Immune Responses to a Streptococcus GapC Chimera and its Potential Use as a Vaccine Candidate for Bovine Mastitis

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ABSTRACT

Although bovine mastitis caused by Streptococcus strains is the most economically important disease affecting the dairy industry worldwide, the development of an effective vaccine has been hampered for many years. In this study, five *S. agalactiae*, three *S. dysgalactiae*, and three *S. uberis* were identified out of thirteen Streptococcus strains isolated during a study of clinical bovine mastitis from seven farms in China. The *gapC* genes, encoding the cell surface-associated GapC proteins of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* were cloned and sequenced. To further identify potential vaccine candidates against Streptococcus-induced bovine mastitis, mice were vaccinated with GapC of *S. agalactiae*, *S. dysgalactiae* and *S. uberis*, resulting in a significant humoral immune for three weeks post-challenge. This observation together with previous studies conducted in our laboratory on GAPDH activity of *Staphylococcus aureus* possibly makes this protein an important target for vaccine development against bovine mastitis.

Keywords: Mastitis; Streptococcus; GapC; Vaccine

INTRODUCTION

Bovine mastitis, an inflammation of the mammary gland, is the single most important factor contributing to economic losses to the dairy industry (1). Several *Streptococcal* species are capable of causing infections that result in mastitis, including *S. agalactiae*, *S. dysgalactiae, S. uberis*. and *S. agalactiae* which are well known worldwide as major contagious pathogens causing bovine sub-clinical mastitis, which may have a substantial impact to the quantity and quality of milk. Pathogens can survive for long periods only within the mammary gland (2). *S. dysgalactiae* and *S. uberis* are more prevalent, infecting mammary glands as favorable conditions arise (3). *S. dysgalactiae* is isolated frequently from intra-mammary infections during lactation and during the non-lactating period. In spite of its high prevalence, little is known about factors that contribute to the virulence of *S. dysgalactiae* (3). *S. uberis* is particularly problematic due to the fact that this so-called 'environmental *Streptococcus*' is ubiquitous in the dairy environment and is predominantly associated with sub-clinical mastitis, resulting in reduced, and poor quality milk yields (4, 5).

Currently, prophylactic practices including antibiotic therapy and teat disinfection are relied upon to minimize the spread of infection. However, these measures are often inadequate, simply because animals are constantly being re-exposed to infection from their surrounding environment. Vaccination is a common and easy strategy for the control of infectious diseases, but none of these vaccines are guaranteed to efficiently control the most common mastitis-causing pathogens. Vaccination is currently used as one such measure with most vaccines composed of killed bacterial cells or bacterial products that elicit protection against a variety of strains. Immunization with autogenous whole cell bacterins have not always resulted in protection against new infec-

tions and specific antigen fractions do not elicit heterologous protection (6, 7). These findings suggest that isolates from different sources encode distinct products and that a vaccine composed of all the different antigens would be impractical to produce. Recently, a new approach has been undertaken consisting of vaccination of a protein which resulted in partial protection against a *Streptococcal* experimental challenge in mice and dairy cows (7, 8). Therefore, as a first step in the development of improved vaccines capable of global protection against *Streptococcal* infections, the identification of conserved antigens produced by all strains must be carried out.

The factors involved in virulence of mastitis isolates of *Streptococcus* are not well understood and they include cell-surface proteins (9, 10), adhesin-like molecules (11, 12), and the Crp family regulatory protein factor (CAMP) (13). Several potential virulence determinants have been described that might contribute to mastitis caused by *Streptococcus*. Resistance to phagocytosis is conferred by a hyaluronic acid situated in the capsule (14), and the organism has also been shown to adhere to and actively invade bovine epithelial cells via a receptor-mediated endocytosis mechanism, where it is able to persist without harming host cells (15). It is reported that the M-like protein Mig of *S. dysgalactiae* confers protection against phagocytosis by bovine polymorphonuclear leukocytes (PMN) (16) and that it is capable of binding to IgA through a region located in the α2-M-binding domain of the protein (8). The virulence factors encoded by *S. uberis* include a trypsin-resistant protein called the R-antigen, similar to the situation in *S. dysgalactiae*, *S. uberis* which possess hyaluronic acid in the capsule which protects the bacteria from phagocytosis (17). Hyaluronidase, neuraminidase, and a lactoferrin-binding protein are also encoded by *S. uberis* (18).

The plasma receptor protein (Plr) (19), surface dehydrogenase protein (SDH) (20), and phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been implicated as a potential *S. pyogenes* virulence factor, which have been attributed to potentially affect host cell gene transcription and thus enhancing disease pathogenicity (21). In addition to the properties mentioned above, it has been suggested that GAPDH proteins might be used as antigens in vaccines to protect against parasitic and microbial infections.

