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Streptococcus iniae CAMP Factor Lost its Co-hemolysis to Fish Red Blood Cells

Mengwei Xiao[1], Kaiyu Wang[1,2], Defang Chen[3], Jun Wang[1], Lanmin Li[1], Xingxing Liu[1], Yi Geng[1,2], Xiaoli Huang[3] and Dan Xiao[4],

[1]Department of Basic Veterinary, Veterinary Medicine College, Sichuan Agricultural University, Ya'an 625014, Sichuan, P.R. China.
[2]Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an 625014, Sichuan, P.R. China.
[3]Department of Aquaculture, College of Animal Sicence& Technology, Sichuan Agricultural University, Ya'an, Sichuan 625014, P.R. China.
[4]Animal Health Research Institute of Tongwei Co., Ltd., Chengdu, Sichuan 610041, PR China.

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Specific primers were used to amplify the gene *cfi* encoding CAMP factor of *Streptococcus iniae*(*S. iniae*) DGX07. CAMP factor ofnatural form of *S. iniae*could be detected by Western-blot with anti-recombinant CAMP factor antiserum. Western-blot suggested that the amount of CAMP factor expressed in*S. iniae*were very low and that might be not virulent enough to trigger immune response in rabbits. Western-blot also demonstrated its non-specific binding ability torabbit immunoglobulin G.CAMP factor showed binding and invasion ability to*Epitheliomapapulosumcyprini* cellsin vitro.However, hemolysis showed the recombinant CAMP factor could exhibit the classic co-hemolytic activity on some mammals'red blood cells but failed to cytolyse fish red blood cells, suggesting that CAMP factor lost its co-hemolysis to fish in *S.iniae*.

Key words: *cfi* gene; molecular analysis; co-hemolysis; cell adherence and invasion; IgG binding.

Streptococcus iniae (*S.iniae*) is a Gram-positive β-hemolytic streptococcusand cannot be classified by the classification scheme of Lancefield[1, 2]. Since firstly isolated from Amazon freshwater dolphin (*Iniageoffrensis*) byPeir and Madin in 1976[3], *S.iniae* have come into sight and have been labeled as a major fish pathogen and opportunistic invasive pathogen to human in the past decades[4-6]. Aquacultures in Israel, Japan, America and China have suffered serious economic losses because of this streptococci[7].

* To whom all correspondence should be addressed. Tel:+86-0835-2885753; Fax: +86-0835-2885302; E-mail:kywangsicau@126.com

Currently, researches on the pathogenesis of *S. iniae* infections are still ongoing.Cytolysis has been employed by numerous bacterial pathogens to degrade, invade host cells, and to resist the host immune attack. *S. iniae* is one of the many bacterial species, including streptococci, which are well-characterized of their hemolysins or cytolysins[8-11].A co-hemolytic phenomenon was first discovered by Christie, Atkins, Munch-Peterson in 1944 in the strain *Streptococcus agalactiae* (*S. agalactiae*)[12]. The term 'CAMP' was named as a combination of the initials of the last name of the original discoverers[12]. In the originalstudy, the classical CAMP reaction was elicited under the combined effects of co-hemolysin CAMP factor, secreted as a pore-forming toxin by *S. agalactiae*, and staphylococcal beta-

hemolysinsphingomyelinase (SMase), derived from neighboring bacterial partners such as *Staphylococcus aureus* (*S. aureus*)[12].CAMP reaction was employed as a diagnostic measure for clinical identification of *S. agalactiae*[13, 14]. The *cfb* gene was ubiquitous in GBS strains so that CAMP test or search of the *cfb* gene by Polymerase Chain Reaction (PCR) was usually used to differentiate GBS from other Streptococcus species. However, previous works on other bacteria have proven that *Streptococcus uberis*, *Streptococcus difficile*, *Streptococcus pyogenes*, *S. iniae*, *Actinobacillus*pleuropneumoniae, *Propionibacterium acnes* also expressed the CAMP factor homologues[15-18]. These data are increasingly questioning the specificity of this *cfb* gene on the clinical GBS identification.

CAMP factor causes cell to lyse when erythrocytes have been first sensitized by pre-incubation with sphingomyelinase (SMase), a protein secreted by *S. aureus*or other bacterial species[19]. SMases sensitize erythrocyte membrane sphingomyelin by hydrolyzing it to ceramide. CAMP factor would not "collaborate" with SMases unless target erythrocytes contain at least 45 mol% of sphingomyelin[20]. The latest study indicated that the GBS CAMP factor's binding to the sheep red blood cells involved the interaction between CAMP factor and GPI (Glycosyl phosphatidy linositol)-anchored proteins on the cell membrane[21].

