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A novel peptide screened by phage display can mimic TRAP antigen epitope against *Staphylococcus aureus* infections

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Running titile: A novel peptide screened by phage display can mimic TRAP antigen epitope against *S.aureus* infection

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Staphylococcus aureus **is a major human pathogen. Pathogenic effects are largely due to production of bacterial toxins, whose synthesis is controlled by an mRNA molecule termed RNAIII. The *S. aureus* protein called RAP (RNAIII activating protein) is secreted and activates RNAIII production by inducing the phosphorylation of its target protein TRAP (target of RAP). Antibodies to TRAP have been shown to suppress exotoxin production by *S. aureus* in vitro, suggesting that TRAP may be a useful vaccine target site.**

Here we showed that a peptide TA21 was identified by screening a phage display library using anti-TRAP antibodies. Mice vaccinated with *E. coli* engineered to express TA21 on their surface (FTA21) were protected from *S. aureus* infections, using sepsis and cellulitis mice models. By sequence analysis, it was found that the TA21 is highly homologous to the C-terminal sequence of TRAP which is conserved among *S. aureus* and *S. epidermidis*, suggesting that peptide TA21 may be a useful broad vaccine to protect from infection caused by various staphylococcal strains.

Staphylococcus aureus is a Gram-positive bacterium that can cause many different types of infectious diseases, ranging from minor skin infection to life-threatening deep infections such as pneumonia, endocarditis, meningitis, postoperative wound infections, septicemia, and toxin shock syndrome (1). Over 500,000 nosocomial infections are reported per year in the USA. The emergence of drug resistance has made many antibiotics ineffective.

Many diseases caused by *S. aureus* have been associated with the toxins the bacteria produce (1). The ability of the bacteria to express toxic exomolecules is due to a complex gene regulatory system. Until now, two staphylococcal quorum sensing systems (SQS1&SQS2) have been described (2). SQS 1 is composed of RAP (3, 7) and its target molecule TRAP (2). RAP can induce the Histidine phosphorylation of TRAP. The phosphorylation of TRAP leads to the synthesis of SQS2 by an unknown mechanism (2,4). SQS 2 comprises the molecules encoded by *agr* (Agr A, B, C, D) (5). AgrD is a pro-peptide that yields an autoinducing peptide (AIP) which can induce the phosphorylation of AgrC to lead the production of RNAIII (5, 6). RNAIII upregulates the expression of *S. aureus* exotoxins. Mice vaccinated with RAP were shown to be protected from *S. aureus* infection (3). RAP binding peptides screened from a random phage-displayed peptide library can inhibit the *S. aureus* pathogenesis both *in vitro* and *in vivo* (8). Antibodies of TRAP are efficient suppressors of *agr*-regulated

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exotoxins produced by *S. aureus* (9). These results suggest that RAP and TRAP may be used as vaccines. Inhibiting the RAP/TRAP quorum sensing system with antibodies or peptide inhibitors has been shown to be highly effective (3, 10-12) and could serve as an alternative to antibiotic therapy. The anti-RAP and anti-TRAP therapeutic approach aims at converting the pathogenic bacteria to the non-pathogenic stage. As one of the cellular components of *S. aureus*, TRAP is highly conserved among staphylococci, like *S. aureus* and *S. epidermidis* (4). Therefore it is reasonable to assume that antibodies against TRAP could protect mice from *S. aureus* and *S. epidermidis* infections.

Peptide vaccines based on relevant epitopes of protective antigens, besides being cost-efficient to prepare, can be used to direct the immune response to cellular or humoral immunity by selection of specific T- and B-cell epitopes (13,14). Coupling of such peptides to carriers and/or the employment of appropriate adjuvants may help enhance the specific immune response (14). Peptide vaccines are attractive also because they may avoid toxic effects associated with the intact immunogen and could be designed to emphasize the immunogenic elements that may be hidden in the native antigen (14). Here we show the development of peptide-based vaccine to *Staphylococcus aureus.*

EXPERIMENTAL PROCEDURES

Bacterial strains, peptide phage display library and animals-Wild type *S. aureus* RN6390B and 04018 were our lab strains (8) grown in CY culture broth Ph.D^{TM} phage peptide library was purchased from New England BioLabs. The complexity of the library was 2.7×10^9 and the titer was 1.5×10^{13}/μl. *E. coli* ER2537 was used as the host strain for the phage library (New England BioLabs). *E. coli* BL21 was our lab strain. *E.coli* GI826 was purchased from Invitrogen Corporation. Immuocompetent hairless BALB/c mice (20-25g, outbred, male) and Balb/c mice (20-25g, outbred, male) were provided by Beijing Research Center of Animals.