We have recently isolated the genes encoding the GapC products of *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*. These GapC proteins possessing GAPDH activity are located on the surface of the bacteria and bind plasmin. Previous work in our laboratory revealed that GapC of *S. dysgalactiae*, a cell surface-associated *Plr* homologue, conferred significant protection against *S. dysgalactiae* infection in dry cows when used as a vaccine (22). The GapC products of *S. agalactiae* and *S. uberis* show significant homology to *S. dysgalactiae*, making it a good candidate for vaccine development.

In this study, we constructed a chimeric GapC protein to create a cross-reactive vaccine antigen that could be used for protection against *S. uberis*, *S. agalactiae*, and *S. dysgalactiae*. This chimeric protein was expressed on the cell surface of *Escherichia coli* for production of large amounts of antigens resulting in considerable savings in production costs and the bacteria derived from these *E. coli* cells could serve as the basis for the preparation of an effective vaccine against bovine *Streptococcus* mastitis.

MATERIALS AND METHODS

Bacterial strains and culture media

During 2005-2008, 100 milk samples from 12 dairy farms were collected from clinical bovine mastitis cases in the provinces of Hebei, Hubei, and Heilongjiang, Tianjin Municipality, and Inner Mongolia autonomous region of China based on one isolate per herd. A total of 13 isolates were identified as *Streptocossus* by conventional microbiological methods including gram stain, colony morphology, and coagulase test with rabbit plasma, as well as species-specific and ubiquitous DNA-based assays reported by Todhunter *et al.* (3). The confirmed *Streptocossus* isolates were stored at -20°C in our laboratory. The biological property of thirteen isolates was listed in Table 1. *E. coli XLI* strain was routinely cultured at 37°C in Luria-Bertani medium (LB; Difco, BD, San Jose USA), Ampicillin (100 μg/mL) or Carbenicillin (50 μg/mL) was added when needed (7). PCR primers were described in Table 2, and the general PCR method was carried out to detected *gapC* gene (23).

Construction of the *gapC* chimeric gene

Genomic DNA from *S. agalactiae*, *S. dysgalactiae*, and *S.uberis* strain were purified with a Nucleospin Tissue Kit (Clontech, Palo Alto, CA,USA) with the addition of 5 U/mL lysozyme (Sigma, St. Louis MO, USA) and used as a PCR template (23). PCR reactions were carried out using specific primers (synthesized by Sangon Biotech, Shanghai, China) (Table 2).

The PCR reaction contained 10 × *Taq* DNA polymerase buffer with $(NH_4)_2SO_4$ 2.5 μL, 2.5 mM dNTP 2 μL, 25 μM

Table 1: Biochemical identifying features of *Streptococcus* Species

Bacteria	Hippurate	Esculin	Mannitol	Sorbierite	Lactose	Synanthrin	Raffinose	Trehalose	Salicin	6.5%NaCl
7-2	+	-	-	-	+	-	-	+	+	-
CH	+	-	-	-	+	-	-	+	+	-
10-2-1	+	-	-	-	+	-	-	+	+	-
SD0309	+	-	-	-	+	-	-	+	+	-
SD0303	+	+	+	+	+	+	-	+	+	-
LS0312	-	-	-	-	+	-	-	+	+	-
LS0351	-	-	-	+	+	-	-	+	-	-
LS0311	+	-	-	-	+	-	-	+	+	-
LS0303	+	-	-	-	+	-	-	-	-	-
LS0313	-	-	-	+	+	-	-	+	+	-
LS0341	+	-	-	-	+	+	+	-	-	-
SD0306	+	+	+	+	+	+	-	+	-	-
LS0302	-	+	-	-	+	-	+	-	-	-

Table 2: Primers used in this study

Bacteria	Sequence [a]	PCR product sizes
S. agalactiae	F: 5'-GCTAGGCTCCATTGAATC-3' R: 5'-TTAACCTAGTTTCTTTAAAACTAGAA-3'	162 bp
S. dysgalactiae	F: 5'-GAACACGTTAGGGTCGTC-3' R: 5'-AGTATATCTTAACTAGAAAAACTATTG-3'	264 bp
S. uberis	F: 5'-TAAGGAACACGTTGGTTAAG-3' R: 5'-TCCAGTCCTTAGACCTTCT-3'	451 bp
S. agalactiae	P1: 5′- CGCGGATCCATGGTAGTTAAAGTTGGTATT -3′ P2: 5′- GACGTCGACAGCGATTTTTGCAAAGTACTC -3′	1005 bP
S. dysgalactiae	P3: 5′- CGCGGATCCATGGTAGTTAAAGTTGGTATT -3′ P4: 5′- GACGTCGACAGCGATTTTTGCAAAATACTC -3′	1005 bP
S. uberis	P1: 5′- CGCGGATCCATGGTAGTTAAAGTTGGTATT -3′ P2: 5′- GACGTCGACAGCGATTTTTGCAAAGTACTC -3′	1005 bP

