RESEARCH LETTER

Distribution of virulence-associated genes in *Streptococcus uberis* isolated from bovine mastitis

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Abstract

Streptococcus uberis is an important pathogen that has been implicated in bovine mastitis but the virulence factors associated with pathogenesis are not well understood. The aim of this work was to examine 11 putative and known virulence-associated genes by PCR in 78 *S. uberis* strains isolated from infected animals in Argentina. Additionally, the distribution of virulence patterns over various herds was determined. Not all genes were present in the strains but all of the detected virulence-associated genes were present in combination. Forty-seven (60.3%) isolates carried seven to 10 virulence-associated genes. Further analysis revealed 58 virulence patterns. Different patterns were found within the same herd and among herds, demonstrating that strains with different virulence patterns were able to cause mastitis. Despite the large number of strains with different virulence patterns, strains with identical patterns was found. Detection of virulence-associated genes in individual *S. uberis* strains isolated from infected animals revealed one to 10 virulence genes. This may indicate that other virulence factors could be involved. The present study reveals the occurrence and distribution of 11 virulence-associated genes among *S. uberis* isolates from bovine mastitis in various herds and contributes to a better understanding of the pathogenicity of this bacterium.

Introduction

Mastitis is a worldwide disease of dairy cattle and is caused by a wide variety of organisms that affect milk quality and yield, resulting in major economic losses. These losses can be attributed to a reduction in milk production, the associated costs of treatment and the culling of persistently infected and repeatedly infected cows. Mastitis pathogens are commonly divided into those that show a contagious route of transmission and those that also frequently infect the udder from an environmental reservoir. Several streptococcal species are among the most frequently isolated as udder pathogens. *Streptococcus uberis* is an important pathogen implicated in bovine mastitis and is predominantly associated with subclinical and clinical intramammary infections in both lactating and nonlactating cows. This species is particularly problematic due to the fact that it is ubiquitous in the dairy environment (Bramley & Dodd, 1984). Although the prevalence of mastitis with contagious pathogens has been reduced by improved milking hygiene, this has had little effect on environmental species (Leigh *et al.*, 1999).

Despite the severe economic impact caused by the high prevalence of *S. uberis* in many well-managed dairy herds, virulence factors associated with pathogenesis are not well understood and constitute a major obstacle for the development of strategies to control this important mastitis pathogen (Oliver *et al.*, 1998). Several putative virulence-associated genes of *S. uberis* have been described. Among these, resistance to phagocytosis conferred by a hyaluronic acid capsule (Ward *et al.*, 2001), plasminogen activator proteins such as PauA (Rosey *et al.*, 1999), PauB (Ward & Leigh, 2002) and SK (Johnsen *et al.*, 1999), lactoferrin-binding proteins (Moshynskyy *et al.*, 2003), adherence to and invasion of epithelial cells mediated by SUAM (Almeida *et al.*, 2006), CAMP factor (Jiang *et al.*, 1996), a surface dehydrogenase protein GapC (Pancholi *et al.*, 1993) and Opp proteins involved in the active transport of solutes

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essential for growth in milk (Smith *et al.*, 2002) have been found.

As yet, nothing has been reported about the occurrence of virulence-associated genes among *S. uberis* isolates from cattle with mastitis in Argentina, and about the possible distribution of virulence patterns at various dairy herds. The aim here was to examine 11 putative and known virulence-associated genes by PCR in 78 *S. uberis* strains isolated from cattle with bovine mastitis in Argentina. In addition, the distribution of virulence patterns at various herds was determined. Although many studies relating the distribution of one or a few virulence-associated genes have been reported, to our knowledge this is the first study that investigates the presence of a greater number of virulence determinants.

Materials and methods

Bacterial isolates

Milk samples were obtained from 2359 milk-producing cows. Seventy-eight isolates were collected from udders of 78 cows with mastitis ($> 250\,000$ cells mL^{-1}) from 21 dairy herds (I–XXI) between 2005 and 2006. One to 17 isolates were isolated from each herd. The size of the herds included in the study varied from 79 to 204 cows. The isolates included in this study are representative of those that cause bovine mastitis in Argentina as they were obtained from the four major dairy provinces (Buenos Aires, Córdoba, Entre Ríos and Santa Fé) located in the east-central region of Argentina. The shortest distance between herds was 24 miles, and the greatest distance between herds was 203 miles.

