Research Article

Detection of *Streptococcus thoraltensis* in Raw Milk with Special Reference to Their Antibiogram

Hams M.A. Mohamed[1], Mona A. El-Zamkan[2*]

[1]Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, 83523, Egypt; [2]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, South Valley University, Qena,83523, Egypt.

Abstract | The genus *Streptococcus* comprises a variety of pathogenic and commensal bacteria that show a surprising capacity for adaptation to new hosts and antimicrobial resistance, resulting in the spread of infection all over the world, leading to huge health loss. A total of 100 raw milk samples collected from small scale producers, farmers and markets at Qena city, Egypt, were examined for the presence of *Streptococcus* species. The preliminary identification was confirmed by using VITEK®2 system which revealed the presence of four isolates of *S. thoraltensis*. The antimicrobial susceptibility of the obtained isolates was detected phenotypically and genotypically, in addition to the ability of these isolates to produce biofilm and protease enzyme. All the isolates displayed resistance to penicillin and oxacillin, while, two isolates were resistant to vancomycin and erythromycin, and three isolates were resistant to linezolid. None of the isolates were resistant to tetracycline, chloramphenicol or clindamycin. B-lactams drug resistance encoded by *pbp1*A gene could be detected in all the isolates, while one isolate harbored *van*A and *optr*A gene, and no isolate harbored *erm*B gene. Two isolates were biofilm producers, one of them possessed *lmb* gene while both lacked *brp*A gene. Only one isolate expressed a proteolytic activity. It was thought that *S. thoraltensis* is non-pathogenic in humans, however, recently, it was implicated in many infections and its isolation from raw milk represents a potential risk for human health. According to our best efforts of research, it is the first time to isolate *S. thoraltensis* from raw milk in Egypt.

Keywords | Raw milk, *Streptococcus thoraltensis*, Antimicrobial resistance, Biofilm production, Proteolytic activity

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***Correspondence** | Mona A. El-Zamkan, Department of Food Hygiene and Control (Milk Hygiene), Faculty of Veterinary Medicine, South Valley University, Qena, 83523, Egypt; **Email:** m_zam@vet.svu.edu.eg
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INTRODUCTION

Streptococcus is a genus that includes a wide variety of Gram-positive, coccus-shaped, chain-forming, facultative anaerobic and catalase-negative species (Hardie and Whiley, 1997). Now, this genus contains 129 recognised species (Parte et al., 2020), because of better phenotypic and genetic identification techniques.

The genus *Streptococcus* plays a major role in animal and human medicine due to its zoonotic potential (Renzhammer et al., 2020). The unusual *S. thoraltensis* organism is a recently identified strain of *Streptococci* and was first described in 1997, where it was isolated from the intestinal tracts of swine by Devriese et al. (1997), and later from rabbit feces by Borø et al. (2010). It belongs to the large, unusual *Streptococcus* spp. group of Gram-positive, catalase-negative and esculin-hydrolysing cocci, and do not have defined group antigens (Facklam, 2002). Based on the distances computed by the approximately-maximum-likelihood algorithm, it was found that *S. thoraltensis* forms a group with *S. hyovaginalis*, *S. halotolerans*, and *S. pluranimalium* in the genus *Streptococcus* termed the *pluranimalium* group (Pan et al., 2018).

Streptococcus thoraltensis has been isolated from the nasal and oropharyngeal mucosa of healthy people (Dhotre et al., 2016; Al-Wakeel, 2017; Al-Tamimi et al., 2019), water

pools (Alaidarous et al., 2017), and from meat (Araby et al., 2020). It was thought that *S. thoraltensis* is non-pathogenic in humans, but recently, very little is known about the pathogenic potential of such bacterium to humans. However, the first chorioamnionitis of human infection by *S. thoraltensis* has been described in 2015 (Vukonich et al., 2015) followed by many case reports of bacteraemia, endocarditis, pneumonia, fever of unknown origin, and abscess (Petridis et al., 2018; Bustami et al., 2019; Wazir et al., 2019; Hai et al., 2020). The increase in human infections case reports could emphasize its impact on the public health. There are few available records about the resistance rates of *S. thoraltensis* towards commonly applied antibiotics. Therefore, more knowledge about the susceptibility patterns of these bacteria is needed, especially due to the ability of this species to cause diseases in animals and humans.

