ISSN: 2577-8455

Review Article

Archives of Infectious Diseases & Therapy

# PCR- based detection and identification of mastitis-causing bacteria: A review

**Dejenie Mengistie**

*National Agricultural Biotechnology Research center, P.O. Box:31, Holeta, Ethiopia*

***Corresponding author**

Dejenie Mengistie, National Agricultural Biotechnology Research center, P.O. Box:31, Holeta, Ethiopia.

**Submitted:** 09 May 2022; **Accepted:** 19 May 2022; **Published:** 24 May 2022.

***Citation:*** *Dejenie Mengistie. (2022). PCR- based detection and identification of mastitis-causing bacteria: A review. Archives of Infect Diseases & Therapy, 6(2), 163-168.*

***Summery***

*Mastitis is the most common and commercially significant infectious disease affecting dairy cattle. Mastitis is a global issue since it harms animal health, milk quality, and milk production economics, affecting every country and resulting in significant financial losses. Mastitis pathogens can be categorized into two types based on their epidemiology: infectious and environmental. The identifying procedures used in most clinical laboratories are microbiological culturing of milk and biochemical assays. However, microbiological culture has several drawbacks, including the possibility of bacteria not being isolated from subclinically infected glands due to a low number of pathogens and the presence of residual therapeutic antibiotics in the collected milk, which may limit bacterial development. As a result, non-culture-based diagnostic approaches are required. The use of PCR as a technique to detect bacterial species often linked with mastitis is evaluated in this article. To identify the bacterial species, traditional procedures based on biochemical and physiological features of bacteria will be used. Then, using species-specific primers, the PCR panel will be tuned for simultaneous detection of bacterial species. It is necessary to assess the test agreement between culture and PCR-based identification methods.*

**Keywords:** Infection, Detection, Mastitis, Pcr-Based, Subclinical

## Introduction

Various diseases might infect and harm cattle populations, with mastitis being the most frequent [1]. Bovine mastitis is a common disease that affects dairy cows all over the world and is caused by inflammation of the mammary gland. It is the most important infectious disease in dairy cattle in terms of economics. Mastitis causes significant losses in milk yield (due to physical and chemical changes in milk), degradation of its nutritional and technical properties, fertility problems, and even systemic sickness, in addition to mammary gland health problems [2].

Mastitis is regarded as the most complicated disease due to its multifaceted causes [3]. Exposure to pathogens, the efficacy of udder defense mechanisms, and the presence of environmental risk factors, as well as interactions between these factors, all influence the likelihood of it occurring [4]. The causal agents of bovine mastitis have been identified as a variety of bacteria. Even though pathogenic bacteria are the most common cause, bovine mastitis can be caused by more than 130 microorganisms including viruses, protozoa, and other chemical and mechanical factors [1]. Mastitis pathogens can be categorized into two types based on their epidemiology: infectious and environmental. Environmental causes include *(Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis, Enterococcus sp., and coagulase-negative staphylococci)*, whereas contagious causes include *(Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis, Enterococcus sp*., and coagulas *(Mycoplasma bovis, Staphylococcus aureus, and Streptococcus agalactiae)* [5]. Although a large range of bacteria has been discovered as mastitis causative agents, only a few staphylococci, streptococci, and coliforms species are responsible for the bulk of infections, making them economically and epidemiologically significant [4]. Mastitis is classified into two types: clinical and subclinical. Because of the typical indications on the affected udder/quarter and concomitant changes in milk composition with clots and flakes formation, the clinical mastitis type is easily observable and diagnosed by farmers. Subclinical mastitis is difficult to detect visually; however, it is defined by a decrease in milk output and a change in milk composition [6]. Mastitis is a complex disease with no easy cure, therefore understanding its prevalence, associated risk factors, and the mastitogenic microorganisms involved are critical components in devising a control strategy [7]. Mastitis is the most economically destructive disease for the dairy sector globally, despite years of research, and it has public health implications by serving as a vehicle for the spread of diseases such as TB, staphylococcal food poisoning, and brucellosis [8]. As a result, the goal of this review is to assess the usage of PCR for the identification of microorganisms that cause bovine mastitis.

