Original Article

First Isolation of Bacillus licheniformis from Bovine Mastitis in Iran

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ABSTRACT

Bacillus licheniformis is a gram-positive, endospore-forming, saprophytic and facultative anaerobe that is resistant to heat and environmental conditions. This study was the first to isolate and confirm *B. licheniformis* as a cause of bovine mastitis in Iran. In the summer of 2020, 105 samples of mastitic milk were collected from dairy farms around Tehran and sent to the microbiology laboratory of the Faculty of Veterinary Medicine at the University of Tehran. The bacterial pathogens were identified using selective and differential culture media and confirmed by PCR to contain the toxin synthetase genes *lic*A, *lic*B and *lic*C in mastitic isolates of *B. licheniformis*. Resistance patterns to 19 antibiotics were determined for two isolates of *B. licheniformis*. *Staphylococcus aureus* and *Escherichia coli* were identified as the most important organisms in the samples. *B. licheniformis* was isolated from the two samples containing all three genes. Both isolates were resistant to streptomycin, trimethoprim-sulfamethoxazole, cefixime, ampicillin, bacitracin, clindamycin, and gentamicin. *B. licheniformis* was reported for the first time in Iran as a cause of bovine mastitis with clinical symptoms. The first isolation of toxin-producing strains of *B. licheniformis* from mastitic cows in Iran raises concerns about the safety of dairy products. In principle, selected strains with toxigenic potential should not be used as feed additives and animal feed. However, whole genome sequencing is proposed to search for genes coding for toxins.

Keywords: *Bacillus licheniformis*, Bovine, Isolation, Mastitis, Toxin synthetase genes

1. Introduction

Bacillus spp. are facultative, gram-positive, rod-shaped, endospore-forming anaerobes. They are found everywhere in the environment and can survive severe conditions in the form of highly resistant endospores (1). Due to this ability, *Bacillus spp*. are more resistant than enteropathogenic bacteria (2). Its fermentative ability and low toxicity make *Bacillus licheniformis* a good bacterium that has long been used safely in industry for products such as enzymes, antibiotics, amino acids and insecticides (2, 3). Bovine toxaemia and abortions in cattle and sheep have been reported, and B. licheniformis has been experimentally shown to infect the bovine placenta (4).

Bacillus spp. has also been implicated as a cause of mastitis in animals, including cows (2), and is known to be one of the most commonly detected putrefactive agents in dairy products in many countries. *B. licheniformis* is the most common species isolated in raw milk, especially in winter, when cows are kept indoors (3). In addition, outbreaks of foodborne pathogenic *B. licheniformis* have been associated with cooked meat, raw milk and vegetables (3, 5).

Bacillus spp. produce heat-stable lipopeptide toxins that have been isolated from dairy products (6). *B. licheniformis* has also been shown to produce a toxic lipopeptide substance called lichenysin A in food poisoning (1). One source of toxigenic heat-resistant endospore-forming bacteria in raw milk could be cows with a history of mastitis (6).

In the present study, *B. licheniformis* was isolated from mastitic cows for the first time in Iran and the presence of lichenysin synthetase genes in the mastitis-induced strains was detected by PCR. This study was conducted to show how toxic *B. licheniformis* can be isolated from raw milk and cause mastitis in cows.

2. Materials and Methods

2.1. Isolation and identification of *B. licheniformis*

The study was conducted on 105 mastitic cows from five farms in the Tehran area that showed clinical symptoms of mastitis. The milk samples were collected in individual sterile containers over the course of three months from June to August 2020. The containers were stored in iced coolers and were sent to the bacteriology laboratory for cultivation and identification of potentially infectious pathogens.

Samples were cultured on blood agar and McConky agar (Merck; Germany) and were at 37°C for 24 h. Suspect *B. licheniformis* colonies were selected and subjected to preliminary biochemical identification using esculin, urease, nitrate reduction, ONPG, egg yolk reaction, motility, glucose acid, ribose, fructose, mannose, mannitol and maltose tests (Merck, Germany) (7). Islated bacterial strains other than *B. licheniformis* were also were by morphological comparison and biochemical tests.