Biofilm is one of the virulence factors and is considered as an important source of contamination of milk and milk products with spoilage and/or pathogenic microorganisms, and spoilage enzymes (Teh et al., 2014). Protease enzyme; a virulence factor of the bacteria causing endocarditis has the ability to cause unfavourable changes in milk (ICMSF, 1980; Al-Salih et al., 2012). So, this work aims to provide an overview of phenotypic characters, the antimicrobial susceptibility of *S. thoraltensis* isolates obtained from raw milk and their ability to form biofilms and produce protease enzyme.

MATERIALS AND METHODS

Ethical approval

Ethical approval was not required for this study.

Isolation and identification

One hundred raw milk samples of large ruminants (cows and buffaloes) and small ruminants (sheep and goats) (50 samples each) were collected from small scale producers, farmers and markets. The samples were examined for the presence of *Streptococcus* species. The milk samples were cultured on Modified Edwards Medium (Thermo Scientific™, CM0027B) supplemented with sheep blood (5-7% v/v) and incubated at 39°C for 48-72h under microaerophilic conditions (5% CO_2) (Borø et al., 2010). The preliminary identification scheme was done according to Borø et al. (2010) and Wyder et al. (2011) as following; bacteriological examination including, Gram's staining, culturing on blood agar, catalase test, oxidase test, hydrolysis of arginine and esculin, and CAMP reaction. This identification was confirmed by using the Vitek2 system according to a standard procedure using VITEK® 2 GP ID card (BioMérieux).

Antimicrobial susceptibility testing

The antimicrobial susceptibility profile of the isolates was performed using standard disk diffusion method (Oxoid, Thermo Fisher Scientific, Basingstoke, United Kingdom). The test was performed using eight antibiotics belonging to 7 classes including: β-lactams (penicillin-G, 10 µg, oxacillin OX,1 µg), macrolides (erythromycin (Ery, 15 µg)), glycopeptides (vancomycin (Van, 30µg)), phenicols (chloramphenicol (C, 30µg)), and cyclines (tetracycline (TET, 30µg)), Oxazolidinone (Linezolid (LZD, 30µg), lincosamides (Clindamycin, 2 µg). Briefly, each bacterial suspension at a concentration of 10^5 CFU/mL was inoculated on sheep blood Mueller-Hinton agar plates and incubated under microaerophilic conditions (5% CO_2) for 48-72h hours at 39°C. The percentage of susceptibilities was calculated based on Clinical Laboratory Standards Institute (CLSI, 2018).

Biofilm formation

Biofilm assays were implemented as described by Tenke et al. (2006) and Hatt and Rather (2008) in triplicates. *S. thoraltensis* isolates were incubated in 10 mL of Tryptic Soy Broth (TSB) with 1% glucose for 24 hours at 37°C. Then, 20 µl of each bacterial suspension was transferred to three wells of sterile 96-well polystyrene microtiter plates holding 180 µl of TSB with 1% glucose and 200 µl of uninoculated TSB with 1% glucose broth assigned as a negative control. The microtiter plate was incubated at 39°C for 24 hours. Next, the broth was cautiously withdrawn, and the wells were washed three times with sterile phosphate-buffered saline. Biofilms were then fixed with methanol for 20 minutes, flicked, and air-dried in a flipped position at 50°C for about 30 minutes. Biofilms were stained with crystal violet (2%) for 15 min. The wells were washed twice with distilled water then dried. The dyed adherent cells were resolubilized in 150 µl of acetic acid (33%) for 30 minutes without shaking at room temp. Finally, a microtiter plate reader was used to estimate the OD of each well at 570 nm. The cut-off value (ODc)= average negative control OD + (3 SD of negative control). Each isolate was characterized as one of the following phenotypes: OD <ODc denoted as non-biofilm producers, ODc< OD < 2ODc denoted as weak biofilm producers 2ODc < OD < 4ODc denoted as moderate biofilm producers and OD > 4ODc denoted as strong biofilm producers.

Detection of antimicrobial resistance and virulence genes

Extraction of bacterial DNA

DNA was extracted from the isolates using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH).

PCR amplification

Four antimicrobial resistant genes (*Pbp1*A, *erm*B, *van*B2

and *optr*A), and two biofilm genes (*brp*A and *lmb*) were used. The used primer sequences and length of amplified products were detailed in Table 1. Uniplex PCR reaction was performed in a 25- µl mixture reaction that composed of, Emerald Amp Max PCR Master Mix (Takara, Japan) (12.5 µl), 1 µl of each primer (20 pmol concentration), water (4.5 µl) and DNA template (6 µl). An Applied biosystem 2720 thermal cycler was used to accomplish the reaction, then, followed by electrophoresis on agarose gel (1.5%) (Applichem, Germany, GmbH) used to separate the PCR amplicon.