## Definition and general aspects

Mastitis is described as an inflammation of the mammary gland,

according to the International Dairy Federation (1987). It can have a traumatic or toxic etiology; however, it is almost often caused by a microbiological infection. Mastitis is defined by physical, chemical, and bacteriological abnormalities in the milk, as well as pathological changes in the udder glandular tissue [9].

It's also known as mammary gland parenchyma inflammation, which is caused by bacteria and their toxins. The bacterial contamination of milk from infected cows renders it inappropriate for human consumption and serves as a vector for diseases such as tuberculosis, sore throat, Q-fever, Brucellosis, and Leptospirosis, among others, with zoonotic implications. Mastitis can be classified as acute or chronic according to how long it lasts, and it can also be separated into clinical and subclinical mastitis depending on the symptoms. Clinical mastitis is classified according to the following symptoms: Mild (milk clotting), moderate (milk alterations and visual symptoms of udder inflammation), or severe (milk and udder changes, as well as systemic indicators) [10].

## Major causative agents of mastitis

Mastitis is most commonly caused by intramammary infections. Infections of the mammary gland secretory tissue and/or the ducts and tubules by microorganisms are classified as these. (International Dairy Federation, 2011). Mastitis can be caused by a variety of bacteria, fungi, mycoplasmas, and algae. Although the cause of 20 to 35 % of clinical mastitis infections is uncertain, it is widely accepted that bovine mastitis is mainly bacterial in origin especially *staphylococci, streptococci,* and *coliforms* are the most common pathogens associated with mastitis [7]. Pathogens that cause mastitis are classified as infectious or environmental. Contagious pathogens exist on and in the mammary gland of cows, where they grow and transfer from cow to cow, usually during milking. *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp., and Corynebacteriumbovis* are all contagious pathogens [4]. Environmental mastitis is a broad term that refers to intra-mammary infections (IMI) caused by pathogens whose major reservoir is the cow's environment. *Streptococci,* other than *S. pneumoniae*, is the most commonly isolated environmental pathogens. gram-negative bacteria such as *Escherichia coli and Klebsiella spp*. agalactiae, often known as environmental streptococci (typically S. uberis and S. disgalactiae) as well as Enterobacter spp. Environmental pathogens require moisture, favorable pH, and organic material for survival and they enter the gland through the teat canal [11].

Pathogens in the environment can be found in soil, bedding materials, manure, and other organic waste. As a result, attempts to prevent or control environmental mastitis should concentrate on the cleanliness of a cow's workplace as well as the cleanliness of the cow herself. Mastitis produced by environmental organisms is opportunistic, and it develops if the host's immune system is weakened or if proper cleanliness and hygiene are not followed [12].

## Pathogenesis of mastitis

The teat canal is normally securely closed by sphincter muscles, preventing infections from entering. It's lined with keratin, a waxy material formed from stratified squamous epithelium that obstructs bacterial migration and includes antibacterial compounds like long-chain fatty acids to help fight infection. Keratin's efficiency, on the other hand, is limited. As parturition approaches, fluid builds within the mammary gland, resulting in increased intramammary pressure and mammary gland fragility due to teat canal dilatation and mammary secretion leakage. Additionally, keratin is washed out during milking, resulting in teat canal distention. The sphincter takes two hours to return to its tight posture [13]. Bacteria must also evade the udder's cellular and humoral defensive mechanisms once within the teat. They start multiplying in the mammary gland if they are not removed. They release toxins and cause leukocytes and epithelial cells to release chemoattractants, such as tumor necrosis factor-a (TNFa), interleukin (IL)-8, and IL-1, eicosanoids such as prostaglandin F2a (PGF2a), oxygen radicals, and acute phase proteins (APPs) (e.g. haptoglobin (Hp), serum (SAA) [14].

