International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

Check for updates

Mycoplasmas detection in milk samples ¿are the techniques reliable?

Huitzil-Alvarado T [1], Rivera A [2,*], Giono-Cerezo S [3], Romero-Arenas O [4], Cedillo-Ramírez L [2] and Castañeda-Antonio D [5]

[1] *Faculty of Chemical Sciences, Benemérita Universidad Autónoma de Puebla, México.*
[2] *Laboratory of Mycoplasmas, Benemérita Universidad Autónoma de Puebla, México.*
[3] *National School of Biological Sciences, Instituto Politécnico Nacional, CDMX.*
[4] *Center of Agroecology, Benemérita Universidad Autónoma de Puebla, México.*
[5] *Laboratory of Microbial Molecular Ecology, Benemérita Universidad Autónoma de Puebla, México.*

International Journal of Science and Research Archive, 2024, 11(01), 063–067

Publication history: Received on 28 November 2023; revised on 04 January 2024; accepted on 07 January 2024

Article DOI: https://doi.org/10.30574/ijsra.2024.11.1.0017

Abstract

Food microbiology is an important aspect that must be considered during food manufacturing, which is, considering the possible sources of contamination, including operators. Highlighting that the group of mycoplasmas are little studied and therefore their presence in foods is not considered. Different mollicutes, particularly mycoplasmas, have been associated with economic losses in the poultry and dairy industries. The objective of this work was to determine the presence of the mycoplasma genus in milk samples through specific microbiological culture and to complement the diagnosis with the PCR technique. Two hundred milk samples were analyzed in Puebla city-Mexico. The samples obtained were centrifuge and reseeded in triplicate on SP4 agar, incubating and analyzed by stereoscopic microscopy. From each milk sample, DNA extraction was carried out with Zymo Research Quick-DNA™ Mini prep kit and primers AR1 and AR2 were used. The microbiological study showed positivity for mycoplasmas of 17% and 39%, in the liquid and solid phase, respectively. With the PCR technique, 53% positivity for mycoplasmas was obtained, which allows us to consider the presence of factors associated with samples of animal origin and that may be interfering with the sensitivity of the technique. Analyzing the viability of techniques to detect microorganisms, particularly mycoplasma, in foods is a practice that is recommended for the well-being of consumers.

Keywords: Mollicutes: Contamination; Food; Milk; Techniques; Diagnosis

1. Introduction

The area of food engineering today represents an important field of opportunity from the point of view of process control during food preparation, that is, from its initial source of the process to its final manufacturing. It should be noted that in developed countries the quality controls to which imported foods are subjected are strict, hence the importance of considering standards during food processing, in the first instance for export. Thus, complying with national and international quality standards, in addition to promoting the economic, social, and public health aspects. Nowadays, the rapid identification of pathogens in food is a challenge in the scientific field and particularly around food technology. Techniques are being developed at the nanomolecular level that facilitate assays with reduced times, high sensitivity, portability, and low costs [1].

Food microbiology is an important aspect that must be considered during food manufacturing, which is, considering the possible sources of contamination, including operators. Highlighting that the group of mycoplasmas are little studied and therefore their presence in foods is not considered. With this information we must highlight the importance of

* Corresponding author: Rivera A

health that foods must comply with and highlight the relevance of considering the diagnosis and study of mycoplasmas, due to the importance in veterinary and public health that these microorganisms can imply [2].

Mollicutes are prokaryotes lacking a cell wall, with a reduced genome and size. Compared to other bacteria, they can be commensal parasites, and some are pathogens in insects, plants, animals, and humans [3].

Different mollicutes, particularly mycoplasmas, have been associated with economic losses in the poultry and dairy industries. Their diagnosis represents an obstacle since they require enriched culture media. And in addition to the fact that the media are susceptible to contamination easily, the slow metabolism for its growth is another limitation in the diagnosis. The sector of the food industry where these microorganisms occur has been affected, for this reason, in recent years, research has been carried out aimed at the rapid and sensitive diagnosis of these microorganisms [4]. The objective of this work was to determine the presence of the mycoplasma genus in milk samples through specific microbiological culture and to complement the diagnosis with the PCR technique.

