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Occurrence of virulence-associated genes in *Streptococcus uberis* and *Streptococcus parauberis* isolated from bovine mastitis

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Abstract: *Streptococcus uberis* is one of the most important mastitis-causing pathogens. Although the pathogenesis and virulence factors required for the intramammary infection development are not yet well established, several putative virulence-associated genes have been described. This work aimed to investigate the presence of ten known and putative virulence-associated genes in *S. uberis* isolated from subclinical or clinical mastitis and its closely related species *Streptococcus parauberis* in 135 dairy farms in the Czech Republic. The PCR analysis detected that all the examined isolates possessed at least four virulence genes and most isolates carried eight out of ten virulence genes. All *S. uberis* isolates were positive for the *oppF*, *gapC* and *sua* genes. Among the most prevalent virulence-associated genes *skc* (98%) and *pauA* (97%) were also found. The *hasA* and *hasB* genes were always present together in 94% of the isolates. The genes *cfu* and *lbp* were detected in 6% and 2%, respectively. In the *S. uberis* isolates, 14 different virulence gene profiles were observed. The most frequent profile was *hasA*$^+$ *hasB*$^+$ *sua*$^+$ *skc*$^+$ *pauA*$^+$ *gapC*$^+$ *oppF* with variable *hasC*, observed in 86% of the tested isolates, occurring in 127 out of 135 farms. *S. parauberis* was identified very sporadically and, although it is closely related to *S. uberis*, only a rare occurrence of the examined virulence-associated genes was found.

Keywords: cows; intramammary infections; mammary gland; pathogens; virulence factors

Streptococcus uberis (*S. uberis*) is considered to be one of the major pathogens causing mastitis in many countries throughout the world, which brings about significant economic losses to farmers. While implementation of mastitis control strategies has been effective in decreasing the prevalence of contagious pathogens in well-managed dairy herds (Phuektes et al. 2001), *S. uberis* remains

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responsible for a significant proportion of clinical and mainly subclinical mastitis (Bradley et al. 2007; Vezina et al. 2021).

S. uberis is a ubiquitous bacterium in the cow's environment, in particular in places where cows gather and rest, and it can also be isolated from different parts of the cow's body. These environmental strains are typically associated with transient intramammary infections (IMI). However, some strains of *S. uberis* can be more adapted to the mammary gland and show varying degrees of contagiousness and can spread directly from cow to cow, probably during the milking process. These strains are able to persist in the mammary gland and cause recurrent infections, even during several lactations (Phuektes et al. 2001; Tassi et al. 2013). The understanding of the pathogenesis and virulence factors required for IMI is not well established. Among the most important virulence factors that causes persistence in the mammary gland is the ability to produce the surface adhesive molecule SUAM (*Streptococcus uberis* adhesion molecule). This protein binds the lactoferrin present in the milk and together with a receptor on the surface of bovine mammary epithelial cells creates a molecular bridge, which allows the bacteria to adhere and internalise into epithelial cells, where humoral hosts defences and antimicrobials in the milk are essentially ineffective (Almeida et al. 2011). Other important factors are the biofilm formation and production of hyaluronic acid capsules, which make the bacteria resistant to phagocytosis and reduce the effect of antimicrobials (Ward et al. 2001; Kromker et al. 2014). Several other putative virulence factors have been described in *S. uberis* – plasminogen activator proteins such as PauA (Rosey et al. 1999) and SK (Johnsen et al. 1999), lactoferrin binding proteins (Moshynskyy et al. 2003), the CAMP factor (Jiang et al. 1996), a surface dehydrogenase protein GapC (Pancholi and Fischetti 1993) and Opp proteins involved in the active transport of solutes essential for growth in milk (Smith et al. 2002). The factors of virulence are not known completely, and it is suggested that their expression varies from one strain to the other (Kromker et al. 2014).

