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Streptococcus lutetiensis and *Streptococcus equinus* as potential emerging bovine mastitis pathogens[1]

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ABSTRACT.- Crippa B.L., Rodrigues M.X., Tomazi T., Bicalho R.C., & Silva N.C.C. 2024. ***Streptococcus lutetiensis* and *Streptococcus equinus* as potential emerging bovine mastitis pathogens.** *Pesquisa Veterinária Brasileira 44:e07259, 2024.* Departmento de Ciência de Alimentos e Nutrição, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, SP 13083-862, Brazil. E-mail: ncirone@unicamp.br

The current study characterizes the genetic distribution of virulence and antimicrobial resistance of *Streptococcus lutetiensis* and *Streptococcus equinus* isolated from cows with clinical mastitis using whole genome sequencing (WGS). Although they are not the protagonist species within the genus *Streptococcus*, recent studies have isolated these species associated with bovine mastitis. In addition, these species are reported and isolated from humans and other animals. A total of four strains of *S. lutetiensis* and one of *S. equinus* were isolated from five cows with identified cases of clinical mastitis at a dairy farm near Ithaca, New York. Nineteen genes associated with antimicrobial resistance and 20 genes associated with virulence were identified in the analyzed strains. All strains presented genes associated with resistance: *alr, ddl, gdpD, kasA, murA, lsa(E), msr(D), mef(A), gidB,* and *LiaF.* Resistance genes associated with several different classes of antibiotics have also been reported. Sixteen virulence-associated genes were identified in all strains. Based on our findings, we conclude that the studied species have the potential to cause mastitis in cattle, and further studies are important to elucidate their role.

INDEX TERMS: *Streptococcus lutetiensis*, *Streptococcus equinus*, mastitis, resistance genes, virulence genes, whole genome sequencing.

RESUMO.- [***Streptococcus lutetiensis* e *Streptococcus equinus* como potenciais patógenos emergentes da mastite bovina.**] O presente estudo caracteriza a distribuição genética de virulência e resistência antimicrobiana de *Streptococcus lutetiensis* e *Streptococcus equinus* isolados de vacas com mastite clínica usando sequenciamento completo do genoma. Apesar de não serem as espécies protagonistas dentro do gênero *Streptococcus*, estudos recentes têm isolado essas espécies associadas à mastite bovina. Além disso, essas espécies são relatadas e isoladas de humanos e outros animais. Um total de quatro cepas de *S. lutetiensis* e uma de *S. equinus* foram isoladas de cinco vacas com casos identificados de mastite clínica em uma fazenda leiteira perto de Ithaca, Nova York. Dezenove genes associados à resistência antimicrobiana e 20 genes associados à virulência foram identificados nas cepas analisadas. Todas as linhagens apresentaram genes associados à resistência: *alr*, *ddl*, *gdpD*, *kasA*, *murA, lsa(E), msr(D), mef(A), gidB e LiaF*. Genes de resistência associados a várias classes diferentes de antibióticos também foram relatados. Dezesseis genes associados à virulência foram identificados em todas as cepas. Com base em nossos achados, concluímos que as espécies estudadas têm potencial para causar mastite em bovinos e mais estudos são importantes para elucidar seu papel.

TERMOS DE INDEXAÇÃO: *Streptococcus lutetiensis*, *Streptocccus equinus*, mastite, genes de resistência, genes de virulência, sequenciamento completo do genoma.

INTRODUCTION

Bovine mastitis causes economic losses, and several microorganisms are considered responsible for mastitis. However, the main genera involved are *Streptococcus*,

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Staphylococcus, and the family Enterobacteriaceae (Ishihara et al. 2020). Among the *Streptococcus* spp., the main mastitis pathogens are *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* (Gao et al. 2017, Zhang et al. 2018).

Despite having few reports in the literature where *Streptococcus lutetiensis* is associated with bovine mastitis, recent studies show the isolation of this microorganism (Chen et al. 2021, Kabelitz et al. 2021). Although not frequent, *Streptococcus equinus* has also been reported as a causal agent of bovine mastitis and isolated from cattle intramammary infections (Kabelitz et al. 2021).

Based on its phenotypic and genotypic characteristics, the *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC) was redefined and divided into three biotypes: type I, which includes *Streptococcus gallolyticus* subsp. *gallolyticus* (formerly *S. bovis* bio-type I); type II/1, which includes *S. lutetiensis* and *Streptococcus infantarius* (formerly *S. infantarius* subsp. *coli*); and type II/2, which includes *Streptococcus gallolyticus* subsp. *pasteurianus* (formerly *S. bovis* biotype II/2) (Almuzara et al. 2013). The complex also includes *S. equinus* and *Streptococcus alactolyticus,* plus one more subspecies of the *S. gallolyticus* clade, named *S. gallolyticus* subsp. *macedonicus* (Pompilio et al. 2019).

