16S rRNA gene sequencing. During bacterial enumeration assays, milk obtained from Farm G showed the highest bacterial colony count, while Farm B showed the lowest. The fewer bacterial counts of cow milk from five farms (A, B, D, F, and I) were not significantly different, while the counts of the other four farms (C, E, G and H) were significantly different. The results demonstrated a notable occurrence of *Pseudomonas paralactis* (PP155068) in all the milk samples obtained from the four farms, showing higher bacterial loads. It underscored the importance of controlling this bacterium for maintaining milk quality and safety. The findings contribute valuable insights into the prevalence and potential sources of contamination in the local dairy industry, offering insights for future interventions to enhance dairy product safety.

Keywords: cow milk; colony count; *Pseudomonas paralactis*; dairy industry; control measures

I. INTRODUCTION

Analysing cow milk samples is typically conducted in accredited laboratories using standardised methods and equipment. It is essential for regulatory compliance and ensuring the safety and quality of dairy products for consumers. Additionally, adherence to good agricultural and manufacturing practices is essential in maintaining the purity and integrity of milk samples.

Identification of raw milk microbiota is crucial for understanding its bacteriological quality and preventing contamination, with *Pseudomonas* spp. being the most common contaminants and Enterobacteriaceae are the main spoilage-associated bacteria (Ercolini *et al.,* 2009). *Pseudomonas* is a bacterium known for its significance in the dairy industry, particularly in the context of milk quality and safety. *Pseudomonas* species can potentially affect human health, and preventive measures are needed to minimise contaminations, during milking steps and storing the refrigerated raw milk, especially during rainy seasons (Capodifoglio *et al*., 2016). *Pseudomonas* spp. in goat and cow milk shows potential for alkaline metalloprotease production, with *P. aeruginosa* being the most frequent in cow milk (Ribeiro Júnior *et al*., 2018). Alkaline metalloproteases are enzymes that degrade proteins, particularly at alkaline pH levels, using a metal ion (like zinc) in their catalytic process.

Mastitis is a prevalent and economically important disease in dairy cows worldwide, and *P. aeruginosa* is a key causative agent of subclinical mastitis in dairy herds. It is caused due to improper udder and teat washing methods. Two novel species of *Pseudomonas* bacteria, *P. lactis* and *P. paralactis*, have been identified from bovine raw milk (von Neubeck *et al*., 2017). *Pseudomonas paralactis* is a Gram-negative, aerobic, mesophilic bacterium that forms circular colonies, and in

*Corresponding author's e-mail: nipunik@kln.ac.lk

addition to raw milk, its isolation evidence is mentioned from chicken faeces (Olofinsae *et al*., 2022). Although not as widely studied as *P. aeruginosa*, recent findings suggest that *P. paralactis* may play a role as an opportunistic intramammary pathogen.

In the "Andella" area, Hambantota district of Sri Lanka, where dairy farming is a crucial aspect of the agricultural landscape, understanding the prevalence of bacteria in cow milk samples becomes essential. Therefore, isolating and identifying bacteria in cow milk samples from the Hambantota district is imperative for assessing the extent of contamination and implementing effective control measures. This study aims to contribute to the existing knowledge on the bacteriological aspects of milk quality in this region, laying the groundwork for strategies to enhance the safety and quality of dairy products. This comprehensive methodology aims to accurately isolate and identify bacteria in cow milk samples, providing a robust foundation for understanding the bacterial presence. By conducting a thorough bacteriological analysis, including the isolation and identification of *P. paralactis*, this study will contribute valuable insights into the bacteriological profile of mastitis in dairy cows in the selected locality to manage and prevent the disease effectively.

II. MATERIALS AND METHODS

A. Sample Collection

A total of 36 cow milk samples (10.00 mL each) from cattle breed Jersey were aseptically collected on the same day from nine dairy farms (4 samples from each) in the "Andella" area, Hambantota district, Sri Lanka. Samples were collected from lactating cows in mid-lactation (100 – 200 days of milking) during the month of September 2023. Samples were obtained in sterile containers, ensuring minimal contamination during collection.