PMNs absorb and kill invading microorganisms via both oxygen-dependent and oxygen-independent mechanisms. They have bactericidal peptides, proteins, enzymes (like myeloperoxidase), and neutral and acidic proteases (including elastase, cathepsin G, cathepsin B, and cathepsin D) stored in intracellular granules. The oxidants and proteases are released to kill bacteria and certain epithelial cells, resulting in lower milk production and the release of enzymes including N-acetyl-b-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH). The majority of PMNs are destroyed by apoptosis once their function is completed. The remaining PMNs are then engulfed and ingested by macrophages (Ginsburg *et al*., 2019). Internal swelling within the mammary epithelium, which is not generally evident by an external inspection, might develop if the infection persists. The alveoli of the mammary gland become injured and begin to lose anatomical integrity. Extracellular fluid components such as chloride, sodium, hydrogen, potassium, and hydroxide ions enter the gland and mix with the milk when the blood–milk barrier is breached. Blood can be found in milk when the blood–milk barrier has been severely damaged. This causes noticeable changes to the udder, such as increased external swelling and gland reddening. Increased conductivity, increased pH, elevated water content, and the presence of visible clots and flakes are all changes that occur in the milk [15].

## Types of mastitis

Mastitis can be clinical or subclinical, depending on how closely the cow is examined at the time of diagnosis.

### *Clinical mastitis*

This is a kind of mastitis that has visible signs, such as clots in the milk or discoloration. Only flakes or clots develop in the milk in mild clinical mastitis (CM), whereas severe cases are accompanied by fever, edema, and coloring of the udder, as well as irregular discharge. Systemic responses, such as fever and loss of appetite, can occur in severe CM [16].

### *Subclinical mastitis*

Mastitis can exist without showing visible indications of infection, which is known as subclinical mastitis (SCM). The most common type of mastitis is SCM. Variations in milk composi-

tion, such as somatic cell count (SCC); leukocytes, and epithelial cells, as well as changes in milk pH and ion concentration, are all indicators of subclinical mastitis [17]. Mastitis can be classified as acute or chronic according to how long the infection lasts. Acute mastitis is defined by quick onset, whereas chronic mastitis is defined by an inflammatory process that lasts months and culminates in the progressive formation of fibrous tissue (Boakes *et al*., 2018).

## Diagnostic tests of mastitis

### *Qualitative examination of milk*

The presence of blood (red or brownish) or pus can change the color of milk (yellow). The consistency of the milk may be altered, resulting in thicker, "stickier" milk, or it may be more watery than usual. The presence of flakes and clots is always abnormal. Mastitis can also cause a change in the scent of the discharge [18].

### *California Mastitis Test (CMT)*

This practical test was created in the 1950s as part of a California testing program, and it calculates the SCC of the milk samples. The reagent is a detergent (3 % sodium lauryl sulfate is commonly employed) that ruptures somatic cells in milk, releasing DNA. This causes apparent gelling of the milk by forming a precipitate with other serum components, fat particles, and the CMT reagent. To the reagent, a pH indicator (for example, bromcresolpurple) can be added. The test process is basic and simple: after discarding the stripping milk, a few streams of (front) milk from each quarter are milked into four plastic dishes put on a paddle. The paddle is then nearly vertically tipped to drain any remaining milk. From a plastic squeeze container, an equal volume of reagent is added, and the two components are swirled together [19].

### *Flow cytometry (FC)*

Flow cytometry (FC) is a technique for determining the physical and chemical features of cells or particles as they pass through a sensing point in suspension. This method for quantifying Somatic Cell Counts in milk was just discovered, and it's especially useful for detecting subclinical mastitis [20].

### *Bacterial Culture as a gold standard test*

Bacterial culture is the classic diagnostic procedure for identifying pathogens in milk, with the presence of bacteria shown by their growth on an adequate growth medium after an incubation period. Bacterial species are identified using phenotypic features such as colony shape, serotyping, and enzyme profile analyses. The gold standard for diagnosing mastitis pathogens is now bacterial culture [21].