As these species are rare in bovine mastitis, reservoirs, virulence, and resistance factors are few known. However, there is a possibility that these microorganisms become more prevalent in mastitis once *S. lutetiensis* has the potential to spread through dairy herds and can adapt to bovine mammary cells or tissue (Chen et al. 2021).

In general, whole-genome sequencing (WGS) virulence-associated and antimicrobial resistance genes can be detected. Furthermore, by providing a high level of information, WGS allows to investigate and compare genomes between different pathogens from different populations (Vélez et al. 2017).

This study described the virulence and resistance profile of *S. lutetiensis* and *S. equinus* isolated from cows with clinical mastitis using whole-genome sequencing.

MATERIALS AND METHODS

Animal Ethics. The isolates analyzed in these studies were isolated by Tomazi et al. (2021), and this research was conducted in full compliance with the guidelines outlined in The Animal Welfare Act of 1985 (P.L. 99–198). The study protocol underwent thorough review and received approval from the Institutional Animal Care and Use Committee at Cornell University (protocol number 2018-0097).

Origin of isolates. The isolates belong to a bacterial collection of the Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA. The strains were previously isolated from clinical mastitis cases identified in a large commercial dairy farm near Ithaca/NY. The farm milked approximately 4,100 Holstein cows three times daily in a 100-stall rotary milking parlor. The isolation followed the same method of preview study (Silva et al. 2021). Briefly, an analysis of the total Gram-positive bacteria count was performed using the technique of Agar droplets using a selective and differential culture medium (Accutreat®, FERA Diagnostics, and Biologicals, Ithaca/NY). A single colony was selected from the aforementioned culture plate and streaked onto a CHROMagar *Streptococcus* base (CHROMagar, France) plate, followed by incubation overnight at 37°C. Then, the isolates with *Streptococcus* sp. characteristics were selected for identification by the 16S gene sequencing. In addition to the species isolated and analyzed in this study, other species isolated from clinical mastitis cases were also analyzed (Tomazi et al. 2021, Silva et al. 2021, Crippa et al. 2023).

Bacterial identification. DNA was extracted from each bacterial isolate using the DNeasy PowerFood Microbial Kit (Qiagen, Valencia/CA, USA), following the manufacturer's instructions. The 16S ribosomal DNA gene was then amplified through PCR, and the PCR products were purified using Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta/IA) following the manufacturer's instructions. The purified DNA samples were submitted to the Cornell University Institute of Biotechnology for Sanger sequencing using 8pmol of primer fD1 and 300ng PCR products (Wood et al. 2019). For species identification, FASTA sequences were compared with the sequences stored available in GenBank, using the BLAST algorithm[4].

Whole-genome sequencing (WGS). The concentration of total gDNA of the samples was determined using a Qubit fluorometer (Thermo Fisher Scientific, Waltham/MA). Then, DNA samples were diluted with ultrapure water (Invitrogen, Waltham/MA) to 0.2ng/μL. After standardization, samples were used as input for the Nextera® XT DNA Library Prep Kit (Illumina Inc., USA). Library preparation was carried out according to the manufacturer's protocol, the Nextera® DNA Library Prep Reference Guide. Pair-end sequencing was performed using a MiSeq Reagent Kit v3 (600 cycles) through the MiSeq Platform (Illumina Inc., San Diego/CA).

Genome sequence analysis. The quality of the raw reads was evaluated using FASTQC. Potential contamination of the sequences was checked using Kraken2 (Wood et al. 2019). Sequencing reads were submitted to the comprehensive genome analysis service using the Pathosystems Resource Integration Center (PATRIC). The genomes were annotated using the Rast tool kit found in PATRIC (PATRIC 3.2.96) (Wattam et al. 2017), which is part of the all-bacteria Bioinformatics Resource Center available online (Brettin et al. 2015).

RESULTS

Four isolates of *Streptococcus lutetiensis* and one *Streptococcus equinus* isolate were recovered from clinical mastitis cases at a large commercial dairy farm near Ithaca, New York. The strains were isolated from five different cows. Nineteen antimicrobial resistance genes and sixteen virulence genes were identified in the strains studied using whole-genome sequencing. In addition to the species isolated and analyzed in this study, other species isolated from cases of clinical mastitis were also analyzed, and these results are available in Silva et al. (2021) and Crippa et al. (2023).