Recent observation showed that *S. iniae* caused significant weight loss and bacteremia in a murine model of subcutaneous infection.It was also demonstrated that *S. iniae*exhibited resistance to phagocytic clearance in human whole blood, and were cytotoxic to human endothelial cells in vitro[22].*S. iniae*SiM[23, 24] and α-enolase (data unpublished) were reported being mobilized to adhere and invade fish epithelial cells. Whether CAMP factor could also facilitate to the adherence and invasion of fish epithelial cells is still unclear. Beside thecytolytic activity,CAMP factor were reported to bind immunoglobulins G and M[25]. A recent study on the GBS CAMP factor'snon-specificbinding to human IgG, however, indicated otherwise[26].

At the outset of the present study, we investigated the possibility of a similar hemolytic effect of *S. iniae*CAMP to fish RBC, and examined its role in binding unspecific IgG and abilities in adherence and invasion to *Epithelioma papulosum cyprini* (EPC) cells. Our results supported that *S. iniae* CAMP factor showed direct evidence in fish epithelial cells binding and invasion, and IgG Fc region binding,whereas its role in hemolytic ability was likely a minor one.

MATERIALS AND METHODS

Plasmid, bacterial strains, kits and chemicals

Escherichia coli (*E. coli*) DH5α and BL21 (DE3) as competent cells (TIANGEN BIOTECH CO., LTD.), cloning vector pMD19-T and expression vector pET-32a (+) (TAKARA BIOTECHNOLOGY CO., LTD.) used in this study were employed for gene clone and expression.*S. iniae* strain DGX07 preserved in the authors' laboratory was isolated from diseased channel catfish from an outbreak in Guangxi province, China in 2010[27]. *S. aureus* was purchased from American type culture collection(ATCC2691[2]3).

Sequening of *cfi* gene

Total genomic DNA of *S. iniae*and *S. aureus*were extracted using Tiangen genomic DNA extraction kit according to the manufacturer's instructions (TIANGEN BIOTECH CO., LTD.). The open reading frame of the *cfi* gene was amplified by following primers: forward (SICAMP1), 5'-ATGAACTCTCAACACATTTTACGTC-3'; reverse (SICAMP2), 5'-TTAGTTAAGAG CAGCTGTTAAGGCA-3'. DNA-Sequencing was performed by (Sangon Biotech (Shanghai) Co., Ltd.). BLAST N software and DNAStar (version 7.0) were applied to the alignment and base composition analysis of *cfi* gene[28, 29].

Expression and purification of CAMP factor and *S. aureus* SMase

Signal peptide-free *cfi* gene was amplified using following primers: forward (SICAMP3), 5'-GGATCCCCTCAACATCAAACA-3'; reverse (SICAMP4), 5'-AAGCTTTTAGTTAAG AGCAGCTGTTAA -3' (*BamH*I and *Hind*III restriction sites are underlined, respectively). PCR was conducted with a 2×MasterMix (TIANGEN BIOTECH CO., LTD.) of 12.5µl in a 25µl reaction mixture containing 1µl of each primer (20 pmol each), 1.0µl DNA template (5ng), and 10.5µl water. The PCR conditions were: 94 °C for 4 min; 30 cycles of 94 °C for 0.5 min, 53 °C for 0.5 min, 72 °C for 1 min;

and then a final extension at 72 °C for 10 min. The product was cloned into pMD19-T vectorand the pET-32a (+) vector. pMD19-T-*cfi* was preserved in *E. coli* DH5α and vector pET-32a (+)-*cfi* was expressed in *E. coli*BL21 (DE3).The recombinant CAMP factor was expressed as His-tagged fusion protein and purified by Ni-nitrilotriacetic acid resin affinity chromatography (Profinity™ IMAC Ni-Changed Resin, BIO-RAD).Purified CAMP factor expressed under optimized conditions were confirmed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For *S. aureus*SMase, the PCR primers were designed based on the SMase gene sequence from Genebank (Accession number: CAA43885). The sequences of the primers are: forward primer; 5'-CGGATCCTCTAAGAAAGATGATACTGAT-3', reverse primer; 5'-CCAAGCTTCTATTTACT ATAGGCTTTGAT-3' (*BamH*I and *Hind*III restriction sites are underlined, respectively).PCR was conducted with a 2×MasterMix of 12.5µl in a 25µl reaction mixture containing 1µl of each primer (20 pmol each), 1.0µl DNA template (5ng), and 10.5µl water. The PCR conditions were: 94 °C for 4 min; 30 cycles of 94 °C for 0.5 min, 55 °C for 1.0 min, 72 °C for 1 min; and then a final extension at 72 °C for 10 min.Expression and purification procedure of SMase were performed as described above.