2.2. Antibiotic resistance test

The antibiotic susceptibility of the *B. licheniformis* isolates to 14 antibiotics was determined by the disk diffusion method, as described by the National Committee for Clinical Laboratory Standards (NCCLS) (8). The *B. licheniformis* isolates were cultured on tryptone-soy broth (Merck) and incubated at 37°C for 1to 2 h. They were then calibrated using the 0.5 McFarland BaSo4 turbidity standard. The resulting suspensions were spread evenly on the surface of dry Mueller-Hinton agar (Merck) plates using an impregnate swap. The antimicrobial disks were placed aseptically on the surface of the inoculated agar media and the plates were incubated at 37°C for 24 h. Measures were then taken to determine the size of the inhibition zones. The isolates were classified as susceptible (S), intermediate (I) and resistant (R) according to the guidelines published by MAST.

2.3. DNA extraction and polymerase chain reaction (PCR)

The mastitic *Bacillus* isolates were cultured for 24 h at 37 °C on LB agar plates (Merck), and genomic DNA was extracted using a commercial DNA purification kit according to the manufacturer's

Table 1. PCR primers for 16S rRNA and detection of lichenysin synthetase genes

Primer pair	Primer sequence (5'-3')	Product (bp)
16S r RNA[1]	17 F: GAGTTTGATCCTGGCTCAG 1541R: AAGGAGGTGATCCAGCCGCA	1500
licA	F: GTGCCTGATGTAACGAATG R: CACTTCCTGCCATATACC	735
licB	F: TGATCAGCCGGCCGTTGTCT R: GGCGAATTGTCCGATCATGTCC	904
licC	F: GCCTATCTGCCGATTGAC R: TATATGCATCCGGCACCA	1195

195 (3 min), 35 cycles; 95 (45 s); 58 (60 s); 72 (90 s); 72 (5 min)

instructions (UltraClean Microbial DNA Isolation Kit, MO BIO, USA). The extracted DNA samples were stored at -20°C for further analysis. A PCR assay targeting the 16S rRNA in the genomic DNA was used to confirm the identification of an isolate of *B. licheniformis*, and this sequence was submitted to GenBank (MT279447.1) (Table 1). Three primer pairs specific for *lic*A (735bp), *lic*B (904bp) and *lic*C (1195bp) were used in this study (Table 1) (9, 10).

Amplification reactions were performed in 25 µl volumes containing 0.2 to 0.6 ng template DNA, 1.5 mM $MgCL_2$, 200 µM dNTP, 2.5 µl PCR buffer 10X, 1 U Taq polymerase PCR, and 10 pmol of each primer (Bioneer; Korea). PCR was performed for 30 cycles in a DNA thermal cycler (TC-512; UK), with 2 min initial denaturation at 94°C, 15 s denaturation at 94°C, 30 s annealing at 60°C for primer pairs *lic*A and *lic*B, 57°C for primer pair *lic*C, 50 s extension at 72°C, and 5 min final extension at 72°C. The amplification products (10 µl) were separated by electrophoresis on 1% agarose gel in 1X TBE buffer for 1 h at 100 V. The agarose gel was then stained with 2 µg/mL ethidium bromide (CinnaGen; Iran) and transilluminated with a UV trans-illuminator (Biorad; UK).

3. Results

Of the 105 mastitis milk samples collected from dairy farms in different parts of Tehran province, *Staphylococcus aureus*, *Streptococcus agalactiae*, *E. coli* and *Trueperella pyogenes* were identified as the major organisms (Table 2). Two *B. licheniformis* strains (1.9%) were isolated from the 105 samples collected. The *B. licheniformis* strain showed a clustered, wrinkled, lichen-like appearance on sheep blood agar. Smears of cultured *B. licheniformis* colonies contained gram-positive bacilli and beta - haemolysis, and were positive for catalase, motility, nitrate reduction, gelatine decomposition, and esculin tests in two isolates (Table 3).

