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Application of recombinant Sjc26GST for serodiagnosis of *Schistosoma japonicum* infection in water buffalo (*Bos buffelus*)

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Abstract

Schistosomiasis japonica is currently the most serious parasitic disease in mainland China and it is estimated that several million people are infected. Furthermore, it is also responsible for the deaths of many domestic animals. In order to establish an effective diagnostic method, the gene encoding Sjc26GST was cloned and expressed in *Escherichia coli* as a fusion protein with His-tag. The purified reSjc26GST was used as an antigen for an enzyme-linked immunosorbent assay (ELISA) and for immunoblotting detection of *Schistosoma japonicum* antibodies in water buffaloes. Our results showed that mean OD values of specific serum IgG antibodies from egg-positive buffaloes were 3.37-fold higher than what was found in egg-negative buffaloes from non-endemic areas. The data also showed the OD value of the endemic egg-negative group reached as high as 1.69 times as that found in non-endemic areas. The positivity rate of egg-positive buffaloes was 100%, but was 30.3% in the endemic egg-negative group. Infected bovine antisera also recognized reSjc26GST, a 27 kDa protein as determined by Western blot. These results suggest that the recombinant GST expressed in *E. coli* should be an effective diagnostic reagent for detection of antibody against *S. japonicum* in buffaloes. Due to straightforward production, excellent sensitivity and high specificity, the reSjc26GST described in this study can be considered as a candidate protein for immunological diagnosis of bovine schistosomiasis. Developing reSjc26GST, with its potential diagnostic values, will be useful for diagnosis and surveillance of schistosomiasis in controlling the spread of this parasitic disease in domestic animals.

Keywords: *Schistosoma japonicum*; Serodiagnosis; Recombinant Sjc26GST; ELISA; Western blot; Buffalo

1. Introduction

Schistosomiasis continues to be a serious worldwide public health problem (Chitsulo et al., 2000). It is estimated that several million people are infected in China every year and there is considerable economic loss due to infection of domestic animals (Ross et al., 1997). Improvement in diagnostic techniques is needed if the problem of Schistosomiasis infection is to be alleviated.

Correct diagnosis is a key step in effective disease control. The lack of a reliable and affordable diagnostic tool is one of the factors that can make control difficult to achieve (Hamilton et al., 1998; Bergquist, 2002; Xiang et al., 2003). Conventional methods for diagnosis of schistosomiasis include detection of eggs and hatching of miracidium (Ross et al., 2001). However, these methods are labor-intensive, time-consuming, somewhat messy and less sensitive in detecting light infections (de Vlas and Gryseels, 1992; Wu, 2002).

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Serodiagnosis could be a useful complement to parasitological tests and is more sensitive than conventional methods (Doenhoff et al., 1993, 2004; Hamilton et al., 1998). Antigens prepared from either schistsome eggs (SEA) or adult worms (SWAP) were the first immunodiagnostic tools applied to diagnose schistosomiasis (Wu, 2002; Zhu, 2005). But these assays proved to have unsatisfactory specificity and sensitivity, especially in individual with light infections (Guan and Shi, 1996). The most common criticism of antibody detection by crude worm extracts is that it cannot accurately reflect the intensity of infection (Doenhoff et al., 2004).

Several studies have described the improvement of serodiagnostic assays by using purified antigens, such as the cationic fraction of SEA (CEF6) (Doenhoff et al., 1993). The use of purified and recombinant antigens have been proposed, which might perform better than crude extracts and provide additional information relating to duration of infection, distinctive pathology or protective immunity (Doenhoff et al., 2004). Recently, assays based on recombinant peptide antigens such as rSj26GST, rSj23HD, rSj32 and Sj31-b have been developed for immunodiagnosis in China (Zhu, 2005). In the present study, rSjc26GST was first used for the diagnosis of schistosomiasis japonica among water buffalo (*Bos buffelus*) in endemic area of China and non-endemic area of Taiwan.

