

## Preparation and characterization of transfer factor specific to *Staphylococcus aureus in vitro*

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The present study explored the preparation and characterization of the transfer factor specific to *Staphylococcus aureus* (SA-STF) with *in vitro* method. The optimal culture conditions for the preparation of SA-STF were: SA 0.0002 µg/mL, PHA (phytohemagglutinin) 100 µg/mL, adding immunogen at 6<sup>th</sup> h, and stopping culture at 96<sup>th</sup> h. The physicochemical properties of *in vitro* prepared SA-STF met the standards of Chinese Pharmacopoeia (2005 edn). The phagocytosis and sterilization rates of STF group were 70.9±12.4 and 62.1±12.2%, respectively and both were significantly higher compared to those of NTF (non-specific) group (P>0.05). Further, marked nodules were observed on the skin of STF group of mice, while these were absent in NTF and control groups. The survival rate of STF group was significant higher compared to the values of other three groups (P<0.05). In conclusion, SA-STF was successfully prepared with *in vitro* method, and it could be a potential biological for the prevention or adjunct therapy of the diseases related to SA.

**Keywords:** Immune activity, physicochemical property, specific transfer factor, *Staphylococcus aureus*

### Introduction

*Staphylococcus aureus* (SA) is a common pathogen responsible for health-care-associated and community acquired infections. It is the etiological factor of wide spectrum of infections and serious threat to human health. For instance, SA has been reported as the leading cause of bacterial infections involving bloodstream, lower respiratory tract, skin and soft tissue in many countries<sup>1</sup>. Therapeutic problems are caused by resistance of SA to many antibiotics, especially to methicillin [methicillin-resistant *S. aureus* (MRSA)].

Recently, there has been an increase in the burden of antibiotic-resistant SA within the community setting<sup>2,3</sup>. Besides, SA is also the most abundant cause of hospital-associated infections. Correspondingly, this large number of infections creates a significant financial burden, making detrimental impacts on public healthy system around the world. With approx half a million people acquiring Staphylococcal infections in the USA per year, the cost of SA healthcare-associated infections was estimated to exceed 14 billion dollars

in 2003<sup>4,5</sup>. To combat the situation, there is an urgent need for the development of drugs, an alternative to antibiotic therapeutics or biologics, as adjuvant therapy.

It is known that specific transfer factor (STF) is an immunoregulatory agent consists of a number of amino acid residues and nucleotides. The key biological activity of STF is to transfer immunity pertaining to certain antigen from one individual to another. Meantime, it has the unique feature of having no immunogenicity and genus specificity. In another words, the STF prepared by one genus of animals has the ability to transfer immunological activity to another genera of animals without causing hypersensitivity. Presently, several STFs have been successfully developed and even been used clinically<sup>6-8</sup>. However, no literature has been reported till date about the preparation of transfer factor specific to SA (SA-STF) *in vitro*.

Based on the previous work on transfer factors<sup>9-11</sup>, the present study explores the preparation of SA-STF with the *in vitro* method. Through the study, it is hoped that the outcome could provide valuable information for the development of biologics for prevention or adjuvant therapy of the diseases related to SA.

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## Materials and Methods

### Animals, Instruments and Reagents

Rabbits, cony pigs and all the experimental mice were procured from the Experimental Animal Centre of Chongqing Medical University, China. All the animals were utilized as per the rules related to experimental animals in China. The instruments utilized for the study were ultraspectrophotometer (model number 6405, JENWAY), tissue disintegrator (Fuhua of Jiangsu), Elx-800 microplate-reader (BioTek). The reagents used included phytohemagglutinin (PHA), methy thiazolyl tetrazolium (MTT), and D-ribose. All the reagents were purchased from Sigma Ltd.

### Preparation of Immunogen

Standard SA strain (ATCC 27660) was identified and cultured at the normal conditions. About 10 mL formaldehyde solution (0.4%) was added into each culture medium and kept at 4°C for 5 d. If the sterility test was negative, SA colonies were collected with the L-shaped glass rod and transferred into sterilized centrifuge tubes. The bacterium was then washed according the following procedure: mixed with 5 mL sterile saline, centrifuge at 3000 rpm for 20 min and then discard the supernatant. The operation was repeated five times. Finally, the bacterium was collected and dried at 37°C in an oven for 1 h.

