Toxqui-Munguia et al. | Article 7643
J Pure Appl Microbiol. 2022;16(3):1878-1883. doi: 10.22207/JPAM.16.3.34
Received: 26 February 2022 | Accepted: 05 July 2022
Published Online: 12 August 2022

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Antimicrobial Susceptibility of Bacteria and Yeasts Isolated from the Milk of Dairy Cattle Presenting with Subclinical Mastitis in Puebla, Mexico

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Abstract

This study was designed to identify the bacteria and yeasts from the milk samples of dairy cattle presenting with subclinical mastitis and evaluate their antimicrobial susceptibility. We collected a total of 52 milk samples from cows across three farms in San Salvador El Seco (Puebla, Mexico). Microbial isolation was performed using microbiological techniques followed by taxonomic identification of bacteria and yeasts. Antimicrobial susceptibility was evaluated using the guidelines provided by the Clinical Laboratory Standard Institute (CLSI). 1 We identified three genera and six species of yeasts including *Candida glabrata, C. krusei, C. lipolytica, Cryptococcus laurentii, Rhodotorula rubra,* and *R. glutinis* and five species of bacteria, including *Staphylococcus saprophyticus, S. aureus, S. hominis, S. epidermidis,* and *Streptococcus disgalactiae.* All of the yeast strains were sensitive to amphotericin B; 1/23 (4.3%) were resistant to ketoconazole and nystatin, 10/23 (43%) were resistant to fluconazole, and 13/23 (53%) were resistant to 5-fluorocytosine. The dominant genus isolated was *Candida,* with the most abundant groups being *C glabrata* and *C. krusei.* Resistance to 5-fluorocytosine was observed in all yeasts except *C. lipolytica,* while both *S. aureus* and *S. epidermidis* were resistant to oxacillin and dicloxacillin. *S. hominis* was resistant to gentamicin. These antimicrobials are still used in bovine therapy for mastitis, directly affecting healthy cattle and, therefore, raw milk.

Keywords: Antimicrobial, Bacteria, Yeasts, Mastitis.

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Citation: Toxqui-Munguia M, Avila-Sosa R, Castaneda-Roldan E, et al. Antimicrobial Susceptibility of Bacteria and Yeasts Isolated from the Milk of Dairy Cattle Presenting with Subclinical Mastitis in Puebla, Mexico. *J Pure Appl Microbiol.* 2022 ;16(3):1878-1883. doi: 10.22207/JPAM.16.3.34

INTRODUCTION

Mastitis is one of the most commercially significant infectious diseases in dairy cattle, with outbreaks often resulting in large economic losses.[3] These infections can be classified as subclinical mastitis when a pathogenic or an opportunistic microorganism infects one or more quarters of the mammary gland, but do not damage the alveoli, making it difficult for the milker to detect any changes in the udder or milk. However, despite the reduced damage these low-grade infections reduce milk yield and increase the somatic cell count in these cows.[4] Animals with clinical mastitis present with abnormalities in their mammary glands, characterized by swelling of the udder, pain, and redness. These cases can also present with increased rectal temperature, lethargy, anorexia, and even death, with most of these animals producing abnormal milk.[5] The infection process begins when the organism gains access to the nipple and then encounters a second barrier along the nipple channel, where they can remain or continue to colonize the alveolar tissue. Leukocytes are then deployed via the circulatory system to remove these pathogens from the injured tissue in an effort to keep the infection in check. However, despite the immune response these infections often permanently atrophy the nipple channel, thus destroying the secretory tissue.[2,5] Mastitis causing microorganisms can be found in stools, stables, skin, and secretions of udders, straw, food, soil sediments, and drug compounds. Other forms of contagion rely on unhygienic milking processes, and are driven by the milker, which may include, drying nipples with the same cloth, overmilking, and not cleaning the nipples before milking, amongst others.[6-9] Although mastitis is primarily caused by bacteria such as *Staphylococcus, Streptococcus, Mycoplasma, Corynebacterium, Enterococcus,* and enterobacteria, fungal infections with pathogens such as *Candida, Cryptococcus, Trichosporon,* and *Aspergillus fumigatus* have also been reported as a etiological agents. Bovine mastitis can also be caused by various yeast species with the most common being *Candida albicans* and *Cryptococcus neoformans,* as well as other species of *Candida* such as *C. inconspicua C. kefyr, C. lusitaniae, C. tropicalis, C. guilliermondii, C. catenulata, C. lambica, C. rugosa,* and *C. zeylanoides.*