Proteolytic activity on agar plates

Proteolytic activity of the isolates was determined according to Pailin et al. (2001). The Isolates' suspensions were inoculated as 2 µl spots on the skim milk agar plates and incubated at 35°C for 48 h under microaerophilic condition (5% CO_2). Translucent halos around the colonies were indicative for the proteolytic activity.

RESULTS AND DISCUSSION

Streptococcus species could be detected in 25% of the examined samples. The phenotypic analysis of our isolates showed general characters of *Streptococcus* as Gram-positive cocci grouped into chains, negative for catalase, CAMP negative, Voges-Proskauer; hippurate; alkaline phosphatase was weak, hydrolysis of mannitol. These characters are similar between the species of *Streptococcus* but, there were four suspected isolates that hydrolyzed aragnin, weakly fermentated L-Arabinose with moderate acid production and tolerated 6.5% NaCl in mannitol salt agar, mild alpha hemolysis. These characters differentiate between these four species isolates and other *Streptococcus* spp., also the growth of these isolates was inhibited completely on bile esculin media and these characters differentiate between the four isolates and *Enterococcus* spp. So, the Vitek2 system confirmed these four isolates as *Streptococcus thoraltensis*. Furthermore, two isolates showed strong and moderate ability to form biofilm on microtiter plate and only one isolate showed strong proteolytic activity on skim milk agar (Figure 1). The phenotypic characters including Gram staining and biochemical reactions of the obtained *Streptococcus thoraltensis* isolates are displayed in Table 2.

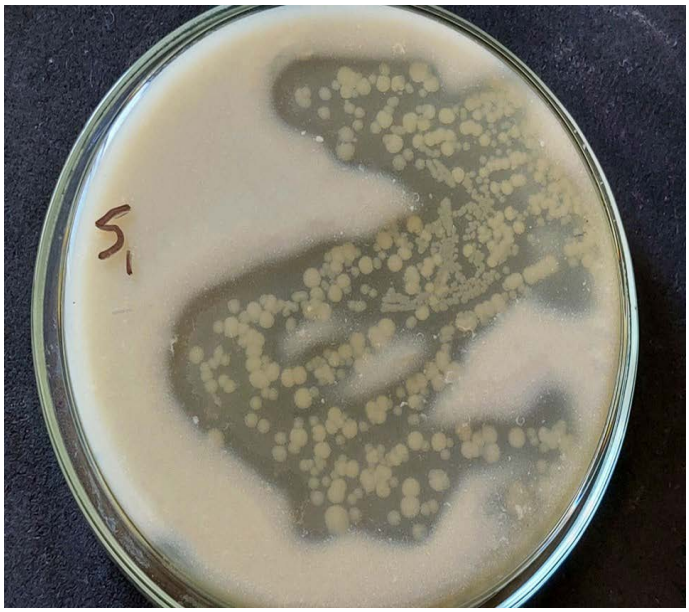

Figure 1: Proteolytic activity of *S. thoraltensis* on skim milk agar.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ermB	CATTTAACGACGAAACTGGC	425	94°C 5 min.	94°C 30 sec.	51°C 40 sec.	72°C 45 sec.	72°C 10 min.	Schlegelova *et al.*, 2008
	GGAACATCTGTGGTATGGCG							
optrA	AGGTGGTCAGCGAACTAA	1395	94°C 5 min.	94°C 30 sec.	53°C 1 min.	72°C 1 min.	72°C 12 min.	Wang *et al.*, 2015
	ATCAACTGTTCCCATTCA							
vanA	GGGAAAACGACAATTGC	732	94°C 5 min.	94°C 30 sec.	50 1min	72°C 50 sec.	72°C 7 min	Al-Tamimi *et al.*, 2019
	GTACAATGTGGCCGTTA							
	TCCCACTGTTCCATATCGTCA							
Pbp1A	AAACAAGGTCGGACTCAACC	430	94°C 5 min.	94°C 30 sec.	57°C 40 sec.	72°C 45 sec.	72°C 10 min.	Mosleh *et al.*, 2014
	AGGTGCTACAAATTGAGAGG							
brpA	TGA AGC TAA GTT GAA TGC TGC	534	94°C 5 min.	94°C 30 sec.	42°C 40 sec.	72°C 45 sec.	72°C 10 min.	Alves-Barroco *et al.*, 2019
	GAA CCA CCA TCA GAC AAG GT							
lmb	AGTCAGCAAACCCCAAACAG	397	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 40 sec.	72°C 10 min.	Kaczorek *et al.*, 2017
	GCTTCCTCACCAGCTAAAACG							

