Enterobacter hormechie- A etiological agent for bovine mastitis identified through PCR method and sequencing

Abstract

Mastitis is an important disease at global level. With steep rise in cases of bovine mastitis, it has become a matter of concern. It is responsible for nearly 70 per cent of the losses in milk production in India and also poses a significant public health risk. Diagnostic methods in field condition are rather too crude relying mainly on the apparent symptoms. Diagnostic kits available for field condition for mastitis fail to give accurate indication about the causative agents, thus increasing antibiotic resistance. Although, molecular methods is not economical, but available as a reliable diagnostic tool for early and accurate assessment of etiological agent. Current study deals with the usage of PCR based diagnostic method coupled with sequencing to find out the causative agent for mastitis in milk sample. In the present study, presence of *Enterobacter hormechie,* was confirmed using PCR method. Sequencing was used to find the base sequence and phylogenetic tree of the causative agent. The 16S rRNA gene based DNA Barcoding of the bacteria found in milk revealed that cattle is infected with Enterobacter hormaechei bacteria. The presence of Enterobacter hormechie is also a matter of concern due to public health importance. More research should be done in future with greater number of sample for prevalence of microorganism from the same region.

Key words- Mastitis, Primer, Enetrobacter hormechie, sequencing, PCR, phylogenetic tree

Introduction

Mastitis refers to inflammation of mammary gland. It includes a wide variety of causative agent. The etiological agents include gram-positive, gram-negative bacteria, viruses, mycoplasma or can also be environmental (e.g., Escherichia coli, coagulase-negative Staphylococcus, Enterococcus spp., Streptococcus uberis) in nature (Cheng *et. al.,* 2020). Depending upon the degree of inflammation, bovine mastitis can be classified as clinical, sub-clinical, and chronic mastitis. Clinical mastitis can further be sub-divided into per-acute, acute, and sub-acute depending on degree of the inflammation. Ironically, sub clinical mastitis, which shows no visible abnormality in the udder or milk, but decreased milk production with an increase in the somatic cell count (Abebe *et. al.,* 2016) and accounts for more financial losses in the herd than do clinical cases. Therefore, early diagnosis of mastitis is necessary so that the quarters of udder don’t get fibrosed. Environmental mastitis is mainly associated with the gram negative bacteria Enterobacteriaceae. The Enterobacteriaceae family has more than 50 genera and over 200 species. Out of 50 genera, Escheria coli, Klebsiella, Enterobactor, Serratia and Proteus are isolated frequently from dairy environment. Thus, identification and characterization of Enterobacteriaceae in dairy production system is necessary to understand the importance of these bacteria as causative agent for mastitis.

Diagnosis of mastitis based on clinical observations is the most common diagnostic method in field condition. Other commonly used methods are identification of the infectious agent, somatic cell count/somatic cell score etc. However, culture and polymerase chain reaction are the modern method for the diagnosis of an intramammary infection, although, both methods have their advantages and disadvantages (Adkin *et. al.,* 2018). Disadvantage in this method is, high cost while advantage is about the accurate and early diagnosis which is necessary in the wake of increasing antibiotics resistance due to its indiscriminate use and also to ensure complete treatment of animal. Now a day, molecular technique such as 16S rRNA sequencing is the gold standard technique for identification of bacteria. Therefore, quality of microbial identification is the key task to control the mastitis in veterinary clinical management. With this hypothesis, present study aimed to identify causative agent of bovine mastitis using PCR technique and sequencing.