Unlike *S. uberis, S. parauberis* is rarely detected in mastitis milk samples (Pitkala et al. 2008). However, differentiation of *S. uberis* from *S. parauberis* is difficult because the biochemical and serological characteristics of the two species are essentially indistinguishable (Jayarao et al. 1991). Identification of these closely related species could be performed by molecular methods (Jayarao et al. 1991), but they could be easily misdiagnosed (Pitkala et al. 2008) using routine conventional tests. The inability to distinguish *S. uberis* from *S. parauberis* could result in erroneous conclusions concerning the epidemiology of *S. uberis*, such as the identification of the reservoirs and mode of transmission (Jayarao et al. 1991). There is a paucity of information on the virulence factors in *S. parauberis* in the literature.

The objective of the current study was to investigate the genotypic variation and distribution of virulence-associated genes of *S. uberis* and *S. parauberis* isolated from mastitis milk samples in the Czech Republic.

MATERIAL AND METHODS

Sample collection

A total of 190 strains of *S. uberis* isolated from subclinical (according to the high number of somatic cells found in the production control programmes, with a cut-off of 400 000 cells per ml) and clinical cow mastitis were used in this study. The samples of mastitis milk were collected from 135 different farms in the Czech Republic from 2019–2021. After sampling, the milk was kept in containers at 6–8 °C and delivered to the laboratory within 2 hours. Only one isolate per farm was used in the study, but if different virulence factor gene profiles were detected on one farm, all of these isolates were included.

Bacterial identification

Ten microlitres of milk samples were plated onto Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood at 37 °C for 48 hours. The isolates were assessed based on the colony appearance, Gram stain reaction and catalase test, and subsequently identified by the phenotypic molecular method using a MALDI-TOF MS mass detector (Bruker Daltonics GmbH, Bremen, Germany).

Subsequently, the strain was confirmed by the detection of the *S. uberis*-specific 16S rRNA gene or *S. parauberis*-specific 16S rRNA gene by polymerase chain reaction (PCR) (Table 1).

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Table 1. Oligonucleotide primers used in this study for the *S. uberis* and *S. parauberis* virulence gene detection

Virulence factor	Genes	Nucleotide sequence (5'-3')	Amplicon size	References
Hyaluronic acid	*hasA*	F: GAAAGGTCTGATGCTGAT R: TCATCCCCTATGCTTACAG	600	Ward et al. (2001)
Hyaluronic acid	*hasB*	F: TCTAGACGCCGATCAAGC R: TGAATTCCYATGCGTCGATC	300	Ward et al. (2001)
Hyaluronic acid	*hasC*	F: TGCTTGGTGACGATTTGATG R: GTCCAATGATAGCAAGGTACAC	300	Field et al. (2003)
Epithelial cell invasion	*sua*	F: ACGCAAGGTGCTCAAGAGTT R: TGAACAAGCGATTCGTCAAG	776	Reinoso et al. (2011)
Surface dehydrogenase protein	*gapC*	F: GCTCCTGGTGGAGATGATGT R: GTCACCAGTGTAAGCGTGGA	200	Reinoso et al. (2011)
Lactoferrin binding protein	*lbp*	F: CGACCCTTCAGATTGGATTC R: TAGCAGCATCACGTTCTTCG	698	Reinoso et al. (2011)
Solvent active transfer	*oppF*	F: GGCCTAACCAAAACGAAACA R: GGCTCTGGAATTGCTGAAAG	419	Smith et al. (2002)
Plasminogen activator proteins	*pauA*	F: GAGATTCCTCTCTAGATATCA R: GGGCTGCAGATCCGTTAAAAAATGACATTAATAT	1 200	Rosey et al. (1999)
Plasminogen activator	*skc*	F: CTCCTCTCCAACAAAGAGG R: GAAGGCCTTCCCCTTTGAAA	800	Johnsen et al. (1999)
CAMP factor	*cfu*	F: TATCCCGATTTGCAGCCTAC R: CCTGGTCAACTTGTGCAACTG	205	Reinoso et al. (2011)
S. uberis specific	16S rRNA ub	F: CGCATGACAAT GGGTACA R: GCCTTTAACTTCAGACTTATCA	445	Hassan et al. (2001)
S. parauberis specific	16S rRNA paraub	F: CATGACAATTAAGTACTCATGTACTA R: CACCACCTGTCACCTCTGTC	884	Hassan et al. (2001)