B. Enumeration and Isolation of Bacteria

Milk samples were subjected to standard bacteriological procedures. The bacterial colonies were quantified with a few modifications to a previously described method (Hossain *et al*., 2017). Tenfold serially diluted samples were pour-plated using Milk Plate Count Agar (MPCA, Oxoid Ltd., Basingstoke, UK) supplemented with Cycloheximide (100 µg mL^{-1}) to enumerate total viable bacteria. The inoculated MPCA plates were incubated at 30 ± 2 °C, for 48 hours. All samples were analysed in duplicates, and the mean values of colonies were calculated.

The number of bacteria present in the test sample was expressed as the mean from two successive dilutions using the formula given in ISO 7218:2007/Amd 1:2013, with 95% confidence interval limits, $\frac{\sum c}{V\,[n_1 + 0.1 n_2\,]d} \pm \frac{1.96\sqrt{\sum c}}{V\,[n_1 + 0.1 n_2\,]d}$

where, $\sum c$ refers to the sum of the colonies counted on all the plates, n_1 is the number of plates retained in the first selected dilution, n_2 is the number of plates retained in the second selected dilution, d is the dilution factor corresponding to the first selected dilution and V is the volume of the sample inoculated onto each plate (in millilitres).

Afterwards, predominantly detected and morphologically distinct colonies from these culture plates were isolated into pure cultures. For bacterial isolation, isolation streaks were drawn on Nutrient Agar (NA, Oxoid Ltd., Basingstoke, UK) supplemented with Cycloheximide (100 µg mL^{-1}). Plates were incubated at 37 ± 2 °C for 24 hours. The bacterial colonies were stored on NA slants and kept at 4 °C for further characterization and identification.

C. Identification of Bacteria

Biochemical tests were carried out on the pure culture of each isolate during the identification of bacterial isolates, following microscopic examinations. Results were interpreted with the identification schemes of Bergey (1994). DNA-based bacterial species confirmation was achieved by comparing the sequences of the 16S rRNA gene using universal primers 27F and 1492R.

Molecular biological identification of bacteria was based on DNA extraction, amplification and sequencing techniques. The amplified PCR products were sequenced using Sanger dideoxy sequencing technology at Genetech Institute, Colombo, Sri Lanka. Sequences were manually edited using BioEdit sequence Alignment Editor (Version 7.2.5) and were compared with the sequences available in the GenBank using BLAST to assess homology (Perera *et al*., 2022). Phylogenetic trees were constructed using MEGA11 software (MEGA Software Inc., USA) to elucidate genetic relationships. DNA

sequences of the identified bacterial species were deposited in the NCBI database, and accession numbers were obtained.

D. Statistical Interpretation and Analysis Techniques

The bacterial counts in milk samples were analysed using a completely randomised design. One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test were used to identify significant differences in bacterial counts across the nine farms. Data analysis was performed using SPSS software (IBM, Armonk, NY), and differences were considered statistically significant at a P-value of less than 0.05.

III. RESULT AND DISCUSSION

The present study investigated the bacterial presence in cow milk samples from semi-intensively managed nine dairy farms in the Andella area, Hambantota District, Sri Lanka. The bacterial colony counts revealed that farms A, B, D, F, and I had significantly lower bacterial counts compared to farms C, E, G, and H.

The dominant bacterium isolated from the milk samples with higher bacterial loads was identified as *Pseudomonas paralactis*, which was confirmed through 16S rRNA gene sequencing. This species was isolated from all the high-contamination farms, underscoring its prevalence in this region. The findings align with previous studies on *Pseudomonas* species in raw milk, which have highlighted the spoilage potential of this bacterium due to its proteolytic and lipolytic activities. The biochemical tests revealed that the isolate was Gram-negative, oxidase-positive, and capable of hydrolyzing gelatin, which is consistent with the characteristics of *Pseudomonas paralactis*.

Interestingly, *P. paralactis* has previously been isolated from raw milk in other regions, but its presence in Sri Lankan dairy farms has not been well-documented. This study, therefore, provides the first report of *P. paralactis* in cow milk samples from Sri Lanka. The molecular confirmation of the bacterium (GenBank accession: PP155068) adds to the growing body of knowledge regarding the geographical distribution of *Pseudomonas* species in dairy environments.