However, despite these reports, the importance of yeasts as participants in the pathogenesis of this disease remains largely unrecognized.[10-14] Milking processes using machines and workers' hands are one of the largest inducers of mastitis making hygiene a critical concern in these environments. The main objective of this investigation was to identify the yeasts and bacteria associated with subclinical mastitis in dairy cows across Puebla, Mexico and evaluate their antimicrobial susceptibility.

MATERIALS AND METHODS

Biological Material

Cows with subclinical mastitis were randomly selected for a physical examination based on the appearance of their milk (clots or blood), the condition of their udders, and the general condition of the animals (udder palpation) as proposed by Barkema in 1998.[11] Milk samples taken just before milking (n = 14 Holstein cows) were obtained from the udders of infected cows across three farms in San Salvador El Seco County, Puebla, Mexico, on which livestock were stabled and milking was performed manually. Each of the teats were disinfected with 70% ethanol and dried with absorbent paper prior to sample collection and the first jet of milk was discarded. A total of 40 mL of milk from each of the four quarters of each bovine was deposited into a container containing 0.5 mL of Azidiol preservative. All milk samples (N=52) were collected from lactating cows and stored in sterile vials at 4°C as described in the Official Mexican Standard, NOM-110-SSA1-1994. All samples were collected between March and October 2015.

Culture Examination and Isolate Identification

Yeast-like fungi were isolated via microbiological methods using Sabouraud dextrose agar (DIFCO, Mexico) and Mycosel®, and plates were incubated at 28°C for 5–7 days. Yeast colonies were stained with lactophenol cotton blue for microscopic observation (40x), and reseeding was used to obtain axenic cultures. *Candida* species, *Rhodotorula* sp. and *Cryptococcus* sp.

were identified based on morphological and biochemical tests (CHROMagar Candida BD®, Auxacolor Bio-Rad). Each milk sample (10 µL) was seeded onto blood agar plates (DIFCO, Mexico) supplemented with sheep blood nutrient agar (5%) for bacterial isolation and the plates were then incubated for 24–48 h at 37°C. Based on the morphology, each colony was identified by Gram staining, and catalase and oxidase tests were performed. Conventional biochemical techniques were used for further identification.[17]

Antifungal and Antibiotic Susceptibility Testing

Antifungal and antibiotic susceptibility profiles were determined using the disk diffusion method according to CLSI document M44-A.[18] Commercially available sensitive discs (Bio-Rad, Hercules, CA, USA) were used in all cases and we evaluated antifungals tested from the following groups: macrolides, amphotericin B (100 µg), imidazoles, ketoconazole (50 µg), fluconazole (25 µg), nystatin (100 IU), and fluorinated pyrimidine, 5-fluorocytosine (1 µg). We also evaluated the following antibiotics using the same method: oxacillin (1 µg), vancomycin (30 µg), dicloxacillin (1 µg), tetracycline (30 µg), erythromycin (15 µg) and gentamicin (10 µg). All selections were based on frequency of application within the study region. We used reference strains, *C. albicans* ATCC 65027 and ATCC 60193 as controls and the data obtained in this study were analyzed using statistical descriptive frequencies and percentages.

