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Isolation and Molecular Detection of Enterobacteraiceae (*Hafnia alvei*) in Cow's and Buffalo's Raw Milk at Basrah Governorate

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ABSTRACT

The current study was aimed at isolating and molecular characterization of *Hafnia alvei* implicated in pathogenic list of mastitis in cows and buffaloes milk. Six (20%) and four (13.33%) isolates of *Hafnia alvei* were obtained from raw milk of cows and buffaloes, respectively. The differences between cows and buffalo milk crude were not statistically significant. In the molecular diagnosis, the positive results for cows and buffaloes, respectively, were 5 (16.66%) in cows and 3 (10%) samples in buffaloes when diagnosed with the RB89-F/RB90-R gene. It was clearly shown with the 450 base pair gene.

Key words: Isolate, *Hafnia alvei*, cows, buffaloes

INTRODUCTION

Hafnia alvei is rod-shaped, Gram-negative bacterial agent belonging to Enterobacteraiceae having a diameter of roughly 1 and 2-5 mm of length. The catabolization of carbohydrates especially D-glucose resulting in production of acids without or with gas. Additionally, almost strains are positive to catalase, methyl red and Voges-Proskauer; but negative to Simmons citrate, Indole and oxidase (Padilla *et al.*, 2015). *H. alvei* is facultatively anaerobic and generates biofilms depending on development phase, strain tested, culture media and temperature. The origin of bacterial name is from "Havn", a city in Copenhagen (Denmark), for genus and from the Latin noun alveus that means "beehive", for species. Worldwide, little available reports are conducted to *Hafnia* genus numerically through the principle of numerical taxonomy (Kibegwa *et al.*, 2020). However, studies based on three different methods of numerical classification revealed that there were 15 strains of *H. alvei* which existed as separately and distinctly branched within the *Klebsiellae* tribe (Nakano *et al.*, 2022). In animals especially mammals, digestive system appears the site of growth and existence of the bacterium. In Michigan and Ohio, Paleomicrobiology investigations for 12,000-year-old mastodon sediments and intestinal samples detected the presence of *H. alvei* (Fagernäs and Warinner, 2022). One previous study referred to *Hafnia* as an etiology of enteritis; however, recent demonstrated data remain low and need to be supported. In humans are often regarded as an opportunistic bacterium that can cause infections related with underlying conditions or predisposing conditions in immunocompromised persons, producing septicemia, endocarditis, meningitis, pneumonia, abscesses, urinary infections, peritonitis, endophthalmitis, cholecystitis, intestinal disorders and postenteritic arthritis (Rossi *et al.*, 2019; Nde, 2020; Suvarna and Mahon, 2022).

In veterinary medicine, in spite of a fact that *H. alvei* has been recognized in past five decades; but relatively, there are scarce available data about the importance of this bacterium in different diseases of animals (Janda and Abbott, 2021). Also, this bacterium could be related with illness outbreaks among different species of animals such bovine, caprine, ovine and birds. In one study, the findings showed that *H. alvei* could cause a significant retardation in skin and wool properties (colour, texture, elasticity and odour) of merino sheep with causing cellular infiltration and hyperemia in dermal tissues. Khan *et al.* (2016) studied a group of goats with varying degrees of pneumonia and accounted that 9.83% of samples were positive to this bacterium. In cattle, results obtained by

another study referred to the role of this pathogen in chronic mastitis (Khan *et al.*, 2016). Mastitis in cattle is a common etiology for great economic losses in Turkish dairies (Nimbalkar *et al.*, 2020), and even worldwild estimated to be more than 28 million USD. Different bacteria have been demonstrated to be main etiology for mastitis. According to their epidemiological association, additional classification of bacterial agents categorized them to environmental and contagious pathogens (Van Eenennaam *et al.*, 2021).

The last category includes *Staphylococcus aureus*, *Staphylococcus agalactiae* and *Mycoplasma* spp. It has been observed that food-borne pathogens lead to outbreaks of infection regardless of the region from which they came. Thus, rapid detection becomes important to reduce the risk of pathogen spreading before an epidemic occurs. Various techniques have been developed to improve the methods of its detection. Microbiology can be studied by conventional and chemical methods or by using molecular biology methods (polymerase chain reaction technology) (Ndivhuwo and Sciences, 2020; Dyson *et al.*, 2022). Therefore, this study was conducted to get reliable, quick, sensitive, specific and effective tool to detect *H. alvei* in samples of raw cow's and buffalo milk contaminated with it. This study aimed at isolating and molecularly characterizing the *Hafnia alvei* from mastitis in cows and buffaloes in Basra governorate.