The significant variation in bacterial counts between the farms indicates the potential role of farm-specific practices in bacterial contamination. Farms with higher bacterial counts may have less stringent hygiene practices during milking, inadequate refrigeration, or poor herd management, all known risk factors for milk contamination. Further studies are needed to investigate these practices and implement effective control measures to mitigate bacterial contamination, particularly from *P. paralactis*.

The prevalence of *Pseudomonas paralactis* in these samples also highlights the need for regular monitoring and improved hygiene measures on dairy farms. This study's findings provide essential data to guide interventions in the local dairy industry, emphasising the importance of addressing bacterial contamination to reduce spoilage and improve public health outcomes.

The nine farms selected in the current study were semi-intensively managed in the "Andella" area, Hambantota district, Sri Lanka. These farms were selected since they reared the cattle breeds considered in the current study. Cattle breeds were determined through observation of their physical traits and verified using the farms' breeding records.

By collecting all the milk samples on the same day, we ensured consistent comparisons of the bacterial populations. Since factors such as the cow's diet, farm management practices, season, parity number, and stage of lactation can influence bacterial populations, we selected cows with the same parity number (3) and at the mid-lactation stage for this study. In total, milk samples were collected from 36 lactating cows (four cows from each farm) during the morning milking session (between 5.00 AM - 5.45 AM). Milk samples were collected into sterilised containers, sealed, and stored under refrigerated conditions (4 °C) until analysis.

In this study, we enumerated, isolated and identified culturable, mesophilic, aerobic bacteria from all 36 cow milk samples. The findings of this study shall shed light on the bacterial communities present in these milk samples.

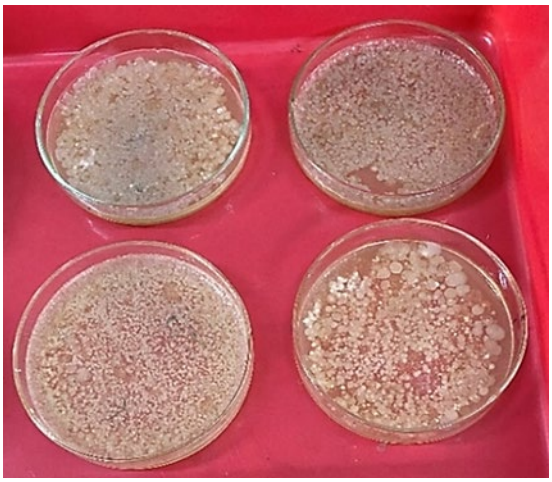

Figure 1. Enumeration assays on culturable, mesophilic, aerobic bacteria from the milk samples

Enumeration assays on culturable, mesophilic, aerobic bacteria from the milk samples were carried out as indicated in Figure 1, and quantitative determinations were made based on colony-forming units per millilitre (CFU mL^{-1}) of cow milk, as shown in Table 1.