Table 2: Phenotypic characters of isolates with comparison to the properties reported for the other previous *S. thoraltensis* isolates:

Phenotypic characters		Isolates	*S. thoraltensis*	References
Gram staining and biochemical reaction				
Gram staining		4/4	Gram-positive cocci grouped into chains	Facklam, (2002), Borø *et al.* (2010)
hemolysis on blood agar		4/4	Partial hemolysis(alpha) on blood agar	Devriese *et al.* (1997), Al-Tamimi *et al.* (2019)
Growth on mannitol salt agar		4/4	Positive tolerance to 6.5% NaCl	Al-Tamimi *et al.* (2019)
Catalase activity	-ve	4/4	Negative result	Hardie and Whiley (1997), Wazir *et al.* (2019)
Aragnin hydrolysis	+ve	4/4	Positive result	Devriese *et al.* (1997)
L-arabinose	Moderate fermentation and weak acid production	4/4	weak acid production	Devriese *et al.* (1997)
Hydrolysis of esculin	+ve	4/4	Positive result	Borø *et al* (2010)
Acidification of lactose	+ve	4/4	Postive result	Wazir *et al.* (2019)
Voges-Proskauer	Weak +ve	4/4	Positive results	Devriese *et al.* (1997)
Urease test	-ve	3/4		Borø *et al.* (2010)
	+ve	1/4		Borø *et al.* (2010)
Hippurate hydrolysis	W +ve	4/4	positive result	Devriese *et al.* (1997)
alkaline phosphatase	V	2/4	positive result	Devriese *et al.* (1997)
	W +ve	2/4		
Indol	-ve	4/4	Negative result	Borø *et al.* (2010)
Biofilm formation				
Microtiter plate	Strong	1/4	NR*	
	Intermediate	1/4		
Proteolytic activity	Translucent halos around the colonies	1/4	NR*	

NR: not recorded.

The antibiogram of all the isolates showed resistance to B-lactams (penicillin and oxacillin), while, two isolates were resistant to erythromycin, vancomycin. Three isolates showed resistance to linezolid while four isolates showed intermediate resistance to tetracycline, respectively. All the isolates were susceptible to chloramphenicol and clindamycin (Table 3).

Screening of *S. thoraltensis* isolates for the presence of antimicrobial resistance genes revealed that *Streptococcus thoraltensis* harbored different antimicrobial genes as, *Pbp1*A gene encoding resistance for B-lactams drugs, *van*A and *optr*A genes encoding vancomycin and linezolid resistance, respectively. While *erm*B gene encoding erythromycin resistance could not be detected in the isolates (Figure 2). Also, it was found that *lmb* gene was detected in one isolate however, *brpA* gene could not be detected in any isolate (Figure 3). The phenotypic and genotypic characters of the obtained *S. thoraltensis* isolates are summarized in Figure 4.

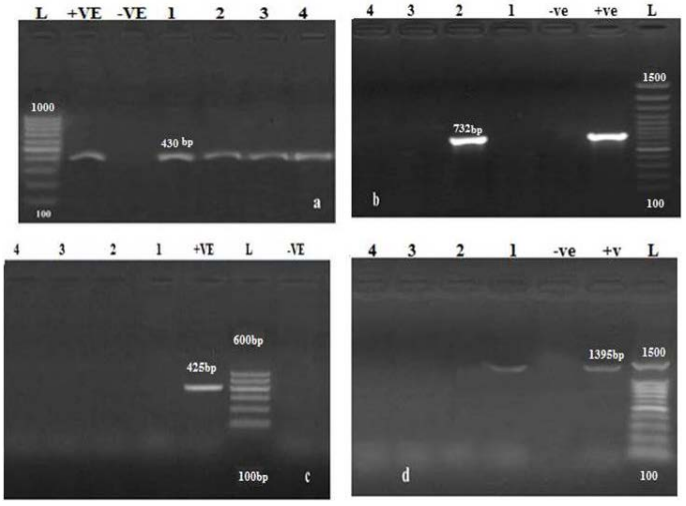

Figure 2: PCR products of the antimicrobial resistance genes (a, b, c and d) identified in *S. thoraltensis* visualized on agarose gel electrophoresis. The expected molecular size of amplified DNA: 430 bp for *Pbp1*A gene (a), 732 bp for *van*A gene (b), 425bp for *erm*B gene (c) and 1395 bp for *optr*A gene (d). Lane 1-4: samples, Lane (L): DNA ladder, Lane (+ve): positive control and Lane (-ve): negative control.