### *PCR as a diagnostic test*

The polymerase chain reaction is a molecular technology that can be used to identify pathogens as an alternate test. PCR is a technique for detecting DNA sequences that are particular to a species or a group of bacteria. As a result, the presence or absence of bacterial DNA in both live and non-viable organisms may be confirmed. The invention of a PCR assay to detect mastitis-causing microorganisms was prompted by the need for a quick and accurate diagnostic. The number of pathogens that can be discovered in a test is determined by the type of PCR utilized. Simplex PCR identifies one target per PCR reaction, but multiplex PCR, which allows the simultaneous identification of numerous pathogens in a single experiment, is now used in most mastitis PCR assays [21].

Each multiplex PCR reaction requires numerous primer sets, each intended to amplify DNA from a single species or species group. When compared to simplex PCR, which uses the same primers for each target, multiplex PCR may result in a drop in analytical sensitivity (10-100 fold). This could be due to reagent rivalry between individual reactions. Multiplex PCR, on the other hand, allows for a lower cost per sample because more pathogens can be discovered in a single response [22]. Even though the PCR assay is a promising diagnostic technique for mastitis diagnosis and control, it has some limitations, including (a) the lack of specific guidelines or cut-off points for the definition of sample contamination, unlike BC; and (b) its use in developing countries is limited in comparison to developed countries (economic reasons), (c) the potential for false-positive results due to milk carryover (defined as the transfer of a small amount of milk from one cow sample to the next at the time of collection due to the presence of residual milk in the milking unit, milk meter, or milk sampler), (d) the applicability of pre-sampling procedures, and (e) the inability to distinguish between viable and nonviable bacterial cells [23].

## Consequences of mastitis

Mastitis has a significant economic impact on milk producers because it affects several essential aspects of cow and herd performance. The costs incurred are both direct and indirect. Veterinary expenditures, additional labor requirements, rejected milk (during treatment), and reduced milk yield and quality are all direct costs. Indirect expenses, sometimes known as hidden costs, are those that are not necessarily visible to the milk producer. Greater risk of following problems, decreased fertility (additional services per conception and, as a result, a protracted calving interval), increased risk of culling, and, on rare occasions, mortality are among them [8].

### *Milk production losses*

Intramammary infection, even at subclinical levels, has been shown to have a deleterious impact on milk production. Physical injury to the breast parenchyma of the damaged mammary gland is mostly to blame for the decrease in milk outputMilk output is reduced in both clinical and sub-clinical mastitis. Furthermore, the reduction in milk production does not occur only during the mastitis case; even after the mastitis case is treated, the cow's milk production remains lower. Because this is milk that was never produced and thus never seen, the producer is unaware of the loss in milk production. The financial impact of decreasing milk production per cow is determined by the farming business's structure [15].

## Discarded milk

Milk must be removed on treatment days and waiting periods due to the treatment of a clinical case. Milk was thought to have to be thrown for 6 days in general: 3 days of therapy and 3 days

of no treatment (Liang et al., 2018).

### *Treatment costs*

The cost of therapy is divided into two parts: veterinary expenses and drug costs. These two costs differ by country. A veterinarian may have to spend time diagnosing a (clinical) mastitis condition or providing supportive care in addition to giving medications (in many countries) [7].

## Effect on Milk quality

Mastitis has an impact on milk quality. Some of these changes result in less efficient milk processing, which may lead to products with less desirable qualities. Milk with an unstable and rancid flavor, a lower cheese output, and a shorter shelf life are just a few examples [14].

### *Pre-mature Culling and replacement*

Mastitis-affected cows are more likely to be culled. One of the most significant areas of economic loss is the cost of the early replacement of animals owing to mastitis. There is, however, a hidden cost. Direct costs are the costs of rearing or purchasing a replacement animal when a cow is culled (mostly heifers). Because the milk yield of multiparous cows is higher than that of primiparous cows, indirect costs include a decreased efficiency of milk production by the replacement animal. Furthermore, a heifer's milk output may be unsatisfactory (heifers have a relatively high culling rate). On the other hand, there are potential returns from culling a cow, primarily in the form of increased meat prices. Depending on milk output, parity, lactation stage, and reproductive status, the costs of forcible culling of a cow vary over time. The cost of growing or purchasing a new animal is included in the direct costs. On the same hand, there are benefits to culling a cow, the most important of which is the meat price. Indirect expenses could include the replacement animal's milk production efficiency being reduced, as a multiparous cow is often more productive than a primiparous cow [25].