3.1. Antimicrobial susceptibility test

Two isolates of *B. licheniformis* were highly sensitive to enrofloxacin, norfloxacin, ceftiofur, polymyxin B,

Table 2. Microorganisms isolated from mastitis in dairy cattle

Isolates	No.	%
Staphylococcus aureus	22	21
Streptococcus agalactiae	17	16.2
Staphylococcus intermedius	8	7.6
E. coli	19	18.1
Trueperella pyogenes	12	11.4
Streptococcus α haemolytic	7	6.7
Klebsiella sp.	3	2.8
Proteus mirabilis	7	6.7
Enterococcus faecalis	5	4.8
Bacillus cereus	3	2.8
Bacillus licheniformis	2	1.9
Total	105	100

Table 3. Biochemical properties of *B. licheniformis* isolates

Test or Characteristic	Result
Catalase production	+
Glucose/ Ribose/ Fructose	+
Mannose/ Mannitol/ Maltose	+
Nitrate reduction	+
ONPG	+
Motility	+
Esculin	+
Egg yolk reaction	+
Urease	-
Gelatine decomposition	+

azithromycin, erythromycin, vancomycin, penicillin G, tetracycline and ciprofloxacin. High resistance rates were found against trimethoprim sulfamethoxazole, streptomycin, cefixime, ampicillin, bacitracin, clindamycin and gentamycin (Table 4).

3.2. Genetic characterization of *B. licheniformis*

The 16S rRNA sequence of the isolated bacterial strains was compared and aligned with known 16SrRNA sequences of other bacteria available in the GenBank database. The isolated bacterial strain was found to be 100% similar to different strains of *B. licheniformis*. A sequence from one isolate was submitted to GenBank with the following accession number MT279447. *B. licheniformis* isolated from

Table 4. Antibiotic resistance results for 19 antibiotics

Antibiotics	isolate2	isolate3
Streptomycin (S10)	R	R
Trimethoprim/Sulfamethoxazole (SXT)	R	R
Enrofloxacin (NFX5)	S	S
Norfloxacin (NOR10)	S	S
Ceftiofur (CFTIO30)	S	S
Polymyxin B (PB300)	S	S
Novobiocin (NB5)	I	I
Azithromycin (ATM30)	S	S
Erythromycin (E15)	S	S
Vancomycin (VA30)	S	S
Cefixime (CFM5)	R	R
Kanamycin (K30)	I	S
Penicillin (P10)	S	S
Ampicillin (AM10)	R	R
Tetracycline (TE30)	S	S
Bacitracin (BA10)	R	R
Clindamycin (CD2)	R	R
Ciprofloxacin (CIP5)	S	S
Gentamicin (GM100)	R	R

S, susceptible; R, resistant; I, intermediate

Figure 1. Lane M: Ladder 100 bp and 1 kb; Lanes 2 and 3: PCR analysis of virulence genes; Lane C+: B. licheniformis ATCC; Lane C-: control negative

mastitis had identical bands of 735, 904 and 1195 bp in size, representing *lic*A, *lic*B and *lic*C, respectively. Two isolates had all three *lic*A, l*ic*B and *lic*C genes (figure 1).

4. Discussion

Although *B. licheniformis* is a widely used bacterium for various biotechnological applications, it can also be associated with bovine septicaemia, peritonitis, ophthalmitis, toxaemia, mastitis, abortion and food poisoning in humans and animals (3). *B. licheniformis* is a common contaminant of dairy products, producing (11) toxins that are toxic to mammalian cells and cause food poisoning (9). Raw milk can be contaminated by endospores of *Bacillus spp*. via the soil, unclean livestock bedding, inadequately cleaned milking equipment, bovine mastitis, and *Bacillus* isolates can successfully form biofilm types in milk (2). Consequently, biofilm formation in milk by Bacillus isolates from the dairy industry can serve as an adaptation to the environmental conditions in the dairy industry (12). According to studies conducted worldwide, *B. licheniformis* is the most common mesophilic spore former in raw milk. Despite regional, seasonal and methodological differences, *B. licheniformis* is usually predominant in raw milk. *B. licheniformis*, which can