The isolates were cultured on blood agar plates with 5% bovine blood and they were presumptively identified as representing *S. uberis* based on colonial appearance, Gram stain reaction and catalase test (National Mastitis Council, 2004) and by conventional identification (Odierno *et al.*, 2006). The selected colonies were maintained frozen at $-20\,^{\circ}C$ in Todd–Hewitt broth (Sigma-Aldrich Co.) containing 20% glycerol for further characterization. Isolates were identified as representing *S. uberis* by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene according to Jayarao *et al.* (1992). All the isolates were additionally confirmed by RFLP analysis of the 16S rRNA gene, using the restriction enzymes RsaI and AvaII (Khan *et al.*, 2003). *Streptococcus uberis* ATCC 27958 and *Streptococcus parauberis* ATCC 13386 were used as reference strains. The target genes, the oligonucleotide primers used and the sizes of the amplicons are summarized in Table 1. Synergistic CAMP-like haemolytic activities were determined together with a β-toxin-producing *Staphylococcus aureus* on sheep blood agar plates (Odierno *et al.*, 2006).

DNA extraction

Genomic DNA was isolated as described by Jayarao *et al.* (1992), purified by ethanol precipitation and dissolved in a buffer containing 10 mM Tris/HCl (pH 7.6) and 0.1 mM EDTA.

PCR amplification

Specific oligonucleotide primers for the detection of the *cfu*, *lbp* and *sua* genes of *S. uberis* were designed for this study with PRIMER3 software (http://frodo.wi.mit.edu/primer3/). DNA amplification for the *hasA*, *hasB*, *hasC*, *oppF*, *pauA*/*B* and *skc* genes was performed using oligonucleotide primers derived from published sequences. All the oligonucleotides were synthesized by Promega Corporation. The PCR was standardized for the detection of each virulence-associated gene following the methodologies described with suitable modifications to optimize the different conditions that affect the sensitivity and specificity of the reaction. Details of the primer sequences are shown in Table 1. To amplify the genes, 50 μL of reaction mixture was made containing 20 ng template DNA, 1 μM oligonucleotide primers, 0.4 μM of each of the four dNTPs, 1.50 U *Taq* polymerase and 1.5 mM $MgCl_2$. The annealing temperature was varied from 48 to 58 °C depending on the gene being amplified. The reactions were carried out in a thermal cycler and genes of each isolate were tested at least twice. A positive and a negative control were included in each run. PCR products were resolved on 1.2% agarose gel at 70 V for 1.5 h. Gels were stained with ethidium bromide solution (0.5 mg mL^{-1}) and photographed under UV light with MiniBisPRO gel documentation.

Results

RFLP analysis of the 16S rRNA gene successfully identified 78 isolates as *S. uberis* at the molecular level based on comparisons with reference strain *S. uberis* ATCC 27958 (Fig. 1).

A synergistic haemolytic CAMP-like reaction on sheep blood agar within the zone of staphylococcal β-toxin could be observed for 18 of the 78 (23%) *S. uberis* strains.

The standardized PCR allowed the amplification of putative and known virulence-associated genes of *S. uberis*, namely *cfu*, *gapC*, *hasA*, *hasB*, *hasC*, *lbp*, *oppF*, *pauA*/*B*, *skc*, *sua*, to their respective base pairs, each represented by a single band in the corresponding region of the DNA marker ladder.

