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Original Article

Prevalence of Virulence Factors and Antimicrobial Resistance of *Streptococcus agalactiae* and *Streptococcus uberis* in Ruminant Sub-clinical Mastitic Milk in Iran

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Background and Aims: The aim of this study was to determine the virulence and antibiotic resistance properties of *Streptococcus agalactiae* (*S. agalactiae*) and *Streptococcus uberis* (*S. uberis*) isolated from the ruminant's sub-clinical mastitic milk in Iran.

Materials and Methods: Positive samples in the CMT test were cultured and *S. agalactiae* and *S. uberis* have been identified using biochemical tests and polymerase chain reaction (PCR). Virulence and antibiotic resistance genes were studied using PCR. A total of 350 milk samples were collected randomly from 4 major provinces of Iran. We chose 55 bovine, 43 ovine, and 32 caprine herds from these regions.

Results: Totally, 34.52% of bovine, 25% of ovine, and 25% of caprine milk samples were found to be contaminated with *S. agalactiae* and also, 27.38% of bovine, 25% of ovine, and 25% of caprine milk samples were found to be contaminated with *S. uberis*. The most commonly detected virulence genes of *S. agalactiae* were *cfb* (68.75%), *scpB* and *pavA* (43.75%). Also, the most commonly detected pathogenic genes of *S. uberis* were *sua* and *hasB* (83.33%), *cfu* (75.00%) and *skc* (70.83%). Totally, *tetO* (89.28%), *ermA* (82.14%), *mefA* (73.21%) and *tetM* (69.64%) were the most common antibiotic resistance genes in *Streptococcus* isolates. The most resistance were detected against tetracycline (87.5%) and erythromycin (83.92%), respectively.

Conclusions: Our results showed that animal milk samples should be controlled, especially in colder seasons of the year in order to study the presences of *S. agalactiae* and *S. uberis*.

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Introduction

Mastitis is one of the main health issues in dairy production. Its losses are not only economic, but also issues such as animal health and welfare, milk quality, antibiotic usage and the image of the dairy sector are important reasons to focus on the mastitis control. Accordingly, mastitis is a topic that is well studied worldwide [1]. Mastitis is considered as one of the most costly diseases in dairy herds because the high volume of discarded milk and diminished milk production amount to approximately 80% of financial losses. The remaining 20% of financial losses are attributed to treatment costs, veterinary fees, labor costs, early culling, and death [2].

The major cause of ruminant mastitis is the infection of the udder by pathogenic bacteria among which *Streptococcus agalactiae* (*S. agalactiae*) and *Streptococcus uberis* (*S. uberis*) are the most common pathogens [3, 4]. Several virulence factors, including fibrinogen binding protein (*fnb*), laminin-binding protein (*lmb*), fibronectin-binding protein (*pavA*), β-C protein (*cba*), capsule, C5a peptidase (*scp*), hyaluronate lyase, α-C protein, β- hemolysin/ cytolysin, and CAMP factor (*cfb*) are responsible for *S. agalactie* infections [5-7]. The *cps* gene cluster coding for the capsule and the *spB* gene coding for surface enzyme ScpB (a C5a peptidase), which causes impairing of neutrophil recruitment and binds fibronectin to promote bacterial invasion of epithelial cells [5]. The C5a peptidase genes were found only in group B *streptococci* (*scpB* gene) and group A streptococci (*scpA* gene) [6]. The *cba is* a *gene* codes for alpha-C protein, which is a surface protein that helps the bacteria to enter the host cells [7]. The *lmb* gene codes for laminine-binding protein (lmb), a surface protein that plays a role in the invasion of the damaged epithelium [8]. Presences of fibronectin-binding proteins (FnbA, FnbB and FnB) have been shown to correspond to the capability of streptococci to adhere to the skin fibroblast [9]. The *S. uberis* virulence factors, including hyaluronic acid capsule, M-like protein, R-like protein, plasminogen activator (pauA/skc), and CAMP factor (cfu) [10, 11].

Adherence, internalization, and intracellular persistence are important for environmental streptococci like *S. uberis* to establish intramammary infection [11-13]. The *S. uberis* adhesion molecule gene (*sua*), previously referred to as lactoferrin-binding protein was found to play a central role in adherence of *S. uberis* to bovine mammary epithelial cells [14, 15] and thus aid in establishing persistent infection.

