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Research Article

Development and Evaluation of Lateral Flow Test Strips for Fish Pathogenic *Streptococcus Agalactiae*

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ABSTRACT

Sterptococcus agalactiae (S. agalactiae) is a major bacterial pathogen to humans, and also to aquatic animals. Recently, S. agalactiae has become one of the major causes of streptococcicosis in aquaculture. In order to detect S. agalactiae more effectively and easily, the rapid test strip of S. agalactiae was developed by using specific monoclonal antibodies. Surface immunogenic protein (Sip) of S. agalactiae was expressed and used for immunization. The hybridoma technique was employed to produce hybridoma cell lines which can secret monoclonal antibodies (mAbs) against Sip of S. agalactiae. Two strains of hybridomas named NB1a and CG10 were screened out. The subtypes of the mAbs were IgG_{2b} and IgG_1, respectively and were confirmed by indirect enzyme linked immunosorbent assay (ELISA). The titers of NB1a were $1:10^5$ and the titer of CG10 was $1:10^6$. Rapid test strips were developed based on these mAbs for the detection of overnight culture of S. agalactiae and a detection limit of 10^7CFU/ml. The strips showed no cross reaction with 28 other bacterial samples and one bovine-originated S. agalactiae. The clinical specimen tests in this study showed that the strips could be used for the detection of S. agalactiae-infected fish, but not for pond water or sediment. The results of specificity, sensitivity and repeatability tests showed the strips could effectively serve for the identification for S. agalactiae in aquaculture.

INTRODUCTION

Streptococcus agalactiae, also called Group B Streptococcus (GBS), belongs to Streptococcace, Streptococcus. It is Gram-positive cocci, which can cause infection of wild and farmed aquatic animals [1,2]. Besides, GBS is also a pathogen for human, bovine etc.. Although GBS was isolated from a different host, gene expression of GBS originating from fish plays important roles in its adaptation to the fish host [3]. It was reported that GBS from different origins can cause the fish infection [4], but the pathogenicity to fish varied [5]. GBS is distributed widely in the world, and most of the outbreaks caused by GBS happened when the water temperature was higher than 26°C. So far, more and more attention was attracted to the fish disease caused by

GBS. Due to its high morbidity and mortality, the disease caused by GBS hinders the sound development of aquaculture leading to sever loss to the fish farms.

Some fish diseases share the same clinical signs, for instance, exophthalmos in fish might be caused by bacterial infection or vitamin E deficiency. Therefore, the diagnosis of fish diseases could not simply rely on the clinical signs, but needs the support of other identification methods, such as biochemical tests and PCRs [1,6,7]. Matsui et al. [8] reported a lateral flow test targeting surface immunogenic protein (Sip) of S. agalactiae isolated from human, which is 99.6% specific and 93.1% sensitive. According to previous reports, Sip, found in nine serotypes of S. agalactiae, can provide cross-protection to mice against the challenge of different serotype of S. agalactiae [9]. However, there is less report regarding to lateral flow test for S. agalactiae from fish origins so far. Therefore, we developed specific monoclonal antibodies and lateral flow tests against Sip, in order to provide tools for S. agalactiae detection.

MATERIALS AND METHODS

Bacteria

Purified Streptococcus agalactiae strain TW3 will be inoculated in BHI broth at 37°C for 24h. The samples of Streptococcus agalactiae (ATCC 51487), Streptococcus agalactiae C918 (Bovine origin), Streptococcus agalactiae TW7 (fish origin) , Streptococcus agalactiae TW10 (fish origin), Edwardsiella ictaluri, Enterococcus faecalis, Strepstococcus iniae, Aeromonas caviae, Salmonella typhimurium, Staphylococcus aureus, Vibrio cholerae, Vibrio parahaemolyticus, Aeromonas hydrophila, Bacillus subtilis, Vibrio alginolyticus, Enterobacter cloacae, Shewanella putrefaciens and Klebsiella pneumoniae were provided by Tongwei Group, Sichuan Province, China. Helicobacter pylori, Stenotrophomonas maltophilia, Proteus mirabilis, Candida tropicalis, Salmonella typhi, Salmonella paratyphi , Serratia marcescens, Escherichia coli, Candida albicans, Citrobacter freundii , Pseudomonas aeruginosa, Staphylococcus haemolyticus, Acinetobacter baumannii, and Neisseria gonorrhoeae were kept in Artron R & D lab (Artron BioResearch Inc., Canada).