Glutathione-*S*-transferases (GSTs) are a family of enzymes that play an important role in the detoxification of xenobiotics and function as intracellular binding proteins (Mannervik, 1985; Precious and Barrett, 1989). These enzymes are distributed on the surface and gut of schistosomula, adult worms and eggs (Trottein et al., 1990; Liu et al., 1993). GSTs from schistosome have been shown to exist as 26 and 28 kDa molecules, each consisting of several isoforms (Trottein et al., 1992; Tiu et al., 1988). The 28 and 26 kDa GST of *Schistosoma japonicum* have been cloned, expressed and well characterized (Henkle et al., 1990; Taylor et al., 1998). It had been shown that immunization of mice, pigs and buffaloes with recombinant Sj26GST (reSjc26GST) was effective in reducing worm burden, female fecundity and egg viability after experimental infection with Chinese *S. japonicum* (S. Liu et al., 1995; S.X. Liu et al., 1995; Shuxian et al., 1997). These studies also showed that rSjc26GST has satisfactory immunogenicity and antigenicity for diagnosis.

We reported that Sjc26GST was capable of inducing effective immunity in mice following challenge infection with *S. japonicum* (Lee et al., 1996, 1998). In the endemic areas of China, bovines such as buffalo are the most important reservoir for schistosome and are considered the major source for human schistiosomiasis (Wang et al., 1998; Davis et al., 2002). Highly sensitive and specific antibody-detecting techniques for diagnosis of schistosomiasis in buffalo are especially required in national disease surveillance programs to eradicate the disease (Ross et al., 2001).

In the present study we not only confirmed the cut-off value of reSjc26GST for immunodiagnosis of bovine schistosomiasis, but we also developed a rapid and economic test kit for use in schistosomiasis prevention. The use of GST–ELISA for diagnosing bovine schistosomiasis is important because its characteristics would make it an important tool for seroepidemiological screening of buffalo sera from different provinces of China, which in turn would contribute to the control of this disease.

2. Materials and methods

2.1. Recombinant Sjc26GST

Sjc26GST was prepared using a modified version of previously described protocols (Trottein et al., 1990; S. Liu et al., 1995). Briefly, total RNA from 100 adult Chinese *S. japonicum* were isolated by using TRIzol reagent (Invitrogen, Carlsbad, USA). A cDNA fragment encoding the 26 kDa GST of Chinese *S. japonicum* was obtained by *in vitro* reverse transcription of the total RNA using MuLV reverse transcriptase (Roche) and amplification by a standard PCR protocol using 2 primers. The forward primer contained a flanking BamHI site (5′-CGG GAT CCC GTC ATG TCC CCT ATA CTA GGT TAT-3′) and the reverse primer contained a KpnI restriction site (5′-GGG GTA CCC CTT TAT TTT GGA GGA TGG TCG CCA-3′) and ProTaq DNA polymerase (Protech). The two primers were used according to the published nucleotide sequence of the cDNA encoding the Sj26 GST of Philippine *S. juponicum* (GenBank accession no. M26914). The PCR-amplified Sjc26cDNA was sequenced using the standard procedure and an ABI PRISM 337-96 DNA Sequencer. The amplified cDNA was cloned into the expression vector pQE31 (Qiagen Inc., Hilden, Germany) and expressed in the *Escherichia coli* host strain M15[pREP4] (Qiagen, Hilden, Germany). Purified reSjc26GST was obtained by loading a 25 g cell lysate on His-bind purification kits with Ni-charged His-bind resin (EMD Biosciences Inc., Darmstadt, Germany). ReSjc26GST was eluted and dialyzed using CelluSep® membrane (Membrane Filtration Products Inc.) against 10 mM PBS at 4 °C

overnight and stored at −80 °C within 1 week before use.