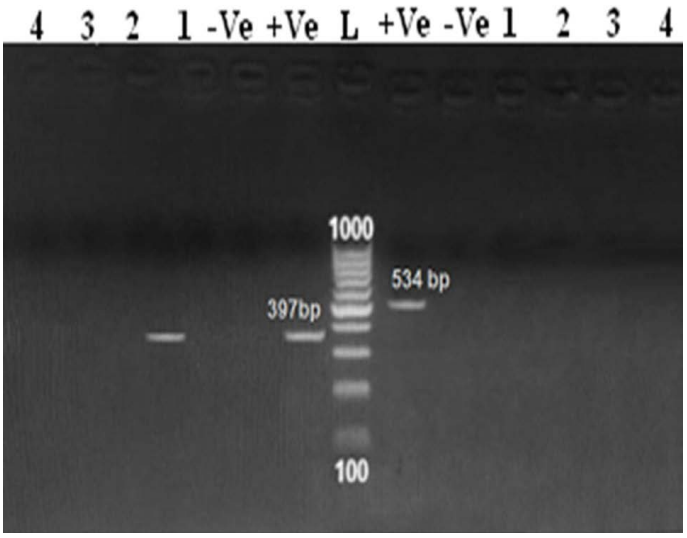

Figure 3: PCR products of the genes encoding for biofilm production (a and b) identified in *S. thoraltensis* visualized on agarose gel electrophoresis. The expected molecular size of amplified DNA: 534 bp for *brp*A gene (a) and 397 bp for *lmb* gene (b). Lane 1 and 2: samples, Lane (L): DNA ladder, Lane (+ve): positive control and Lane (-ve): negative control.

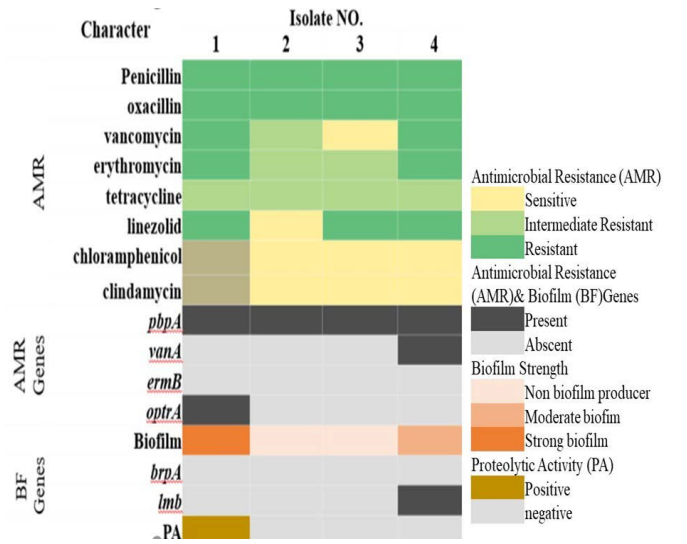

Figure 4: Heat map summary of phenotypic and genotypic characters of *S. thoraltensis* isolates.

Our study describes the presence of *Streptococcus thoraltensis* in raw milk samples. This was possible by the use Vitek2 system as an identification method that results in a rapid and reliable modern technique to identify veterinary bacteria (Garcia-Garrote et al., 2000; Hernández-Durán et al., 2017). It wasn't possible to identify the isolates by conventional methods of *Streptococci* identification due to the absence of precise specificity and accuracy biochemical tests that resulted in species definitions that are often qualified by a number of exceptions; moreover, these organisms grow slowly and may need extra factors for isolation and characterization (Collins et al., 1984; Facklam, 2002). Many studies illustrated the role of Vitek2 system in identifying *S. thoraltensis* (Dhotre et al., 2014; Petridis et al., 2018; Al-Tamimi et al., 2019).