2. Materials and methods

Two hundred milk samples were analyzed during the months of January-March 2022, which were obtained at Emiliano Zapata market in Puebla city-Mexico. The samples obtained had a volume of 10 mL each and were placed with automatic micropipettes in sterile BIOLOGIX 10-9151 centrifuge tubes. The samples are transported to the laboratory at 4°C. In tubes with SP4 broth (9 mL), 1 mL of each of the milk samples was placed, in duplicate. Allowing it to incubate at 37°C/72 hours, 5 µL of each of the samples were subsequently reseeded in triplicate on SP4 agar, incubating at 37°C/72 hours. The reseedings on agar were analyzed by stereoscopic microscopy to demonstrate the characteristic growth of mycoplasmas.

From each milk sample, 5 mL was centrifuged at 1000 rpm/10 minutes, the resulting button was subjected to DNA extraction (Zymo Research Quick-DNA™ Mini prep kit) under the following protocol: 1. Add 4 volumes of Genomic Lysis Buffer to each volume of sample (4:1), mix briefly by vortexing, then let stand at room temperature for 5-10 minutes. 2. Transfer the mixture to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10000 rpm/1 minute, Discard the Collection Tube with the flow through. 3. Transfer the Zymo-Spin™ IICR Column to a new Collection Tube. Add 200 µL of DNA Pre-Wash Buffer to the spin column. Centrifuge at 10000 rpm/ 1 minute. 4. Add 500 µL of g-DNA Wash Buffer to the spin column. Centrifuge at 10000 rpm/1 minute. 5. Transfer the spin to a clean microcentrifuge tube. Add 50 µL DNA Elution Buffer to the spin column. Incubate 2-5 minutes at room temperature and then centrifuge at 10000 rpm/30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored -20°C for future use.

Primers AR1 and AR2 were used, which amplify a 301bp fragment of the 16S rRNA gene sequence in thirty mycoplasma species [5]. The reaction mixture for a volume of 50 µL contained 10 µL of PyroStart™, Fast PCR Master Mix (2X), 32 µL of water, 1.5 µL of the primer AR1 and 1.5 µL of the primer AR2 and 5 µL of the test DNA. The amplification conditions (Techne TC-412) were: 95°C/1 minute, 40 cycles (95°C/1 minute, 50°C/1 minute, 72°C/1 minute) 72°C/5 minutes. The amplified products were run on a 2% agarose gel for 70 volts/40 minutes, stained with RedGel and visualized on a photodocumenter (SYNGENE Bio Imaging System).

3. Results and discussion

The microbiological study showed positivity for mycoplasmas of 17% and 39%, in the liquid and solid phase, respectively. The low sensitivity in traditional microbiological culture for mycoplasmas is due to the lack of turbidity that liquid phase mycoplasma cultures present. In the solid phase there is another limitation, which is related to the low light transmission that occurs in microbiological analysis. This low light transmission occurs due to the opacity that is experienced in the medium when the sample containing it is placed, milk this makes vision difficult and does not allow an accurate diagnosis.

With the PCR technique, 53% positivity for mycoplasmas was obtained, which allows us to consider the presence of factors associated with samples of animal origin and that may be interfering with the sensitivity of the technique. In relation to the above, we document a series of investigations that provide an explanation and, above all, new trends in the diagnosis of mycoplasmas in samples of both animal and plant origin, highlighting the importance that this implies around food health.

In dairy and cheese producing farms, microbial communities were analyzed using culture-dependent and culture-independent techniques. All isolated bacteria were grouped using randomly amplified polymorphic DNA (RAPD) and identified by 16S rRNA gene sequencing, species-specific PCR, and multiplex PCR. A total of 26 different species were identified, the majority belonging to lactic acid bacteria. Using the temporal-temperature-gradient gel electrophoresis (TTGE) technique with two 16S rRNA genes and rpoB, low species diversity was revealed. Furthermore, with the TTGE technique, *Leuconostoc lactis* and *Mycoplasma agalactiae* that had not been isolated by culture were detected. These results allow us to recommend that complementing diagnostic methods offers a more complete vision of the microbial diversity in the analyzed samples (2).

Mycoplasma agalactiae has historically been considered the main etiological agent of contagious agalactia, specifically in sheep. The clinical signs are mastitis, arthritis, keratoconjunctivitis and occasionally it causes abortions. A total of 110 milk samples (90 positive and 20 negative) were analyzed to optimize the Loop-Mediated Isothermal Amplification (LAMP) technique based on the p40 gene. LAMP turned out to be ten times more sensitive than real-time PCR, with a detection limit higher than 103 CFU/mL. The LAMP assay detected *Mycoplasma agalactiae* in 81/90 (90%) positive milk samples, compared to 69/90 (77%) positive samples detected by real-time PCR. Therefore, the potential of the LAMP assay as an effective tool for the diagnosis of contagious agalactia has recently been indicated (4). PCR is a technique that is routinely used for the early detection of mycoplasmas in bovine milk samples, with an average diagnosis time of 3 days. Therefore, recently different techniques have been evaluated to improve diagnosis. LAMP and PURE have been evaluated, which facilitates the filtration and elimination of components that inhibit DNA amplification. Demonstrating that the limit detection of *Mycoplasma bovis* was 102 CFU/mL in milk using PURE-LAMP. It is suggested to replace PCR with PURE-LAMP for the detection of mycoplasmas in milk samples and in the presence of mastitis (6).