grow at both thermophilic and mesophilic temperatures, is found throughout milk production and processing (12, 13). *B. licheniformis* has been reported in 18 countries as the second most common thermophilic spore producer in milk powder after *A. flavithermus* (14). *Bacillus* strains that produce heat-stable endospores and toxins pose a potential risk to dairy products as they can survive common dairy processes such as pasteurization (typically 74 °C 15–20 s) and whey evaporation at 50–70 °C. One source of toxicogenic heat-resistant endospore-forming bacteria in raw milk could be cows that have suffered from mastitis in the past. *Bacillus* strains originating from the milk of cows with clinical mastitis should not be present in dairies, as mastitis milk is generally not supplied to dairies (9). Mastitis remains a major challenge for the dairy industry despite the widespread use of control strategies (15).

Occasionally, *Bacillus* species have been reported to cause mastitis. In the present study, *B. licheniformis* was isolated from 2 of 105 mastitis milk samples and antibiotic susceptibility tests were performed. The isolates were highly sensitive to ciprofloxacin**,** enrofloxacin and tetracycline used in this study. High resistance rates were observed to trimethoprim-sulfamethoxazole, streptomycin, cefixime, ampicillin, bacitracin, clindamycin and gentamicin (Table 4). Awareness of the sensitivity of this bacterium to antimicrobial agents is important for choosing the appropriate treatment. It is possible that the bacteria causing mastitis may lose their sensitivity to antibiotics over time or even acquire this feature. The lack of strict regulations and the lack of monitoring of the distribution and use of antimicrobial drugs in veterinary institutions are influential factors in the increase of microbial resistance. It is believed that it is important to keep an eye on the pathogens that lead to mastitis in order to identify patterns of antimicrobial resistance. In addition, careful use of antibiotics can prevent the development and spread of microbial resistance in animals caused by antibiotics. The treatment of mastitis caused by these strains with numerous antibiotic resistances is a challenge. The emergence of antibiotic resistance in bacteria has raised health and veterinary concerns (8, 9, 16).

Adimpong et al. (2012) state that there is limited information on the resistance of *Bacillus spp*. to various antibiotics and that they appear to be able to transfer their antimicrobial resistance genes (17). Allam et al. (2017) showed 100% susceptibility to gentamicin and ofloxacin and also found 90% susceptibility to cloxacillin, as the most effective alternative (18).

In 2019, Suliman and Salih isolated three *Bacillus* species (two *B. licheniformis* and one *B. mycoides*) from 50 milk samples from clinical and subclinical mastitis cases. They compared the percentage incidence of mastitis caused by *Bacillus spp*. such as *B. licheniformis* and *Staphylococcus spp.* in acute and chronic mastitis (19). Sadashiv and Kaliwal (2014) isolated *Bacillus spp*. from clinical and subclinical bovine mastitis and showed the highest antibiotic resistance to methicillin (100%), penicillin G (91.40%) and oxacillin (80.54%). As shown in the study, all *Bacillus spp.* were sensitive to vancomycin (16).

David et al. (2012) isolated *B. licheniformis* (N-38) and *Bacillus sonorensis* (N-18) from starters for Sudanese bread production. All strains were sensitive to tetracycline (8.0 mg/L), vancomycin (4.0 mg/L), and gentamicin (4.0 mg/L), but resistant to streptomycin. Banyko proved that contamination of milk with *Bacillus spp*. can occur during dairy production processes (20). *Bacillus spp*. can produce the heat-stable lipopeptide lichenysin, which causes food poisoning and mastitis in cattle. These toxic bacilli with heat-resistant spores have become a problem in the dairy industry, where they are widely used as probiotics and food additives. Scheldeman et al. (2006) isolated *B. licheniformis* sporadically during a survey of Belgian dairies for the presence of potentially highly heat-resistant spores in milk (21). Taylor et al. 2005 found that *B. licheniformis* strains can produce a heat-stable toxin that does not vacuolize. They also cited a study in which *B.*

licheniformis isolates were collected that produced toxin. In this study, 53 strains of *B. licheniformis* were isolated from various sources, including bovine mastitis (22).