[a] The restriction enzyme sites (Bam *HI* and Sal *I*) used for the construction of the chimera are indicated using underline.

gapC forward primer 1 µL, 25 µM *gapC* reverse primer 1 µL, 25 mM $MgCl_2$ 2 µL, Genomic DNA 1 µL, 5 U/µL *Taq* DNA polymerase 0.20 µL, and ddH_2O 15 µL with 20 µL of the total volume. The PCR conditions were as follows: initial denaturation step at 95°C for 4 min; followed by 40 cycles of 95°C for 1 min; 50°C for 1 min; 72°C, for min each; and completed by incubation at 4°C for 1 h. All amplified PCR products were detected on 1% agarose gels electrophoresis and stained with ethidium bromide. PCR products were reclaimed and purified using Biospin GeL Extraction Kit (Bioer, Hangzhou, China) according to the manufacturer's instruction.

The gene encoding the gapC chimera was constructed as follows: the PCR fragment was digested with *Bam HI* and *Sal I*. The resultant 1005 bp fragment was cloned into pQE-30 (Qiagen, Hilden, German), an *E. coli* expression vector that adds a 6-histidine tag at the NH_2 terminus of the GapC protein for purification by affinity chromatography. The resultant plasmids were named pQE-30-TR/GapC (*S. agalactiae gapC* gene), pQE-30-WR/GapC (*S. dysgalactiae gapC* gene), and pQE-30-RF/GapC (*S.uberis gapC* gene). The *gapC* chimeric gene was sequenced and analyzed (23).

Expression and purification of the GapC

The expression and purification of CapC was carried out according to the method of Brassard *et al.* (23). The *E. coli XLI* (Takara, Dalian, China) strain was used as a host for the cloning and expression study. Prior to transformation, a $CaCl_2$

method (24) was carried out to prepare *E. coli XLI* competent cell. The resulting recombinant plasmids (pQE-30-TR/GapC, pQE-30-WR/GapC, and pQE-30-RF/GapC) were transformed into *Escherichia coli XLI*. A positive clone was selected, inoculated into Luria Broth media containing ampicillin BD, (San Jose USA), and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma, CA, USA) at 37°C for 1-4 h. The cells were centrifuged and the pellet was resuspended in 5 mL 50 mM sodium phosphate pH 8.0 containing 300 mM NaCl, 10 mM imidazole and 5 mg lysozyme, and then the whole mixture were incubated on ice for 30 min. The bacterial cells were disrupted by sonication on ice. The lysate was centrifuged at 10,000 g for 30 min and the supernatant was saved for the purification of the six-histidine (His_6) tag fusion protein using Ni-NTA His·bind^R Resin purification system (Novagen, CA, USA) according to the products guideline. Non-recombinant *E. coli XLI* cells and non-induced recombinant clone were used as negative controls.

SDS-PAGE and western blot analysis

SDS-PAGE and western blot analysis was carried out according to the previous study (24). Bacterial lysates were subjected to 12% gel of the SDS-PAGE protocol and transferred to nitrocellulose membrane Hybond^{TM}-C (Amersham, Uppsala Sweden) as described previously (25). The primary antibody was mouse anti-*Streptococcus* polyclonal antibody (1:1500 dilution, prepared in our laboratory) or mouse anti-His_6 monoclonal antibody (1:2000 dilution, Invitrogen, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse antibodies were used as the second antibody. The protein bands were visualized by 3, 3'-diaminobenzidine (DAB, Zhongshan, Beijing, China) as recommended by the manufacturer.

Determination of GAPDH activity

The purified protein (His_6GAPDH) was assayed for GAPDH activity according to the protocol of Brassard (23) with some modifications. Purified protein (50 µL) was added to 100 µL of 10 mM DL-glyceraldehyde-3-phosphate (DL-GAP), 100 µL 10 mM NAD^+ and 750 µL of assay buffer (40 mM triethanolamine, 50 mM Na_2HPO4, 0.2 mM EDTA, 20 mM 2-mercaptoethanol and 0.1% (v/v) Tween-20, pH 8.6). Negative control assays were performed as above without the addition of DL-GAP. The reduction of NAD^+ to NADH was monitored spectrophotometrically at optical density (OD) 340 at 20 s intervals for 4 min using NovaspecII (Pharmacia, CA, USA). Activity calculation was based on a molar absorption coefficient of 6.22×10^{-3} mol^{-1} cm^{-1} for NADH at 340 nm. Protein concentration was determined using a colorimetric Bradford assay (Bio-Rad, CA, USA).

Mice immunizations and immune assays

BALB/c mice (SPF, 4-6 weeks, 18-22 g, healthy) were provided by Experimental Animal Center, Harbin Veterinary Research Institute, The Chinese Academy of Agricultural Sciences, China. Animal experiments were approved by Ethics Committee of Experimental Animal Center, Heilongjiang Bayi Agricultural University.