2.2. Serum samples

In collaboration with the Chinese Center for Disease Control and Prevention (China CDC) and Hunan Institute of Parasite Disease, China, 530 serum samples of water buffaloes in the *S. japonicum* endemic area of Hualong County, Hunan Province were surveyed. Among these samples, 130 were infected with *S. japonicum* with eggs detected in their feces. We selected 33 sera of buffaloes from the endemic area without *S. japonicum* eggs found during fecal examination to be assigned to the egg-negative control group. In addition, these 33 buffaloes were checked regularly to confirm their feces remained egg-negative during those 2 weeks. All fecal samples were examined by the miracidium hatching method (see below). Twenty-six sera of uninfected buffaloes in Taiwan were also included as control group in non-endemic area of schistosomiasis.

2.3. Egg-examination and egg-hatching

The miracidium hatching method was used to check the infection of *S. japonicum* in buffaloes (as described above), as follows. Two grams of feces were homogenized in distilled water and then filtered over double layer gauze to remove larger detritus, repeating the filtration until a clear filtrate was obtained. This suspension was then transferred to a volumetric flask and exposed to light. This way the moving miracidium in the supernatant could be checked by means of a magnifying glass.

2.4. Preparation of buffalo serum

A total of 15–20 ml venous blood was taken from the ears of each buffalo. The samples were centrifuged twice (500 × *g*, at 4 °C, for 10 min) for sera preparation and sodium azide was added to give a 0.1% solution. Sera were then stored frozen at −80 °C. The anti-reSjc26GST antibodies in sera were detected by GST–ELISA and GST-immunoblotting.

2.5. GST–ELISA

Capability of the antigen to detect specific antibodies in the bovine sera of different groups was monitored by the enzyme-linked immunosorbent assay (ELISA). Five micrograms per millilitre reSjc26GST protein was diluted with a coating buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$, 0.85% NaCl, pH 7.4). The diluted proteins were added to the wells of microtiter plates in 100 μl aliquots. These plates were incubated overnight at 4 °C and then blocked with 0.5% non-fat skim milk in PBS for 30 min at 37 °C. After removing the blocking solution, 1:80 diluted sera was added into wells and the plates were incubated for 2 h at 37 °C. After four washes with PBS containing 0.05% Tween-20, 1:2000 diluted goat-anti-bovine IgG (The Binding Site, Birmingham, UK) conjugated with affinity purified horseradish peroxidase (HRP) were added into each well of plates. These plates were incubated for 30 min at 37 °C, washed five times and 100 μl of ABTS peroxidase substrate solution (Zymed, S. San Francisco, US) containing hydrogen peroxide (H_2O_2) was added to each well. The optical density of each well was read with 405 nm after incubating at room temperature (RT) for 30 min.

2.6. GST-immunoblotting

Western blotting was carried out to confirm the specificity of reSjc26GST as recognized by antibodies in the bovine sera. Aliquots of 100 μg/ml proteins resolved by 13.5% homologous SDS-PAGE were electrophoretically transferred into a polyvinylidene difluoride (PVDF, MSI, Westborough, US) membrane. Strips were cut and blocked with 5% non-fat skim milk in PBS for 30 min at 37 °C and then washed. Serum sample collected from different infected groups was added to each strip and incubated at 37 °C for 60 min. These strips were washed and incubated with HRP-conjugated goat anti-bovine IgG (The Binding Site, Birmingham, UK) at 37° for 35 min. After washing, color development was initiated by adding tetramethyl benzidine (TMB) solution (Zymed, S. San Francisco, US) and stopped by adding ddH_2O.