Expression and purification of His-tagged rSip

DNA was extracted from purified Streptococcus agalactiae strain TW3 by using Promega DNA extraction kit (PROMEGA). According to the sequence of sip gene from S. agalactiae (tilapia origin; HQ878436.1, Genbank), primers were designed as listed in Table 1. The sip gene was amplified by PCR using the pair of primers in Table 1, containing the BamH I and Sall restriction enzymes sites, respectively. PCR will be carried out under the following conditions. After initial denaturation of DNA at 94°C for 5 min with a thermal cycler, the program will be set at 94°C for 30 s, 55°C for 35 s, and 72°C for 1min 18s for a total of 30 cycles and then at 72°C for 10 min for the final extension.

Table 1: Primers for expression of sip gene (Amplicon ~1302bp).

Primers	Sequence (5'-3')	Restriction enzyme cutting sites
Sip-bamh1-fw	5'-GCCGGATCCATGGAAATGAATAAAAAGGTAC -3'	BamHI
Sip-sal1-re	5'-GCGGTCGACTTAGTTAAAGGATACGTGAACGTGG -3'	SalI

Amplified sip DNA was ligated to the pET32a vector containing a sequence encoding hexahistidine. Escherichia coli cells BL21 was transformed with pET32a-sip. The transformants was cultured in LB broth containing (100 µg/mL) of kanamycin at 37°C. Hexahistidine-tagged recombinant Sip (rSip) will be expressed by adding 0.8mM isopropyl-beta-D-thiogalactopyranoside (IPTG), and the culture will be incubated at 37°C for an additional 5 h. The soluble fraction containing rSip was extracted by ultrasonic oscillation and then applied to and eluted from a nickel-ion immobilized-metal affinity chromatography (IMAC) resin column. The protein concentration was quantified by the bicinchoninic acid assay kit (Sigma-Aldrich, Canada) according to the protocol and used as an immunogen for the production of mAbs and as an antigen in ELISA.

Electrophoresis and Western blotting

Purified rSip was subjected to sodium dodecyl sulfatepolyacrylamide gel (12%) electrophoresis (SDS-PAGE). Protein bands was visualized by staining with Coomassie brilliant blue or the Western blotting method using the

polyclonal antibodies against S. agalactiae in order to test the activity of rSip.

Immunization Schedule

Female BALB/c mice (8 weeks old) were immunized intraperitoneally with 25μg, 50μg, 100μg and 150μg of rSip protein emulsified in Freund's complete adjuvant (Sigma-Aldrich, Canada), respectively. After 4 and 8 weeks, the mice were injected intraperitoneally with the same antigen emulsified in Freund's incomplete adjuvant (Sigma-Aldrich). At 10 weeks, the serum of each immunized mice were collected and the titer of the serum was detected by indirect ELISA. The mouse with highest titer level received a final immunization intraperitoneally with rSip protein alone.

Hybridoma generation and Production of mAbs to Sip protein.

Splenocytes of immunized mice were harvested three days after the final booster injection. These cells were fused with the myeloma cell line SP2/0 at a ratio of 5:1 by 50% (w/v) polyethyleneglycol 4000 (Sigma-Aldrich) according to the protocol described previously by Situ and Wu [10]. The resulting hybridoma cells were cultured in a hypoxanthine-aminopterin-thymidine medium, and the culture supernatants of hybridoma cells were then screened for the production of antibody by an ELISA using 5μg/ml of rSip as the coating antigen. The positive hybridoma cells identified by ELISA were cloned twice by limiting dilution and then amplified in the ascites of BALB/c mice. The ascites were collected and the anti-rSip mAbs in the ascites were purified by Protein G Sepharose affinity chromatography (GE Healthcare Life Sciences, Canada) according to the instruction.

Subtype and titer of mAbs

Following the instructions of the manufacture, the class and subclass of the anti- S. agalactiae mAbs were determined using indirect ELISA with specific antibodies in six isotypes, goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA (supplied by Sigma-Aldrich, Canada). The purified antibodies in different dilutions were measured by indirect ELISA to get the titer of the antibodies.