MATERIALS AND METHODS

Totally, 60 cows and buffaloes raw milk samples were obtained amongst different household animals and local markets throughout the Basrah governorate. Before sampling, udder of each animal was cleaned by warm water, dried and the milk samples were drained into sterile plastic container which were transported to laboratory using cooled box and kept frozen (4°C) until analyzed. Five different locations were used to collect the samples.

To isolate *H. alvei*, 0.5 ml raw milk sample was injected in 4.5 ml medium buffer peptone water for 24 h, then 1 ml from the previous medium was inoculated onto MacConkey Eosin Methylene Blue agars at 37°C (1 day). The bacterial cultures were purified by taking a type of bacterial colonies that were different in appearance, shape, colour and size. Conducting phenotypic and biochemical tests (Formalized paraphrase) the sample was swabbed onto a disposable glass-slide that subjected for Gram's staining and visualization by light microscopy.

Oxidase test (1.0% 4 tetramethyl), Simmon's citrate (Oxoid-UK) and Carbohydrate oxidative, urease (Himedia – India), TSI, methyl red, Vogues proskauer, gelatin hydrolysis tests were used to determine phenotypic characteristics (Nde, 2020; Yaqoob *et al.*, 2022) Until pure cultures could be established, the isolates were sub-cultured on the same medium. The isolates were grown onto nutrient broth to be kept as stock cultures at -20°C in 15% (v/v) glycerol for further examination (Sengupta and Bhowal, 2022).

All raw milk samples were subjected for extraction of DNAs by the Genomic DNA Extraction Kit (G-spin Total) to demonstrate existance of *H. alvei* DNAs. Targeting the 16S rDNA gene, the primers of many Enterobacteriaceae spp. like *Shigella* and others species; RB89-F AAG TTC TGA CGC GAT TGG, RB90-R TGT ACG CGA TCA AGA ATC CC were designed. The Mastermix tubes were prepared at a final volume of 25 µl [5 µl DNA, 2.5 µl for each F and R primers and 15 µl free-nuclease water], and PCR reaction was conducted in Thermocycler system (Techne, UK) following these conditions: 1 cycle initial denaturation (95°C/5 min), 35 cycles for denaturation (95°C/ 45 sec), annealing (50°C/45 sec) and extension (72°C/45 sec), and 1 cycle for final extension (72°C/6 min).

The amplified PCR products subjected for analysis by Ethidium Bromide stained 1% agarose gel, recognized by electrophoresis, and band sizes were detected based on the ladder marker (1000-1 bp; Higashiura *et al.*, 2019). Sequencing of positive PCR products was carried out in the Macrogene Compant (Korea), and the received data were analyzed by the Parbi-Doua and NCBI BLAST programs, aligned and compared to isolates of NCBI-GenBank. Chi-square test in the SPSS was used to detect significant differences at $P<0.05$ (Jaafar Al-Gharban, 2017).

RESULTS AND DISCUSSION

Based on morphological and staining properties of colonies, 4-6 isolates were found positive to cow and buffalo samples, respectively. All these

appeared as G-negative rod-shaped isolates. Phenotypic characteristics confirmed the high percentage (50-46.66%) of bacterial isolates plating inoculation, as well as biochemical tests identified 6 (20%) and 4 (13.33%) respectively. However, insignificant variation was seen between the values of positive cow and buffalo (P>0.05) samples (Table 1).

Table 1. Total results of positive *Hafnia alvei* isolates by bacteriology

Animal species	Numbers tested	Conventional bacteriological analysis number (%)	
		Plating characterization	Biochemical characterization
Cow	30	15 (50%)	6 (20%)
Buffalo	30	14 (46.66%)	4 (13.33%)
Total	60	29 (48.33%)	10 (16.66%)

Raw milk from six cows and four buffalos was recorded positive to *16S rDNA* gene of *H. alvei* isolates. Five out six tested samples (83.3%) showed positive results, while the number of samples that showed a positive result in buffaloes was three samples out of four tested, with a percentage (75%; Fig. 1).