### *Subsequent Disorders*

Mastitis has been linked to an increased incidence of lameness, and clinical mastitis has been linked to the diagnosis of ketosis, displaced abomasum, and non-parturient paresis at the same time or later. Although clinical mastitis is not a risk factor for reproductive abnormalities, both CM and SCM have been shown to harm reproductive function [26].

## PCR Primer design and standardization

ClustalW (https://www.genome.jp/tools-bin/clustalw) or other sequence alignment tools will be used to align the sequences of the target bacterial strains available in the NCBI database, and primers will be constructed to amplify conserved sections of distinct targets specific to each target organism. All PCR primers will be designed to have melting temperatures that are similar to those required for multiplex PCR. To optimize, PCR has to be performed independently for each target microorganism. Different amounts of Taq polymerase, MgCl2, and a combination of various primer concentrations will be utilized to standardize the multiplex PCR composition [27].

## Accuracy evaluation of the multiplex PCR assay

Reference strains and strains generated from subclinical mastitis cases will be used in this study to assess the accuracy of multiplex PCR for species identification. Pasteurized milk will be spiked individually with a reference bacterial strain and used for standardization to determine the detection threshold. The target bacteria will be spiked in milk after that. The inoculated milk samples will be serially diluted ten times using uninoculated pasteurized milk as a diluent. In distilled water, parallel dilutions of each organism will be prepared, and cell count will be determined using the Standard Plate Count method (CFU ml-1). DNA will be extracted from spiked milk dilutions, and PCR thresholds will be compared on dilutions containing organisms with 104–1CFU ml-1. Similarly, the concentration of purified DNA extracted from each target bacterial species will be evaluated using nanodrop, and 10-fold serial dilutions of purified DNA will be made and then exposed to multiplex PCR to determine the detection limit of DNA.

## Determining the comparative efficiency of detection

All gathered milk samples will be processed simultaneously for culture-based bacterial isolation and DNA extraction from milk to establish comparative efficiency. Biochemical tests will be used to identify the species of selected colonies collected using the culture method. The detection of target pathogens will be carried out utilizing DNA isolated from milk in the multiplex PCR method.

## Discussion

Identification methods in most clinical laboratories are based on the microbiological culture of milk and biochemical assays for subsequent identification. Microbiological culture, on the other hand, has several drawbacks. Due to the low quantity of pathogens present when samples are obtained, milk culture may not yield any bacteria from subclinically infected glands. Due to the presence of residual therapeutic antibiotics in the analyzed milk, microorganisms may not be identified from mastitic milk. These approaches are both time-consuming and labor-intensive procedures. It takes roughly a week to complete the treatment. Although additional methods such as electrical conductivity (EC), California Mastitis Test (CMT), and Somatic Cell Count (SCC) are available, they can only detect a limited number of pathogens or provide limited information about the infection. These approaches likewise focus solely on whether or not mastitis occurred, and their sensitivity and specificity are impacted by a variety of circumstances. Molecular techniques like polymerase chain reaction (PCR) have been developed to identify distinct mastitis pathogens due to the limitations of culture and other traditional approaches. The development of PCR-based technologies offers a promising option for identifying germs quickly. This technology allows for the identification of bacterial infections in hours rather than the days that traditional cultural procedures need. Due to its great sensitivity, PCR can also boost detection levels. Furthermore, PCR may detect bacteria in the presence of preservatives or residual therapeutic antibodies in milk, avoiding the false-negative result caused by a lack of bacterial growth, which is a major drawback of traditional approaches. For the specific and sensitive identification of mastitis pathogens in milk, various PCR-based approaches have been

developed. Because mastitis is caused by a variety of species, multiplex PCR as a diagnostic approach could be useful for detecting main mastitis-causing pathogens in a specific, quick, and simultaneous manner [29-32].