PCR amplifications of virulence-associated genes and species-specific genes

The regions in the virulence-associated genes and *S. uberis*-specific gene were amplified using three multiplex PCRs: 1) *hasA, hasB, hasC, sua*; 2) *cfu, lbp, skc, and* the *S. uberis*-specific 16S rRNA gene; 3) *pauA, gapC,* and *oppF*. The multiplex PCRs were previously optimised for the detection of each set of genes. The *S. parauberis*-specific 16S rRNA gene was detected in the simplex PCR in the case of a negative reaction with the *S. uberis*-specific 16S rRNA gene.

A few colonies of a pure bacterial culture were resuspended in 50 µl of sterile distilled water. The suspension was incubated for 10 min at 100 °C and centrifuged for 10 min at 10 000 × *g*. The supernatant was used in the PCR reaction as the template DNA. The 20-µl reaction mixture contained 10 µl of a HotStarTaq Plus Master Mix 2 ×, 1 µl of primers (10 pmol/µl) *(*primer sequences and their product size are shown in Table 1), 2 µl of a CoralLoad Concentrate 10 × (Qiagen, Hilden, Germany), 4 µl of DNase-free water and 2 µl of DNA.

The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 90 s, extension at 72 °C for 90 s and final extension at 72 °C for 10 minutes. Ten microlitres of the PCR product was electrophoresed on a 2% agarose gel stained with ethidium bromide (Sigma Aldrich, St. Louis, MO, USA) and the PCR products were visualised under ultraviolet light.

https://doi.org/10.17221/95/2021-VETMED

RESULTS

A total of 190 isolates from 135 farms, where subclinical or clinical mastitis caused by *S. uberis* occurred, were included in our study and the presence of ten virulence-associated genes was determined. The PCR analysis revealed that all the examined isolates possessed at least four virulence genes, and most isolates carried eight out of ten virulence genes. All the *S. uberis* isolates were positive for the *oppF*, *gapC* and *sua* genes. *Skc* (98%) and *pauA* (97%) were also among the most prevalent virulence-associated genes. The *hasA* and *hasB* genes were always present together in 94% of the isolates. The *hasC* gene was very variable, 62% of the isolates possessed this gene and the isolates $hasC^+$ and $hasC^-$ were often detected in one farm at the same time. The *cfu* and *lbp* genes were detected in 6% and 2% of the isolates, respectively. The prevalence of the virulence factor genes is summarised in Table 2.

In our study, fourteen different virulence gene profiles were observed. The most frequent profile was $hasA^+$ $hasB^+$ $hasC^+$ sua^+ cfu^- lbp^- skc^+ $pauA^+$ $gapC^+$ $oppF^+$ (profile A) observed in 54% of the tested isolates in 103 farms. The profile $hasA^+$ $hasB^+$ $hasC^-$ sua^+ cfu^- lbp^- skc^+ $pauA^+$ $gapC^+$ $oppF^+$ (profile B) was observed in 32% of the isolates in 62 farms. The other profiles occurred in only 1–2% of the tested isolates. The profile occurrence is shown in Table 2.

Of the 135 farms, *S. parauberis* was identified in only five farms – three isolates were detected in the mastitis milk and two in the environment. All the isolates were $gapC^+$ and one isolate was skc^+, and no other gene was detected in the *S. parauberis* isolates (Table 3).