Blood agar plate hemolysis analysis

Hemolysis of the recombinant protein on the blood agar plates (BAP) were performed following the procedure of agar diffusion assay with slight modification[30]. 5% rabbit blood LB plates were prepared and samples were placed in wells punched by a perforex. Recombinant SMase(0.2mg/ml), recombinant CAMP factor(0.1mg/ml), CAMP factor with1:100 dilutedanti-CAMP factor antiserum (see M&M 2.5), and PBSwere added as demonstrated in the Results. In an additional hemolysis analysis, CAMP factors were replaced by a serious of *E. coli* BL21 (DE3) cells. *E. coli* BL21 without pET-32a(+) plasmids, BL21 with pET-32a(+) plasmids, BL21-pET-32a(+)-*cfi*with same volumes of 0.8mM IPTG, SMase were added into the wells as described in Results. Plates were incubated at 28°C overnight.

Liquid hemolysis assay was employed to investigate species specificity[30]. Defibrinated red blood cells (RBCs) of sheep, fresh RBCs of swine, rabbit and tilapia (*Oreochromis mossambicus*) and channel catfish (*Ictalurus punctatus*)were sedimented (8,000 g in a microcentrifuge for 5 min) and washed by centrifugation in hemolytic buffer (10mmol/lTris, 0.9% NaCl, 10mM $MgCl_2$, 1%Bovine Serum Albumin (BSA)) until the supernatant is clear. 50µl 100% suspension of erythrocytes suspension was added into the 1.5 ml mirco-tube and then mixed with 100µl purified SMase. Mixture was pre-incubated for 30min at 37 °C before 100µl of serially diluted purified CAMP factor. Hemolytic buffer was supplementarily added to 1ml and incubated for another 30min at 37 °C. The mixture were then centrifuged at 8,000 g for 5min and 800µl supernatant fluid was carefully removed and placed in 800µl hemolytic buffer, unless otherwise stated. The optical density (OD) value of the sample obtained above were spectrophotometrically determined at 540µm. Total hemolysis (positive control) values were obtained by adding 50µl three times washed erythrocytes suspension into 950µl 5mM phosphate buffer with 10mM $MgCl_2$ and 1%BSA. Negative controls were performed without CAMP factor and all the procedures remained the same. The indicator of hemolysis was established as described in liquid hemolysis assay[30].

The influence of incubation time of RBC with CAMP factor was also investigated. RBC were incubated with 100µg of CAMP factor but different incubation time. The rest procedures were performed as described as above.

Generation of antiserum against CAMP factor and *S. iniae*

All the animals in the study were carefully treated. Two healthy New Zealand male rabbits (1 month old) were injected with 2ml of equal volume mixture of CAMP factor (0.5mg/ml) and Freund complete adjuvant (FCA, Sigma) at the first time. Another two rabbits were injected with the 0.8% formaldehyde-inactivated *S. iniae* (1.0×10^7 CFU/ml). Negative sera were obtained right before the first injection. Continuous injections with 2ml of equal volume mixture of purified proteins and Freund incomplete adjuvant (FCA, Sigma) were carried out at weekly intervals for another 4 weeks, followed by two extra injections of 2 ml of CAMP factor (0.5mg/ml) or inactivated *S. iniae* (1.0×10^7 CFU/ml) alone. The rabbits were bled by cardiac puncture, under anesthesia. Titers of the sera of the recombinant protein at each injection interval

were immediately tested by gel-double-immunodiffusion assay. The antiserums were purified by gradient precipitation of ammonia sulfate.

Antiserums were tested for specificity by Western-blot. CAMP factor were subjected to SDS-PAGE and then electroblotted onto PVDF membranes according to standard procedures. PVDF membranes were subsequently washed three times containing TBST (100 mMTris, 150 mMNaCl, 0.1% Tween 20, pH 7.5), and then blocked with TBST with 3% bovine serum albumin at 37°C for 1 h. After washed with TBST three times, the membrane was incubated with 1:100 diluted anti-CAMP factor antiserum in the same buffer at 4°C overnight. After three times washed with TBST, the membranes were then incubated with HRP goat anti-rabbit IgG antibody (WuXiAppTec) 37°C for 1 h. DAB (TIANGEN BIOTECH CO., LTD.) was employed to visualize the specific antigen-bound antibody according to the manufacturer's construction.

Immunoblot analysis for CAMP factor expression under natural growing environment

In order to test whether *S. iniae* could express CAMP factor under natural growing environment, *S. iniae* CGX07 were cultured in Brain Hearth Infusion (BHI) and harvested at OD value 0.6. Pellets were ultrasonic treated on ice for 3×10 min (SCIENTZ, Ningbo Scientz Biotechnology CO., LTD.) and subjected to SDS-PAGE and then electroblotted onto PVDF membranes as described above. The membranes were incubated with 1:100 diluted anti-CAMP factor antiserum (titer of 1:32) in the same buffer at 4°C overnight.