The ability to control these infections depends on a detailed knowledge of the epidemiology of the organisms and their environment. Detection of various virulence genes of *S. agalactiae* and *S. uberis* in various resources helps us to know more about the bacterial epidemiology in various parts and resources. However, many reports showed the high presences of antibiotic resistant in these bacteria [16-18]. The high numbers of antibiotic resistance patterns among *S. agalactiae* and *S. uberis* have been coded by of several antibiotic resistance genes [19]. As far as we know, the epidemiology and

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prevalence of *S. agalactiae* and *S. uberis* in ruminant mastitic milk is essentially unknown in Iran. Also, veterinarians do not pay attention to proper antibiotic prescription. Therefore, the present study was carried out in order to study the molecular characterization, antibiotic resistance properties and seasonal distribution of *S. agalactiae* and *S. uberis* isolated from ruminant's sub-clinical mastitic milk in Iran.

Materials and Methods

Samples

In order to study the risk factors (origin of milk sample collection and various seasons) for determination the prevalence of *S. agalactiae* and *S. uberis*, from April 2011 to April 2012 (in various seasons of the year), a total of 350 milk samples were collected randomly from 4 major provinces of Iran (Khozestan, Isfahan, Fars and Chaharmahal va Bakhtiary). There were 110 bovine, 88 ovine, and 65 caprine herds in these areas and we choose 55 bovine, 43 ovine, and 32 caprine herds from these regions (Table 1). The animals, which their milk samples were collected for this study were clinically healthy and the milk samples showed normal physical characteristics. Samples were collected under sterile hygienic conditions and were immediately transported to the microbiology and biotechnology laboratories of Islamic Azad University, Shahrekord Branch, Iran. The study was approved by Ethics Committee of Islamic Azad university of Shahrekord branch, Iran.

Table 1. Herds size and number of milk samples, which were collected for study the distribution of *S. uberis* and *S. agalactiae* in four provinces of Iran.

Provinces	No. herds in the study			No. herds studied			No. samples per herd			No. milk samples		
	Bovine	Ovine	Caprine	Bovine	Ovine	Caprine	Bovine	Ovine	Caprine	Bovine	Ovine	Caprine
Khozestan	30	20	25	16	8	13	2-4	2-4	1-2	60	28	20
Isfahan	30	16	10	16	7	6	2-4	2-3	2-3	40	19	14
Fars	30	38	15	15	19	8	2-5	2-3	2-3	50	35	18
Chaharmahal va Bakhtiary	20	14	15	8	9	5	3-5	1-2	3-4	30	18	18
Total	110	88	65	55	43	32	2-5	1-4	1-4	180	100	70

California mastitis test (CMT) and identification of *S. agalactiae* and *S. uberis*

All milk samples were tested using the CMT method [20]. All positive samples were collected through CMT technique was studied for the presences of *S. agalactiae* and *S. uberis*. For isolation of *Streptococcus* species, 0.5 ml of pooled milk sample was enriched in 5 ml of streptococcal selection broth (HiMedia Laboratories, Mumbai, India) and incubated at 37°C for 6 hrs in 5% CO_2 condition. After enrichment, the samples were streaked onto 5% sheep blood agar (Merck, Darmstadt, Germany) and incubated at 37°C for 24-48 hrs in 5% CO_2 condition. The suspected streptococcal colonies were purified on BHI agar (Merck, Darmstadt, Germany). The purified cultures were

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tentatively identified based on Gram's staining and biochemical tests namely, catalase, oxidase, and bile esculin hydrolysis. To differentiate the suspected *S. agalactiae* and *S. uberis* isolated from *Enterococci*, a selective media, KF streptococcal agar (Merck, Darmstadt, Germany) was also used. The colonies were also confirmed using the polymerase chain reaction (PCR) based on the detection of *16SrRNA* gene region of *S. agalactiae and S. uberis* described previously [21, 22]. The PCR reaction mixture contained 2.5 µl of 10×Taq polymerase buffer (1.5 mM MgCl); 1.0 µl of forward primer (10 µM); 1.0 µl of reverse primer (10 µM); 0.2 µl of dNTP (25 mM), 0.1 µl of Taq polymerase (0.25 u); 5 µl of DNA (50 to 100 ng/µl); add ddH2O (sterile) to total volume 25 µl. The reaction was carried out in a PCR thermocycler, as follows: 94°C for 4 min.; five cycles of 94°C, 58°C and 72°C for 45 s each step; 20 cycles of 94°C, 58°C, 72°C for 45 s each step; and a step of 72°C for 5 min., at the end of the reaction. Strain of *S. agalactiae* PTCC 1447 (Iranian Research Organization for Science and Technology, Iran) was used as a positive control.