The Preparation of test strips

The preparation of the mixture of anti- rSip mAbs and colloidal gold: Purified mAb (concentration 2mg/ml) was conjugated with the colloidal gold liquid, kept in room temperature (RT) for 5 min. The solution was centrifuged by 8000 rpm for 30 min as soon as 5% PEG was added. The red deposition was collected in a 12ml centrifuge tube, suspended with the colloidal gold torpent, and diluted into the working concentration (commonly OD_{532}= 30-50). Then solution was kept in 4°C.

The preparation of reaction membrane: The other mAbs were diluted to 2mg/ml by using 0.1M PBS. The goat anti-mouse IgG was diluted to 1.0mg/ml by using 0.1M PBS. The former was used to coat the test line, while the latter for the control line on NC membrane. Then membrane was kept in 37°C for 24h. After being blocked by BB buffer (Artron BioResearch Inc, Canada), the membrane was washed by WB buffer (Artron BioResearch Inc, Canada) and then dried and kept in room temperature.

Assembly of strips: The test strips were assembled with the sample pad, conjugate pad, reaction membrane, and absorbent pad [11]. The strips were cut into 3mm pieces by auto-stripcutter.

Selection of the best match-pairs of mAbs for S. agalactiae

All conjugated-mAb were matched with the coated-mAb for the strip preparation. The sample pad of the strip was dipped into the samples of S. agalactiae until half of the NC membrane was soaked in the liquid. The color development of a control line and a test line was observed after 3-10 min in RT. The match-pairs showing the strongest positive signal for the detection of S. agalactiae but negative results for the other tested bacteria were used for further specificity and sensitivity tests.

Characteristics of test strips

Sensitivity test by strips: The sample of S. agalactiae was diluted by multiproportion from 6×10^{10} CFU/ml to 6×10^{6} CFU/ml, and then the strips were tested to value its minimum detection dose.

Specificity test by strips: The samples of Streptococcus agalactiae (ATCC 51487), Streptococcus agalactiae C918 (Bovine origin), Edwardsiella ictaluri, Enterococcus faecalis, Strepstococcus iniae, Aeromonas caviae, Salmonella typhimurium, Staphylococcus aureus, Vibrio cholerae, Vibrio parahaemolyticus, Aeromonas hydrophila, Bacillus subtilis, Vibrio alginolyticus, Enterobacter cloacae, Shewanella putrefaciens, Klebsiella pneumoniae, Helicobacter pylori,

Stenotrophomonas maltophilia, Proteus mirabilis, Candida tropicalis, Salmonella typhi, Salmonella paratyphi , Serratia marcescens, Escherichia coli, Candida albicans, Citrobacter freundii , Pseudomonas aeruginosa, Staphylococcus haemolyticus, Acinetobacter baumannii, and Neisseria gonorrhoeae were tested by strips.

Repeatability test by strips: Three samples of S. agalactiae and three samples of negative control were tested by strips respectively with the ten replicates per sample. The Test was performed and recorded by two different operators.

Detection of clinical samples: Sick tilapia samples were collected from a fish farm located in Wenchang, Hainan Province, China. Sick tilapia (N=10) was confirmed of the infection of S. agalactiae by using PCR method and was used for strip tests. Samples from healthy tilapia (N=5) were also used for strip tests. The liver/ brain were removed from fish and were homogenized separately. 1 ml of sterile physiological saline were added and mixed with the tissue homogenates. The mixture was detected by using the strips.

The 10 ml of water and sediment where sick tilapia was present were collected and detected directly by using strips.

RESULTS

Amplification of sip gene

S. agalactiae was used as template to successfully amplified a fragment between 1000-1500bp.

Purification of rSip protein

rSip was expressed in BL21 and successfully purified. The expressed protein is about 70Kda (including the target rSip ~50KDa) (Figure 2). The result of western blotting showed that rSip reacted specifically with polyclonal antibody against whole-cell S. agalactiae (Figure 3).

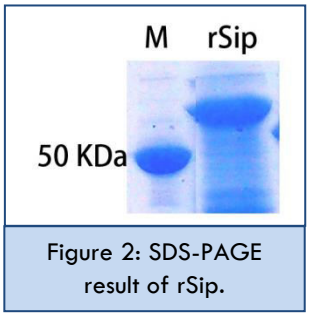

Figure 2: SDS-PAGE result of rSip.