In the present study, toxic B. licheniformis was isolated for the first time from bovine mastitis in Iran, and it was demonstrated that heat-resistant endospores can be transmitted to humans and cause food poisoning. Since the spores of *B. licheniformis* are known for their thermal stability and resistance to hydrogen peroxide, they can survive in cleaning and dairy processes such as spray evaporation. Milk from subclinical carriers of these bacteria (*B. licheniformis)* can pose a risk to the safety of powdered milk products (9).

Farmers can suffer severe economic losses as a result of mastitis and there is a possibility that milk from affected cows may be contaminated with bacteria and therefore unfit for human consumption. Multifactorial mastitis has serious public health implications. It serves, serving as a medium for the transmission of various zoonotic diseases (9, 17). Bovine mastitis is the most important infection in dairy cows and is a growing problem due to the decision to increase milk production and broad-spectrum antibiotics. The bacteria causing mastitis are traditionally divided into three main groups: infectious, opportunistic and environmental (20, 23). *B. licheniformis* is also found in the environment as a saprophytic bacterium.

Lichenysins are surface-active lipopeptides with antibiotic properties that are produced nonribosomally by different strains of *B. licheniformis*. In contrast to well-defined methods for the isolation and structural properties of lichenysin, little is known about the biosynthetic mechanisms of Lichenysin production. *B. licheniformis*, which has a well-conserved secretory system and no polyketide biosynthesis, was introduced in 2004 by Veith et al. as a producer of the lipopeptide lichenicin (24). In the present study, three lichenysin synthetase genes, *lic*A, *lic*B and *lic*C, were confirmed based on the PCR results..

Madslien et al. (2013) showed that lichenysin is produced by most *B. licheniformis* strains. There is a strong correlation between lichenysin concentration and toxicity in boar spermatozoa, erythrocytes and Vero cells. In principle, selected strains with toxic potential should not be used as feed additives and animal feed. To find the genes that code for the toxins, a complete sequencing of the genome is advisable (25).

In the study by Nieminen (2007) , 23 Bacillus strains were isolated from 100 mastitis milk samples, including the pathogens of *B. licheniformis*. Using the boar sperm motility test, they identified heat-resistant toxins. Using exclusive primers, the *Bacillus* mastitis isolates were analysed for the presence of known non-ribosomal synthetase peptide genes. In seven strains of *B. licheniformis* tested, including strains with mastitis toxin, primers targeting the lichenicin synthetase genes *lch*AA, *lch*AB and *lch*AC gave amplitudes of the expected magnitude. Two strains of *B. licheniformis* mastitis showed similar ribopatterns to those previously found in infant food (9).

As there are few reports of *B. licheniformis* isolation from mastitis infections worldwide, the presence of this bacterium in this infection is indisputable. Obviously, these reports are alarming and further research needs to be carried out. It is necessary to conduct further studies on the possible risks and problems of the presence of this organism and its toxins in the development and spread of mastitis. We hope that new perspectives and approaches can be developed to combat mastitis.

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Authors' Contribution

Study concept and design: B. N. F.
Acquisition of data: I. A. T.
Analysis and interpretation of data: B. N. F.

Drafting of the manuscript: S. M. J.

Critical revision of the manuscript for important intellectual content: I. A. T.

Administrative, technical, and material support: B. N. F.

Ethics

The authors of this study hereby declare that all the ethical standards were followed in the procedure of preparing the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of Data and Materials

The data generated and/or analysed during the current study are available from the corresponding author on request.

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