The immune responses to the GapC proteins were determined in Balb/c mice immunized with the purified GapC proteins. Proteins (10 mg dose) were emulsified with Freund Adjuvant Complete F5881 (Sigma, CA, USA) at ratio of 1:1, and administered via the intramuscular route. The trial consisted of a total of seventy mice divided into seven groups of ten each. Three groups were immunized with pQE-30-TR/GapC (Group 2), pQE-30-WR/GapC (Group 3) and pQE-30-RF/GapC (Group 4), respectively. A placebo group (Group 1) immunized with PBS was included. The other groups consisted of immunized with killed *S. agalactiae* (Group 5), *S. dysgalactiae* (Group 6) and *S. uberis* (Group 7). In all cases, first vaccinations were carried out, and then boosted after three weeks. Serum samples were collected at days 0, 7, 14, 21, 28, 35, 42, 56 and tested by ELISA for the presence of total IgG antibodies against the GapC proteins using alkaline-phosphatase-conjugated polyclonal antibody against mouse IgG. GapC proteins in serum were detected by Western blot assays described above. A micro-agglutination test utilizing *S. agalactiae*, *S. dysgalactiae*, *S. uberis*is as antigen for detecting and measuring serum agglutinins against bacteria were performed.

Lethal challenge

Two weeks after boosting, control and GapC-immunized mice were intraperitoneally challenged using 100 µL lethal dose of *S. agalactiae* LS0310, *S. dysgalactiae* LS0312 and *S. uberis* SD0306, respectively. The animals were monitored three times a day. Once the animals displayed serious clinical signs (moribund), they were euthanized and counted as dead. The animals that survived after challenge were sacrificed

after a week post challenge. Surviving number of animals was recorded for up to 7 successive days.

RESULTS

Construction of the chimeric *gap* gene

Based on the genome sequences of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* (Accession Nos. AF421899, AF375662 and AF421900), PCR primers (Table 2) were designed to amplify the *gapC* gene of a 1005 bp fragment (Fig. 1A) and these fragments were cloned into the expression plasmid pQE-30 to generate plasmid pQE-30-TR/GapC, pQE-30-WR/GapC, and pQE-30-RF/GapC. As expected, the obtained products could be cut with both Bam *HI* and Sac *I*, and the fragments of the expected size (1005 bp) were detected (Fig. 1B). Sequencing of the insert in pQE-30-TR/GapC, pQE-30-WR/GapC, and pQE-30-RF/GapC revealed the presence of a 1005 bp open-reading frame (ORF) encoding a putative protein of 335 amino acids with a predicted molecular weight of 38 KDa. A BLAST search of the protein indicated that *S. agalactiae* (pQE-30-TR/GapC) shared 97% homology with the GapC proteins of the reference strain, with changes being Ala 104→Gly, Ala 105 deletion, Ala 112→Glu, Val 187→Ile, Ala 203→Gly, Ala 177→Pro, Ala 322→Ser in the protein. *S. dysgalactiae* (pQE-30-WR/GapC) and *S. uberis* (pQE-30-RF/GapC) shared 100% homology with the GapC proteins of the reference strains, although 2 and 39 changes of nucleic acids, respectively, were present.

SDS-PAGE and Western Blot

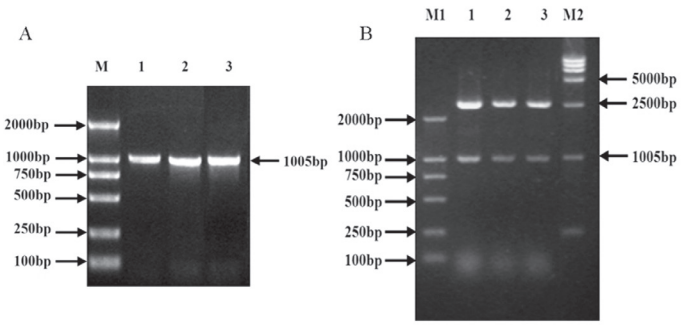

Figure 1: Amplification and cloning of *gapC* gene. (A) The *gapC* gene was amplified by PCR (1005 bp). Lane M: DNA Marker DL2K plus (Transgen, Beijing, China); lanes 1-3: pQE-30-RF/GapC, pQE-30-WR/GapC, and pQE-30-TR/GapC. (B) Recombinant plasmid was identified with *Sal* I and *Hind* III (2500 bp and 1005 bp) digestion. Lane M: DNA Marker DL2K plus; lane 1-3: pQE-30-RF/GapC, pQE-30-WR/GapC, and pQE-30-TR/GapC.