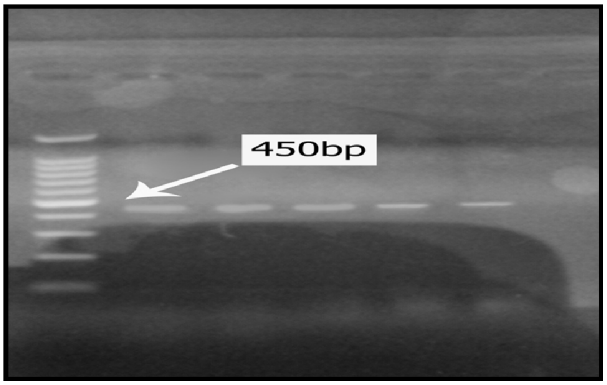

Fig. 1. The PCR amplification of RB89-F/RB90-R gene products of *Hafnia alvei* isolates (450-bp).

Shut-Alarab, Al-Zubair, Al-Qurna, Basrah center and Abi-Elkhasib are the Basrah districts where raw milk from cows and buffalos was located. The highest ratio of *H. alvei* contamination in cow raw milk was found in Al-Zubair (50%), as well as in buffalo (22.2%), while the lowest ratio in cow milk was found in AL-Qurna (0%), while the lowest ratio in buffalo milk was recorded in Shut-Alarab, Basrah center and Abi-Elkhasib (0%) for all (Table 2). The difference in raw milk *H. alvei* contamination among Basrah districts was not significant (P>0.05).

Table 2. Distribution of cow and buffalo milk *H. alvei* isolates according to Basrah districts

Districts	Number (%) of isolates			
	Cow		Buffalo	
	Tested	Positive	Tested	Positive
Shut-Alarab	7	0	3	0
AL-Zubair	6	3 (50)	9	2 (22.2)
AL-Qurna	5	0	0	1 (10)
Basrah center	8	1 (12.5)	5	0
Abi-Elkhasib	4	1 (25)	3	0
Total	30	5 (16.66)	30	3 (10)

Raw milk from cows and buffaloes was tested in October, November and December (Table 3). The highest ratio of contamination in *H. alvei* in cows raw milk was found in October, 2020 (23%), while the lowest was found in December, 2020 (11.11%). The highest ratio of raw milk *H. alvei* contamination in buffaloes was found in December (20%), and the lowest in October (11.11%).

Table 3. Distribution of *H.alvei* isolates in cow and buffalo raw milk according to months of sampling

Month	Number (%) of isolates			
	Cow		Buffalo	
	Tested	Positive	Tested	Positive
October	13	3 (23)	9	1 (11.11)
November	8	1 (12.5)	16	2 (12.5)
December	9	1 (11.11)	5	1 (20)
Total	30	5 (16.66)	30	3 (10)

PCR products of RB89-F/RB90-R gene products of *H. alvei* isolates were sent to Korea-macrogene. Data of sequenced DNAs were applied to BLAST-NCBI software to determine identity between *H. alvei* isolates. In this study, complete sequencing of genes RB89-F/RB90-R gene was compared with the ones reported in GenBank. In the present study, four sequnces confirmed similarity to sequences producing alignments to CP015379.1 from Spain which underwent analysis of the genetic tree (Figs. 2, 3 and 4).

The role and function of mastitis pathogens

Hafnia alvei strain HUMV-5920, complete genome

Sequence ID: CP015379.1 **Length:** 4542863 **Number of Matches:** 1

Range 1: 4521969 to 4522204 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
353 bits(191)	3e-93	221/236(94%)	0/236(0%)	Plus/Minus

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Query  1        AGTTGCTGTTTATTCTCCAAGGACAGGGCATAACGGCCATTGGAAAACCTATCCCTGCAG  60
               ||||||||||||||| ||||||||||||||  ||||||||  ||||||||||||||||||||
Sbjct  4522204  AGTTGCTGTTTATTCGCCAAGGACAGGGCAGAACGGCCAGCGGAAAACCTATCCCTGCAG  4522145

Query  61       TCACTATCGTGCGCGGCGATGGCGACCAACACCGCTTTGGGTTGGCTGATCGTGTAGCTT  120
               ||||||||||||||| |||||  ||||||||||||||||  ||||||||||||| |||||
Sbjct  4522144  TCACTATCGTGCGCGGTGATGGTGACCAACACCGCTTTGGATTGGCTGATCGTGGAGCTT  4522085