LAMP is an isothermal nucleic acid amplification technique, unlike polymerase chain reaction (PCR) technology, in which the reaction is carried out by alternating a series of steps or temperature cycles, isothermal amplification. It is carried out at a constant temperature and does not require a thermal cycler. You need at least four primers, but you can use six, 2 external primers F3 and B3, 2 internal primers FIP (F1C, F2) and BIP (B1C, B2) that have both sense and antisense sequences in such a way that aids in the formation of a loop and 2 loop primers F(FLP) and B(BLP) designed to accelerate the amplification reaction by additional binding to sites that are not accessible by the internal primers. Recognizing a total of 8 different sequences of the target DNA (Figure 1), all these primers are what give specificity to the method (7).

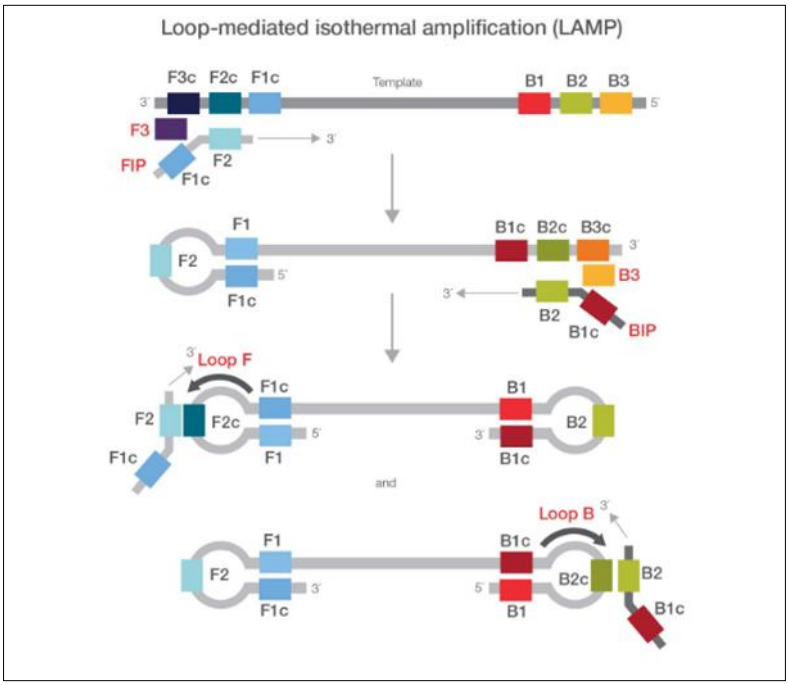

Figure 1 Two external primers F3 and B3, 2 internal primers FIP (F1C, F2) and BIP (B1C, B2) that have both sense and antisense sequences as such. way that helps in the formation of a loop and 2 loop primers F(FLP) and B(BLP) designed to accelerate the amplification reaction by additional binding to sites that are not accessible by the internal primers (Source: https://www.thermofisher.com/mx/es/home/life-science/pcr/isothermal-nucleic-acid-amplification/loop-mediated-isothermal-amplification.html).

The detection of mycoplasmas is commonly related to the low sensitivity of the microbiological cultures used. It is proposed that the sedimentation of mycoplasma species is achieved by centrifuging and subsequently reseeding small volumes on agar plates, which increases the ability to detect mycoplasmas compared to conventional culture (8).

In 1982, isolations of acholeplasmas and mycoplasmas were reported from vegetable samples; the cultures were liquid and contained lysozyme. Ampicillin and thallium acetate. Cultures were filtered (0.4 μm) and these samples were grown in SP4 broth. Of the 35 samples analyzed, 11 were positive for *Acholeplasma laidlawii*, *Acholepalsma axanthum*, *Acholeplasma oculi* and *Mycoplasma verecudum* (9).