The distribution of virulence-associated genes in 78 *S. uberis* strains was determined. PCR analysis detected the *hasC* gene in 70 (89.7%) of the strains, the most common gene in the examined isolates. The *sua* gene was found in 65 strains (83.3%), *gapC* in 62 (79.4%), *cfu* in 60 (76.9%), *hasA* in 58 (74.3%), *hasB* in 52 (66.6%), *skc* in 51 (65.3%), *oppF* in

Table 1. PCR primers, annealing temperature and expected PCR for *Streptococcus uberis* strains

Target gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	References
16S rRNA gene	CCAAGCTTGCTCAGGACGAACGCT CGGGATCCCGCCCGGGAACGTATTCAC	56	1430	Jayarao *et al*. (1992)
16S rRNA gene	GAGAGTTTGATCCTGGCTCAGGA CGGGTGTTACAAACTCTCGTGGT	55	1400	Khan *et al*. (2003)
cfu	TATCCCGATTTGCAGCCTAC CCTGGTCAACTTGTGCAACTG	59	205	Accession number U34322
hasA	GAAAGGTCTGATGCTGAT TCATCCCCTATGCTTACAG	58	600	Ward *et al*. (2001)
hasB	TCTAGACGCCGATCAAGC TGAATTCCYATGCGTCGATC	58	300	Ward *et al*. (2001)
hasC	TGCTTGGTGACGATTTGATG GTCCAATGATAGCAAGGTACAC	58	300	Field *et al*. (2003)
gapC	GCTCCTGGTGGAGATGATGT GTCACCAGTGTAAGCGTGGA	55	200	Accession number AF421900
lbp	CGACCCTTCAGATTGGATTC TAGCAGCATCACGTTCTTCG	53	698	Accession number AY376838
oppF	GGCCTAACCAAAACGAAACA GGCTCTGGAATTGCTGAAAG	54	419	Smith *et al*. (2002)
pauA/pauB	GAGATTCCTCTCTAGATATCA GGGCTGCAGATCCGTTAAAAAATGACATTAATAT	50	1200	Rosey *et al*. (1999), Ward & Leigh (2002)
skc	CTCCTCTCCAACAAAGAGG GAAGGCCTTCCCCTTTGAAA	52	800	Johnsen *et al*. (1999)
sua	ACGCAAGGTGCTCAAGAGTT TGAACAAGCGATTCGTCAAG	58	776	Accession number DQ232760

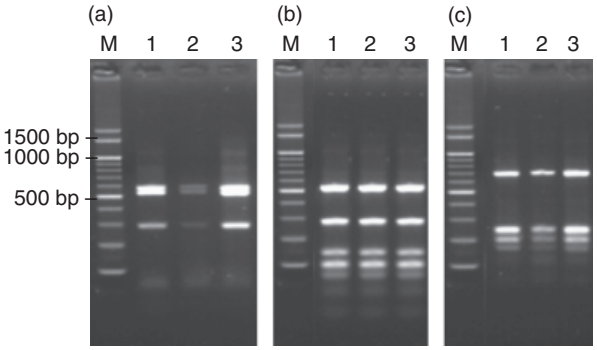

Fig. 1. Typical fragments of the PCR-amplified 16S rRNA gene of *Streptococcus uberis* after digestion with the restriction enzymes HhaI (a), MspI (b) and RsaI (c). M, a 100-bp ladder size marker.

50 (64.1%), *pauA* in 48 (61.5%) and *lbp* in nine (11.5%). Evidence of *pauB* was not found. The capsular genotype *hasABC* was found in 48 (61.5%) strains.

Results revealed that not all genes were present in the strains but all of the detected virulence-associated genes were present in combination. Of 78 strains examined, 47 (60.2%) isolates possessed seven to 10 virulence-associated virulence genes. Table 2 provides further details of the numbers involved.

Table 2. Detection of virulence-associated genes in individual *Streptococcus uberis* strains

No. of virulence-associated genes	1	3	4	5	6	7	8	9	10
No. of isolates	1	5	4	7	14	17	17	11	2

Further analysis showed that 58 different virulence patterns (initially named with a capital letter from A to Z, and then with two capital letters to BF) were found in all 78 *S. uberis* isolates, and 33 (42.3%) strains belonged to the 12 most frequent patterns. Data regarding these 12 most frequent virulence patterns are summarized in Table 3.