All the recombinant GapC proteins were expressed as 6-histadine-tagged fusion proteins in *E. coli XLI* host cells. The *gapC* chimeric gene was cloned in front of a histidine tag for purification. Analysis of the purified, recombinant 6-histadine GapC proteins by SDS-PAGE indicated molecular weight of 38 kDa (Fig. 2A), which closely matches the predicted molecular weight based on predicted fusion protein sequences. The resultant protein reacted with a monoclonal antibody against the histidine tag, a polyclonal antibody against GapC (Fig. 2B). This protein was purified and used in vaccination trials as described below.

GAPDH activity

The mastitis-causing *S. agalactiae*, *S. dysgalactiae* and *S. uberis* encodes GapC, a surface-exposed protein with GAPDH activity (6). To compare the enzymatic activities of the various GAPDH isoforms in the purified GapC protein and bacteria, we examined dynamic catalysis during a 1 minute time frame. Our data revealed no activity was detected for GapC in the control trial. GapC encoded by pQE-30-TR/GapC (0.358), pQE-30-WR/GapC (0.320), and pQE-30-RF/GapC (0.324) were significantly higher ($P<0.05$) than the *S. agalactiae* (0.120), *S. dysgalactiae* (0.107), and *S.uberis* (0.116) (Figure 3).

Humoral immune responses to the GapC

In order to verify whether the recombinant GapC protein could simultaneously induce strong humoral responses against bacteria, mice were immunized with the recombinant proteins and their immune responses to the three proteins were determined with ELISA by measuring total IgG in serum at one week intervals; the results were presented in

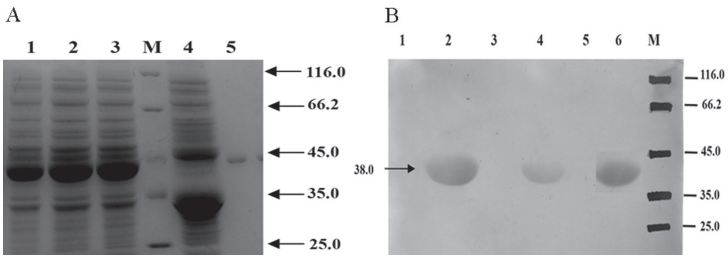

Figure 2: SDS-PAGE and Western blotting analysis of the recombinant protein. (A) Lane 1: pQE-30-RF/GapC; Lane 2: pQE-30-WR/GapC; Lane 3: pQE-30-TR/GapC, Lane 4: Control, and Lane 5: purified GapC protein. (B) Lane 1, 3 and 5: Control, Lane 2: pQE-30-RF/GapC; Lane4: pQE-30-WR/GapC; Lane6: pQE-30-TR/GapC.

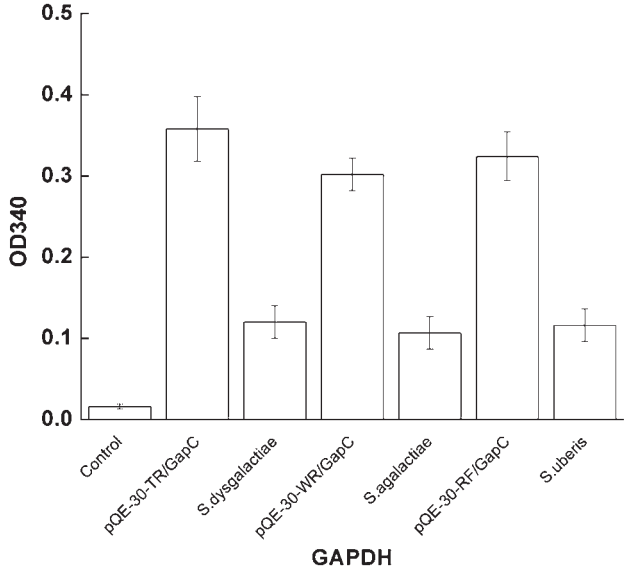

Figure 3: GAPDH activity analysis of the recombinant pQE-30-TR/GapC, pQE-30-WR/GapC, and pQE-30-RF/GapC protein.

Table 3. Compared to the control group, the GapC-specific total IgG serum titers increased significantly in mice immunized with GapC (P<0.001). Preliminary vaccination induced significant increase of antibody, and after boosting for two weeks, the antibody titer reached the highest level of 1:128000, 1:64000, and 1:128000, respectively. Thereafter it fell back slowly until day 56.