Material and methods

The animals were screened and selected from Organized dairy farm in and around Hazaribagh and Ramgarh District, Veterinary Clinical Complex (RVC) and Instructional livestock farm complex, College of Veterinary Science and Animal Husbandry, Kanke, Ranchi. Selections of the animals were done on the basis of History, Physical and clinical examination of udder. Indirect tests were performed after collections of milk samples from selected animals on the basis of above-mentioned criteria for the diagnosis of mastitis. Screening of mastitis cow was done by California Mastitis Test (CMT)), strip cup test and modified white side test. Out of the milk samples of selected animal, four random samples were taken for PCR based analysis and sequencing. This was a part of M.V.Sc. work in the department of Veterinary Medicine, CoVSc. & A.H on the topic “Therapeutic efficacy of Psidium guajava and Punica granatum against coliform mastitis in cattle”. Current research article is based on the molecular analysis part of the research. The purpose of PCR based assay was to emphasize on molecular diagnostic technique and to be able to decipher the holistic phylogenetic detail of the causative organism of the mastitis in current experimental units. The following work was done by outsourcing in Akriti Biotech Research & Development Centre, Kathal More, Ranchi, Jharkahnd.

Four milk samples were received and labeled as LF, RF, RH1 and LH1 (Figure1). After serial dilution they were inoculated (Figure 2a, 2b, 2c and 2d). Milk samples were serially diluted and the highest dilution was spread on EMB agar plates and incubated at 37°C for overnight. No bacterial growth observed in sample RF and RH1. Single colony obtained on the EMB agar plates of samples LF1 and LH (Figure 3a, 3b, 3c and 3d) was inoculated in Lysogeny Broth (LB) and incubated at 37°C for overnight. Genomic DNA was isolated from the respective bacteria cultures (Figure 4a and 4b) using Hi Media bacterial DNA isolation kit (Figure 5). Universal 16S rRNA gene primer set was used to amplify the 16S rRNA gene and sent for sequencing. Two PCR reactions failed for unknown reasons, so our internal primer set was used (Figure 6).

Composition of the PCR Mix was as follows-

**PCR Composition (for 50 l reaction):**

Master Mix (Promega)– 25µl

Forward Primer– 2.5 µl

Reverse Primer– 2.5µl

Template DNA– 2 µl

Nuclease free water for final volume – 18ul

**PCR programme was run in the following steps-**

Initial denaturation 94 0C for 5minutes

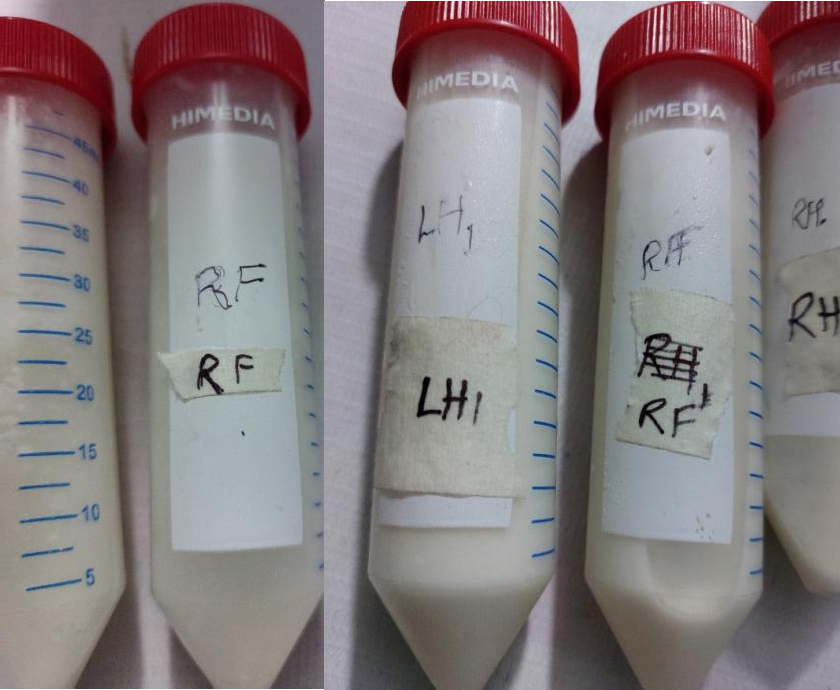
Denaturation 940C for 40seconds

Annealing 450C for 45seconds

Extension 720C for 1minute

Final Extension 720C for 10minutes

Sequencing of the PCR amplicon thus obtained was performed on Applied Biosystems platform. Sequence analysis was done online on NCBI Database.