Query  121      ATACCGGCGTCACGGCGAGCTGGTTAAATACGCGAGAGCCAAAGAAAAAAGAGCAGGTAG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4522084  ATACCGGCGTCACGGCGAGCTGGTTAAATACGCGAGAGCCAAAGAAAAAAGAGCAGGTAG  4522025

Query  181      CGGGCCAGCGTAAACGCAACACGAAAATTCCGGCGAAACCCAAGGAGCCGGAGGCT  236
               ||| | |||||||||||||| |||||||    ||||||||||||||||||||||||
Sbjct  4522024  CGGTCAAGCGTAAACGCAAGACGAAAAGCAAGGCGAAACCCAAGGAGCCGGAGGCT  4521969
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Fig. 2. Blast of partial sequence of *H. alvei* strain MJ01 against *H. alvei* strain HUMV-5920.

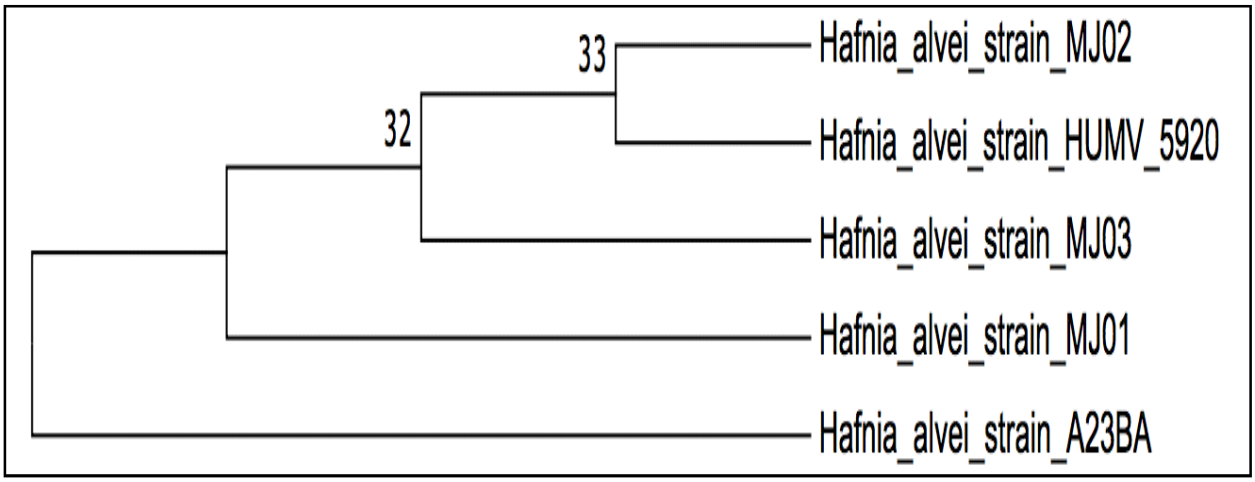

Fig. 3. Evolutionary relationships of *H. alvei* strains registered in the NCBI database.