A weak but significant (p<0.01) cross-immune response to GapC was observed in mice for *S. agalactiae* , *S. dysgalactiae*, and *S. uberis* (data not shown). These results indicated that the recombinant protein simultaneously induced high levels of predefined cross-reacting GapC antibodies. All immune sera produced with the GapC-immunized rabbits reacted with the three above mentioned strains. The agglutination

Table 3: Antibody IgG titres of recombinant GapC protein in immunized serums

Days	pQE-30-TR/GapC	pQE-30-WR/GapC	pQE-30-RF/GapC
0	0	0	0
7	1:2000	1:2000	1:1000
14	1:8000	1:4000	1:4000
21	1:16000	1:8000	1:8000
28	1:32000	1:16000	1:16000
35	1:64000	1:32000	1:64000
42	1:128000	1:64000	1:128000
49	1:128000	1:64000	1:64000
56	1:64000	1:32000	1:64000

Table 4: Agglutination potency in immunized serums

Groups	Agglutination antigen	Value
pQE-30-TR/GapC	LS-10-1	2^{11}
	LS-12-2	$>2^{12}$
	SD-6	$>2^{12}$
pQE-30-WR/GapC	LS-10-1	$>2^{12}$
	LS-12-2	$>2^{12}$
	SD-6	2^{11}
pQE-30-RF/GapC	LS-10-1	$>2^{12}$
	LS-12-2	2^{12}
	SD-6	$>2^{12}$

potency was found to be greater than 2^{11} (Table 4), and cross agglutination was observed, which implied there probably existed common agglutination antigens.

Immunoprotection

In general it was evaluated that the GapC product may be successfully used in a vaccine trial. The goal of this research was to investigate the immune responses to GapC proteins of *Streptococcus* to the response obtained with the *gapC* chimera. The protective efficacy of the GapC was evaluated in terms of survival numbers. As shown in Table 5, none of challenged control animals survived in each experiment after a week post-challenge. In comparison to the control, more than 3 animals immunized with GapC survived after challenge, indicating the immune protective potential of GapC.

DISCUSSION

Table 5: Protection from *S. agalactiae*, *S. dysgalactiae*, *S. uberis* challenge

Group	Strains	Survival number	Immunoprotection
Control	LS-10-1	0/10	0%
	SD-6	0/10	0%
	LS-12-2	0/10	0%
pQE-30-TR/GapC	LS-10-1	7/10	70%
	SD-6	7/10	70%
	LS-12-2	8/10	80%
pQE-30-WR/GapC	LS-10-1	8/10	80%
	SD-6	9/10	90%
	LS-12-2	10/10	100%
pQE-30-RF/GapC	LS-10-1	6/10	60%
	SD-6	9/10	90%
	LS-12-2	7/10	70%

Mastitis caused by *Streptococcus* remains one of the more costly diseases for dairy farmers. Although antibiotic therapy and culling of infected animals are the most effective approaches to control of the disease, the success rate of treatment against *Streptococcus* infections varies considerably. When the treatment costs and losses due to reduced milk production, discarding of the milk and culling of the animals are calculated, it is clear that new approaches such as vaccination are needed to control the disease. In the last few years, it has been proposed that immunization with recombinant proteins might be a viable alternative to the more traditional approach of using whole cell lysates as the antigenic component of a vaccine (6, 25). With a view to identifying cross-protective protein antigens for use as potential vaccines against mastitis-causing *Streptococci*, we have found that the pathogenic *Streptococci S. dysgalactiae*, *S. uberis* and *S. agalactiae* all possess cell wall-associated GapC. Therefore, the objective of the present study was to compare the quantity and quality of the immune response to *Streptococci* antigens encoded by *gapC* gene.

The outer bacterial surface proteins play an important role in transport of nutrients, cellular metabolism as well as virulence-related functions such as evasion of host defenses, adhesion and invasion (26). The families of proteins with GAPDH activity are conserved at the DNA and protein sequence level and have been shown to be potential components of vaccines against a variety of bacterial, mycotic, and parasitic infections (6, 7, 27). We initiated this work by constructing plasmids encoding the GapC of *S. dysgalactiae*, *S. uberis* and *S. agalactiae*, respectively. Three protein chimeras were constructed by PCR amplification of the *gapC* genes of mastitis-isolates of *S. agalactiae*, *S. dysgalactiae*, *S. uberis*is followed by tandem ligation of the PCR products. Then we characterized *gapC*, the gene encoding for the 38 kDa protein GAPDH. The 6-histidine tag GapC was purified and used to determine whether its previously observed protective capacity against *S. dysgalactiae*, *S. uberis* and *S. agalactiae* infection, would also result in cross-species protection due to the more than 98% homology observed between the three proteins. The nucleotide sequence for *gapC* was determined and an open reading frame of 1005 bp was identified. The nucleotide sequence homologies showed that the GapC shares a degree of identity with GAPDH of other *Streptococci*. The amino acid sequence seemed to be conserved among these *Streptococci*. The GapC exhibited a significant similarity to the GAPDH of *S. agalactiae* (97%), *S. dysgalactiae* (100%) and *S. uberis* (100%), respectively. The protein was expressed in *E. coli* and it reacted to specific GapC antisera. In addition, relatively high levels of GAPDH activity in comparison with the non-recombinant protein have been found in the soluble protein fraction after sonication of cells (23). Similar values have been obtained when using gentler cell disruption methods (i.e. homogenization with a Sorvall blender or a Potter-Elvehjem device), whereas no measurable activity was found associated with the non-recombinant protein obtained after centrifugation, which supported the evidence that all the GAPDH was located in the purified proteins, as was later confirmed by immunological studies.