Resistance factors

The WGS of these isolates showed a wide variety of resistance genes associated with different classes of antimicrobials. All strains exhibited ten resistance genes: *alr, ddl, gdpD, kasA, murA, lsa(E), msr(D), mef(A), gidB,* and *liaF*. Two genes were present only in *S. lutetiensis*, *fabK,* and *lnu(B)*, while four were present in *S. equinus*: *dxr*, *mtrA*, *fabL-like,* and *rho*. The most prevalent classes of antibiotics associated with the resistance genes found in this study were peptide antibiotics, triclosan, and macrolides. The distribution of antimicrobial classes and their respective products are shown in Table 1.

[4] Available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> Accessed on May 24, 2021.

Virulence factors

Sixteen virulence-associated genes were observed in all strains. These were: *purN*, *clpP*, *cpsY*, *pepC*, *glnA*, *rpoE*, *fba*, *leuS*, SPy_1633, *vicK*, *purH*, *purL*, *luxS*, *purB*, *guaA* and *lepA*. As with the distribution of resistance genes, some virulence genes were only present in one species. The *ciaR* and *ccpA* genes were present only in *S. lutetiensis*, while the *lsp* and *carB* genes were present only in *S. equinus*. The distribution of the main virulence-associated genes and their respective products is presented in Table 2.

DISCUSSION

Streptococcus lutetiensis and *Streptococcus equinus* belong to *Streptococcus bovis/Streptococcus equinus* complex (SBSEC), a non-enterococcal group D *Streptococcus* spp. complex (Pompilio et al. 2019). In clinical sources, the SBSEC has antibiotic resistance genes diffused (Jans et al. 2015). Our results showed strains that showed genes associated with resistance to macrolides, lincosamides, and peptide antibiotics, among others.

Considering that these species are not the most important in bovine mastitis, we can consider its importance as a reservoir of resistance genes because of the possibility of transfer to the streptococcal species, which commonly cause infections in humans and animals (Van Hoek et al. 2011, Chancey et al. 2012, Jans et al. 2016, Alves-Barroco et al. 2020, Park et al. 2021).

Antimicrobial peptides (AMP) are important in combating infections caused by Gram-positive pathogens. However, it is known that these pathogens can develop mechanisms that block AMP action. Mechanisms such as the ability to modify the cell wall, while virulence factors or surface proteins are also a part of the strategy used by bacteria against AMP actions (Assoni et al. 2020). Virulence genes associated with biofilm formation (*luxS*, *purN*, *purL, purH* and *purB*) were found in the strains analyzed in this study. It is known that biofilm formation protects bacteria from the action of antibiotics (Jefferson 2004).

Streptococcus's *mef*(A) and *erm* genes are significant determinants for resistance to macrolides, lincosamides, and streptogramin B (Poole 2005, Zhou et al. 2019). Specifically, regarding lincosamide resistance, the genes of the *lnu* family are the mediators that encode nucleotide transferases and then catalyze the adenylation of lincosamides (Zhou et al. 2019). Resistance genes associated with different classes of antibiotics were observed in this study, as well as a resistance gene associated with triclosan, which is a biocide. This resistance to various antimicrobials can be mediated by efflux pumps. These mechanisms determining resistance to antimicrobials may be specific and/or multidrug, including antibiotics and biocides (Poole 2005).

The virulence factors are important for bacteria during infection, where they act to nullify the host defense mechanisms. These virulence factors can include toxins and enzymes that overcome effective non-specific host defense measures and structural components (Calvinho et al. 1998).

Chen et al. (2021) studied *S. lutetiensis* isolated from cases of bovine clinical mastitis and, through PCR analysis, determined that the most prevalent virulence genes were *bca*, *speG*, *hly*, *scpB*, and *ssa* (Chen et al. 2021). None of these genes identified in the study by Chen et al. (2021) were found in the

Table 1. Distribution of genes associated with resistance, product and antimicrobial class among *Streptococcus lutetiensis* and *Streptococcus equinus* isolated from cows with clinical mastitis