1. Marker ; 2.rSip

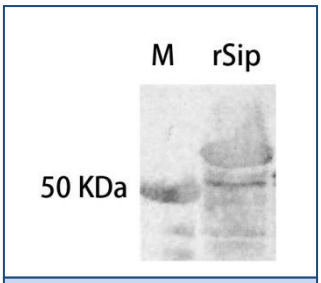

Figure 3: Results of Western blotting

Immunization

Eight BALB/c mice were used for immunization at different dosages of rSip of 25μg, 50 μg, 100 μg and 150 μg. The ELISA results showed that the serum titer of rSip-immunized mice reached 1:10^6 when using rSip as coated antigen; while the serum titer reached 1:10^4 when using whole-cell S. agalactiae as coated antigen.

Production of mAbs to S. agalactiae and match-pair selection

In total, 84 positive cell lines were obtained after the cell fusion. All the 84 mAbs were purified, and used as coating-Ab/ conjugated-Ab for the strip assembly. After 7056 times of match-pair tests, match-pair NB1a-CG10 which showed strong signal to S. agalactiae and negative results to other bacteria were selected out for further tests.

Subtype and titer of mAbs

NB1a belonged to subtype IgG_{2b}, and CG10 belonged to subtype IgG_1 (Table 2). The titers of mAb CG10 are 10^{-6}, and the titer of NB1a is slightly lower as 10^{-5} (Table 2).

Table 2: Titers and Subtypes of mAbs by indirect ELISA.

mAb No.	titre	subtype
NB1a	10^{-5}	IgG_{2b}
CG10	10^{-6}	IgG_1

Western blot using anti-S. agalactiae monoclonal antibodies

S. agalactiae and other common bacteria in aquaculture were tested in western blot. Three anti-S. agalactiae monoclonal antibodies were used as primary antibody for western blot respectively. The results showed both mAb NB1a and CG10 can specifically recognize S. agalactiae without any cross-reaction with the ones from other tested bacteria (Figure 4).

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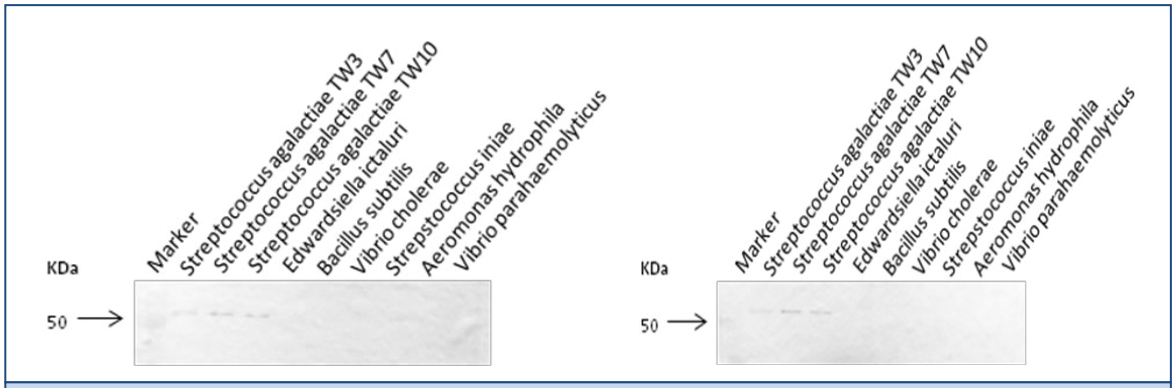

Figure 4: Western-blot result of common bacteria in aquaculture

1-A Primary Ab: NB1a;1-B Primary Ab: CG10

Characteristics of S. agalactiae rapid test strips

The mAb NB1a was used as coating-mAb and mAb CG10 was used as conjugated-mAb. Overnight culture of S. agalactiae in Brain Heart Infusion (BHI) medium has been tested directly. The result showed positive in both cultured S. agalactiae TW3 sample and BHI supernatant sample from cultured S. agalactiae (Figure 5). The characteristics of the strips were further tested.