2.7. Statistical analysis

Statistical differences between groups were determined using a Student's *t*-test and expressed as *P*-values. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Purification of reSjc26GST

The sequences of both the nucleotide obtained from reverse transcription and amino acids expressed in *E. coli* system were 100% identical with those of the

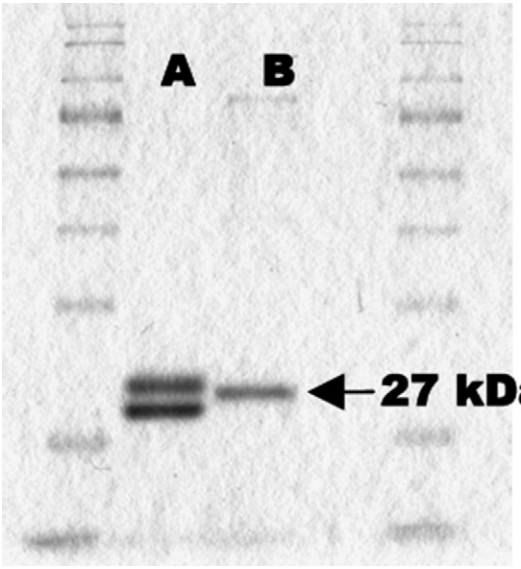

Fig. 1. SDS-PAGE of purified recombinant Sjc26GST in a 13% polyacrylamide gel stained with Coomassie blue. (A) Native GST purified from adult worms; (B) recombinant Sjc26GST. The lanes on the left and right show the protein size ladder.

Chinese strain (GenBank accession no. BU711548). The recombinant Sjc26GST was expressed in M15[pREP4] *E. coli* cloned using amplified cDNA and cultured in LB medium. The expressed protein was induced by 1 mM IPTG and purified by a Hisbind resin affinity column (EMD Biosciences) at a final concentration of 2.5 mg/ml. To characterize the properties of recombinant Sjc26GST, SDS-PAGE and immunoblotting assays were performed. As shown in Fig. 1, a single band approximately 27 kDa of reSjc26GST (lane B) was identified and the size was in-between native GST and its isomer (lane A). There were no significant differences in immunoblotting assay by mouse antiserum between reSjc26GST and native Sjc26GST (data not shown).

3.2. Immunodiagnostic assays by ELISA

To evaluate whether reSjc26GST could be a suitable antigen for diagnosis of bovine schistosoma infection, the purified proteins were tested using an ELISA. The optimal conditions for ELISA assay using the recombinant GST were determined by a serial of titration experiments. The concentration of reSjc26GST at 5 μg/ml and the serum dilution factor of 1:80 were used for this study. As shown in Table 1, the antibody levels of anti-reSjc26GST for bovine, either with or without ova of schistosoma in the endemic area, were significantly higher than those in the non-endemic area ($P < 0.05$). The mean OD value of specific IgG antibodies from egg-positive buffaloes was 3.37-fold higher than that of egg-negative buffaloes from the non-endemic area. The data also showed the mean OD value for the egg-negative group in the endemic area was 1.69 times of that of the same group in the non-endemic area. The mean OD ± standard deviation (S.D.) values of non-endemic area was 0.269 ± 0.076, which resulted in a cut-off value of 0.497. The cut-off value was established based on the mean absorbance of negative control sera from the non-endemic group plus three standard deviations. By comparison with the cut-off value, none of the samples from egg-positive buffaloes had OD lower than the cut-off, giving a 100% positive rate, whereas 10 of 33 samples from egg-negative buffaloes had an OD value above the cut-off, giving a 30.3% positive rate for this group.

The distribution of absorption values for all test samples from the endemic and non-endemic areas are shown in Fig. 2. All of the OD values of the ova-positive buffaloes within the endemic area were above the cut-off value. Thirty-three samples without ova, detected from the same area, could be separated into two parts by mean OD values. Among them, 10 with higher OD than the cut-off value and located in the upper part, whereas the other 23 had OD lower than the cut-off value and under the mean OD value. The values of OD for bovine

Table 1
Results of ELISA assay using recombinant Sjc26GST for antibodies of buffaloes in the endemic and the non-endemic area

Bovine groups	Number tested	Mean (OD ± S.D.)	Positivity rate (%)[a]
Endemic area			
Egg-positive sera	130	0.907 ± 0.125*	100
Egg-negative sera	33	0.455 ± 0.254*	30.3
Non-endemic area			
Egg-negative sera	26	0.269 ± 0.076[b]	

Student's *t*-test compared to non-endemic control; *$P < 0.001$.