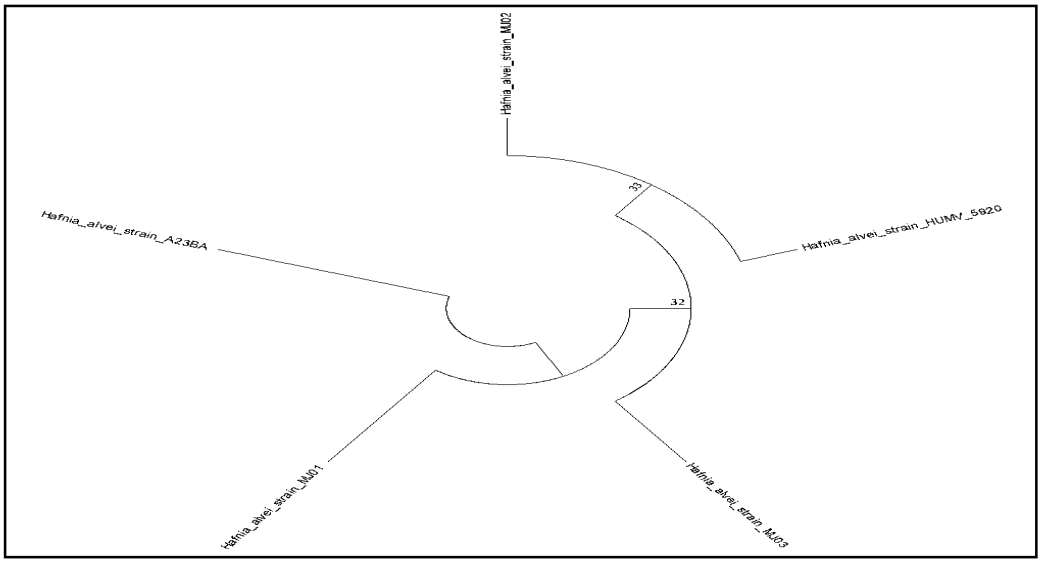

Fig. 4. Evolutionary relationships of *H. alvei* strains registered in the NCBI database.

as possible zoonotic agents was intensively investigated in order to enhance public awareness of food safety issues. In poor nations, diseases are more prevalent than in developed nations. It is critical because it has a huge economic and health impact. Early detection can aid in preventing the spread of the disease (Cantas and Suer, 2014). Among the most prevalent zoonosis connected with cattle are gastrointestinal diseases. *Listeria*, *Enterohaemorrhagic*, *Campylobacter*, *Shigella*, *Yersinia*, *Streptococcus*, *Salmonella* and *E. coli* are among the bacteria that cause human food poisoning. Certain causative agents might exist in products of milk due to incorrect treatment or food preparation (Samardžija *et al.*, 2017). At the animal level, lactation stage, breed, age, level of cleanliness, productivity and somatic cell counts (SCCs) could act as individual risk variables. Under the same holding conditions, particular breeds have high susceptibility to mastitis. However, mastitis risk might be increased significantly in contaminated environments and cows having greater higher SCCs (Hiitiö *et al.*, 2017). Following the CNS species, coliform bacteria are a prevalent environmental pathogen. *H. alvei* was the most prevalent coliform in this investigation. It's a substance that can cause persistent mastitis in cows (Mugo, 2020). The bulk of the coliform population in pasteurized fluid milk was found to be *Enterobacter*, *Hafnia*, *Citrobacter*, *Serratia* and *Raoultella*, according to a research (Masiello *et al.*, 2016). Until recently, *Hafnia* had been demonstrated to have on species, *H. alvei* that actually new *H. paralvei* species, with additional strains formerly categorized as *Obesumbacterium proteus*, which is now obsolete. The initials are *H. even* an enteropathogen later identified as a new species, *Escherichia albertii*, was placed in Alvei sensu lato *H. alvei* despite being a very diverse cluster, it was isolated from wide source ranges such as animals, water and soil (Janda and Abbott, 2021; Foster-Nyarko and Pallen, 2022). Microbiological testing is a frequent diagnostic approach that has evolved into the gold standard in mammary gland immunological function research. Mastitis diagnosis based on gene analysis is becoming increasingly prevalent. Gene analysis can apply for previous investigation of isolates and to identify an existence of bacteria (Martins *et al.*, 2019). After employing selective plating, morphological and biochemical characterization to detect *H. alvei* species, appllied the polymerase chain reaction methodology using *H. alvei* primers in order to reach a simple, rapid and specific aim of quality and sensitivity of 100%. No matter how low the concentration, milk samples obtained from various places and for various durations of time give false positive or false negative findings. Because this approach produces no false negative findings, even if only one sample is detected, it is possible to examine *H. alvei*, especially because the FAO considers all samples positive if only one sample is positive (D'Amore *et al.*, 2020). The Neighbour-Joining technique had applied for inferring an evolutionary history to showing bootstrap consensus tree that initiated from 500 replicates (de Villiers *et al.*, 2019). Branches that corresponded for partitions could be replicated at <50% of collapsed bootstrap replicates. Next to the branches are the percentages of duplicate trees in which the related taxa clustered together in the bootstrap test (500 repetitions; Hammond *et al.*, 2019). The evolutionary distance could be calculated by Jukes-Cantor method and expressed in base substitutions per site unit. Five nucleotide sequences were examined (Kumar *et al.*, 2021). 1st + 2nd + 3rd + noncoding codon locations were included. Gaps and missing data were removed from all positions. The total number of positions in the final dataset was 191. MEGA7 was used to perform evolutionary analysis.

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