Genes	*Streptococcus lutetiensis* (n=4)	*Streptococcus equinus* (n=1)	Product	Antimicrobial class
alr	4	1	Alanine racemase (EC 5.1.1.1)	Cycloserine
ddl	4	1	D-alanine--D-alanine ligase (EC 6.3.2.4)	Cycloserine
gdpD	4	1	Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46)	Peptide antibiotics
kasA	4	1	3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.179)	Isoniazid, Triclosan
murA	4	1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	Fosfomycin
lsa(E)	4	1	ABC-F type ribosomal protection protein => Lsa(E)	Lincosamides, Pleuromutilins
msr(D)	4	1	ABC-F type ribosomal protection protein => Msr(D)	Erythromycin, Telithromycin, Streptogramin
mef(A)	4	1	Macrolide resistance, MFS efflux pump => Mef(A)	Macrolides
gidB	4	1	16S rRNA (guanine(527)-N(7))-methyltransferase (EC 2.1.1.170)	Aminoglycosides
LiaF	4	1	Membrane protein LiaF(VraT), specific inhibitor of LiaRS(VraRS) signaling pathway	Peptide antibiotics
fabK	4	0	Enoyl-[acyl-carrier-protein] reductase [FMN, NADH] (EC 1.3.1.9), FabK => refractory to triclosan	Triclosan
lnu(B)	4	0	Lincosamide nucleotidyltransferase => Lnu(B)	Lincosamides
folA, Dfr	3	1	Dihydrofolate reductase (EC 1.5.1.3)	Diaminopyrimidines
folP	3	1	Dihydropteroate synthase (EC 2.5.1.15)	Sulfonamides
erm(G)	1	1	23S rRNA (adenine(2058)-N(6))-dimethyltransferase (EC 2.1.1.184) => Erm(G)	Lincosamides, Streptogramins, Macrolides
dxr	0	1	1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267)	Fosmidomycin
mtrA	0	1	Two-component system response regulator MtrA	Macrolides, Penams
fabL-like	0	1	Enoyl-[acyl-carrier-protein] reductase (EC 1.3.1.104), FabL-like, predicted	Triclosan
rho	0	1	Transcription termination factor Rho	Bicyclomycins

samples from our study. The difference in the pathogenicity of the isolates can explain this.

In our study, both the *S. lutetiensis* and the *S. equinus* isolates presented a wide variety of genes associated with virulence. This demonstrates the ability of these species to cause infections and negatively affect animal health.

In many Gram-negative and Gram-positive bacteria, the *luxS* gene is highly conserved, and *luxS* was determined in all isolates of this study. The enzyme S-ribosyl-homocysteine-lyase (*luxS*) produces chemical signals called autoinducers (AIs). These signals are responsible for the method of communication between bacteria, called quorum sensing (QS). In biofilm formation, bacteria regulate their gene expression in response to changes in cell population through QS (He et al. 2015).

The *purN*, purL, *purH* and *purB* genes were identified from the two *Streptococcus* species analyzed in this study. These genes are involved in purine (*pur*) biosynthesis and have been considered essential for motility in other bacterial species (Blaschke et al. 2021). In addition, *pur* genes have also been shown to be essential for biofilm formation in *Bacillus cereus* and several other species; *De novo* purine biosynthesis (DNPB) has also been shown to be necessary for virulent species (Blaschke et al. 2021).

A study investigated the role of the *clp*P gene in another species of the genus *Streptococcus* and demonstrated that this gene was associated with thermotolerance. Furthermore, the presence of this gene was indispensable for the survival of the strains under stress conditions (Ibrahim et al. 2005).

Other studies also report that these species have been isolated from human, animal and food sources (Table 3). These studies show the potential of these species to cause diseases in humans and animals, in addition to offering resistance to antimicrobials (Choi et al. 2003, Park et al. 2021). However, there is an extremely low number of results on the analysis of the genetic profile of resistance and virulence of *S. lutetiensis* and *S. equinus*, which makes it difficult to compare results with other studies.

Pompilio et al. (2019) analyzed manuscripts published from the 2000s to 2019 and found only 16 manuscripts dealing with the study of antibiotic resistance among SBSEC isolates. For the virulence profile, the same difficulty occurs. Thus, the results presented in this study contribute to filling this gap about the resistance and virulence profile of these two species. Also, according to Pompilio et al. (2019), tetracycline, erythromycin, and clindamycin were the antimicrobials that showed the highest resistance rates. *S. lutetiensis* showed higher rates of resistance to erythromycin, and when compared to our study, our strains of *S. lutetiensis* also showed genes responsible for conferring resistance to erythromycin. In the study by Almuzara et al. (2013), *S. lutetiensis* was 60% resistant to erythromycin and clindamycin.

In the same work by Pompilio et al. (2019) cited above, the authors also reinforce the importance of whole genome studies, as these are useful to improve the accuracy of identification and identify specific virulence factors associated with specific diseases. Thus, the results of this study can contribute to the studies of these SBSEC.