Table 3: Antibiogram of the obtained *S. thoraltensis* isolates.

Antibiogram	Results	Number of isolates (N/4)
Penicillin	R	4/4
Oxacillin	R	4/4
Vancomycin	R	2/4
	IR	1/4
Erythromycin	R	2/4
	IR	2/4
Tetracycline	IR	4/4
Linezolid	S	1/4
	R	3/4
chloramphenicol	S	4/4
clindamycin	S	4/4

World Health Organization stated that *Streptococcus* infections are still one of the most serious problems facing recent medicine (Krzyściak et al., 2013). Antibiotic therapy is one of the principal choices in the treatment of human and animal infections. Consequently, antibiotic resistance problem has long been the focus of numerous research teams around the world as a source of genes linked to drug resistance in people and animals (Pol and Ruegg, 2007; Thomas et al., 2015). However, the available records about *S. thoraltensis* resistance to antibiotics are limited. The present study represented the antibiotic susceptibility profile as shown in Table 2. Therefore, knowledge about susceptibility patterns of this bacteria is needed, especially due to its potential hazards to animals and humans. The antimicrobial resistance profile of *S. thoraltensis* was variable among studies (Vukonich et al., 2015; Dhotre et al., 2016; Petridis et al., 2018; Bustami et al., 2019; Wazir et al., 2019; Renzhammer et al., 2020; Hai et al., 2020).

Streptococcus spp. are, in general, sensitive to penicillin, and this compound remains the drug of choice, but unfortunately, these genera of pathogenic bacteria are quickly developing resistance to this antibiotic (Käppeli et al., 2019). This makes it imperative to expedite the search for new antimicrobials and technologies that can be used for therapy in veterinary medicine. Furthermore, Al-Tamimi et al. (2019) found that all *S. thoraltensis* isolates were fully resistant to most antibiotics that had been used in including penicillin, methicillin, and vancomycin, also Park et al. (2014) recorded a sensitivity of *S. thoraltensis* isolates to tetracyclines despite of their high resistance to vancomycin.

In the current study, the unjustifiable misuse of antimicrobials may have led to resistance of *S. thoraltensis* to most antibiotics, which may cause treatment failure. Also, the resistance to vancomycin would be a disastrous problem because it is the antibiotic of latest recourse for a huge number of multiple-antibiotic-resistant strains.

In addition to the phenotypic method, genotypic detection of the antibiotic resistance, using selected genes showed the presence of penicillin-binding protein gene *(pbp1*A) among the isolates. The beta-lactams are the most widely used and efficient of all antibiotics. However, widespread resistance has emerged among most common pathogens over the past few decades, (Hakenbeck et al., 1999). Navarre and Schneewind (1999) found that one of the common resistance mechanisms to β-lactam antibiotics is the alterations of penicillin-binding proteins (PBPs) that catalyze polymerization, and the cross-linking peptidoglycan precursors in the bacterial cell wall biosynthesis step.

In the present work, molecular analysis indicated the presence of *van*A gene in *S. thoraltensis* isolates. This may be due to *van*A is the most common gene isolated from vancomycin-resistant *Enterococci* and owing to taxonomical and structural resemblances between *Enterococci* and *Streptococci*. The possibility of vancomycin resistance of *S. thoraltensis* to be mediated by *van*A was considered (Park et al., 2014). Unlike, Desjardins et al. (2004) who detected *erm* genes in different species of *Streptococcus* belonging to different groups (8%), our isolates lacked *erm*B gene. Leclercq (2002) elucidated that this resistance may be due to methylation of the ribosomal drug binding site, which resulted in macrolides, lincosamides, and streptogramin resistance, also methylases are encoded by the *erm* genes and may be inducible or constitutively expressed. Macrolide resistance here may be due to the expression of other erm gene classes than ermB.

The multidrug resistance of both two *S. thoraltensis* isolates could be due to their ability to form biofilms as the detected strong and moderate biofilms on microtiter plate. Biofilm is defined as "a matrix-enclosed microbial accretions which can adhere both to biological and non-biological surfaces" (Kaczorek et al., 2017). Once bacterial biofilms attached, biofilm cells can withstand unfavourable environmental conditions, such as nutrient depletion or treatment with antimicrobial substances (Jahid and Ha, 2014). Also biofilms on processing equipments/ surfaces are the main source of contamination of dairy products (Srey et al., 2013; Cappitelli et al., 2014).