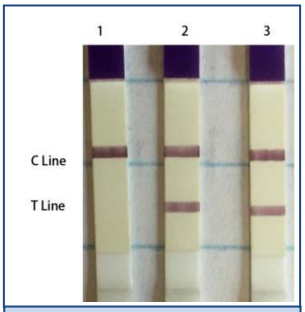

Figure 5: Test strips for detection of S. agalactiae.

1 BHI medium (negative control); 2 cultured S. agalactiae TW 3 in BHI; 3 BHI supernatant after enrichment of S. agalactiae culture.

C Line: Control line; T Line: Test line

Results of sensitivity test: Culture solution of S. agalactiae was 10-time diluted from 6×10^{10} CFU/ml to 6×10^{6} CFU/ml in order to test the sensitivity of the strips. Weak T cline could be observed when the concentration is 6×10^{7} CFU/ml, but the T line did not appear when concentration continued dropping to 6×10^{6}CFU/ml (Figure 6). Figure 1 showed that the detection limit of this match-pair is 6×10^{7} CFU/ml.

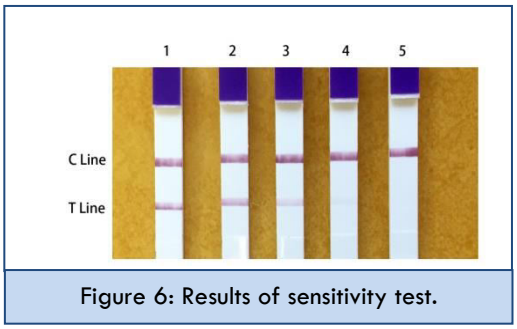

Figure 6: Results of sensitivity test.

1. 6×1010CFU/ml; 2. 6×109CFU/ml; 3. 6×108CFU/ml; 4. 6×107CFU/ml; 5. 6×106CFU/ml

C Line: Control line; T Line: Test line

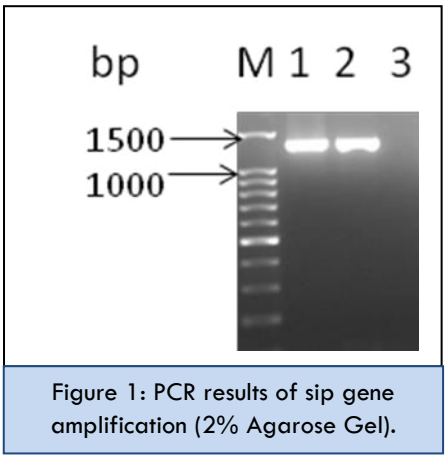

Figure 1: PCR results of sip gene amplification (2% Agarose Gel).

Marker: 100bp DNA Ladder (TAKARA), Lane 1-2 Group 1 sip gene; Lane 3 Group 1 negative control

Results of specificity tests: The rapid test strips of both two match-pairs showed high specificity to S. agalactiae and no cross-reaction with other tested bacteria species (Figure 7 and Table 3).

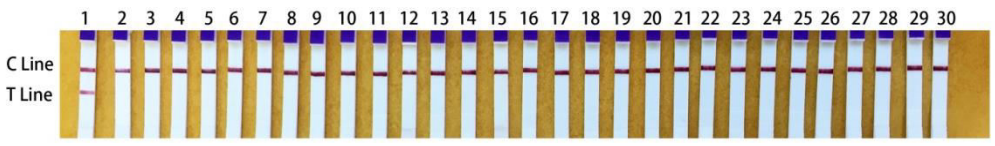

Figure 7: Specificity tests of strips.

1. Streptococcus agalactiae (ATCC 51487), 2. Streptococcus agalactiae C918 (Bovine origin), 3. Edwardsiella ictaluri, 4. Enterococcus faecalis, 5. Strepstococcus iniae, 6. Aeromonas caviae, 7. Salmonella typhimurium, 8. Staphylococcus aureus, 9. Vibrio cholerae, 10. Vibrio parahaemolyticus, 11. Aeromonas hydrophila, 12. Bacillus subtilis, 13. Vibrio alginolyticus, 14. Enterobacter cloacae, 15. Shewanella putrefaciens, 16. Klebsiella pneumoniae, 17. Helicobacter pylori, 18. Stenotrophomonas maltophilia, 19. Proteus mirabilis, 20. Candida tropicalis, 21. Salmonella typhi, 22. Salmonella paratyphi, 23. Serratia marcescens, 24. Escherichia coli, 25. Candida albicans, 26. Citrobacter freundii, 27. Pseudomonas aeruginosa, 28. Staphylococcus haemolyticus, 29. Acinetobacter baumannii, 30. Neisseria gonorrhoeae

Table 3-1: Detection limit of strips for S. agalctiae.