DNA extraction, PCR amplification and gel electrophoresis

Purification of DNA directly from typical colonies was achieved using a DNA extraction kit (DNP™, CinnaGen, Iran) according to the manufacturer's instructions. The concentration of the purified DNA was determined using spectrophotometer (Biophotometer, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and stored at -20°C until further use. Oligonucleotide primers for detection of virulence factors of *S. agalactiae* and *S .uberis* have been shown in table 2. The *S. agalactiae* isolates were screened for *cfb*, *fnbB*, *pavA*, *lmb*, *scpB*, and *cba* virulence genes, and *S. uberis* isolates were screened for *cfu*, *skc*, *sua*, *lbp*, *hasA*, *hasB*, *hasC*, *gapC* and *oppF* genes. To amplify the virulence genes of *S. agalactiae* and *S. uberis* isolated from ruminant mastitic milk samples, 50 µl of reaction mixture was made containing 20 ng DNA, 1 mM oligonucleotide primers, 0.4 mM of each of dNTPs, 1.5 U Taq polymerase and 1.5 mM $MgCl_2$. The annealing temperature was varied from 50 to 60°C based on the gene being amplified. The reaction was carried out in a PCR thermocycler, as follows: 94°C for 4 min.; 35 cycles of 94°C, 50-60°C and 72°C for 55 s each step; and a step of 72°C for 8 min., at the end of the reaction. Finally, all PCR products were resolved on 1.5% agarose gel at 90V for 80 min. Gels were stained with ethidium bromide solution (0.5 mg/mL) and photographed under UV light with Uvitech gel documentation (UK).

Antibiotic resistance genes

The macrolide resistance genes *mefA*, *ermA*, and *ermB*, were detected by PCR [23] among all *S. agalactiae* and *S. uberis* isolates. Tetracycline resistance genes *tetM*, *tetO*, *tetT*, *tetW*, *tetL*, *tetQ*, *tetK* and *tetS* were tested by PCR [23-25]. All isolates were tested for the presence of the lincosamide resistance gene *linB* [26]. All isolates were further screened by PCR for the presence of the streptogramin, a resistance gene *vgaB* [27]. All negative results were confirmed in at least three independent assays. Since the positive control sample was not used in regard with detection of some genes, to confirm or

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reject the PCR results, the PCR products of the primary positive samples were purified using a PCR product purification kit (Roche Applied Science, Germany) and sent to the Macrogen Co. (South Korea) for sequencing and sequenced with ABI 3730 XL device and Sanger sequencing method.

Table 2. A set of oligonucleotide primers for potential virulence factors of *S. agalactiae* and *S .uberis*.

Bacteria	Target gene	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	References
S. agalactiae	*Cfb*	F-GCTGTTTGAAGTGCTGCTTG R-GACTTCATTGCGTGCCAAC	288	60	23
	fnbB	F-TGATGCTGCAAAAGAATTGC R-TTACAGCCCCTTTTTGAGGA	629	53	23
	pavA	F-TTCCCATGATTTCAACAACAAG R-AACCTTTTGACCATGAATTGGTA	495	58	23
	Lmb	F-AGTCAGCAAACCCCAAACAG R-GCTTCCTCACCAGCTAAAACG	397	57	23
	scpB	F-AGTTGCTTCTTACAGCCCAGA R-GGCGCAGACATACTAGTTCCA	567	58	23
	Cba	F-TGCACCGAAGTTACCAGATG R-CAGCCAACTCTTTCGTCGTT	149	56	23
S. uberis	*Cfu*	F-TATCCCGATTTGCAGCCTAC R-CCTGGTCAACTTGTGCAACTG	205	59	24
	Skc	F-CTCCTCTCCAACAAAGAGG R-GAAGGCCTTCCCCTTTGAAA	800	52	25
	Sua	F-ACGCAAGGTGCTCAAGAGTT R-TGAACAAGCGATTCGTCAAG	776	58	24
	oppF	F-GGCCTAACCAAAACGAAACA R-GGCTCTGGAATTGCTGAAAG	419	54	4
	Lbp	F-CGACCCTTCAGATTGGATTC R-TAGCAGCATCACGTTCTTCG	698	53	24
	hasA	F-GAAAGGTCTGATGCTGAT R-TCATCCCCTATGCTTACAG	600	58	26
	hasB	F-TCTAGACGCCGATCAAGC R-TGAATTCCYATGCGTCGATC	300	58	26
	hasC	F-TGCTTGGTGACGATTTGATG R-GTCCAATGATAGCAAGGTACAC	300	58	27
	gapC	F-GCTCCTGGTGGAGATGATGT R-GTCACCAGTGTAAGCGTGGA	200	55	24