In order to test whether the CAMP factor could trigger the host immune response under natural growing environment, CAMP factor were subjected to SDS-PAGE and then electroblotted onto PVDF membranes as described above. The membranes was then incubated with 1:100 diluted anti-inactivated *S. iniae* CGX07 antiserum in the same buffer at 4°C overnight.

Immunoblot analysis for CAMP factor unspecific binding to immunoglobulins

Recombinant GBS α-enolase (and *S. iniae* α-enolase) were electroblotted onto PVDF membranes as described above. PVDF membranes were washed three times and then blocked with TBST with BSA. After washed with TBST, the membrane was incubated with 1:100 diluted anti-α-enolase antiserum pre-incubated with different amount of CAMP factor (50 or 100μg) for an hour at 37°C. The following procedures were as described above. The anti-α-enolase antiserum used in controls were pre-incubated with same volume of PBS.

Antiserum inhibits adherence and invasion of EPC cells by *S. iniae*

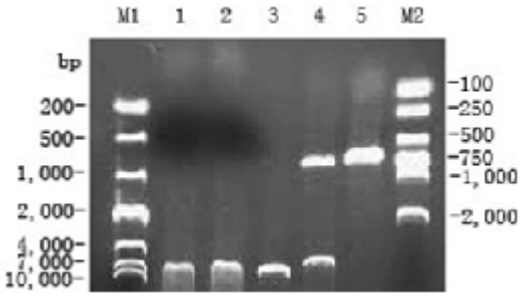
EPC cells were cultured in M199 medium supplemented with 10% fetal calf serum (FCS) (Life Technologies) containing 5 μg/ml penicillin and 100μg/ml streptomycin[31]. *S. iniae* (10^7 CFU) were incubated with equal concentrations of either 1: 100 diluted normal rabbit IgG or purifed rabbit anti-CAMP factor IgG for 1 h at 37°C in M199 medium without any antibiotics. Monolayers were infected with *S. iniae* treated with or without anti-CAMP factor IgG. For adherence assay, the number of CFU associated with the monolayer after incubation for 2 h at 37°C in a CO_2 incubator was determined by viable counts to evaluate adherence[32]. For invasion assay, After 2 h incubation procedure described above, infected monolayers were washed three times with phosphate-buffered saline (PBS), followed by incubation with M199 medium supplemented with 10% fetal calf serum (FCS) containing 5 μg/ml penicillin and 100μg/ml streptomycin for an additional 1 h to kill uninvaded (unadhered) bacteria. Levels of internalization of bacteria to ECP cells were determined by intracellular CFU divided by initial inoculum of CFU.

RESULTS

Clone and sequencing of *cfi* gene

Sequencing of the DGX07 *cfi* geneshowed that this gene had 771bp. The sequence was submitted to the NCBI GeneBank (Accession No. KC132870).The insert gene was verified by digestion with restriction enzymes *BamHI*and *HindIII* (Fig. 1).

Online BLAST showed that *S. iniae* DGX07 *cfi* gene encoded a peptide that was 100% identical to that of *S. iniae* (gi: 406658292) and was predicted to belong to the CAMP factor superfamily. The less identical proteins in order were those from *S. canis* (63%), *S. pyogenes* (62%), *S. agalactiae* (61%), *S. pseudoporcinus* (60%) and



M1, DNA Marker (DL15000); Lane 1,pET-32a(+); Lane2, pET-32a(+) digestion with *BamHI*and *HindIII*; Lane 3, digestion of the recombinant plasmid with *BamHI*; Lane 4, digestion of the recombinant plasmid with *BamHI*and *HindIII*; Lane 5, bacterial colony PCR; M2. DNA Marker (DL2000)

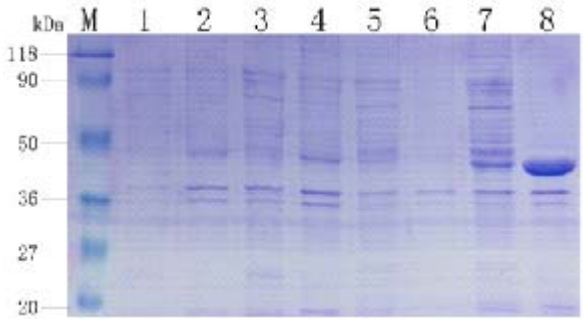
Fig.1. Identification of the positive pET-32a(+)-*cfi*by bacterial colony PCR and digestion with restriction enzymes *BamHI*and *HindIII*

S. uberis (57%). Clustal X 2.0 and Mega 4.1 were used to construct the phylogenetic tree with the most similar peptide sequences. Among them, DGX07 *cfi* gene coding peptide sequence shared 100% homology with that of *S. iniae* (gi: 406658292) and was significantly homologous to almost all the CAMP factor protein sequences of Streptococcal species, whereas less homologous to those of non-Streptococcal species (Fig. 2).