S. agalactiae samples	Serotype	Isolation origin	Detection Limit
TW3	Ia	Fish	6×10^{7}CFU/ml
TW6	Ia	Fish	1.1×10^{7}CFU/ml
TW7	Ia	Fish	1.3×10^{7}CFU/ml
TW9	Ia	Fish	1.1×10^{6}CFU/ml
TW10	Ia	Fish	1×10^{7}CFU/ml
TW31	II	bovine origin	negative

Table 3-2: Specificity test of S. agalactiae

	Match-pair
(Conjugated-mAb)	CG10
(Coating-mAb)	NB1a
Streptococcus agalactiae (fish origin)	+++
Streptococcus agalactiae (bovine origin)	-
Edwardsiella ictaluri	-
Enterococcus faecalis	-
Strepstococcus iniae	-
Aeromonas caviae	-
Salmonella typhimurium	-
Staphylococcus aureus	-
Vibrio cholerae	-
Vibrio parahaemolyticus	-
Aeromonas hydrophila	-
Bacillus subtilis	-
Vibrio alginolyticus	-
Enterobacter cloacae	-
Shewanella putrefaciens	-
Klebsiella pneumoniae	-
Helicobacter pylori	-
Stenotrophomonas maltophilia	-
Proteus mirabilis	-
Candida tropicalis	-
Salmonella typhi	-
Salmonella paratyphi	-
Serratia marcescens	-
Escherichia coli	-
Candida albicans	-
Citrobacter freundii	-
Pseudomonas aeruginosa	-
Staphylococcus haemolyticus	-
Acinetobacter baumannii	-
Neisseria gonorrhoeae	-

Results of repeatability test: It was showed that the positive and negative coincidence were both 100% (Table 4).

Table 4-1: Results of repeatability test (by Observer 1).

Type Samples	Results	Total	Coincidence （%）
Positive Samples	30(+)	30	100(30/30)
Negative Samples	30(-)	30	100(30/30)

Table 4-2: Results of repeatability test (by Observer 2).

Type Samples	Results	Total	Coincidence （%）
Positive Samples	30(+)	30	100(30/30)
Negative Samples	30(-)	30	100(30/30)

Results of clinical sample test: Sick tilapias from all farms were found positive by using the strips for S. agalactiae (Figure 8). Samples from healthy fish did not show any positive results. However, no positive results were observed for water and sediment samples from the pond where sick fish were present (Figure 8).

DISCUSSIONS

Streptococcus agalactiae has been isolated from human [12] and other animals [13,14]. The first report about the isolation of S. agalactiae in aquaculture was in 1966 [15], and since then, S. agalactiae has been found to be related to many kinds of aquatic animal infection [16]. So far, ten different serotypes of S. agalactiae have been reported [17], including Ia, III and V, which are pathogenic serotype to humans [18], and Ia, Ib and III were proved to be virulent to fish [19,20]. The outbreak of disease caused by S. agalactiae led to sever loss to

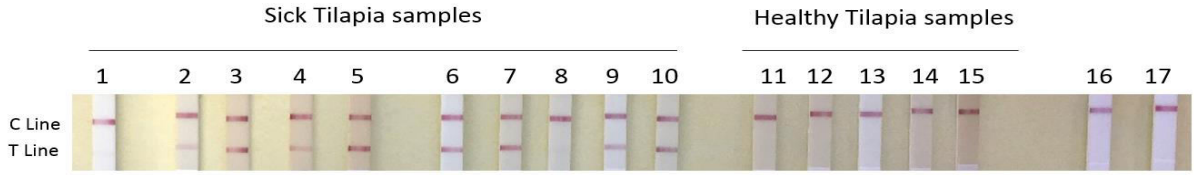

Figure 8: Results of clinical sample tests.

1 -10 Liver/Brain samples from sick tilapia; 11-15 Liver/brain samples from healthy tilapia; 16 sediment sample; 17 water sample.