**Figure 1: Milk Samples**

Figure 2a

Figure 2b

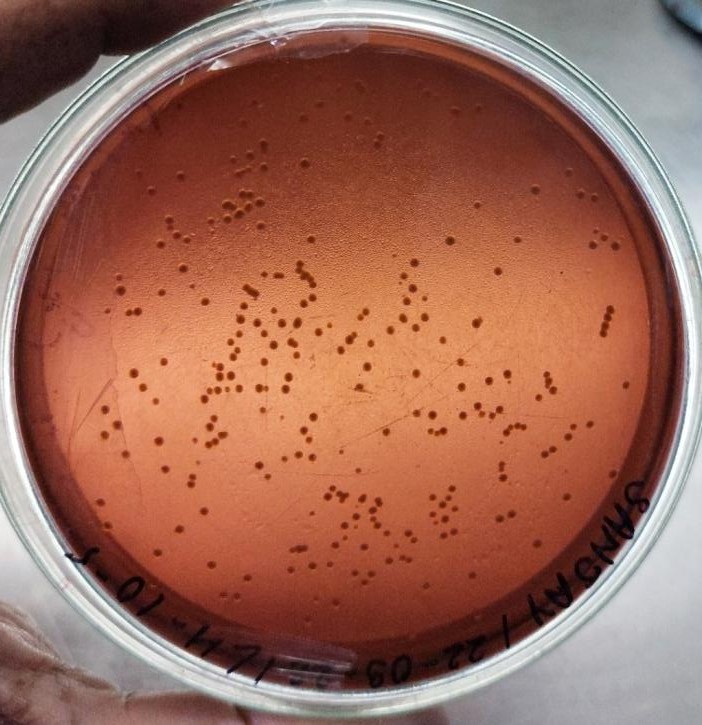
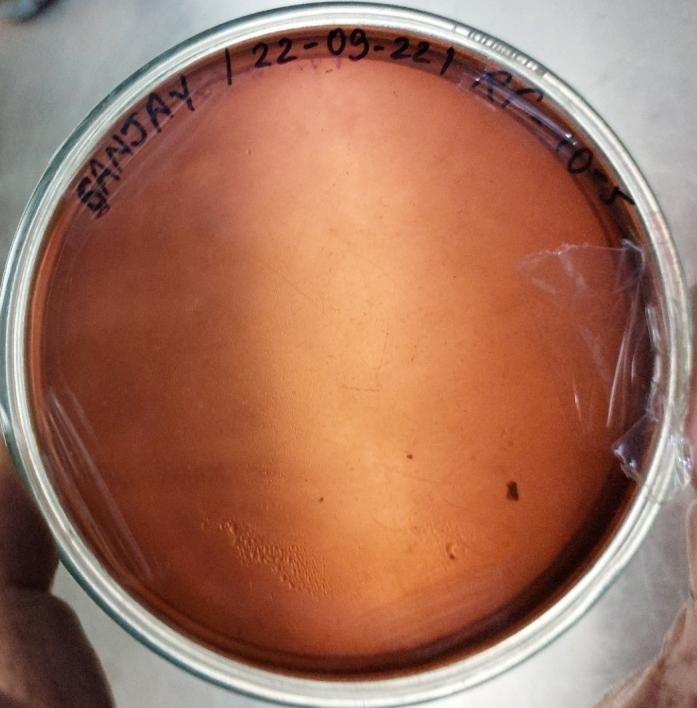
Figure 2c

Figure 2d

**Result and Discussion**

**Fig 2 : Serial dilution technique**

Out of Four milk samples were received and labeled as LF, RF, RH1 and LH1. The milk samples LH1 and LF were found to be contaminated with bacteria as evident by the growth on EMB agar plates, however sample LH1 being highly infected as shown in Fig 3c and 3d. Milk samples RH1 and RF had not bacterial contamination as no growth of bacteria was observed on the plate (Fig. 3a and b).