RESULTS

Strain identifications for each of the 52 samples evaluated in this study are summarized in Table 1. Initial evaluations revealed that 62% of the total samples included microorganisms known to induce subclinical mastitis, with two of the three farms including samples with yeast contamination. Each animal underwent a physical examination by udder palpation, and no mammary glands presented signs or symptoms of clinical mastitis, such as pain on contact, anorexia, hyperthermia, lumps, or milk clots. We identified a total of five genera and 11 species of microorganisms in this study (Table 2). *Staphylococcus (S. aureus, S. hominis, S. saprofiticus,* and *S. epidermidis*) and

Table 1. Number of sampled cows, glands and positive samples causing mastitis from three dairy farms in San Salvador El Seco, Puebla, Mexico

Farm	Number of sampled cows	Number of sampled glands	Positive samples
1	5	20	10
2	4	16	11
3	4	16	11
Total	13	52	32

Table 2. Mastitis-causing bacteria and yeast species (N) isolated from positive samples (T) from three dairy farms in San Salvador El Seco, Puebla, Mexico

Microorganism	N/T
Streptococcus disgalactiae	10/32
Staphylococcus aureus	8/32
Staphylococcus hominis	6/32
Staphylococcus saprofiticus	5/32
Staphylococcus epidermidis	4/32
Candida glabrata	9/32
Candida kruzei	5/32
Rhodotorula rubra	2/32
Candida lipolityca	1/32
Rhodotorula glutins	1/32
Cryptococcus laurentii	1/32

Candida (*C. glabrata, C. kruzei,* and *C. lipolytica*) species were the most prevalent.

We then evaluated the susceptibility of the yeast strains to various antifungals (Table 3). All strains were sensitive to amphotericin B, 4.3% were resistant to ketoconazole and nystatin, and 43% were resistant to fluconazole. The highest percentage of resistance (53%) was observed for 5-fluorocytosine, with 69% of the yeast species found on Farm 2 demonstrating clear resistance to this antifungal compound. In addition, *C. laurentii* was isolated from farm 1, and there were no yeast positive samples from farm 3. Bacterial susceptibility to antibiotic agents (Table 4) varied between farms, with gentamycin (80%) producing the highest susceptibility rates. Bacterial susceptibility to erythromycin, tetracycline, and oxacillin was 60% and the highest level of resistance (60% of the isolated bacterial species) was towards dicloxacillin.

Table 3. Percentage antifungal susceptibility of mastitis-causing yeasts in positive samples from three dairy farms in San Salvador El Seco, Puebla, Mexico

Yeasts	Antifungal agents (concentration)				
	5-fluorocytosine (1 µg)	Fluconazole (25 µg)	Ketoconazole (50 µg)	Nystatin (100 IU)	Amphotericin-B (100 µg)
C. glabrata	45.45%	36.36%	9.09%	9.09%	0%
C. krusei	50%	16.66%	0%	0%	0%
R. rubra	100%	100%	0%	0%	0%
C. lipolytica	0%	0%	100%	0%	0%
R. glutinis	100%	100%	0%	0%	0%
C. laurentii	100%	100%	0%	0%	0%

Table 4. Percentage antibiotic susceptibility of mastitis-causing bacteria isolated from positive samples from three dairy farms in San Salvador El Seco, Puebla, Mexico

Bacteria	Antibiotic agents (concentration)					
	Oxacillin (1 µg)	Vancomycin (30 µg)	Dicloxacillin (1 µg)	Tetracyclin e (30 µg)	Erythromyci n (15 µg)	Gentamycin (10 µg)
S. aureus	0%	40%	0%	0%	0%	100%
S. epidermidis	0%	66.3%	0%	0%	0%	100%
S. saprofiticus	100%	100%	100%	100%	100%	100%
S. hominis	100%	0%	0%	100%	100%	0%
S. disgalactiae	100%	0%	100%	100%	100%	100%