Table 1. Bacterial colony counts of the milk samples

Identity of the cow		**Bacterial colony counts (CFU mL^{-1})**	**Mean bacterial colony counts (CFU mL^{-1}) for farms**
Farm A	no. C1	$(2.71 \pm 0.22) \times 10^4$	30,550 [a]
	no. C2	$(3.29 \pm 0.24) \times 10^4$	
	no. C3	$(3.23 \pm 0.24) \times 10^4$	
	no. C4	$(2.99 \pm 0.23) \times 10^4$	
Farm B	no. C1	$(2.60 \pm 0.21) \times 10^4$	26,200 [a]
	no. C2	$(2.25 \pm 0.20) \times 10^4$	
	no. C3	$(2.76 \pm 0.22) \times 10^4$	
	no. C4	$(2.87 \pm 0.22) \times 10^4$	
Farm C	no. C1	$(1.95 \pm 0.18) \times 10^5$	194,500 [c]
	no. C2	$(1.89 \pm 0.18) \times 10^5$	
	no. C3	$(1.90 \pm 0.18) \times 10^5$	
	no. C4	$(2.04 \pm 0.19) \times 10^5$	
Farm D	no. C1	$(3.32 \pm 0.24) \times 10^4$	29,875 [a]
	no. C2	$(3.13 \pm 0.23) \times 10^4$	
	no. C3	$(2.76 \pm 0.22) \times 10^4$	
	no. C4	$(2.74 \pm 0.22) \times 10^4$	
Farm E	no. C1	$(1.40 \pm 0.16) \times 10^5$	138,750 [b]
	no. C2	$(1.54 \pm 0.16) \times 10^5$	
	no. C3	$(1.26 \pm 0.15) \times 10^5$	
	no. C4	$(1.35 \pm 0.15) \times 10^5$	
Farm F	no. C1	$(2.61 \pm 0.21) \times 10^4$	29,800 [a]
	no. C2	$(3.38 \pm 0.24) \times 10^4$	
	no. C3	$(3.20 \pm 0.24) \times 10^4$	
	no. C4	$(2.73 \pm 0.22) \times 10^4$	
Farm G	no. C1	$(3.28 \pm 0.24) \times 10^5$	331,500 [e]
	no. C2	$(3.24 \pm 0.24) \times 10^5$	
	no. C3	$(3.38 \pm 0.24) \times 10^5$	
	no. C4	$(3.36 \pm 0.24) \times 10^5$	
Farm H	no. C1	$(2.20 \pm 0.20) \times 10^5$	234,250 [d]
	no. C2	$(2.45 \pm 0.21) \times 10^5$	
	no. C3	$(2.34 \pm 0.20) \times 10^5$	
	no. C4	$(2.38 \pm 0.20) \times 10^5$	
Farm I	no. C1	$(3.46 \pm 0.25) \times 10^4$	30,775 [a]
	no. C2	$(2.62 \pm 0.21) \times 10^4$	
	no. C3	$(3.30 \pm 0.24) \times 10^4$	
	no. C4	$(2.93 \pm 0.23) \times 10^4$	

Means that share a common superscript letter in the last column are not significantly different ($P>0.05$).

Table 1 shows that bacterial colony counts in the milk from five farms (A, B, D, F, and I) have lower bacterial loads, while the other four have higher counts. In line with our numerical values, Champagne *et al.* (2009) have enumerated the contaminating bacterial community in unfermented pasteurised milk samples.

Using the Tukey method for grouping information, the bacterial counts of cow milk from five farms (A, B, D, F, and I) were found to be statistically similar and not significantly different from each other. In contrast, the bacterial counts from the other four farms (C, E, G, and H) were significantly different from those of the first group and from each other. Statistical analysis of the results indicated a notable prevalence of bacteria in the samples, highlighting the importance of addressing these bacteria in the local dairy

industry. The data can also be used to explore the potential correlations between bacterial presence and various environmental or farming practices, offering insights into the sources of contamination.

Afterwards, predominantly detected and morphologically distinct colonies from these culture plates were isolated into pure cultures. Interestingly, in the present study, a common bacterial isolate was dominantly seen in all the milk samples obtained from the four farms, which showed higher bacterial loads (Figure 2).