Previous work has shown that vaccination of dairy cows against mastitis pathogens can result in a rapid and intense inflammatory and immune response (6). In addition, a previous study showed that vaccination of mice and rats with a 22 aa B cell epitopic region of *S. mansoni* GAPDH resulted in partial protection against challenge with *S. mansoni* (28), suggesting that BALB/c mice are a good model for investigating the vaccine and the significance of the humoral antibodies in the protection against infection. Most animal vaccines employ killed bacterial cells, or crude mixtures of proteins to elicit protection, however, in this study we have used a defined subunit vaccine to elicit greater protection than has been observed in previous trials (29-31).

We firstly detected expression of recombinant GapC in transformed cells (Fig. 2), and we observed significant humoral and very low cell-mediated immune responses. We further test if an additional boost with the recombinant proteins would enhance the quality and quantity of the immune responses in mice. Two weeks after boosting, GapC-immunized mice were challenged and more than 3 animals immunized with GapC survived indicating the immunoprotective potential of GapC. Vaccination with 6-histidine tag GapC resulted in cross protection against mastitis. This may be because the 6-histidine tag GapC is as protective as that of *S. uberis*, because of its sharing the 98% similarity.

In conclusion, we showed that the *Streptococcus* GapC protein is important for establishment of humoral immunity. This observation together with previous studies conducted in our laboratory on GAPDH proteins of *Staphylococcus aureus* makes this protein an important target for vaccine development against bovine mastitis.