### Preparation of Spleen Cell Suspension

Adult, male rabbits (12 in number), each weighing 2-3 kg, were sacrificed under a general anesthetic. Then spleens were collected, cut into pieces and ground through stainless steel sieve of 200-mesh. Single-cell clusters were taken and used to prepare the suspension with a concentration of  $5 \times 10^6$  spleen-cells/mL according to a reference<sup>9</sup>.

### Determination of Conditions for Immunizing Spleen Cell *In Vitro*

#### Best Concentration of SA

The dried SA was diluted using RPIM 1640 medium and different concentrations (0.2, 0.02, 0.002, 0.0002 & 0.00002 µg/mL) of SA were prepared. Eighteen culture bottles were divided into six groups and each one was filled with 1 mL spleen cell suspension ( $5 \times 10^6$  cells/mL) and 0.5 mL PHA (100 µg/mL). Then 1 mL of different concentrations of SA suspension was added to 1<sup>st</sup> to 5<sup>th</sup> group; while 1 mL of RPIM medium without SA was added to the 6<sup>th</sup> group as control. The cultural bottles were then

cultured for 5 d at 37°C in 5% CO<sub>2</sub> atmosphere. Since it has been shown that STF gives a special absorbance peak at 260 nm (OD<sub>260</sub>)<sup>9-13</sup>, the best concentration of SA was also confirmed by taking OD<sub>260</sub> at different concentrations of SA.

#### Optimal Quantity of PHA

Nine culture bottles were divided into three groups, and filled with 1 mL suspension of spleen cells ( $5 \times 10^6$  cells/mL) and 1 mL best concentration of SA. From 1<sup>st</sup> to 3<sup>rd</sup> group, the bottles were added with 1 mL gradient concentrations of PHA (50, 100 & 150 µg/mL) and bottles were cultured for 5 d at 37°C in 5% CO<sub>2</sub> atmosphere.

#### Most Appropriate Time to Add Immunogen

Eighteen culture bottles were divided into six groups; each one was filled with 1 mL PHA (optimal concentration) and 1 mL spleen cell suspension ( $5 \times 10^6$  cells/mL). Simultaneously, 1 mL SA (optimal concentration) was added into the bottles of the first group; while at 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup> and 12<sup>th</sup> h, 1 mL SA (optimal concentration) was added into the bottles of second to sixth group. Then bottles of all groups were cultured for 5 d at 37°C in 5% CO<sub>2</sub> atmosphere.

#### Proper Culture Time

The spleen cell suspension ( $5 \times 10^6$  cells/mL), PHA (optimal concentration) and the most appropriate quantity of SA were mixed and cultured at 37°C in 5% CO<sub>2</sub> atmosphere. Then culture was stopped at the 48<sup>th</sup>, 72<sup>nd</sup>, 96<sup>th</sup>, 120<sup>th</sup> and 144<sup>th</sup> h, respectively.

#### Preparation of SA-STF under Best Conditions

Under the optimum conditions (as detected from the above experiments), spleen cells, PHA and SA were mixed and cultured at 37°C in 5% CO<sub>2</sub> atmosphere. Finally, the cultured cells were collected to prepare SA-STF.

SA-STF was prepared according to the procedure reported in the earlier study<sup>11</sup>. Since the key components of STF were polypeptide and ribose<sup>12,13</sup>, the concentrations of polypeptide and ribose were detected to verify the quality of SA-STF. The quality of SA-STF was routinely detected by taking OD values at the wavelength of 260 nm (OD<sub>260</sub>) and 280 nm (OD<sub>280</sub>) simultaneously.

#### Preparation of Nonspecific TF (NTF)

The spleen of adult, healthy rabbit was collected and directly used to prepare NTF with the same technique as used in case of SA-STF.

**General Physicochemical Properties of SA-STF**

In accordance with the standards for biological products published in Chinese Pharmacopoeia (2005 edn)<sup>14</sup>, the physicochemical properties of SA-STF were measured. The content included multi-wavelength scanning, colour, pH, absorption peak, content of polypeptides and ribose, sterility test, pyrogen test and safety test.