Antimicrobial resistance pattern

All *S. uberis* and *S. agalactiae* isolates were tested by Kirby–Bauer simple disk diffusion method according to the guidelines from the Clinical and Laboratory Standards Institute [28] for antimicrobial susceptibility tests for bacteria isolated from sub-clinical mastitic milk samples. The following antimicrobials were selected for testing, based on licensing for mastitis treatment in cattle and widespread using [P10= penicillin (10 u/disk); TE30= tetracycline (30 µg/disk); S10= streptomycin (10 µg/disk); C30=chloramphenicol (30 µg/disk); SXT= sulfamethoxazol (25 µg/disk); GM10= gentamycin (10 µg/disk); ERT10= erythromycin (10 µg/disk), NFX5= enrofloxacin (5 µg/disk); L2= lincomycin (2 µg/disk); CF30= cephalothin (30 µg/disk); CIP5= ciprofloxacin (5 µg/disk); TMP5=trimethoprim (5 µg/disk); F/M300= nitrofurantoin (300 µg/disk); AM10=ampicillin

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(10 u/disk)]. Resistance was determined by measurement of inhibition of growth around the antimicrobial disk according to the zone diameter interpretative standards of Clinical and Laboratory Standards Institute [28], and when not available, according to the antimicrobials manufacturers' instructions. *S. uberis* ATCC 27958 and *S. parauberis* ATCC 13386 were used as reference strains.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), Chi-square test and Fisher's exact two-tailed test analysis was performed and the differences were considered significant at values of $p<0.05$.

Results

Table 3 presents the prevalence of CMT positive milk samples, which were collected from 4 major provinces of Iran. In this study, 84 out of 180 (46.66%), 8 out of 100 (8%), and 4 out of 70 (5.71%) bovine, ovine and caprine milk samples were found to be positive in CMT test. There were significant differences in the prevalence rates of CMT positive milk samples (p=0.032). Bovine milk samples were the most common positive CMT samples.

Table 3. Distribution of sub-clinical mastitic milk in ruminants of 4 major province of Iran using the CMT assay

Provinces	No. milk samples			CMT positive (%)		
	Bovine	Ovine	Caprine	Bovine	Ovine	Caprine
Khozastan	60	28	20	28 (46.66)	3 (10.71)	2 (10)
Isfahan	40	19	14	17 (42.5)	2 (10.52)	-
Fars	50	35	18	25 (50)	2 (5.71)	1 (5.55)
Chaharmahal va Bakhtiary	30	18	18	14 (46.66)	1 (5.55)	1 (5.55)
Total	180	100	70	84 (46.66)	8 (8)	4 (5.71)

Table 4 presents the prevalence of *16SrRNA* gene of *S. agalactiae* and *S. uberis* in bovine, ovine, and caprine CMT positive milk samples. Totally, 29 out of 84 bovine (34.52%), 2 out of 8 ovine (25%), and 1 out of 4 caprine (25%) milk samples were found to be contaminated with *S. agalactiae* and also, 23 out of 84 bovine (27.38%), and 1 out of 4 caprine (25%) milk samples were found to be contaminated with *S. uberis*. Totally, the milk samples of Khozestan and Fars provinces were the most contaminated with *S. agalactiae* and *S. uberis* while the milk samples of Chaharmahal va Bakhtiary province were the less contaminated. There were significant differences for the prevalence of *S. agalactiae* and *S. uberis* (p=0.021) and there were significant differences for the prevalence of *S. agalactiae* and *S. uberis* in bovine with ovine and caprine milk samples (p=0.029).

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Table 4. Detection of the *16SrRNA* genes of *S. uberis* and *S. agalactiae* in CMT positive ruminant milk samples using the PCR technique.

Provinces	CMT positive milk samples			PCR positive (%)					
				S. agalactiae 16SrRNA gene			*S. uberis 16SrRNA* gene		
	Bovine	Ovine	Caprine	Bovine	Ovine	Caprine	Bovine	Ovine	Caprine
Khozastan	28	3	2	10 (35.71)	1 (33.33)	1 (50)	8 (28.57)	-	1 (50)
Isfahan	17	2	-	6 (35.29)	-	-	4 (23.52)	-	-
Fars	25	2	1	8 (32)	1 (50)	-	7 (28)	-	-
Chaharmahal va Bakhtiary	14	1	1	5 (35.71)	-	-	4 (28.57)	-	-
Total	84	8	4	29 (34.52)	2 (25)	1 (25)	23 (27.38)	-	1 (25)