Table 2. Prevalence of the virulence factor genes and the gene profiles of the *S. uberis* isolates (n = 190)

Profile	No. isolates	%	*hasA*	*hasB*	*hasC*	*sua*	*cfu*	*lbp*	*skc*	*pauA*	*gapC*	*oppF*	No. genes
A	103	54	+	+	+	+	–	–	+	+	+	+	8
B	62	32	+	+	–	+	–	–	+	+	+	+	7
C	4	2	–	–	–	+	–	–	+	+	+	+	5
D	4	2	+	+	+	+	+	–	+	+	+	+	9
E	3	2	+	+	+	+	–	–	+	–	+	+	7
F	3	2	+	+	–	+	+	–	+	+	+	+	8
G	2	1	–	–	+	+	–	–	+	+	+	+	6
H	2	1	–	–	–	+	+	–	–	–	+	+	4
I	2	1	+	+	+	+	–	+	+	+	+	+	9
J	1	1	+	+	–	+	–	+	+	+	+	+	8
K	1	1	–	–	–	+	+	–	+	+	+	+	6
L	1	1	–	–	+	+	+	–	–	–	+	+	5
M	1	1	–	–	+	+	+	–	+	+	+	+	7
N	1	1	+	+	+	+	–	–	–	+	+	+	7
Gene prevalence (%)			94	94	62	100	6	2	98	97	100	100	

(+) = gene detected; (–) = gene not detected

Table 3. Prevalence of the virulence factor genes and the gene profiles in the *S. parauberis* isolates (n = 5)

Profile	No. isolates	%	*hasA*	*hasB*	*hasC*	*sua*	*cfu*	*lbp*	*skc*	*pauA*	*gapC*	*oppF*	No. genes
AA	4	80	–	–	–	–	–	–	–	–	+	–	1
BB	1	20	–	–	–	–	–	–	+	–	+	–	2
Gene prevalence (%)			0	0	0	0	0	0	20	0	100	0	

(+) = gene detected; (–) = gene not detected

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DISCUSSION

S. uberis is a particularly problematic mammary pathogen due to its ubiquitous occurrence and the ability of some strains to persist in the mammary gland and resist mammary gland defence mechanisms and antimicrobials. *S. uberis* is a genetically highly variable bacterium [635 ST types are described in the multi-locus sequence typing (MLST) database] (Davies et al. 2016). Even within one farm, a wide range of genetic variants of *S. uberis* isolates can be detected, some of which are able to infect the mammary gland and cause transient infections, some even persist in the mammary gland over several lactations and cause chronic recurrent infections, while other strains are unable to overcome the defence mechanisms of the teat canal and the mammary gland. It has also been shown that some strains are more resistant to antibiotic treatment than other strains, although they are equally sensitive to the antimicrobial in the laboratory. This is probably due to the ability to form a biofilm and the ability to adhere to and penetrate epithelial cells of the mammary gland, where it is more difficult to achieve an effective concentration of the antimicrobial. Many factors are known or suspected to be involved in the pathogenesis of a mammary gland infection, and it is obvious that different *S. uberis* strains differ in possession of the virulence-associated genes.

In the present study, 190 *S. uberis* isolates (from 135 farms) originating from clinical and mainly subclinical mastitis cases, were investigated for the presence of genes encoding virulence factors and the gene profiles were determined.

The most prevalent virulence-associated genes were *sua, gapC* and *oppF*. The *sua* gene, encoding the SUAM surface protein which, after lactoferrin binding, is responsible for adherence to and internalisation into mammary epithelial cells, was detected in all the *S. uberis* strains isolated from the clinical and subclinical mastitis. The high prevalence of *sua* gene in mastitis isolates has also been shown in other studies (about 98%) (Perrig et al. 2015; Kaczorek et al. 2017) confirming the importance of these pathogenic strategies in *S. uberis* mastitis and the SUAM is also considered a good vaccine candidate against mastitis (Almeida et al. 2015). None of the *S. parauberis* isolates harboured the *sua* gene, and this may also be the reason why *S. parauberis* is not as successful and is a less prevalent cause of IMI compared to *S. uberis*.