[a] Positivity rates were determined by percentage of number of tested sample with the OD values higher than cut-off value.

[b] Cut-off value (optical densities) was 0.497, determined with mean +3 S.D.

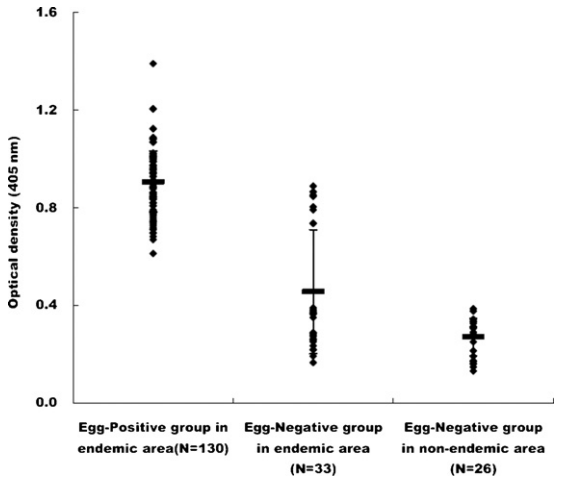

Fig. 2. Distribution of optical density for 189 test samples. Each point represents the mean of triplicate values for the same sera. The horizontal black lane represents the mean of total values on that group.

in the non-endemic area were all bellow the cut-off value.

3.3. Immunoblot assays

The immunospecificity of sera from buffaloes of different groups was further checked by Western blotting with reSjc26GST protein. The results showed that reSjc26GST protein was clearly and significantly recognized by the sera of egg-positive buffaloes, while sera of egg-negative buffaloes did not. The reactivity of reSjc26GST with sera from the egg-negative group in endemic area was very weak and absent in the group from the non-endemic area (Fig. 3). This data indicated a specific antibody response against reSjc26GST.

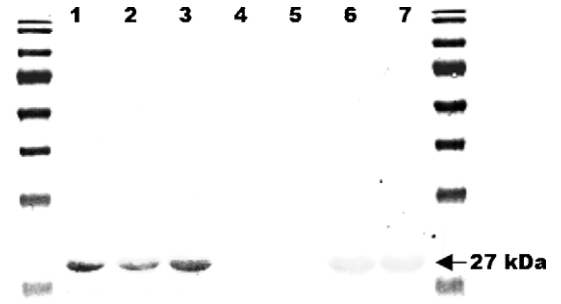

Fig. 3. Western blotting analyses of reSjc26GST with bovine sera from egg-positive group and egg-negative group in endemic area. (1–3) Three individual bovine sera from egg-positive group; (4 and 5) two individual bovine sera from egg-negative group in non-endemic area; (6 and 7) two individual bovine sera from egg-negative group in endemic area. Standard protein size ladder was shown to the left and right with molecular weights as 24,000, 33,000, 40,000, 55,000, 72,000, 100,000, 130,000, and 170,000 D (lane M).

4. Discussion

Schistosomiasis japonica is a typical zoonosis. Domestic animals, especially bovines and pigs, play an important role in the transmission and epidemiology of this parasitic disease in China (Henkle et al., 1990; Davis et al., 2002). In order to develop a rapid and simple immunodiagnostic assay for schistosomiasis japonica, an ELISA test was performed, so that antibodies in the sera of buffalo could be detected.