**Immunological Activity of STF**

*Phagocytosis and Sterilization tests*

Sixty Kunming (KM) mice were divided into four groups randomly: STF, NTF, normal saline (NS) and control groups. Phagocytosis and sterilization tests were carried out according to a reference<sup>15</sup>.

*Delayed-type Hypersensitivity (DTH) Test*

Thirty KM mice were randomly divided into STF, NTF and NS groups. Referring Maddisons' reports<sup>16</sup>, DTH test was performed.

*Immune Protection Test*

First, the median lethal dose (LD50) of SA to KM mice was determined using Karber test<sup>17</sup>. Then 125 KM mice were randomly divided into four groups (STF, NTF, NS & control) and immune protection test of each group was carried out according to the previous study<sup>11</sup>.

**Statistical Analysis**

All data were analyzed with SPSS15.0 software. Quantitative data was presented as  $\bar{x} \pm s$  (mean $\pm$ SD). Single elemental data analysis was performed using chi square test, whereas t-test was used for comparing paired sample sets. Statistical significance was at  $P \leq 0.05$ .

**Results**

**Optimum Conditions for SA-STF Preparation *In Vitro***

The optimum conditions for preparing SA-STF were found to be as follows: SA 0.0002  $\mu\text{g/mL}$ , PHA 100  $\mu\text{g/mL}$ , adding immunogen at 6<sup>th</sup> h, and stopping culture at 96<sup>th</sup> h (Fig. 1). In the test for determining the best concentration of SA, the concentrations of polypeptide and ribose of SA-STF were found to be in accordance to its OD<sub>260</sub> values (Fig. 2).

**Physicochemical Properties of SA-STF**

SA-STF had a maximum absorption peak at 260 $\pm$ 4 nm (Fig. 3). The ratio of OD<sub>260</sub>/OD<sub>280</sub> was 2.04 $\pm$ 0.19. Its pH value was 7.1 $\pm$ 0.4. The concentration of polypeptide and ribose was 2.26 $\pm$ 0.29 mg/mL and 0.684 $\pm$ 0.094 mg/mL,

respectively. The results of protein qualitative test, sterility test, pyrogen test and safety test were all negative (Table 1). All the physicochemical properties were in accordance with the standards of Chinese Pharmacopoeia (2005 edn).

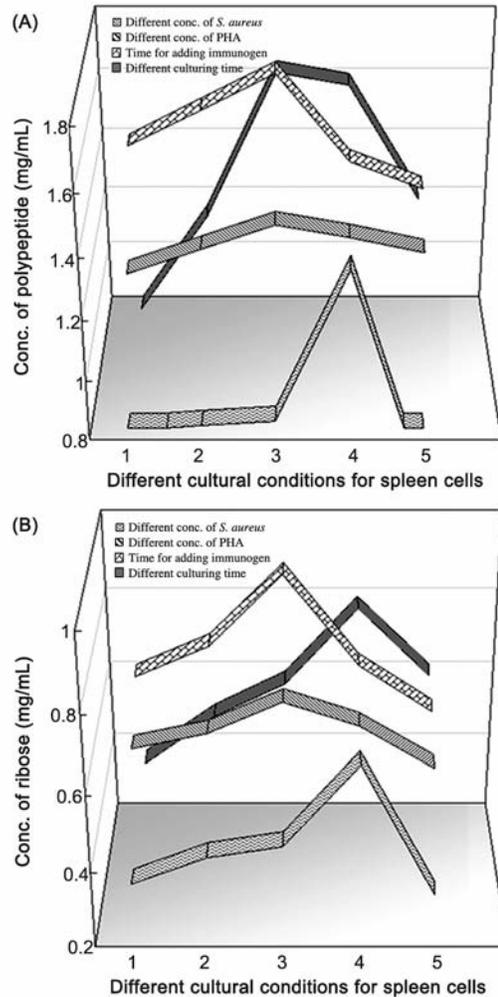


Fig. 1 (A & B)—Determination of the best conditions for culture of spleen cells for SA-STF production with optimal concentrations of polypeptide (A) and ribose (B).