Another gene that was present in 100% of our *S. uberis* isolates and also in all the *S. parauberis* isolates was the housekeeping gene *gapC*. It encodes the GAPDH protein (glyceraldehyde-3-phosphate dehydrogenase), which is described as possibly being associated with the virulence due to its ability to bind some host proteins or to confer resistance against reactive oxygen species produced by the host phagocytic cells (Pancholi and Fischetti 1992; Reinoso et al. 2011). In other studies, a high prevalence of *gapC* gene has also been found (90–100% of isolates) (Zadoks et al. 2005; Kaczorek et al. 2017; Boonyayatra et al. 2018). GAPDH is also considered a good immunomodulatory protein and has been a focus of vaccine studies (Fontaine et al. 2002).

Oligopeptide permease, encoded by the *oppF* gene, is necessary for the utilisation of amino acids from specific peptides, allowing bacterial growth in milk (Smith et al. 2002). We detected the *oppF* gene in all 190 isolates, but some other studies reported that the *oppF* gene may not be amplified from all the strains (Zadoks et al. 2005; Boonyayatra et al. 2018), and in the study of Reinoso et al. (2011) in Argentina, the *oppF* gene was detected in only 64% of the *S. uberis* isolates.

Different frequencies of the *lbp* gene detected in *S. uberis* have been reported in many countries, ranging from 0% in Poland, 2% in our study in the Czech Republic, to 11.5% in Argentina and even 78.4% in Thailand (Reinoso et al. 2011; Kaczorek et al. 2017; Boonyayatra et al. 2018). A similar situation exists with the *cfu* gene encoding the CAMP factor that forms pores in the host-cell membrane and thus causes its lysis (Jiang et al. 1996). Prevalence ranges from 4% in Germany, 6% in our study, 19% in Poland to 35% in Thailand or 77% in Argentina (Khan et al. 2003; Reinoso et al. 2011; Kaczorek et al. 2017; Boonyayatra et al. 2018). Possession of these two putative virulence-associated genes does not appear to be necessary for a successful infection, due to its low detection frequency in mastitis strains.

One of the known bacterial defence mechanisms is capsule production which is dependent on the *has* operon. In *S. uberis*, two discrete loci comprising homologues of either *hasAB* or *hasC* were identified and all three genes are essential for the capsule formation (Ward et al. 2001). Our results were in accordance with this statement, because the *hasA* and *hasB* genes were always present together,

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specifically in 94% of the isolates. The occurrence of *hasC* (in 62% of isolates) was independent of the occurrence of *hasAB*. The co-occurrence of *hasA* and *hasB* in *S. uberis* has been observed in other studies [Boonyayatra et al. (2018), in 55.7% of the isolates], whereas some other studies described their independent occurrence (Reinoso et al. 2011; Kaczorek et al. 2017). In our experiment, a higher prevalence of *hasAB* and a lower prevalence of *hasC* were detected compared to other studies, in which *hasA* ranging from 17% to 74%, *hasB* from 56% to 79% vs. 94% in our study, and *hasC* ranging from 83% to 92% vs. 62% in our study were found. The co-occurrence of all three genes in the *has* operon, which is probably necessary for the capsule formation, was shown by 60% of the isolates, and similar results were obtained in the study of Reinoso et al. (2011). Although *hasABC* genes occurred at a higher frequency in isolates associated with a disease, suggesting that the capsule is required for some aspects of IMI and the pathogenesis, 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 mammary gland and resistance to the phagocytosis was ascribed to an undefined component unconnected with the capsular phenotype. In addition, Field et al. (2003) also stated that non-capsulated strains of *S. uberis* are still able to resist the phagocytosis by neutrophils and cause mastitis in dairy cows.