**Fig. 3a- Milk sample from RH1 on culture media**

**Fig. 3b- Milk sample from RF on culture media**

**Fig. 3c- Milk sample from LH1 on culture media**

**Fig. 3d- Milk sample from LF on culture media**

**Genomic DNA yield**: The genomic DNA yield from the respective pure bacterial cultures (Fig. 4a and 4b) was found to be 10 µg (Fig. 5) as analyzed by spectrophotometry analysis.

**PCR Amplification:** The PCR reaction of 16S rRNA gene gave an amplification of 1.5 kb (Fig.6)



1

2



1

2

3

4

5

Fig. 5-DNA Extracted

Fig. 6-PCR amplification of 16S rRNA gene. Lane1:100bp Ladder, Lane 2: LH1; Lane 3: LF, Lane 4: Positive control, Lane 5: Negative control

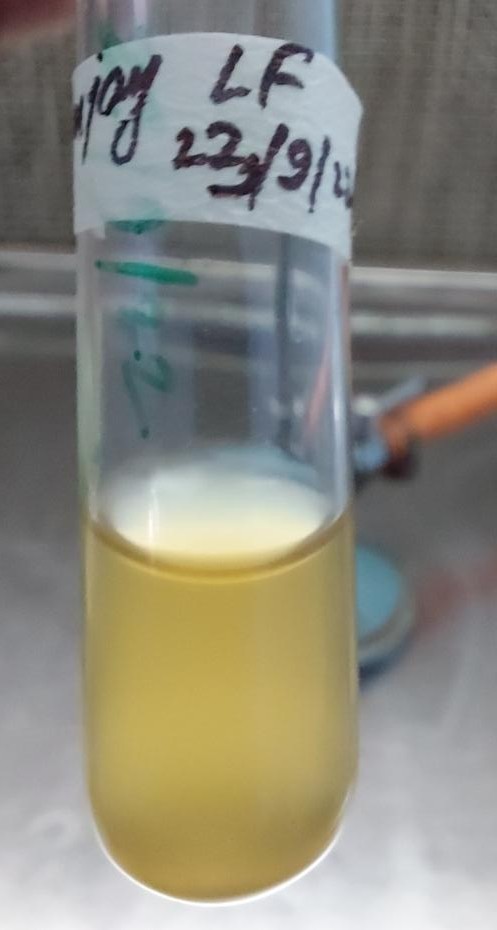
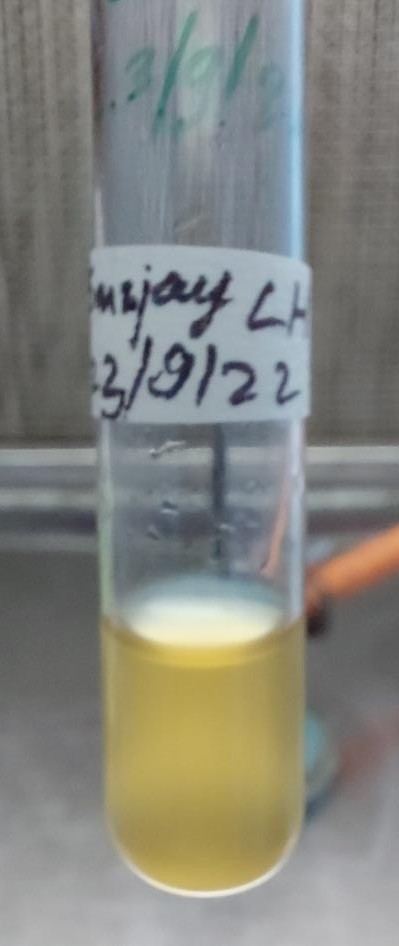


Figure 4a: Pure culture Bacteria from LH1

Figure 4b: Pure culture of Bacteria from LF

**Sequencing**: The DNA sequence obtained after sequencing, when analyzed using the NCBI Database, revealed that both the milk samples were infected with Enterobacter hormaechei, a common pathogen that is a recognized contaminant of raw milk and dairy products.