Table 5 shows the results of PCR assays for detection of genes encoding the virulence factors of *S. agalactiae* and *S. uberis* in bovine, ovine, and caprine milk samples. The PCR product of some these genes are shown in Figure 1 and 2. The most commonly detected genes of *S. agalactiae* were *cfb* (68.75%), *scpB* and *pavA* (43.75%). Also, the most commonly detected genes of *S. uberis* were *sua* (83.33%), *cfu* (75.00%) and *skc* (70.83%). Bovine milk samples had the highest virulence genes while ovine and caprine milk samples had the lowest virulence genes of *S. agalactiae* and *S. uberis*. There were significant differences (p=0.036) for the prevalence of *cfb* gene of *S. agalactiae* with *fnbB* and *lmb* genes and also p=0.019 for the prevalence of *sua* and *cfu* of *S. uberis* with *lbp* gene.

Table 5. Distribution of virulence genes of *S. uberis* and *S. agalactiae* in sub-clinical mastitic milk samples.

Species	*S. agalactiae* virulence genes							*S. uberis* virulence genes							
	cfb	*fnbB*	*pavA*	*lmb*	*scpB*	*cba*	*cfu*	*skc*	*sua*	*lbp*	*hasA*	*hasB*	*hasC*	*gapC*	*oppF*
Bovine	20/29	3/29	14/29	2/29	14/29	4/29	18/23	17/23	19/23	3/23	4/23	19/23	16/23	13/23	15/23
Ovine	1/2	0/2	0/2	0/2	0/2	0/2	0	0	0	0	0	0	0	0	0
Caprine	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1	0/1
Total	22/32	3/32	14/32	2/32	14/32	4/41	18/24	17/24	20/24	3/24	4/24	20/24	16/24	13/24	15/24

Table 6 presents distribution of antibiotic resistance genes of *Streptococcal* strains isolated from ruminant sub-clinical mastitic milk samples. The PCR product of some these genes are shown in figure 3. The most common detected antibiotic resistance genes were *tetO* (89.28%), *ermA* (82.14%), *mefA* (73.21%) and *tetM* (69.64%), which were encoding tetracycline and macrolides antibiotics, respectively. Also, *linB* and *vgaB* genes were detected in six (10.71%) and seven (12.50%) isolates, respectively, which were the less common antibiotic resistance genes. There were significant differences (p=0.041) for the prevalence of *tetO* with *tetK*, *tetS*, *linB* and *vgaB* genes and also, p=0.033 for the prevalence of *ermA* with *linB* and *vgaB* genes of *Streptococcus* strains. The bovine isolates had the highest amounts of antibiotic resistance genes.

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Table 6. Distribution of antibiotic resistance genes of *Streptococcus* strains isolated from ruminant sub-clinical mastitic milk samples.

Species	Antibiotic resistance genes												
	mefA	***ermA***	***ermB***	***tetM***	***tetO***	***tetT***	***tetW***	***tetL***	***tetQ***	***tetK***	***tetS***	***linB***	***vgaB***
Bovine	40/52	44/52	15/52	39/52	46/52	28/52	19/52	15/52	12/52	11/52	11/52	6/52	7/52
Ovine	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Caprine	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Total	41/56	46/56	15/56	39/56	50/56	28/56	19/56	15/56	12/56	11/56	11/56	6/56	7/56

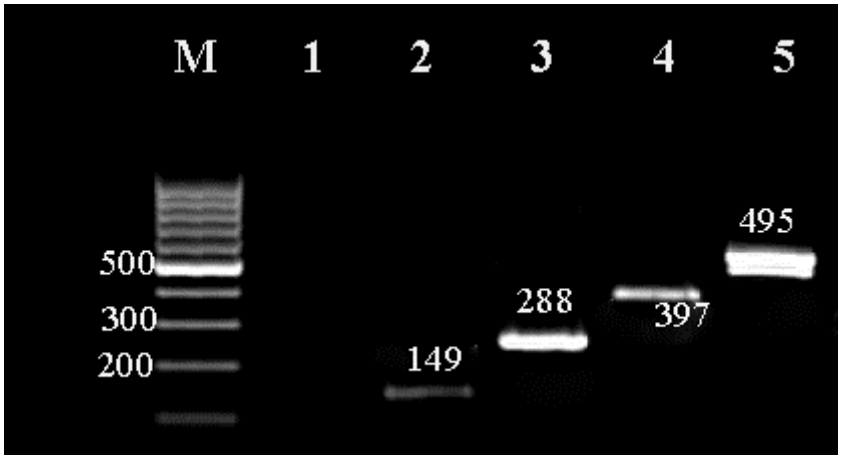

Fig. 1. Agarose gel electrophoresis of PCR products amplified for the *cfb* (288 bp), *pavA* (495 bp), *lmb* (397 bp), and *cba* (149 bp) genes from *S. agalactiae*. M=100bp DNA ladder