Table 2. Distribution of virulence-associated genes and their respective products among *Streptococcus lutetiensis* and *Streptococcus equinus* strains isolated from mastitis-affected cows

Genes	*Streptococcus lutetiensis* (n=4)	*Streptococcus equinus* (n=1)	Product
purN	4	1	Phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)
clpP	4	1	ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92)
cpsY	4	1	Methionine biosynthesis and transport regulator MtaR, LysR family
pepC	4	1	Aminopeptidase C (EC 3.4.22.40)
glnA	4	1	Glutamine synthetase type I (EC 6.3.1.2)
rpoE	4	1	DNA-directed RNA polymerase delta subunit (EC 2.7.7.6)
fba	4	1	Fructose-bisphosphate aldolase class II (EC 4.1.2.13)
leuS	4	1	Leucyl-tRNA synthetase (EC 6.1.1.4)
SPy_1633	4	1	Ribonuclease Y
vicK	4	1	Two-component sensor kinase SA14-24
purH	4	1	IMP cyclohydrolase (EC 3.5.4.10) / Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3)
purL	4	1	Phosphoribosylformylglycinamidine synthase, synthetase subunit (EC 6.3.5.3)/ Phosphoribosylformylglycinamidine synthase, glutamine amidotransferase subunit (EC 6.3.5.3)
luxS	4	1	S-ribosylhomocysteine lyase (EC 4.4.1.21) @ Autoinducer-2 production protein LuxS
purB	4	1	Adenylosuccinate lyase (EC 4.3.2.2) @ SAICAR lyase (EC 4.3.2.2)
guaA	4	1	GMP synthase [glutamine-hydrolyzing], amidotransferase subunit (EC 6.3.5.2)/GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit (EC 6.3.5.2)
lepA	4	1	Translation elongation factor LepA
ciaR	4	0	Two-component system response regulator CiaR
ccpA	4	0	Catabolite control protein A
lsp	0	1	Lipoprotein signal peptidase (EC 3.4.23.36)
carB	0	1	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)

Table 3. *Streptococcus lutetiensis* and *Streptococcus equinus* isolated from human, animal and food samples

Microorganism	Origin	References
S. lutetinensis	Cheese	Özkan et al. (2021)
S. lutetinensis	Bovine mastitis	Chen et al. (2021)
S. lutetinensis	Cat with intestinal lymphoma	Piva et al. (2019)
S. lutetiensis	Rumen of Nelore bulls	Oliveira et al. (2022)
S. lutetinensis	Endocarditis in humans	Chongprasertpon et al. (2019)
S. lutetinensis	Diarrhea in children	Choi et al. (2003)
S. lutetinensis	Present in blood samples (from a patient with cholangitis)	Almuzara et al. (2013)
S. lutetiensis	Non-colorectal cancer	Corredoira et al. (2013)
S. lutetiensis	Human blood	Jans et al. (2016)
S. lutetiensis	Neonatal bacteremia and meningitis with empyema	Yu et al. (2021)
S. lutetiensis and *S. equinus*	Beef and dairy cattle	De Sousa et al. (2021)
S. lutetiensis and *S. equinus*	Domestic ruminants	Park et al. (2021)
S. equinus	Rumen	Jans et al. (2015)
S. equinus	Equine faeces	Jans et al. (2015)
S. equinus	Raw milk	Choi et al. (2003)
S. equinus	Ileostomy effluent	Jans et al. (2015)

CONCLUSION

Although *Streptococcus lutetiensis* and *Streptococcus equinus* are not species commonly related to mastitis in cattle, they possess several virulence and resistance genes, mainly resistance genes associated with antibiotics used to treat mastitis caused by the genus *Streptococcus*. In addition, understanding the genetic profile of these isolates can contribute to future studies, providing information about the possible role of these species as pathogens that cause mastitis and other diseases, whether in humans or animals. The ease with which these genes can arise, whether by mutation or acquisition, is worrying. Therefore, further studies are needed to elucidate the role of these species in causing bovine mastitis.

Authors' contributions.- Bruna L. Crippa wrote and revised the manuscript; Nathália C.C. Silva and Marjory X. Rodrigues performed bacteria isolation and DNA sequencing; Tiago Tomazi collected samples and farm data; Rodrigo C. Bicalho and Nathalia C.C. Silva conceived the study and performed data analysis. All authors reviewed the manuscript.

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Conflict of interest statement.- The authors declare no conflict of interest for this article.

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