Sequencing Result

**Sample:** LF bacteria **Identity**:Enterobacter*hormaechei*

# Sequence:

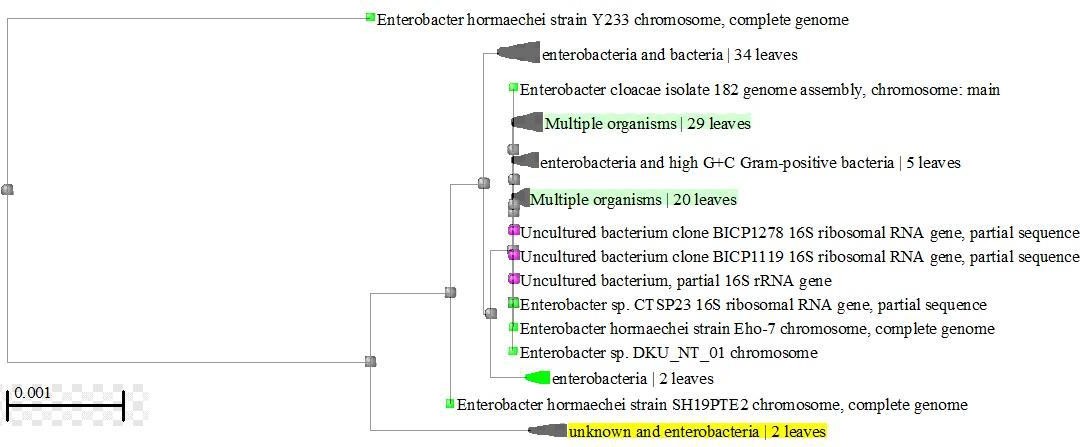
GCTGAGGCGGCTACACATGCAGTCGAACGGTAACAGGAAGCAGCTATGCTAGCTATTGCTAGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTCGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCC CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTACCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAAACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCATTTGTTGCCGGGGGTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAACTGGAGGAAGGGGGGGATGACGTCAAGTCATCAGGCCCTTACGAGTAGGGCTCCCCCCTGCTAAATGGCGCTTCAAGAAAAAGCGACCT

**Sample:** LH bacteria **Identity: *Enterobacter hormaechei*** Sequence:

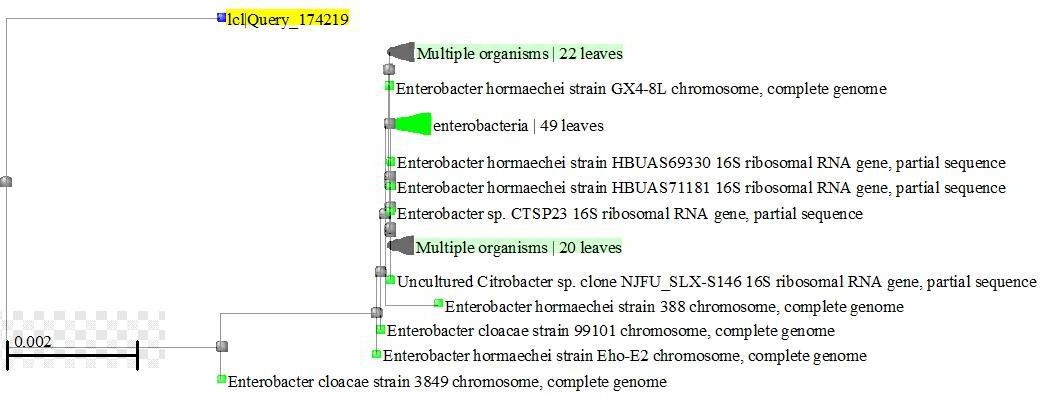
CCGGGGCGGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTACCAGAGATG CTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAGGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACCACCTGCTACATGGGCCATACAAGAAGAAGCGACTTCGCGA AAACAAGCGGACCTCATAAAATGCTTCCAATCCGAATGAAAT