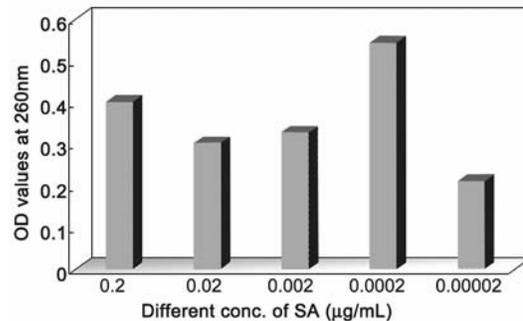


Fig. 2—The OD values of SA-STF prepared with different concentrations of SA at 260 nm wavelength.

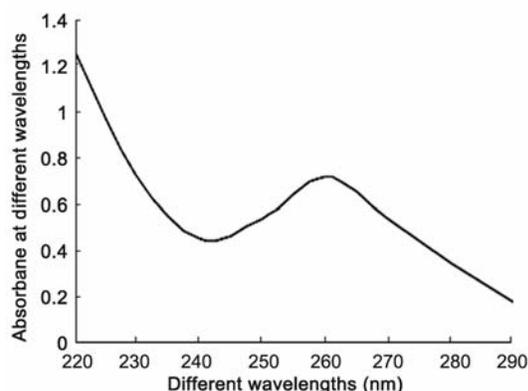


Fig. 3—The scanning curve of SA-STF drawn by the absorbance at different wavelength.

Table 1—Physicochemical properties of SA-STF prepared *in vitro*

Items	SA-STF	NTF
Colour	Colourless	Colourless
Protein qualitative test	Negative	Negative
Sterile test	Negative	Negative
Safety test	Negative*	Negative*
Pyrogen test	Negative*	Negative*
pH	7.1±0.4	6.9±0.4
OD <sub>260</sub> /OD <sub>280</sub>	2.04±0.19	1.94±0.27
Maximum absorbance	260±4	260±4
Conc. of polypeptide (mg/mL)	2.26±0.29	2.13±0.24
Conc. of ribose (mg/mL)	0.684±0.094	0.669±0.078

\*It qualifies the Chinese Pharmacopoeia 2005 edn

### Immunological Activity of SA-STF

#### Phagocytosis and Sterilization Test

The phagocytosis and sterilization rates of STF and NTF groups were higher as compared to those of NS and control groups ( $P < 0.05$ ). However, no significant difference was observed for the two indices between STF and NTF groups ( $P > 0.05$ ) (Tables 2 & 3).

#### Skin-delayed Type Hypersensitivity Test

In STF group, the mice skin was inflamed at the injection site and marked nodules were formed; while no nodules were observed in case of both NTF and control groups.

#### Immune Protection Test

LD<sub>50</sub> of SA to KM mice was determined as  $4.6 \times 10^9$  SA/0.2 mL (Table 4). After being injected twice LD<sub>50</sub> dose of SA, the survival rate of each group was observed. As shown in Table 5, the survival rate of STF-1 group was significantly higher compared to those of the other three groups ( $P < 0.05$ ); while differences in survival rates of NTF, NS and Control groups were not significant (all  $P > 0.05$ ).

Table 2—The results of phagocytosis test

Group	Phagocytosis rate (%)	P value
STF	70.9±12.4	0.029 <sup>a</sup>
NTF	64.1±10.6	0.074 <sup>b</sup>
NS	46.3±11.7	0.035 <sup>c</sup>
Control	44.8±10.3	0.022 <sup>d</sup>

<sup>a</sup>Comparison with NS group ; <sup>b</sup>Comparison with STF group; <sup>c</sup>Comparison with NTF group; <sup>d</sup>Comparison with STF group

Table 3—The results of sterilization test

Groups	Sterilization rate (%)	P value
STF	62.1±12.2	0.031 <sup>a</sup>
NTF	58.4±11.7	0.069 <sup>b</sup>
NS	42.6±10.9	0.038 <sup>c</sup>
Control	40.9±11.2	0.028 <sup>d</sup>