Both *pauA* and *skc* are genes encoding the plasminogen activator proteins which we detected in 98% of the *S. uberis* isolates. It is suggested that the activation of plasminogen to plasmin could result in the hydrolysis of milk proteins and, thus, increase the availability of peptides and amino acids for rapid bacterial growth in the lactating mammary gland during the very early stages of infection. Moreover, the proteolytic activity of plasmin facilitates the bacterial penetration and dissemination into the tissues (Leigh 1993). The high prevalence of *pauA* and *skc* genes in mastitis isolates (our study; Kaczorek et al. 2017) indicates high importance for IMI and is reasonably considered a good candidate for vaccine development (McVey et al. 2005).

In the present study, in the *S. uberis* strains isolated from the clinical and subclinical mastitis, fourteen different virulence gene profiles were observed. The PCR analysis showed that all the examined isolates possess at least four virulence genes, with most isolates carrying eight out of ten virulence genes. The vast majority of the isolates showed the profile $hasA^{+}$ $hasB^{+}$ sua^{+} cfu^{-} lbp^{-} skc^{+} $pauA^{+}$ $gapC^{+}$ $oppF^{+}$ with the variable *hasC* ($hasC^{+}$ or $hacC^{-}$), observed in 86% of the tested isolates, occurring in 127 out of 135 farms. Profiles A and B (only different in *hasC*) were often detected in one farm at the same time (in 39 farms). The combination of these factors in profile A and B appears to be beneficial to the strain and increases the likelihood of causing mastitis.

During the almost three-year period of the sample collection in 135 farms, *S. parauberis* was identified in only five farms and, in addition, two isolates originated from the environment, not from the mastitis milk samples. Other studies also showed a low prevalence, Pitkala et al. (2008) reported only two isolates in 111 different farms in Finland, Bentley et al. (1993) reported only three *S. parauberis* isolates of the 206 gram-positive cocci isolated from mastitic milk, Khan et al. (2003) identified only one of 131 *Streptococcus* isolates as *S. parauberis* in Germany. These studies also pointed to the difficulty of distinguishing *S. parauberis* from *S. uberis* by conventional testing, which can cause complications in evaluating the epidemiological data and antibiotic resistance reports, but the occurrence of *S. parauberis* is so low that it is not of critical importance in mastitis diagnostics. In our study, we solved this diagnostic problem by detecting species-specific genes using the PCR technique (Table 1).

In *S. parauberis*, we detected very few virulence-associated genes, the gene *gapC* was amplified in all five isolates and the gene *skc* was amplified in one isolate. Despite the very small number of *S. parauberis* isolates, comparison with *S. uberis* shows that these virulence genes are only minimally present in this species, which is in accordance with the fact that *S. parauberis* rarely causes mammary gland inflammation. However, the results indicate that *S. parauberis* is also genetically variable and different isolates carry different virulence-associated genes, and it is also likely that only some isolates are able to penetrate the mammary gland and cause mastitis.

S. uberis is a genetically highly variable bacterium with different levels of virulence. In 135 dairy farms, fourteen virulence gene profiles associated with IMI were detected, and the profiles varied between farms, but also within the same herd. One of the profiles significantly predominated, suggest-

https://doi.org/10.17221/95/2021-VETMED

ing that the profile *hasA*$^+$ *hasB*$^+$ *sua*$^+$ *cfu*$^-$ *lbp*$^-$ *skc*$^+$ *pauA*$^+$ *gapC*$^+$ *oppF*$^+$ with the variable *hasC* appears to be beneficial to the strain and increases the probability of causing mastitis.

Several virulence-associated genes are considered as good targets for vaccine development due to frequent occurrence and their highly conserved nature. However, the involvement of all important virulence factors in the pathogenesis of *S. uberis* mastitis has not been fully elucidated upon, which is a major obstacle for the development of strategies to control this important mastitis pathogen. Further research is needed to discover other important virulence related factors and elucidate upon all the important steps in the pathogenesis of *S. uberis* intramammary infection.

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Conflict of interest

The authors declare no conflict of interest.

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