CAMP factor functions as a co-hemolysin

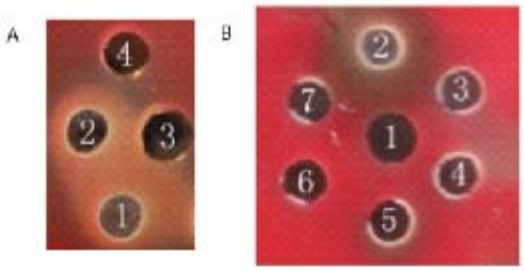
To determine whether the CAMP factor from the fish pathogenic *S. iniae* DGX07 has similar co-hemolytic characteristics, the classical agar plate assay was carried out with slight change[33]. Thepurified recombinant CAMP factor was verified by SDS-PAGE (Fig. 3). Tests on the blood agar plates showed that the recombinant CAMP factor could act as the classic co-hemolysin. As was

Fig. 2. Phylogenetic tree analysis of the bacterial strains that contained the protein of CAMP factor



M, protein marker; Lane 1, BL21-pET-32a(+) (pellet); Lane 2, the uninducedBL21-pET-32a(+) (supernatant); Lane 3, the induced BL21-pET-32a(+) (pellet); Lane 4, the induced BL21-pET-32a(+) (supernatant); Lane 5, the BL21-pET-32a(+)-*cfi*(pellet); Lane 6, BL21-pET-32a(+)-*cfi* (supernatant); Lane 7, the induced BL21-pET-32a(+)-*cfi*(pellet); Lane 8, the induced BL21-pET-32a(+)-*cfi* (supernatant).

Fig. 3. Expression analysis of the recombinant CAMP factor

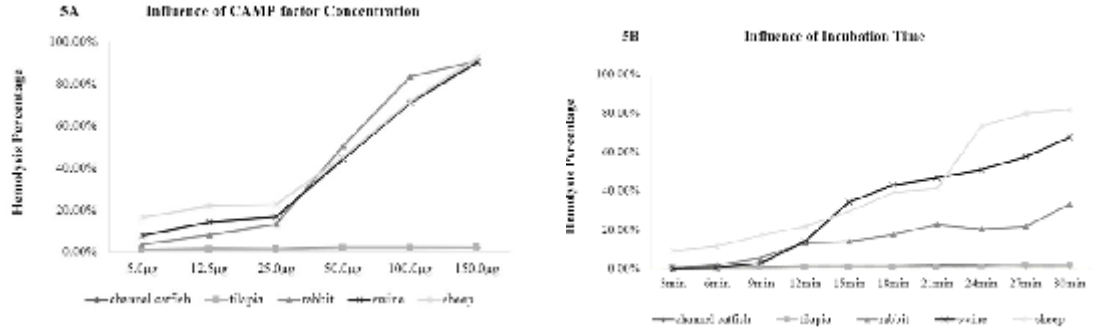


A, 1,*S. aureus*SMase; 2, CAMP factor of of 1mg/ml; 3, CAMP factor of of 1mg/ml with 1:32 diluted antiserum; 4, Negative control samples (PBS)
B, 1. *S. aureus*; 2, BL21-pET-32a(+)-*cfi* with 0.8mM IPTG; 3, BL21-pET-32a(+) with 0.8mM IPTG; 4, BL21 without pET plasmids with 0.8mM IPTG; 5, BL21-pET-32a(+)-*cfi*; 6, BL21-pET-32a(+); 7, BL21 without pET plasmids.

Fig. 4. Co-hemolytic activity assay of the CAMP factor and of its neutralized form and on BL21 cells

observed in Figure 4A, CAMP factor or *S. aureus*SMase alone could not induce any red blood cell lyzing unless these two substances were adjacently added on the blood agar plates. Another tests in which CAMP factor was replaced by the bacterium BL21 (DE3)-pET-32a(+)-*cfi*also confirmed this co-hemolysis (Fig.4B). All the results of the co-hemolytic studies supported the conclusion that the *S. iniae* CAMP factor could exhibit the co-hemolytic activity.

It could be noted that varied CAMP factor concentration employed shared almost the same shape of hemolysis curves, for the rabbit, guinea-pig and sheep RBCs. These curve showed a same lag period varying in duration with the concentration of CAMP factor not more than 25μg, and in which very little hernolysis occurs.