## Conclusion

Rapid approaches that can deliver a conclusive result in less than 24 hours or even during the initial examination of the animal have been a key driver in any disease diagnostics. The approaches must meet the requirements of speed, simplicity, sensitivity, specificity, reproducibility, and low cost to attain such rapidity. For the diagnosis, surveillance, and treatment of this economically significant disease in dairy cows, accurate and cost-effective methods of identifying mastitis microorganisms are critical.

## References


1. Abebe, R., Hatiya, H., Abera, M., Megersa, B., & Asmare, K. (2016). Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. BMC veterinary research, 12(1), 1-11.
2. Cervinkova, D., Vlkova, H., Borodacova, I., Makovcova, J., Babak, V., Lorencova, A., ... & Jaglic, Z. (2013). Prevalence of mastitis pathogens in milk from clinically healthy cows. Veterinarni medicina, 58(11), 567-575.
3. Liang, D., Arnold, L. M., Stowe, C. J., Harmon, R. J., & Bewley, J. M. (2017). Estimating US dairy clinical disease costs with a stochastic simulation model. Journal of dairy science, 100(2), 1472-1486.
4. Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: Risk factors, therapeutic strategies, and alternative treatments—A review. Asian-Australasian journal of animal sciences, 33(11), 1699.
5. Bi, Y., Wang, Y. J., Qin, Y., Guix Vallverdú, R., Maldonado García, J., Sun, W., ... & Cao, Z. (2016). Prevalence of bovine mastitis pathogens in bulk tank milk in China. PLoS One, 11(5), e0155621.
6. Gaddi, R. M., Isloor, S., Rathnamma, D., Avinash, B., Veeregowda, B. M., Bhaskar, R., & Rao, S. (2016). Multiplex-PCR to detect pathogens and analysis of relation of age and stage of lactation of cows to sub-clinical mastitis.
7. FAO. (2014). Impact of mastitis in small scale dairy production systems. Animal Production and Health Working Paper. No. 13.
8. Cobirka, M., Tancin, V., & Slama, P. (2020). Epidemiology and classification of mastitis. Animals, 10(12), 2212.
9. Sharma, N., Maiti, S. K., & Sharma, K. K. (2007). Prevalence, etiology and antibiogram of microorganisms associated with Sub-clinical mastitis in buffaloes in Durg, Chhattisgarh State (India). International Journal of Dairy Science, 2(2), 145-151.
10. Klaas, I. C., & Zadoks, R. N. (2018). An update on environmental mastitis: Challenging perceptions. Transboundary and emerging diseases, 65, 166-185.
11. Etifu, M. (2012). Study on prevalence of mastitis and associated risk factors with isolation and anitimicrobial susceptibility of major pathogs in alage state dairy farm.
12. Bekuma, A., & Galmessa, U. (2018). Review on hygienic milk products practice and occurrence of mastitis in cow's milk. Agricultural Research & Technology: Open Access Journal, 18(2), 1-11.
13. Alves Simões, P. B. (2018). Intramammary infection in heifers: the application of infrared thermography as an early diagnostic tool (Doctoral dissertation, University of Glasgow).
14. Ezzat Alnakip, M., Quintela-Baluja, M., Böhme, K., Fernández-No, I., Caamaño-Antelo, S., Calo-Mata, P., & Barros-Velázquez, J. (2014). The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. Journal of veterinary medicine, 2014.
15. Zigo, F., Vasil, M., Ondrašovičová, S., Výrostková, J., Bujok, J., & Pecka-Kielb, E. (2021). Maintaining optimal mammary gland health and prevention of mastitis. Frontiers in Veterinary Science, 8, 69.
16. Royster, E., & Wagner, S. (2015). Treatment of mastitis in cattle. Veterinary Clinics: Food Animal Practice, 31(1), 17-46.
17. Sumon, S. M. R., Parvin, M. S., Ehsan, M. A., & Islam, M. T. (2020). Relationship between somatic cell counts and subclinical mastitis in lactating dairy cows. Veterinary World, 13(8), 1709.
18. Kibebew, K. (2017). Bovine mastitis: A review of causes and epidemiological point of view. J Biol Agric Healthc, 7(2), 1-14.
19. Tanni, N. S., Islam, M. S., Kabir, M., Parvin, M. S., Ehsan, M. A., & Islam, M. T. (2021). Evaluation of sodium lauryl sulfate for the development of cow-side mastitis screening test. Veterinary World, 14(8), 2290.
20. Martins, S. A., Martins, V. C., Cardoso, F. A., Germano, J., Rodrigues, M., Duarte, C., ... & Freitas, P. P. (2019). Biosensors for on-farm diagnosis of mastitis. Frontiers in bioengineering and biotechnology, 186.
21. Giuliano, C., Patel, C. R., & Kale-Pradhan, P. B. (2019). A guide to bacterial culture identification and results interpretation. Pharmacy and Therapeutics, 44(4), 192.
22. Dupas, E., Briand, M., Jacques, M. A., & Cesbron, S. (2019). Novel tetraplex quantitative PCR assays for simultaneous detection and identification of Xylella fastidiosa subspecies in plant tissues. Frontiers in plant science, 1732.
23. Mahmmod, Y. (2013). The future of PCR technologies in diagnosis of bovine mastitis pathogens. Advances in Dairy Research, 2, e106.
24. Murphy, S. C., Martin, N. H., Barbano, D. M., & Wiedmann, M. (2016). Influence of raw milk quality on processed dairy products: How do raw milk quality test results relate to product quality and yield?. Journal of Dairy Science, 99(12), 10128-10149.
25. Azooz, M. F., El-Wakeel, S. A., & Yousef, H. M. (2020). Financial and economic analyses of the impact of cattle mastitis on the profitability of Egyptian dairy farms. Veterinary World, 13(9), 1750.
26. Mellado, M., García, J. E., Véliz Deras, F. G., de Santiago, M. D. L. Á., Mellado, J., Gaytán, L. R., & Ángel-García, O. (2018). The effects of periparturient events, mastitis, lameness and ketosis on reproductive performance of Holstein cows in a hot environment. Austral journal of veterinary sciences, 50(1), 1-8.
27. Shiriaev, D. I., Sofronova, A. A., Berdnikovich, E. A., Lukianov, D. A., Komarova, E. S., Marina, V. I., ... & Dontsova, O. A. (2021). Nybomycin Inhibits both Fluoroquino-