C line: control line

T line: test line

aquaculture, especially to the breeding industry of tilapia [21,22]. Therefore, only correct and rapid diagnosis and identification can provide the foundation for immediate and efficient treatment. Until now, the most popular ways of identifying S. agalactiae in aquaculture is the conventional bacterial isolation and identification, as well as PCR identification, which is time-consuming, requires the professional person and equipments to perform. Rapid test strips for the detection of S. agalactiae are easy to use and may be a good way to save time. Development of monoclonal antibodies specifically targeting S. agalactiae lays the basis for the rapid test strips. Since monoclonal antibody (mAb) production was first introduced to the world [23], various kinds of mAbs have been developed so far. They are widely used in clinical diagnosis with obvious advantages, such as high specificity of the detection. mAbs can be used in enzyme-linked immunosorbent assay (ELISA), western blotting, immunoturbidimetric assay and lateral flow assay and so on. In aquaculture, many mAbs were developed for fish pathogens [24]. However, there are fewer reports about the development of mAbs targeting to fish pathogenic S. agalactiae. In our previous study, formalin-killed whole cell of S. agalactiae was used as the antigen to produce mAb but was not successful because of the low positive rate of hybridoma cell lines (unpublished data). Five mAbs against S. agalactiae from other different companies showed low titer ($\leq 1 : 10^3$) to both S. agalactiae ATCC 12386 and S. agalactiae TW3 used in this study (unpublished data). Hence, the protein from S. agalactiae was considered to be the antigen for immunization. In order to produce the specific antibody targeting to S. agalactiae, the protein should be found only in S. agalcatiae and have good antigenicity. However, the protein fit for the requirement is not easy to find. Virulence factors of S. agalactiae are distributed to the surface of the bacteriumand a variety of different proteins leads to variable immuno-protection, such as capsular polysaccharide which has good antigenicity, but the specific antibodies induced by capsular polysaccharide is type-dependent [25]. Several cell surface proteins of S. agalactiae have been reported as potential vaccine candidates, such as Cα, Cβ and Rib protein, but these proteins do not present in all serotype of S. agalactiae isolates [26]. Surface immunogenic protein (Sip) is a highly conserved surface exposed protein [26] and found to be present in nine serotype of GBS isolates [27]. It has been reported that the DNA vaccine SL/pVAX1-sip was effective in protecting tilapias against S. agalactiae infection [28]. Therefore, Sip was chosen to be used as the antigen for immunization and further selection. For each recombinant protein, it is important to make sure it shows good activity. Western blotting was employed to check the reaction of rSip with a polyclonal antibody against S.agalactiae. The results indicated that the rSip established in this study showed good activity and can be used in the further tests.

Two positive hybridoma cell lines secreting monoclonal antibodies against rSip were selected out and these mAbs can also be used in western blotting with no cross-reaction to other common bacteria in aquaculture. These two antibodies were proved later that they can detect whole cell of S. agalactiae when used as match-pairs in the lateral flow tests with the

detection limit ranging from 10^6- 10^7 CFU/ml, which probably results from a different concentration of Sip in S. agalactiae. Matsui et al. [8] have developed the strips for detection of S. agalactiae from human by using mAbs against rSip, which allowed the detection limit of 10^5-10^6 CFU/ml. So far, few reports about strips for S. agalactiae from fish have been found. In our study, untreated S. agalactiae was used for all the tests, while Triton-X 100 was used to pretreat the bacterial samples before loaded on the strips in Matsui et al.'s report [8], which may lead to the 10-fold difference of detection limit between our strips and the strips developed by Matsui et al. [8]. Strips developed in this study can be applied for the detection of enriched S. agalactiae culture. As the supernatant of bacterial culture showed positive results in strip tests indicating that Sip is also a secreted protein which can be found in the cultural supernatant. The clinical specimen tests in this study showed that the strips could be used for the detection of S. agalactiae- infected fish, but not for pond water or sediment. It might because the bacterial concentration in water/sediment sample was lower than the detection limit of our strips. Detection of S. agalactiae using strips developed in this study is time-saving and the result is easy to observe. Above all, **the anti- S. agalactiae** colloidal gold strips **provided another way for rapid** identification, diagnosis and **epidemic** surveillance of **S. agalactiae in aquaculture**, so that efficient treatment could be applied in aquaculture.

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