Different concentration of CAMP factor (5.0~150.0¼g) (Fig. 5A) or incubation time (3~30min) (Fig. 5B) was employed on five species. All RBCs were pre-incubated with same amount of purified SMase (100¼g) for 30min at 37 °C and then incubated with different concentration of CAMP factor for 30min at 37 °C (Fig. 5A) or with different incubation time with 100¼g CAMP factor at 37 °C (Fig. 5B) . Hemolysis percentage was estimated using the formula in the Materials and Methods. All the hemolysis tests were repeated for three times.

Fig. 5. Liquid hemolysis of different incubation time and concentration of CAMP factor on several species

Following the lag period (≤ 25μg), hemolysis became quite rapid and were more than 90% complete when the amount of CAMP factor reached above 100μg. For the influence of CAMP factor incubation time, it could be seen that rabbit, guinea-pig and sheep RBCs were not steadily lysed as the increment of incubation time (Fig. 5A & 5B).

However, tests on fish RBCS showed that tilapia (*Oreochromis mossambicus*) and channel catfish (*Ictalurus punctatus*) RBCs were completely resistant upon CAMP factor attack, whether with high concentration of CAMP factor or with long incubation time (highest percentage of hemolysis <2.3%). These results indicated that CAMP factor could not contribute to *S. iniae* hemolysis in fish host infection (Fig. 5A & 5B).

***S.iniae* expresses low amount of CAMP factor**

The antigenicity of the recombinant CAMP factor was analyzed by western-blot. The anti-CAMP factor antiserumused in the Western-blot analyseswere confirmed by gel-double-immunodiffusion test and a titer of 1:32 was identified (Fig.6). CAMP factor was separated by SDS-PAGE, electro-blotted onto PVDF membranes. Results showed that anti-CAMP factor antiserum demonstrated that *S. iniae* CGX07 CAMP factor had antigenicity(Fig. 7A). In additional western-blots, anti-CAMP factor antiserum bound to the ultrasonic treated *S. iniae* CGX07 (Fig. 7B), indicating that *S. iniae* CGX07 expressed CAMP

factor under natural growing environment. Anti-inactivated *S. iniae* CGX07 antiserum,however, could not bound CAMP factor on the PVDF membrane (data not show), suggesting that the amount of CAMP factor expressed under natural environment were very low and that might be less virulent to trigger immune response in rabbits.

CAMP factor unspecifically binds to immunoglobulins

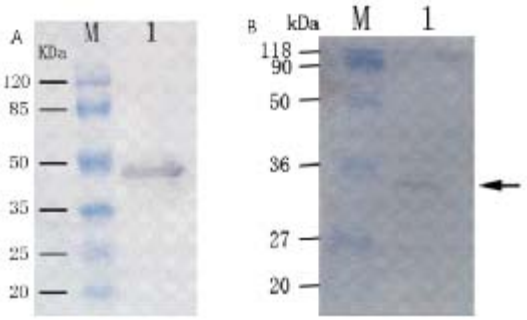
The Recombinant GBS and *S. iniae* α-enolase were prepared in another researches (data not published). In the preliminary tests, these two serum could not detect CAMP factor on the PVDF membranes. The western-blot results demonstrated that the anti-α-enolase serum pre-incubated with CAMP factor of both species showed a limited binding ability to the α-enolase compared to those pre-incubated with PBS. The level of inhibition showed a dose-dependent pattern of CAMP factor, indicating its binding to the Fc domains of rabbit non-unspecific antibodies (Fig. 8A & 8B).

CAMP factor facilitates adherence and invasion of *S. iniae* to EPC cell line

Early reports showed that *S. iniae*

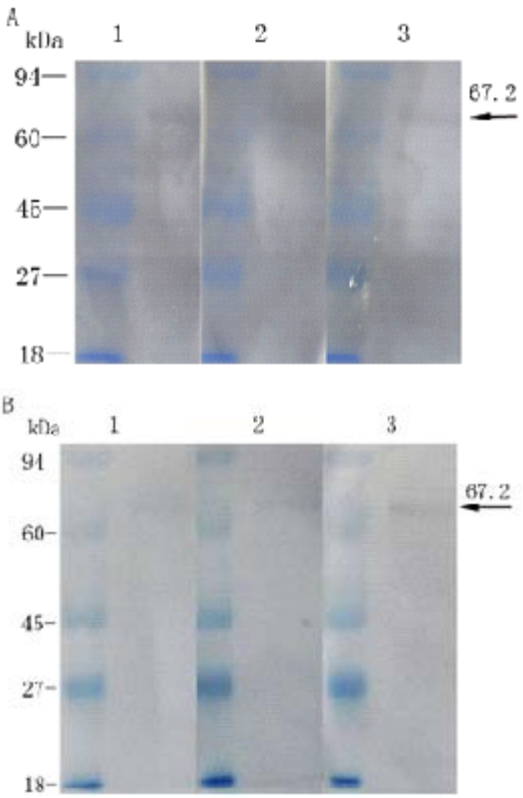
1) 2-fold diluted antiserum. 2) 4-fold diluted antiserum. 3) 8-fold diluted antiserum. 4) 16-fold diluted antiserum. 5) 32-fold diluted antiserum. 6) control of negative rabbit sera. 7) purified recombinant protein