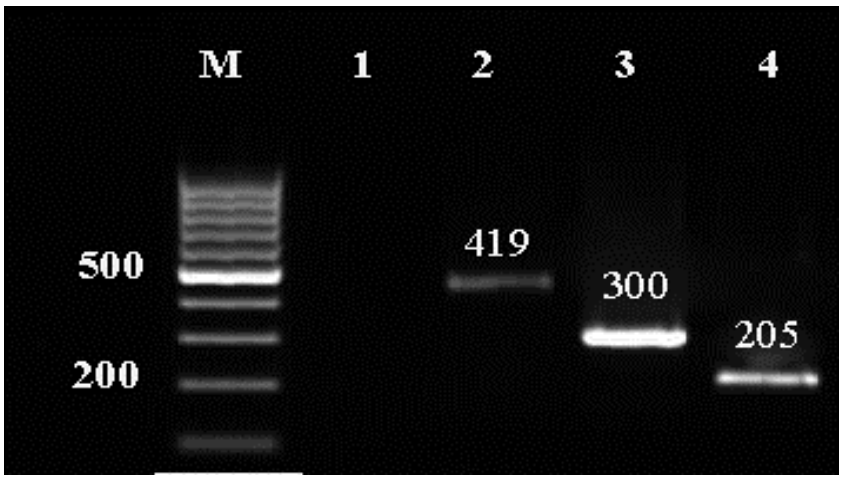

Fig. 2. Agarose gel electrophoresis of PCR products amplified for the *cfu* (205 bp), *oppF* (419 bp), and *hasB* (300 bp) genes from *S. uberis*. M=100bp DNA ladder

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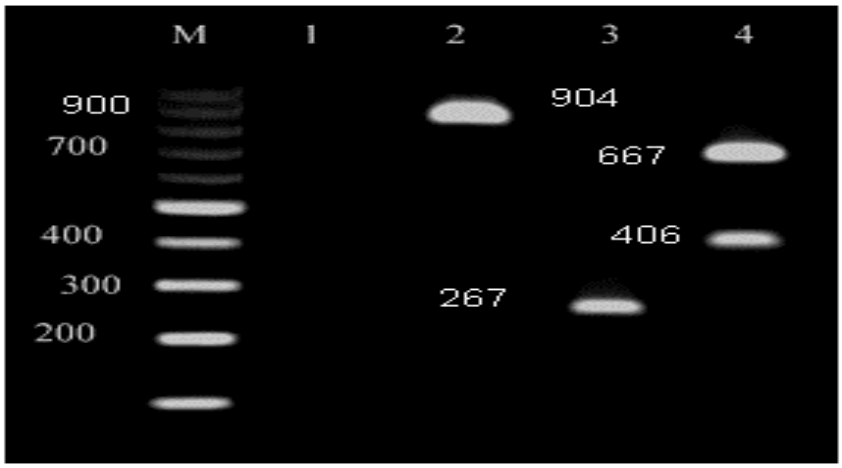

Fig. 3. Agarose gel electrophoresis of PCR products amplified for the *tetL* (267 bp), *tetM* (406 bp), *tetS* (667 bp), and *tetQ* (904 bp) genes from *Streptococcus* strains. M=100bp DNA ladder

Table 7 presents the antibiotic resistance pattern of Streptococcal strains isolated from sub-clinical mastitic milk samples. *Streptococcus* strains had the highest antibiotic resistance to tetracycline (87.50%) and erythromycin (83.92%). Lincomycin and streptomycin due to their low antibiotic resistance (5.35% and 7.14%, respectively) are the best drugs for treatment of the cases with *Streptococcus*. Statistical differences were significant between the resistance of *Streptococcus* against tetracycline with lincomycin and streptomycin and erythromycin with lincomycin (p=0.018).

Table 7. Antibiotic resistance pattern of *Streptococcus* strains isolated from ruminant sub-clinical mastitic milk samples (Disk Diffusion Method).