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REFERENCES

1. Kossaibati, M.A. and Esslemont, R.J.: The costs of production diseases in dairy herds in England. Vet. J. 154: 41-51, 1997.
2. Erskine, R.J., Bartlett, P.C., Johnson, G.L. and Halbert, L.W.: Intramuscular administration of ceftiofur sodium versus intramammary infusion of penicillin novobiocin for treatment of Streptococcus agalactiae mastitis in dairy cows. J. Am. Vet. Med. Assoc. 208: 258-260, 1996.
3. Todhunter, D.A., Smith, K.L. and Hogan, J.S.: Environmental streptococcal intramammary infections of the bovine mammary gland. J. Dairy Sci. 78: 2366-2374, 1995.
4. Bramley, A.J.: Sources of *Streptococcus-Uberis* in the Dairy-Herd .1. Isolation from Bovine feces and from straw bedding of cattle. J. Dairy Res. 49: 369-373, 1982.
5. Kruze, J. and Bramley, A.J.: Sources of *Streptococcus uberis* in the dairy-herd .2. Evidence of Colonization of the Bovine Intestine by *Str-Uberis.* J. Dairy Res. 49: 375-379, 1982.
6. Fontaine, M.C., Perez-Casal, J., Song, X.M., Shelford, J., Willson, P.J. and Potter, A.A.: Immunisation of dairy cattle with recombinant S*treptococcus uberis* GapC or a chimeric CAMP antigen confers protection against heterologous bacterial challenge. Vaccine. 20: 2278-2286, 2002.
7. Bolton, A., Song, X.M., Willson, P., Fontaine, M.C., Potter, A.A. and Perez-Casal, J.: Use of the surface proteins GapC and Mig of S*treptococcus dysgalactiae* as potential protective antigens against bovine mastitis. Can. J. Microbiol. 50: 423-432, 2004.
8. Song, X.M., Perez-Casal, J., Fontaine, M.C. and Potter, A.A.: Bovine immunoglobulin A (IgA)-binding activities of the surface-expressed Mig protein of *Streptococcus dysgalactiae*. Microbiol-Sgm. 148: 2055-2064, 2002.
9. Stalhammarcarlemalm, M., Stenberg, L. and Lindahl, G.: Protein Rib - a novel Group-B streptococcal cell-surface protein that confers protective immunity and is expressed by most strains causing invasive infections. J. Exp. Med. 177: 1593-1603, 1993.
10. Wibawan, I.W.T. and Lammler, C.: Properties of Group-B Streptococci with protein surface antigen-X and antigen-R. J. Clin. Microbiol. 28: 2834-2836, 1990.
11. Wibawan, I.W.T., Lammler, C., Seleim, R.S. and Pasaribu, F.H.: A hemagglutinating adhesin of Group-B streptococci isolated from cases of bovine mastitis mediates adherence to Hela-Cells. J. Gen. Microbiol. 139: 2173-2178, 1993.
12. Spellerberg, B., Rozdzinski, E., Martin, S., Weber-Heynemann, J., Schnitzler, N., Lutticken, R. and Podbielski, A.: Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. Infect. Immun. 67: 871-878, 1999.
13. Ruhlmann, J., Wittmannliebold, B., Jurgens, D. and Fehrenbach, F.J.: Complete amino-acid sequence of Protein-B. Febs Lett. 235: 262-266, 1988.
14. Almeida, R.A., and Oliver, S.P.: Antiphagocytic Effect of the Capsule of Streptococcus-Uberis. J. Vet. Med B. 40: 707-714, 1993.
15. Oliver, S.P., Almeida, R.A. and Calvinho, L.F.: Virulence factors of Streptococcus uberis isolated from cows with mastitis. J. Vet. Med. B. 45: 461-471, 1998.
16. Song, X.M., Perez-Casal, J., Bolton, A.., and Potter, A.A.: Surface-expressed mig protein protects *Streptococcus dysgalactiae* against phagocytosis by bovine neutrophils. Infect. Immun. 69: 6030-6037, 2001.
17. Leigh, J.A., Field, T.R. and Williams, M.R.: Two Strains of *Streptococcus uberis*, of differing ability to cause clinical mastitis, differ in their ability to resist some host defense factors. Res Vet. Sci. 49: 85-87, 1990.
18. Schaufuss, P., Sting, R., Schaeg, W. and Blobel, H.: Isolation and characterization of hyaluronidase from *Streptococcus uberis*. Zbl Bakt-Int. J. Med. M. 271: 46-53, 1989.
19. Lottenberg, R., Broder, C.C., Boyle, M.D., Kain, S.J., Schroeder, B.L. and Curtiss, R.: Cloning, sequence analysis, and expression in *E.coli* of a streptococcal plasma receptor. J. Bacteriol. 174: 5204-5210, 1992.
20. Pancholi, V., and Fischetti, V.A.: A major surface protein on Group-A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding-activity. J. Exp. Med. 176: 415-426, 1992.
21. Pancholi, V. and Fischetti, V.A.: Glyceraldehyde-3-phosphate dehydrogenase on the surface of group-a streptococci is also an adp-ribosylating enzyme. P. Natl. Acad. Sci. USA. 90: 8154-8158, 1993.
22. Zhu, H.W., Zhu, Z.B., Cui, Y.D., Zhang, J., Liu, L.F. and Piao, F.Z.: GAPDH activity and immunogenicity of *Staphylococcus aureus* recombinant GapC protein. Chin. J. Biotech. 24: 754-759, 2008.
23. Brassard, J., Gottschalk, M. and Quessy, S.: Cloning and purification of the *Streptococcus suis* serotype 2 glyceraldehyde-3-phosphate dehydrogenase and its involvement as an adhesin. Vet. Microbiol. 102: 87-94, 2004.
24. Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular cloning: a laboratory manual, 2nd edition. Cold spring harbor laboratory press, Cold spring harbor, NY. 2001.
25. Ma, J.Z., Cui, Y.D., Zhang, J., Zhu, Z.B. and Piao, F.Z.: Cloning and expression of *Staphylococcus aureus* surface protein Isdb and its immune experiment in mice. Chin. J. Biotech. 27: 566-571, 2011.
26. Kerro-Dego, O., Prysliak, T., Perez-Casal, J. and Potter, A.A.: Role of GapC in the pathogenesis of *Staphylococcus aureus.* Vet. Microbiol. 156: 443-447, 2012.
27. Rosinha, G.M.S., Myioshi, A., Azevedo, V., Splitter, G.A. and Oliveira, S.C.: Molecular and immunological characterisation of recombinant *Brucella abortus* glyceraldehyde-3-phosphate-dehydrogenase, a T- and B-cell reactive protein that induces partial protection when co-administered with an interleukin-12-expressing plasmid in a DNA vaccine formulation. J. Med. Microbiol. 51: 661-671, 2002.

28. Argiro, L.L., Kohlstadt, S.S., Henri, S.S., Dessein, H.H., Matabiau, V.V., Paris, P.P., Bourgois, A.A. and Dessein, A.J.: Identification of a candidate vaccine peptide on the 37 kDa *Schistosoma mansoni* GAPDH. 18: 2039-48, 2000.
29. Finch, J.M., Hill, A.W., Field, T.R. and Leigh, J.A.: Local Vaccination with Killed *Streptococcus-Uberis* protects the bovine mammary-gland against experimental intramammary challenge with the homologous strain. Infect. Immun. 62: 3599-3603, 1994.
30. Finch, J.M., Winter, A., Walton, A.W. and Leigh, J.A.: Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*. Vaccine. 15: 1138-1143, 1997.
31. Leigh, J.A., Finch, J.M., Field, T.R., Real, N.C., Winter, A., Walton, A.W. and Hodgkinson, S.M.: Vaccination with the plasminogen activator from *Streptococcus uberis* induces an inhibitory response and protects against experimental infection in the dairy cow. Vaccine. 17: 851-857, 1999.