DISCUSSION

Mastitis is a clinically important disease affecting dairy cattle. Many microorganisms, usually bacteria, have been reported as causal agents. That said, many cases of mastitis are caused by yeast infection, although these infections are less well understood.[18,22] Yeasts are often cosmopolitan environmental agents that act as opportunistic pathogens of the mammary glands of dairy cattle. Subclinical mastitis occurs when host defenses are compromised.[20,21] There are many sources of mastitis, including cattle skin, milkers' hands, sanitation solutions, and milking hygiene conditions.[6,8] *Candida* is the microorganism most commonly involved in subclinical mastitis and several species of this genus have been recovered from infected glands.[8,21-23] There have also been several reports describing the isolation of various emerging yeast species as the pathogenic agent from infections in central and northern Europe and Mexico (*C. glabrata, C. krusei, C. zeylanoides, C. norvegica, C. viswanathii, C. guilliermondii, C. tropicalis,* and *C. albicans*) and *C. rugosa* is a common agent that induces mastitis after intramammary antibiotic treatments.[22,23] Our evaluations revealed a high incidence of *Candida* based infections, with *C. glabrata* being the most commonly isolated (45.45%). These species are gaining ground in this condition due to environmental factors such as indiscriminate usage of antibacterial agents, overexploitation of livestock, and poor hygiene habits at milking time.[26] *C. lipolytica* (4%) has also been identified as an emerging agent for bovine subclinical mastitis in Puebla, Mexico. Moreover, *Rhodotorula* can be found on nails, skin, dairy products, and in the environment, making it a common pathogen in immunocompromised animals and humans. *C. laurentii* has also been identified in animals with mastitis, but its frequency of isolation is low compared to *C. neoformans*.[27] The most important bacterial genera identified in this study were *Staphylococcus* and *Streptococcus* with *S. aureus* being an important inducer of mastitis worldwide and is epidemiologically active.[27,28] This is because of its complex pathogenicity and its difficult eradication. In addition, these pathogens easily infect healthy animals making its control even more problematic. We also isolated *S. saprofiticus,* *S. hominis,* and *S. epidermidis* in this study suggesting that these may be emerging pathogens in this environment, creating a novel public health concern for humans who may consume raw milk products from infected cattle.

Evaluations of the antifungal susceptibility of these isolates revealed that, *C. laurentii, R. rubra, R. glutinis, C. glabrata,* and *C. krusei* all retained a high rate of sensitivity to ketoconazole, nystatin, and amphotericin B, suggesting that the restricted use of these agents in cattle has preserved their antibiotic effects in these strains. This is important as this suggests that treatment with these antifungals would guarantee successful mastitis treatment.[29] All of the isolated strains from the yeast species, except for *C. lipolytica*, showed resistance (56%) to 5-fluorocytosine. This finding seems initially counter-intuitive since most yeasts such as *Candida albicans* are usually sensitive to common antifungals, but fluconazole resistance is not rare as many yeasts show intrinsic resistance to this agent. *S. aureus* and *S. epidermidis* showed similar behaviors to those reported by Calderon and Rodriguez [25] suggesting that the gradual exposure of these strains to specific antibiotics is selecting for more widespread resistance, which may complicate therapeutic treatment in the future. Our results highlight the need for accurate causal agent identification even in subclinical mastitis as incorrect identification can reduce therapeutic efficacy and, when combined with favorable environmental conditions, support the development and spread of antimicrobial resistance by bacteria through modification of its genetics and expression of resistance genes.[30]

CONCLUSION

The dominant yeast genus causing subclinical mastitis in dairy cattle in this study was *Candida* with the most abundant species being *C. glabrata.* The resistance of all species to 5-fluorocytosine, except for *C. lipolytica*, was highlighted. This is also the first report to link *C. lipolytica*, which is generally considered a commensal organism, to subclinical mastitis in cattle. The presence of *Staphylococcus* species was largely unsurprising and once again highlights the significant concerns around antimicrobial resistance in these pathogens. In addition, our evaluations highlight the potential risks associated with antibiotic resistance which may directly affect cattle health and, therefore, the raw milk consumer. The presence of opportunistic yeasts and bacteria affects livestock health and the quality of artisanal unpasteurized dairy products.

ACKNOWLEDGMENTS

The authors would like to thank Benemerita Universidad Autonoma de Puebla for providing related support in compiling this work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

The authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication

FUNDING

This work was funded by the Rectory Research and Postgraduate Studies (VIEP/2018/00302) of the Benemerita Universidad Autonoma de Puebla.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, Postgraduate in Environmental Sciences, of the Benemerita Universidad Autonoma de Puebla with approval number 001, 01/20/2018.

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