Species	Antibiotic resistance pattern													
	P10	**TE30**	**S10**	**C30**	**SXT**	**GM10**	**NFX5**	**L2**	**CF30**	**CIP5**	**TMP5**	**F/M300**	**AM10**	**ERT5**
Bovine	14/52	45/52	4/52	18/52	24/52	27/52	26/52	3/52	27/52	24/52	14/52	12/52	24/52	43/52
Ovine	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2
Caprine	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2	2/2
Total	14/56	49/56	4/56	18/56	24/56	27/56	26/56	3/56	27/56	24/56	14/56	12/56	25/56	47/56

P10=penicillin (10 u/disk); TE30=tetracycline (30 µg/disk); S10=streptomycin (10 µg/disk); C30=chloramphenicol (30 µg/disk); SXT=sulfamethoxazol (25 µg/disk); GM10=gentamycin (10 µg/disk); NFX5=enrofloxacin (5 µg/disk); L2=lincomycin (2 µg/disk); CF30=cephalothin (30 µg/disk); CIP5=ciprofloxacin (5 µg/disk); TMP5=trimethoprim (5 µg/disk); F/M300=nitrofurantoin (300 µg/disk); AM10=ampicillin (10 u/disk); ERT5=erythromycin (5 µg/disk)

Table 8 presents the seasonal distribution of *S. uberis* and *S. agalactiae* isolated from ruminant sub-clinical mastitic milk. The milk samples, which were collected in winter season, had the highest contamination rate while the milks, which were collected in autumn and spring had the lowest contamination rate. Totally, 18.75%, 6.25%, 68.75% and 6.25% of *S. agalactiae* strains were isolated from the milks, which were collected in summer, autumn, winter and spring

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seasons and also, 16.66%, 4.16%, 79.16% and 0.0% of *S. uberis* strains were isolated from the milks, which were collected in summer, autumn, winter and spring seasons. There were significant differences (p=0.026) for the rates of contamination between the milk samples, which were collected in winter and spring.

Table 8: Seasonal distribution of *S. uberis* and *S. agalactiae* in ruminant sub-clinical mastitic milk.

Species	No. positive samples		Seasonal distribution							
	S. agalactiae	*S. uberis*	*S. agalactiae* (%)				*S. uberis* (%)			
			Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Bovine	29	23	6 (20.68)	2 (6.89)	19 (65.51)	2 (6.89)	4 (17.39)	1 (4.34)	18 (78.26)	-
Ovine	2	-	-	-	2 (100)	-	-	-	-	-
Caprine	1	1	-	-	1 (100)	-	-	-	1 (100)	-
Total	32	24	6 (18.75)	2 (6.25)	22 (68.75)	2 (6.25)	4 (16.66)	1 (4.16)	19 (79.16)	-

Discussion

As far as we know, this present study is the first prevalence report of virulence and antibiotic resistance properties of *S. agalactiae* and *S. uberis* isolated from ruminant sub-clinical mastitic milk in Iran. The results of this present study showed that *S. agalactiae* and *S. uberis* had the high prevalence in sub-clinical mastitic milk of Iran. Totally, *S. agalactiae* was more prevalent than *S. uberis* and it was agreed with previous studies [3, 21]. Our results showed that there were severe seasonal distribution for prevalence of *S. agalactiae* and *S. uberis* in sub-clinical mastitic milk. Totally, these bacteria were more prevalent in the milk samples, which were collected in winter season and it was in agreement with the previous studies [29, 30]. The main reason for the higher prevalence of these bacteria in winter is the fact that during this time climatic events, heat, rain, and thunderstorms, as well as variation of barometric pressure may have influence on the autonomic nervous system. These events caused reduction in the levels of animal immunity. Therefore, several infections and illnesses have been occurred. Also, animals are more crowded in barn in cold seasons. Therefore, diseases and infections would be disseminated. After analyzing the average temperatures of these 4 provinces in each season (30˚C for spring, 45˚C for summer, 16˚C for autumn and 4˚C for winter), it was recognized that the prevalence rate of *S. agalactiae* and *S. uberis* in each season is related with their average temperatures. Our results showed significant differences between relative temperatures of summer with winter. Therefore, the highest levels of animal health care should be performed on the cold months of year. The prevalence rate of infection with *Streptococcus* species between 11 and 47 percent [31]. Totally, 34.52% and 27.38% of bovine milk, 25% and 0.0% of ovine milk, and 25% and 25% of caprine milk samples of our investigation were found to be infected with *S. agalactiae* and *S. uberis*, respectively. The

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main source of the infection is the udder of infected ruminants. Although, when hygiene is poor, contamination of the environment may also provide an additional source of infection [21, 31]. These are the most common causes of environmental mastitis in animals. *S. uberis* has been isolated from bedding materials of animals and *S. dysgalactiae* can also be found in the environment of dairy herds [31].