Other work showed that the PCR test was used to detect *Mycoplasma gallisepticum* from samples collected from the environment (feed, drinking water, excrement, and dust) and from naturally infected poultry. One hundred and three samples were positive using PCR specific to *Mycoplasma gallisepticum* and 68 were positive using PCR for mycoplasma, spiroplasma, acholeplasma and ureaplasma. Six of these samples were also culture positive. These results indicate the dissemination capacity of *Mycoplasma gallisepticum* and the possibility of using PCR for epidemiological analysis and control of farms for decontamination and introduction of new birds (10).

In addition to the importance of accurate detection of mycoplasma in foods, there is also interest in reducing levels of resistance to various families of antimicrobials, since the frequency of resistance varies according to the species, but also according to the country or groups of animals from which they were sampled, highlighting the relevance of this type of studies. In the world, the relevant issue related to public health is food safety, especially in countries with overpopulation such as China, and the issue of food safety is related to diseases caused by microorganisms. In China, a food contamination monitoring system has been established since 2000. Progress has been made around education and research in food safety. The food industry is focusing on the mitigation of microbial risks through the implementation of the ISO 22000 standard, regulating the application of molecular tools for the rapid detection of contamination (11,12).

4. Conclusion

Studies for quality control or surveillance of food health are required to ensure that a food is hygienic and healthy. Safe food production enables market access and productivity, which drives economic development in societies. It is important that producers implement good practices in production processes to reduce the risks of contamination in vegetables and animals for human consumption. Food safety seeks to avoid diseases acquired through food, conditions of an infectious or toxic nature. For this reason, analyzing the viability of techniques to detect microorganisms, particularly mycoplasma, in foods is a practice that is recommended for the well-being of consumers.

Compliance with ethical standards

Acknowledgments

To the institution for the facilities granted to carry out this work.

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Author's contributions

All authors contributed equally to the conception and development of the work.

References

[1] Umesha S, Manukumar HM. Advanced molecular diagnostic techniques for detection of food-borne pathogens: Current applications and future challenges. Critical Reviews in Food and Nutrition 2018; 58(1): 84-104.

[2] Martin-Platero AM, Maqueda M, Valdivia E, Purswani J, Martinez-Bueno M. Polyphasic study of microbial communities of two Spanish farmhouse goats milk cheeses from Sierra de Aracena. Food Microbiology 2009; 26: 294-304.

[3] Razin S, Yogev D, Naot Y. Molecular biology and pathogenesis of mycoplasmas. Microbiol. Mol. Biol. Rev. 1998; 62(4): 1094-1156.

[4] Tumino S, Tolone M, Parco A, Puleio R, Arcoleo G, Manno C, Nicholas RAJ, Loria GR. Validation of loop-Mediated isothermal amplification (LAMP) field tool for rapid and sensitive diagnosis of contagious agalactia in small ruminants. Animals 2020; 10, 509.

[5] Shidu MK, Rashidbaigi A, Testa D, Liao MJ. Competitor internal standards for quantitative detection of mycoplasma DNA. FEMS Microbiology Letters 1995; 128: 207-212.

[6] Itoh M, Hirano Y, Yamakawa K, Yasutomi I, Kuramoto K, Furuoka M, Yamada K. Combination of procedure for ultra-rapid extraction (PURE) and loop-mediated isothermal amplification (LAMP) for rapid detection of *Mycoplasma bovis* in milk. J. Vet. Med. Sci. 2020; 82(7): 875-880.

[7] Zijenah LS. The world health organization recommended TB diagnostic tools. In: Tuberculosis. IntechOpen, Edited by Jean-Marie Ntumba Kayembe. 2018. Pp. 1-20.

[8] Punyapornwithaya V, Fox LK, Gay GM, Hancock DD, Alldredge JR. Short communication: the effect centrifugation and resuspension on the recovery of Mycoplasma species from milk. J. Dairy. Sci. 2009; 92(9): 4444-4447.

[9] Somerson NL, Kocka JP, Rose D, Del Giudice RA. Isolation of acholeplasmas and mycoplasma from vegetables. Appl. Environ. Microbiol. 1982; 43(2): 412-417.

[10] Marois C, Dufour-Gesbert F, Kempf I. Polymerase chain reaction for detection of *Mycoplasma gallisepticum* in environmental samples. Avian Pathol. 2002; 31(2): 163-168.

[11] Gautier-Bouchardon AV. Antimicrobial resistance in Mycoplasmas spp. Microbiol. Spectr. 2018; 6(4): 1-120.

[12] He S, Shi X. Microbial food safety in China: Past, present, and future. Foodborne Pathog. Dis. 2021.