**Phylogenetic Analysis:**

Bacteria from milk sample LF clearly depicts that the bacteria found in the milk sample is closest to *Enterobacter hormaechei* in terms of evolutionary tree or distance. Whereas, bacteria from milk sample LH1 was closest to *Enterobacter cloacae.* On the contrary, the sequence alignment presented *Enterobacter hormaechei* in the first match.

The 16S rRNA gene based DNA Barcoding of the bacteria found in milk revealed the cattle is infected with *Enterobacter hormaechei* bacteria. The 16S rRNA gene sequence of bacteria isolated from LF milk sample, on sequence alignment analysis matched 97.95% with *Enterobacter hormaechei* and in accordance with the phylogenetic tree of distance tree. In case of bacteria from milk sample LH1 the sequence alignment analysis shows 98.73% identity with *Enterobacter hormaechei.* However, in the phylogenetic tree the query sequence shows closest relation to *Enterobacter cloacae*. But, since the query sequence matches first with *Enterobacter hormaechei*, we consider it to be the correct identity.

**Figure 7- Phylogenetic tree of bacteria isolated from LH1 milk sample**



**Figure 8- Phylogenetic tree of bacteria isolated from LF milk sample**

*Enterobacter* is an oxidase-negative, gram- negative bacterium that belongs to the family *Enterobacteriaceae* formerly known as Enteric group 75 (O'hara *et al.,* 1989). *Enterobacter hormaechei* is a common pathogen that infects both cattle and humans (O'hara *et al.,* 1989). *Enterobacter hormaechei* infection can cause mastitis in cattle as reported earlier in a number of cases (Aslantaş *et al.,* 2022; Mokgaotsi, 2019; Rodrigues *et al.,* 2017; Zhong *et al.,* 2023) as observed in present study also. Enterobacter sp. exists in broad range of environments like soil, water, food processing factories, plantations etc. They are also widely considered as potential human pathogens. A number of clinical cases of *Enterobacter hormaechei* milk infection have been reported in almost every region of the world. The case of *Enterobacter hormaechei* infection was first reported in a 2-months old severely ill calf (Wang *et al.,* 2020). It also infects the buffalos as reported by Fagiolo and Lai (2007). *Enterobacter hormaechei* has also been isolated from animals suffering from polymicrobial bovine pneumonia (Choudhary *et al.,* 2019). In recent times, *E. hormaechei* has evolved as emerging pathogenic bacteria due to its potential to cause bloodstream infection in livestock linked with morbidity, mortality and financial cost (Qian *et al.,* 2020).

Incidence of multi drug resistant strains of *Enterobacter hormaechei* being isolated from cow with reproductive system infection (Zaitsev *et al.,* 2022). It belongs to the Cluster VI of *Enterobacter cloacae* complex (ECC) that is a group of common nosocomial pathogens that are capable of cause a wide variety of infections (Annavajhala *et al.,* 2019). One of the primary reasons for Multi Drug Resistance or Antimicrobial Resistance among pathogens is due to the ability of pathogens to produce biofilm that prevents the drug to reach the bacteria cell (Liu *et al.,* 2022). Since, *Enterobacter hormaechei* belong to the biofilm forming group of pathogens, the reason for its drug resistance is its ability of formation of biofilm (Edris *et al.,* 2023).

**Conclusion**

The finding of the present revealed that milk samples were found to contain *Enterobacter hormechei* bacteria hitherto none very common causative agent for bovine mastitis with zoonotic importance, so it raises alarming public health concern in the wake of milk being global source of food. Further and future research is needed with more number of samples to ascertain and confirm Enetrobacter hormechei as well as to find other causative agent in mastitic milk using PCR method.

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