Several investigations have been performed on the prevalence of *S. agalactiae* and *S. uberis* in Iranian dairy herds [3, 32, 33]. Moatamedi et al. [3] showed that *S. agalactiae* and *S. uberis* were causative agents in 20 and 0.83% of CMT positive milk samples, respectively, which was agreed with our results. Ahmadi et al. [32] reported that *S .agalactiae* was isolated from 8% of the 100 samples and 15% were positive in amplification of the *16SrRNA* gene. Our results showed that *cfb* (68.75%), *scpB* and *pavA* (43.75%) were the most common virulence genes of *S. agalactiae* and were *sua* (83.33%), *cfu* (75.00%) and *skc* (70.83%) were the most common virulence genes of *S. uberis* in ruminant milk samples. Beenu et al. [34] reported that out of 27 isolates of *S. agalactie* none was carrying the *bca* gene, while 6, 7, 8 and 6 isolates were positive for *scpB*, *rib*, *lmb* and *cyiE* genes. All *S. agalactiae* isolates of the Shome et al. [35] study carried the *cfb* gene, only a single isolate possessed the *pavA* gene, while the *cfu* gene was detected in six (85.7 %) *S. uberis* and the *sua* gene was found in four (80 %), which was in line with our results. The *sua* gene of *S. uberis* plays a major role in adherence of bacterium to bovine mammary epithelial cells [14, 15]. The *cba* gene of *S. agalactiae* plays an important role in bacterial penetration into the host cells [7]. The *lmb* gene plays an important role in the invasion of the damaged epithelium [8]. Therefore, those animals, which infected with stereptococcal virulence genes, were more prone to stereptococcal mastitis. Because of the importance of antibiotic prescription for the prevention of invasive stereptococcal infections especially in mastitis and the concern regarding antibiotic resistance, one of the objectives of this study was to determine the antibiotic resistance properties of *Stereptococcus* spp. isolated from ruminant sub-clinical mastitic milk. Our results showed that all of the *Stereptococcus* spp., which was isolated from studied milk samples had one or more antibiotic resistance genes. Totally, *tetO* (89.28%), *ermA* (82.14%), *mefA* (73.21%) and *tetM* (69.64%), which were encoding tetracycline and macrolides were the most common detected antibiotic resistance genes. These results are in aligning with the previous studies [19, 36]. In accordance with other reports from all around the world, antibiotic resistance analysis showed that most of the isolates were resistant to tetracycline [37-39]. Also, resistance to fluoroquinolone has been documented in other countries [40]. Erythromycin resistance had been reported from many other sites of the world, too [19, 41, 42].

Stereptococcal isolates of our study had the highest antibiotic resistance to tetracycline (87.50%) and erythromycin (83.92%) in disk diffusion method, which were aligned with the previous investigations [19, 43]. Dogan et al. [44] reported that resistance to tetracycline and erythromycin was more common among *S. agalactiae* isolates from humans

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than among isolates from cattle. Macrolide and tetracycline resistance among *S. agalactiae* strains have been reported previously from Spain [45]. Resistance against tetracycline, erythromycin, co-trimoxazole, ampicillin and enrofloxacin was 55.5%, 33.3%, 11.1%, 11.1%, and 7.4%, respectively among all mastitic milk borne *S. agalactiae* isolates of previous study [39]. Totally, the presence of antibiotic resistance is different in various geographical regions. Mainly, the high, irregular and unauthorized use of some antibiotics leads to high amounts of antibiotic resistance. It depends on veterinarian's choice of antibiotic for prescription. The high presences of resistance to the studied antibiotic showed the irregular and unauthorized use of it in veterinary treatment in Iran.

Conclusions

Our results showed that animal milk samples should be controlled especially in colder seasons of the year in order to study the presences of *S. agalactiae* and *S. uberis*. All of the mastitic milk samples harbored *cfb*, *scpB*, *pavA*, *sua*, *hasB* and *cfu* virulence genes and *tetO*, *ermA*, *mefA* and *tetM* antibiotic resistance genes. It is better to use antibiotics in regular and authorized manner based on the results of disk diffusion method. Also, to tetracycline and erythromycin should not be used in cases of Stereptococcal infections. It appears that researchers should develop new antibiotics instead of relying on traditional antibiotics for treating animals and even humans with infectious diseases.

Conflict of Interest

All authors declare that there is no conflict of interest.

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