Ten virulence-associated genes were present in two (2.5%) strains and belonged to pattern D. The most frequent virulence pattern (E) was $cfu+gapC+hasAB+hasC+lbp-oppF+pauA/B+/-skc+sua+$ detected in seven (9%) strains. Eight virulence-associated genes were found in 14 (17.9%) strains and belonged to patterns B, G, L, N and AN. Seven genes were found in two (2.5%) strains and belonged to pattern Q, and six genes were found in eight (10.2%) strains belonging to patterns P, AH, AT and AX. The remaining 45 strains were grouped in different virulence patterns, where each pattern grouped only one strain.

Different virulence patterns were found within the same herd and among herds. However, strains with identical

Table 3. Most frequent virulence patterns of *Streptococcus uberis* strains

	Virulence-associated genes											
Virulence pattern	*cfu*	*hasA*	*hasB*	*hasC*	*gapC*	*lbp*	*oppF*	*pauA/B*	*skc*	*sua*	No. of strains	Herd(s)
B	1	1	1	1	1	0	0	1/0	1	1	3	IV
D	1	1	1	1	1	1	1	1/0	1	1	2	XV, XX
E	1	1	1	1	1	0	1	1/0	1	1	7	IV, VI, XII
G	1	1	1	1	1	0	0	1/0	1	1	3	VI
L	0	1	1	1	1	0	1	1/0	1	1	2	XII, XIX
N	0	1	1	1	1	0	1	1/0	1	1	2	IV, XI
P	0	1	1	1	0	0	1	1/0	1	0	2	IV, VIII
Q	1	1	1	1	1	0	1	0/0	0	1	2	V, VII
AH	1	0	0	0	1	1	1	1/0	1	0	2	II, III
AN	1	1	1	1	1	0	1	0/0	1	1	4	IV, XI, XVI
AT	1	0	1	1	0	1	1	0/0	1	0	2	IV
AX	1	1	1	0	1	0	0	0/0	1	1	2	VI

virulence patterns were found in only two herds, and these herds had a high prevalence of *S. uberis*: strains showing patterns B, E, N and AT were found in herd IV; strains showing patterns E, G and AX were found in herd VI. A great diversity of different virulence patterns was present in the remaining herds. On the other hand, strains with identical virulence patterns were found in different herds. For example, pattern E was present in herds IV, VI and XII, and pattern AN was present in herds IV, XI and XVI.

Discussion

Molecular identification of 78 *S. uberis* was performed by RFLP analysis of the 16S rRNA gene. 16S rDNA RFLP analysis has been suggested to be a useful tool for more precise identification of streptococci in bovine milk (Jayarao *et al.*, 1992; Reinoso *et al.*, 2010).

We found that all of the *S. uberis* strains examined harboured at least one virulence-associated gene. Pattern *cfu*+*hasAB*+*hasC*+*gapC*+*lbp* − *oppF*+*pauA/B*+/ − *skc*+*sua*+ was the most frequent. These genes encode virulence factors that promote invasion of host tissue, survival in the host environment, evasion of the host immune response and internalization in the mammary gland cells, suggesting that strains with this pattern may be more virulent and have greater probability of causing disease.

The gene *cfu*, coding for CAMP factor in *S. uberis*, a further putative virulence factor homologous to Fc binding, was found in 76.9% of the strains examined. However, a positive CAMP reaction was observed in 23% of *S. uberis* strains. Our results are in contrast to those of Khan *et al.* (2003), who reported a positive CAMP reaction and detection of the *cfu* gene for five of 128 (3.8%) *S. uberis* strains. Results suggest that the presence of this gene might not be related to expression of the CAMP factor. This would explain the difference observed here and by Khan *et al.* (2003). Ward *et al.* (2009) showed that a coding sequence for CAMP factor was not identified in *S. uberis* 0140J. However, our research analysed 78 strains.