lone-Sensitive and Fluoroquinolone-Resistant Escherichia coli DNA Gyrase. Antimicrobial Agents and Chemotherapy, 65(5), e00777-20.
28. Quinn, P. J., Carter, M. E., Markey, B., & Carter, G. R. (1994). Clinical Veterinary Microbiology Wolfe publisher.
29. Korwin-Kossakowska, A. (2008). An association of BoLA alleles DRB3. 2* 16 and DRB3. 2* 23 with occurrence of mastitis caused by different bacterial species in two herds of dairy cows. Animal Science Papers and Reports, 26(1), 37-48.
30. Quinn, P. J., Carter, M. E., Markey, B. K., & Carter, G. R. (2002). Veterinary microbiology microbial diseases-bacterial causes of bovine mastitis. London: Mosby International Limited, 465-475.
31. Radostits, O.M., Gay, C.C., Hinchchiff, K.W. and Constable, P.D. (2007). A Textbook of the Disease of cattle, sheep, pigs, and goats. 10th ed. London: Ballieer, Tindall.
32. Vianna, P. C. B., Mazal, G., Santos, M. V., Bolini, H. M. A., & Gigante, M. L. (2008). Microbial and sensory changes throughout the ripening of Prato cheese made from milk with different levels of somatic cells. Journal of Dairy Science, 91(5), 1743-1750.