In this study, recombinant GST purified from soluble fractions of *E. coli* extracts was recognized by sera from C57BL/6 mice infected with *S. japonicum* using both ELISA and Western blot analysis. Furthermore, reSjc26GST was also investigated and used in other studies for vaccine development on mice, pigs and buffaloes (S. Liu et al., 1995; S.X. Liu et al., 1995; Shuxian et al., 1997; Wu et al., 2004). It may be feasible to develop reSjc26GST as kits that block the transmission among reservoir hosts which could be expected to have a major impact in the prevention of transmission between reservoir hosts and humans (McManus, 2000; Ross et al., 2001). The results of ELISA tests revealed that mean OD values of IgG antibodies of egg-positive buffaloes are higher than egg-negative groups and both of egg-positive and egg-negative groups in endemic area are higher than group in non-endemic area. The positivity rates were as high as 100 and 30.3% of the cut-off values in the endemic and the non-endemic control groups, respectively. This is indicated as an obvious antigen-specific IgG antibody response to reSjc26GST which can not only clearly judge the egg-examined-positive bovine sera from egg-examined-negative sera, but also can discriminate between sera of endemic and non-endemic areas. The level of anti-Sjc26GST antibody in immunized mice was significantly higher than those in control mice at 6 weeks post-challenge infection (S. Liu et al., 1995). Anti-Sjc26GST antibodies were similarly produced in the immunized buffaloes (Shuxian et al., 1997). The data demonstrated that the reSjc26GST expressed in *E. coli* can be useful as a good diagnostic reagent for detection of antibodies against *S. japonicum* in buffaloes.

It is worthy of noting that there are 10 OD values of sera significantly higher than others and inflate the mean OD value of endemic egg-negative buffaloes. The 10 sera were clustered together so that samples in egg-negative buffaloes of endemic area were divided into two masses instead of showing a normal distribution. If these 10 sera were to be excluded, the mean OD from this group would be decreased almost to the level of the non-endemic area. Many treatments could therefore be

sufficiently effective to cause excreted egg counts to decrease below easily detectable levels, but still leave a residue of infection that is sufficient to maintain antibody positivity (Doenhoff, 1998; Doenhoff et al., 2004). The higher OD displayed by these 10 sera may due to these buffaloes having been infected with schistosoma previously but have been cleared at this time, so that led to the false-positive anti-GST antibodies reaction in serum of buffalo. It is suggested that using reSjc26GST could further select out those infected buffaloes, not found by egg-examination methods. The high proportion of antibody-positive individuals with an egg-negative test, who give the ostensibly poor specificity, is often in the result of samples from endemic areas. This could be largely attributable to the lack of sensitivity found with conventional methods (Whitty et al., 2000). Better correlations with infection intensity could be obtained through the use of more purified antigens and/or assaying for particular immunoglobulin isotypes (Mott and Dixon, 1982; Grogan et al., 1996). Further studies will need to be performed in order to confirm the ELISA using reSjc26GST by comparing the results with commercially available diagnosis kits.

Several investigators have reported on the usefulness of Western blotting (WB) analysis for differentiating between acute and chronic schistosoma infections (Valli et al., 1999), different schistosoma species (Tsang et al., 1984) and for recognizing the isotype in infected children (Noya et al., 1995), but no industrial kit was available. The specificity of these antibodies against bovine schistosomiasis japonicum was further confirmed by the WB analysis using reSjc26GST. Positive antiserum recognized an obvious 27 kDa antigen on WB, but the sera of negative groups reacted either weakly or not at all. The result indicated that GST-immunoblotting has a potential to be developed for use in specific assays and shows good reactivity with bovine schistosoma species.

The present study demonstrates the value of reSjcGST26 in ELISA and WB analysis for integrating immunodiagnostic tools and makes it a candidate protein for the immunological diagnosis of schistosomiasis. The application of reSjcGST26 in immunodiagnosis can be an important tool for utility in field studies, especially in low endemic areas for schistosomiasis. Developing reSjc26GST with potential diagnostic values will be useful for surveillance of schistosomiasis and allow for early treatment which is critically important role in controlling the spread of this parasitic disease in domestic animals and humans.

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