Fig. 6. Detection of the antiserum of the recombinant CAMP factor titer by gel-double-immunodiffusion test



A, M, protein marker; 1, recombinant CAMP factor against the purified CAMP factor induced immune serum. B, M, protein marker; 1, sonicated mixture of the S.iniae against the purified CAMP factor induced immune serum.

Fig. 7. Western-blot analyses of recombinant CAMP factor immunized-rabbit serum against purified CAMP factor and sonicated *S.iniae*

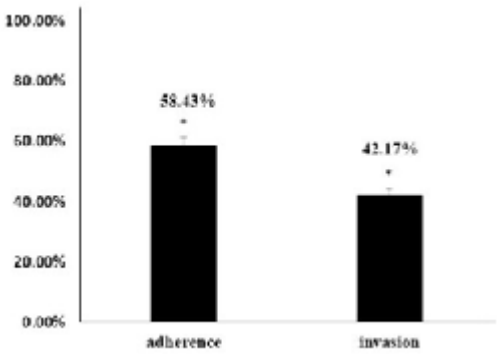


A, anti-GBS α-enolase serum pre-incubated with CAMP factor for 30min at 37 °C and the rest procedure were as that of Western-blots. 1, serum pre-incubated with PBS, 2, serum pre-incubated with 50μg CAMP factor, 3, serum pre-incubated with 100μg CAMP factor. B, anti-*S.iniae* α-enolase serum pre-incubated with CAMP factor for 30min at 37 °C and the rest procedure were as that of Western-blots. 1, serum pre-incubated with PBS, 2, serum pre-incubated with 50μg CAMP factor, 3, serum pre-incubated with 100μg CAMP factor.

Fig. 8. Western-blot analyses of CAMP factor unspecific binding to rabbit IgG Fc region

virulent factors (e.gSiM) facilitated adherence and invasion host cells. Tests were carried out to examine whether *S. iniae* CAMP factor could also facilitate adherence and invasion of epithelial cells by *S. iniae*.

Strains DGX07 were grown to mid-log phase and incubated with 1: 100 diluted normal rabbit serum or rabbit anti-CAMP factor IgG at 37°C for 1 h. Bacteria were then incubated with EPC epithelial cells for 2 h at 37°C. Adherent



The controls (without serum incubation) were standardized as 100 percentage adherence and invasion. Percent adherence was normalized for growth in wells that contained EPC cells and equals CFU associated with the monolayer divided by total CFU in the well at 2 h postinfection. Invasion was intracellular CFU divided by initial inoculum of CFU. The data were means ± standard errors of the means from three independent experiments. The percentage were indicated above the bars. Asterisks denote results with mean differing significantly form control (*S. iniae* incubated with normal rabbit IgG) experiments:*P<0.05.

Fig. 9. Comparison of adherence and invasion of *S. iniae* to EPC cells with or without the help of CAMP factor

bacteria were assayed by viable counts associated with the monolayer. Anti-CAMP factor IgG reduced adherence and internalization of *S. iniae* to EPC epithelial cells to 58.43% and 42.17% respectively (Fig. 9). Results revealed that nearly 40% and 60% the CAMP factor were neutralized by anti-CAMP factor IgG in adherence and invasion assays. These data suggested that CAMP factorremarkably enhanced the binding and infection of fish epithelial cells by *S. iniae* and antibody provided at least 4 0% protection against *S. iniae*adherence to and infection of EPC cells.

DISCUSSION

Whether CAMP factor is a systemic virulent factor is controversial[26, 34]. To date, CAMP factor functions as 1) a co-hemolysin accompanied with *S. aureus* SMase and 2) unspecific binds to the immunoglobulin IgG and IgM Fc fragment. It could therefore be inferred that as a co-hemolysin, CAMP factor could not function in the absence of *S. aureus*SMase. Besides, CAMP factor were reportedly not binding to the human IgG and IgM[26]. Apart from this, CAMP factor was proposed lethal to mice or rabbits using two different injection regiments[35]. However, recent reports showed that CAMP factor gene mutant strain exhibited full virulence in a mouse model infection compared to that of wild type strain, suggesting that CAMP factor expressed in its native context is not essential for systemic virulence of GBS[34]. In our study, as were shown in the Western-blots, recombinant CAMP factor showed its immunogenicity in rabbits and anti-CAMP factor serum could detect this protein in ultrasonic treated *S. iniae*, whereas anti-inactivated *S. iniae* could not detect CAMP factor. These results indicated that only high concentration of purified CAMP factor (0.5mg/ml) could activate immune response in rabbits, but *S. iniae* expressed only a small amount of CAMP factor under native context, which was not enough to exhibit its immunogenicity.