One of the biofilm producing isolates harboured *lmb* gene which its role in biofilm was supported when its mutant resulted in profound defects in biofilm maturation (Boles et al., 2010), while the *brp*A-deficient mutant was found to be a reason for severe defects in biofilm formation when grown in a glucose-containing medium on polystyrene surfaces (Wen and Burn, 2002; Yoshida and Kuramitsu, 2002).

To best our search efforts, we could not find reports regarding the prevalence of biofilm formation among *S. thoraltensis* strains, however, many investigations have been conducted to assess the potential of *Streptococcus* spp. to form biofilms (Boonyayatra and Pata, 2016; Kaczorek et al., 2017; Moliva et al., 2017; Bonsaglia et al., 2019; Pieranski et al., 2021). The production of biofilms was considered as a significant factor in the pathogenesis of numerous diseases, both in humans (e.g., endocarditis) and in animals (e.g. mastitis) (Jung et al., 2012; Gomes et al., 2016). Accordingly, *S. thoraltensis* role as a mastitis initiating bacteria was emphasized in a large study, where it was the only isolated bacterium from 31.45% and 1.34% of 173,345 milk samples collected from clinical and non-clinical mastitis cases, respectively, and was also included in a cluster of pathogens known to be associated with sever mastitis (Díaz Cao et al., 2020). While, Kim et al. (2017) found that *S. thoraltensis* represents 1.6% of bacterial pathogens in mastitis-affected farms.

A few numbers of *Streptococci* have proteolytic activities which lead to bitterness in milk and its products, in addition to unfavorable changes (ICMSF, 1980). One of *S. thoraltensis* isolates was found to have proteolytic power on skimmed milk agar and this isolate is a strong biofilm producer. The proteolysis and the lipolysis produced per dairy bacterial cell were found to be higher within biofilm cells than within planktonic cells (Teh et al., 2012). The accumulation of enzymes within biofilms may also aid in the survival of the dairy bacteria within a dairy environment. Moreover, protease enzyme produced by viridans *Streptococcus* spp. was found to be associated with endocarditis (Straus, 1982; Al-Salih et al., 2012).

Isolation of *S. thoraltensis* from nasal and oropharyngeal mucosa of healthy people (Dhotre et al., 2016; Al-Wakeel, 2017; Al-Tamimi *et al*., 2019) and from water bowels (Alaidarous et al., 2017) makes human, water and/ or dairy utensils the most common source of milk contamination with this microorganism especially with its ability to produce biofilm.

Although *Streptococcus thoraltensis* is recently identified and was known to be non-pathogenic in humans, however, recently very little is known about the pathogenic potential of such bacterium to humans, providing a new insight in the field of microbiology. It is associated with cases of Chorioamnionitis (Vukonich et al., 2015), bacteraemia (Petridis et al., 2018; BHE, 2021; Wazir et al., 2019);

endocarditis (Hai et al., 2020) and genital infection in women (Al-Wandawy et al., 2020) and has been isolated from organs of diseased humans working in the meat industry (Renzhammer et al., 2020). These recent health issues make *S. thoraltensis* represent a higher underestimated risk for zoonotic potential warrants further investigations. Also, its role as a mastitis pathogen deserves closer attention and should be properly identified by veterinary diagnostic laboratories. Furthermore, they express high resistance rates that appear to be directly related to the industry's antimicrobial usage and could be further applied as biological markers for resistance monitoring.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this work reports the isolation of four bacterial strains which appeared to be *S. thoraltensis*. Considering these results, we cannot exclude a potential risk for human health related to the consumption of raw milk. Thus, the prevalence of *S. thoraltensis* in the dairy food chain necessitates more investigations. Additionally, as *S. thoraltensis* appeared to be associated with cases of clinical and subclinical mastitis, the role of this opportunistic pathogen needs to be closely monitored. It is essential to tightly regulate the use of antibiotics to avoid the emergence of antibiotic resistant strains of these bacteria which is a considerable recent hazard to humans.

ACKNOWLEDGMENTS

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NOVELTY STATEMENT

This research highlights the role of raw milk as a source of the recently identified Streptococcus thoraltensis that resulted in increased human infection case reports. S. thoraltensis could be detected in raw milk samples and showed different antimicrobial resistance to different antimicrobials and one isolate showed biofilm formation and proteolytic activity.

AUTHOR'S CONTRIBUTION

Authors contributed equally in the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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