Capsule production is dependent on the *has* operon, which consists of the *hasAB* gene cluster and *hasC* gene (Ward *et al.*, 2001). The *hasA* gene was found in 74.3% of the strains tested herein. According to Coffey *et al.* (2006), this gene is essential for capsule production. Our results show that *hasABC* was present in 61% of the strains; this result agrees with Field *et al.* (2003), who reported that *hasABC* genes occurred at a higher frequency in isolates associated with disease, suggesting that the capsule is required for some aspects of intramammary gland infection and pathogenesis. Here, the *hasC* gene was present in 89.7% of the strains. It is unclear why the *hasC* gene was found at such a high frequency. Recent studies carried out in *S. uberis* 0140J suggested that *hasC* apparently is unrelated to capsule biosynthesis (Ward *et al.*, 2009). Ward *et al.* (2001) and Field *et al.* (2003) have discussed capsule and phagocytosis, and Ward *et al.* (2009) reported that the hyaluronic acid capsule of *S. uberis* plays only a minor role in the early stages of infection of the lactating bovine mammary gland and resistance to phagocytosis was ascribed to an undefined component unconnected with the capsular phenotype.

Another gene included in this study was *oppF*, which has also been shown to play a significant role during growth of *S. uberis* in milk (Smith *et al.*, 2002). We found that the *oppF* gene was found in 64.1% of the strains. To our knowledge only one study, carried out with 50 *S. uberis* isolates, reported that *oppF* cannot be amplified from all strains (Zadoks *et al.*, 2005).

Different serine proteases that activate host plasminogen to plasmin, generating activity needed for the degradation of extracellular matrix proteins and subsequent colonization, have been described. In addition, the activation of endogenous plasminogen present in milk would lead to hydrolysis of

milk proteins and thereby release of peptides from which *S. uberis* could obtain essential amino acids (Kitt & Leigh, 1997). We found that the *skc* gene was harboured by 65.3% of the strains. To our knowledge, only one study has investigated the *skc* gene in *S. uberis* (Johnsen *et al.*, 1999); nine of 10 investigated strains contained *skc* genes with similar structures and properties. Evidence of *pauB* was not found in *S. uberis* herein. Only one report describes the presence of the *pauB* gene in one *S. uberis* strain isolated from a clinical case of bovine mastitis (Ward & Leigh, 2002). Our results showed that 61.5% of the strains harboured the *pauA* gene. In contrast, Ward & Leigh (2004) reported a very high prevalence of *pauA* alleles in field isolates collected from various European locations, which supported the observation that plasminogen activators are likely to confer an advantage with respect to colonization and growth. However, Ward *et al.* (2003) reported that expression of PauA is not essential for infection of the mammary gland, as indicated by the isolation of *pauA*-negative isolates from mastitic cows and by experimental studies. It is unclear why the *pauA* gene was found at low frequency in this work. According to the identification scheme used, 78 strains could be identified as representing *S. uberis*. Although Zadoks *et al.* (2005) reported that *pauA*-negative isolates may represent a novel subtaxon of *S. uberis* that is genetically closely related to *S. parauberis*, this could not be confirmed in our study.

Finally, *gapC*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was included because in several pathogenic bacteria GAPDH protein has been described as being associated with virulence (Ling *et al.*, 2004; Maeda *et al.*, 2004) due to its ability to bind several host proteins (Pancholi & Fischetti, 1992) or to confer resistance against reactive oxygen species produced by host phagocytic cells (Holzmuller *et al.*, 2006). A recent study in *Streptococcus agalactiae* describes GAPDH as a virulence-associated immunomodulatory protein (Madureira *et al.*, 2007). Furthermore, Perez-Casal *et al.* (2004) have suggested that a GapC product may be a good target for *S. uberis* vaccine development. In the present study, the *gapC* gene was found in 79.4% of the strains.

In conclusion, we found a large number of virulence patterns associated with intramammary infections. Different virulence patterns were found within the same herd and among herds, demonstrating that strains with different virulence patterns were able to cause mastitis. Despite the large number of strains with different virulence patterns, strains with identical patterns were found. Nevertheless, it is important to consider that *S. uberis* infections may be likely to be dependent on host factors.

Detection of virulence-associated genes in individual *S. uberis* strains isolated from mastitis showed strains which carried one to 10 virulence genes. This results show that any virulence-associated gene is present in all the strains and therefore reveals the absence of classical virulence factors such as those present in other species of *Streptococcus*. This may indicate that other virulence factors could be involved. Within this context, it will be of great interest to investigate new genes related to virulence in *S. uberis*.

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