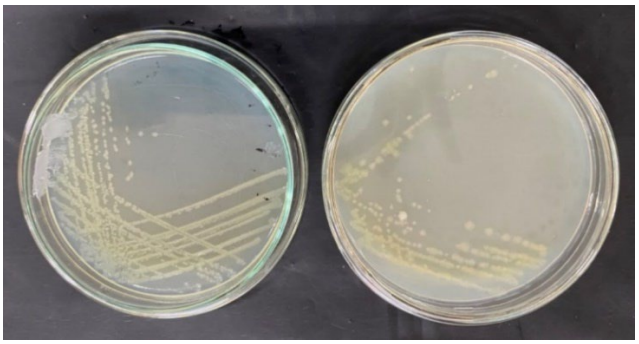

Figure 2. Isolated bacterial colonies grown in pure cultures on Nutrient Agar

The isolate was subjected to bacterial identification assays using morphological and biochemical approaches, and eventually, species confirmation was achieved by comparing gene sequences. Bacteriological analyses, including culture on microbiological media, revealed characteristic colonies indicative of *Pseudomonas* species. Biochemical tests confirmed the identity of the isolates as *P. paralactis,* and molecular techniques, including PCR and gene sequencing, provided additional verification. Bacterial characterisation based on morphological appearance, physiology, and biochemical test results has been found to be unstable, inconsistent, and heavily influenced by subjective interpretation (Sarjito *et al*., 2009; Wijayanti *et al*., 2018). The advancement of DNA barcoding, with its error-free and time-efficient methods, has greatly enhanced microbial identification and our understanding of microbial diversity (Wijayanti *et al*., 2018). In this study, bacterial species were confirmed by comparing the 16S rRNA gene sequence with published GenBank sequences that exhibited the highest homology.

The morphological studies on the bacterial isolate showed a Gram-negative, rod-shaped, motile, catalase and oxidase positive, non-endospore former. The colonies were beige, smooth, round-shaped and around 1 mm in diameter. In the biochemical characterisation of the isolate, it was positive for the utilisation of fructose, glucose, galactose, mannitol, mannose and ribose. It showed lipolysis in tributyrin agar and proteolysis in skim milk agar. Haemolysis was also detected. It was negative for the hydrolysis of starch but positive for the gelatine hydrolysis. H_2S was not produced. It was negative for indole formation, urease production, Voges–Proskauer reaction, and nitrate and nitrite reductions. The growth was not observed under anaerobic conditions.

After biochemical characterisation of the isolate, species confirmation was performed through DNA analysis by comparing the 16S rRNA gene sequence (amplified using the 27F and 1492R primers). With the most similar GenBank records, showing a 99.58% similarity, lead to the identification as *P. paralactis* (NR_156987). The comparative analysis of known sequences helps trace possible contamination sources and evaluate the virulence potential of the identified strain. The DNA sequence of the identified bacterial species has been deposited in the NCBI database as *P. paralactis* (PP155068). Furthermore, the molecular confirmation ensured the accuracy in the identification process, and sequencing data contributed for understanding the isolates' genetic diversity.

The high prevalence of *Pseudomonas paralactis* in cow milk samples underscores the need for targeted control measures. This bacterium is well known for its antimicrobial resistance patterns (Olofinsae *et al*., 2022). Its resistance to disinfectants and antibiotics further complicates treatment and control, particularly in farm environments. The identification of specific sources of contamination can guide interventions, such as improved hygiene practices on farms or modifications to milk processing protocols.

Its close phylogenetic relationship with other pathogenic *Pseudomonas* species suggests it may share common virulence mechanisms such as biofilm formation, which protects the bacteria from host immune responses and antibiotic treatment. Additionally, *P. paralactis* is likely capable of producing exoenzymes, for proteolytic and lipolytic activities, which can degrade milk components and damage mammary tissues. Transmission is often linked to environmental reservoirs such as contaminated water,

milking equipment, or bedding, highlighting the importance of good agricultural and manufacturing practices. While infections caused by *P. paralactis* may result in less severe clinical symptoms, its involvement in subclinical mastitis can lead to considerable economic losses due to decreased milk yield.

Further research and monitoring of milk samples from this area are essential for understanding the scope of the issue and implementing effective control measures. It is imperative to focus on improving the overall health and hygiene practices in dairy farming to mitigate the economic impact of mastitis on the dairy industry. Furthermore, timely diagnosis and screening of cows for mastitis are crucial in limiting the spread of the disease, its impact on milk production and food security. (Fesseha *et al*., 2021). Additionally, the study emphasizes the importance of routine pasteurizing milk to ensure public health. Furthermore, the results of this study contribute to the growing body of knowledge on *P. paralactis*.

IV. CONCLUSION

In conclusion, the isolation and identification of bacteria in the cow milk samples from the "Andella" area in the Hambanthota district of Sri Lanka have provided valuable insights into the prevalence of *P. paralactis* in dairy cows. This knowledge serves as a foundation for developing targeted strategies to mitigate bacterial contamination, enhance milk quality, and ensure the safety of dairy products in the region. Ongoing monitoring and control efforts will be essential for sustaining a healthy and robust dairy industry in Hambantota district, Sri Lanka.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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