<sup>a</sup>Comparison with NS group ; <sup>b</sup>Comparison with STF group; <sup>c</sup>Comparison with NTF group; <sup>d</sup>Comparison with STF group

Table 4—The results of Karber assay

Dilution	No. of live mouse	No. of dead mouse
10 <sup>-2</sup>	10	10
10 <sup>-3</sup>	10	10
10 <sup>-4</sup>	10	6
10 <sup>-5</sup>	10	0
10 <sup>-6</sup>	10	0
Total	50	26
LD <sub>50</sub>	$4.6 \times 10^9$ SA/0.2 mL	

Table 5—The survival rate of KM mice in different groups

Items	STF	NTF	NS	Control
Total live mouse	25	25	25	25
No. of mouse survived	18	9	9	8
Survival rate (%)	72 <sup>a</sup>	36 <sup>b</sup>	36 <sup>b</sup>	32 <sup>b</sup>

<sup>a</sup>Survival rate of STF group is significantly higher compared to those of the other three groups ( $P < 0.05$ ); <sup>b</sup>Differences between NTF, NS and control are not significant ( $P > 0.05$ )

## Discussion

TF was discovered in 1940s and had been extensively studied in the past 70 years. It has been known that TF, especially STF, can transfer cell-mediated immunity from an immune donor to a non-immune recipient. Recently, it was proved that STF played a role in immune regulation to infections<sup>16</sup>, which including viruses, bacteria, and fungal organisms<sup>18-20</sup>. Traditionally, STF was most frequently prepared with *in vivo* method, while only recently *in vitro* method was developed to meet

such a purpose<sup>21</sup>. In the present study, SA-STF was prepared with *in vitro* method with the hope that it would provide methodological reference for *in vitro* preparation of STF and would serve a biological product for adjuvant treatment of SA infection.

In the present study, optimum conditions for preparing SA-STF were determined through the detection of concentrations of polypeptide and ribose of SA-STF. Interestingly, it was found that the OD value, *i.e.*, the concentrations of polypeptide and ribose of SA-STF, were the highest when the concentration of SA was 0.0002 µg/mL. This indicated that the results of the both methods were similar, since measuring OD<sub>260</sub> value was more simple, quick and economical, it could replace detecting concentrations of polypeptide and ribose, and be used to identify the quality of STF.

The physicochemical properties, *viz.*, colour, absorption peak and content of polypeptide and ribose, of SA-STF developed *in vitro* was found similar to other STFs<sup>9,13,22,23</sup>. Thus, the *in vitro* technique for preparing SA-STF is feasible and successful as far as the physicochemical properties are concerned.

In case of phagocytosis and sterilization tests, the phagocytosis rate of STF group was significantly higher compared to that of NS and control groups. This indicated that SA-STF could improve the phagocytic function of phagocytes. However, the immune activity of SA-STF was non-specific because no difference was observed between the phagocytosis rate of SA-STF group and that of NTF group. In the sterilization test, both biological products could enhance the bactericidal action of phagocytes, and no difference was observed between the sterilization rates of both the groups. Hence, phagocytosis and sterilization test were the tests suited to detect non-specific immune activity of STF.

DTH test is a traditional method to detect immune activity of TF<sup>24,25</sup>. In the present study, marked nodules were formed at the injection sites of the STF group's mice skin, while no nodule were observed in the other groups. This showed that SA-STF had the activity of immune transfer specific to SA, and was in accordance with other studies related to STF<sup>20,26</sup>.

As observed in the immune protection test, the survival rate of KM mice in STF group was significantly higher compared to those of the other groups (NTF, NS & control). This signifies that SA-STF prepared with *in vitro* method could

effectively transfer the immune ability specific to SA and could trigger the immune system in the mice to protect against the pathogenesis of SA.

In conclusion, SA-STF was successfully prepared with *in vitro* method and its physicochemical properties matched well with other STFs. SA-STF could be able to transfer immune activity specific to SA and could protect the individuals against the pathogenesis of SA. Therefore, SA-STF could be taken as a potential biological for the prevention or adjuvant therapy of the diseases relates to SA infection.

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