CAMP reaction, confirmed in many streptococcus including *S. iniae* and non-streptococcus, till now, has still been adopted as clinical identification of GBS[13, 14]. Several clinical literatures reportedthe CAMP reaction in*S. iniae*[1, 6, 36]. More reliable detection and identification methods should be employed in GBS diagnosis.In order to gain insight into the different species RBCs sensitivity to CAMP factor, we employed liquid hemolysis assays and RBCs from healthy rabbits, guinea-pigs, sheep and fish were adopted. Interestingly, our liquid hemolysis assay revealed that CAMP factor cytolyzed rabbit and pig RBCs as efficient as sheep RBCs, which contradicted to the previous findings that CAMP factor lyzed RBCs with more than 45 mol% sphingomyelin. Rabbit and guinea-pig RBC, with less than 45 mol% sphingomyelin in the cell membrane[37, 38], which in retrospective were considered not sensitive to

CAMP factor lysis, were lysed as well as sheep RBC, containing 51 mol% sphingomyelin.

*S. iniae*infection to the only known mammals (human, Amazon freshwater dolphins and flying foxes[2, 3, 39, 40]) is rarely reported in the past decades, comparing to the severity and frequency of *S. iniae*infection upon fisheries worldwide. It could therefore be inferred that fish were the primary targets of *S. iniae*. CAMP factor inother streptococcus were known as co-hemolysins, and accordingly, CAMP factor in*S. iniae* was supposed to co-hemolyse RBCs of its susceptible hosts (finfish). Our results of co-hemolysis tests on fish were in sharp contrast to those on mammals'. This total loss of co-hemolysis on fish was difficult to explain, since CAMP factor could lyse RBCs despite the "sphingomyelin" principle in mammals in our study.To date, no evidence have ever shown that *S. aureus* could infect fish. One possible explanation is that CAMP factor malfunctions under the circumstances that sphingomyelin is without. Earlier work highlighted the possibilities that CAMP factor, carried by a mobile genetic element, could be spread among other field pathogenic streptococci of veterinary origin[41]. Our dataalso led to the speculation that without the access to *S. aureus* (or sphingomyelin), *S. iniae* CAMP factor had lost part of its function as a co-hemolysin in the pathogenic progress of *S. iniae* infection to fish. Since CAMP factor does not have significant homology to their counterparts from other streptococci, we proposed that hemolysis function of CAMP factor was not essential to *S. iniae*.

Fc region binding ability is adopted by numerous *Streptococcus* pathogen, rendering themselves more aggressive and higher chances in surviving when confronted host immune systems. SiM proteins may also bind trout immunoglobulin by the Fc region[23]. Evidence showed that CAMP factor unspecifically bound to IgG and IgM[25], but later demonstrated otherwise[26]. Our studies with Western-blot suggested both the anti-GBS α-enolase and anti-*S. iniae* α-enolase serum that pre-incubated with CAMP factor could not retain full binding ability to their antigen, respectively. These results again supported the postulation that CAMP factor were capable of IgG Fc region binding.

Reports indicated that *S. iniae* infection might begin with colonisation of external or gastrointestinal tissues followed by local spread and subsequent invasion of the blood stream. To enter the central nervous system (CNS) from the bloodstream, *S. iniae* might work their way through the blood brain barrier[42]. These procedures requires the ability of epithelial cell binding. When CAMP factor was neutralized by its serum, *S. iniae*showed a decreased abilities in both adherence (58.4% compared to normal) and invasion (42.7% compared to normal) to EPC cell line. The relatively high efficiency in facilitating *S. iniae*of adherence and internalization to epithelial cells suggested that CAMP factor could serve as an invasin for *S. iniae* colonization of epithelium. Failure of CAMP factor to co-hemolyze fish RBCs, the abilities to bind IgG Fc region and epithelium cells, depicted that CAMP factor in *S. iniae* lost its cytolytic function but was compensatorily employed in antigen presentation blocking and epithelium attacking. However, we presumed that *S. iniae* would receive limited assists from CAMP factor, because low amount of CAMP factor were expressed under native context. Further characterization of distribution of CAMP factor and *S. iniae* CAMP factor mutant strain (gene knockout) in the hosts are ongoing.

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