Expression and purification of recombinant TRAPs (rTRAPs)-Two kinds of recombinant TRAP proteins were expressed in *E. coli* by using conventional methods. Briefly, *traP* genes (accession: AF202641 and AY248703) were amplified by PCR using the genome DNAs of *S. aureus* RN6390B and 04018 as templates with the same primers as follows: forward primer (p1 5'-GGAATTCCATATGAAGAAACTATATACA-3') and reverse primer (p2 5'-CCCAAGCTTCTATTCTTTTATTGGGTATAG-3'). The amplified gene was digested by *NdeI* and *HindIII* and inserted into the corresponding sites of vector pET-28a (Invitrogen). Induction of synthesis of recombinant protein was carried by the addition of IPTG. Purification of recombinant protein on a His-tag binding resin was carried out according to the manufacturer's instructions (Amasherm). The recombinant protein was eluted with 0.5M imidazole. To purify rTRAP without His tag, thrombin was used to digest the His tag. Thrombin was then inhibited and removed by benzamidine/Sepharose 4B according to the manufacturer's instructions. The digested His tag was removed by His-tag binding resin.

Production and purification of polyclonal anti-TRAP antibodies -New Zealand white female rabbits were first immunized by subcutaneous injection of 1ml of immunogens (0.1mg of rTRAP1 in PBS mixed with complete Freund`s adjuvant). Repeated immunizations were performed by injection of mixtures of rTRAP1 and incomplete Freund`s adjuvant on the 4th and 7th week. On the eighth week, the sera were collected and

analyzed by ELISA against injected recombinant proteins. Anti-TRAP antibodies were purified by applying the sera to an affinity column prepared by conjugating purified rTRAP1 to CNBr-activated sepharoseTM 4B resin (Amersham Pharmacia). Antibodies were eluted with 0.5M imidazole.

Selection of peptides binding to rTRAP 1 antibodies by phage display-The procedure of selection was in accordance with the protocol of Ph.D^{TM} phage peptide library kit (New England BioLabs). For each round of selection, phages (1×10^{11}) were applied to a 96-well plate pre-coated with anti-rTRAP1 antibodies (10μg/well). The level of specific phage enrichment was calculated by ratio of input and output as described (8). After three rounds of biopanning, positive phage clones were selected and their DNA were sequenced.

Enzyme-linked immunosorbent assay (ELISA)-The specific binding of positive phage clones to polyclonal anti-TRAP antibodies were tested by ELISA. Briefly, TRAP polyclonal antibodies were applied to 96 microtiter well plates (Nunc) (10μg/well) and incubated overnight at 4°C. Unbound TRAP antibodies were removed, and the wells were blocked with 3% BSA in PBS at 37°C for 1 h. Selected phage clones ($1x10^9$ /well) were added and incubated for 2 h at 37°C. Plates were then washed with washing buffer (PBS-0.05% Tween 20) 5 times for 3 min, and anti-phage M13 monoclonal antibody (1:1000) (Amersham Pharmacia) was added and incubated for 1 h at 37°C. After washing as above, the binding of anti-phage antibodies were detected using TMB (3, 3', 5, 5'-Tetramethyl Benzidine dihydrochloride) substrate (Sigma) and color intensity was determined spectrophotometrically at OD 450nm.

Competitive ELISA- the different doses of rTRAP1 protein with the same amount phage ($1x10^9$ /well) were added into each well of 96 microtiter well plates (Nunc) coated with TRAP antibodies (10μg/ml). The titer of the phage that bound was detected as above using M13 monoclonal antibody (1:1000).

TA6 and TA21 were displayed on bacterial surface-E. coli GI826 (F- lacIq ampC::Ptrp cI △fliC △motB *eda*::Tn*10*) was used to host and multiply recombinant plasmids (Invitrogen). pFliTrx7H is a derivative of pFliTrx (Invitrogen), containing unique *Nde* I and *Nco* I sites. The oligonucleotides coding for the structural gene of the epitopes of *traP* were synthesized with one *Cpo I* -overhang at its 5'-end, and one *Nco I* -overhang at its 3'-end. Recombinant DNA techniques were carried out according to a standard protocol. The insertions were verified by restriction analysis, and positive clones were subjected to DNA sequencing. *E. coli* GI826 carrying the recombinant plasmids were amplified and induced according to the manufacturer's instructions (Invitrogen).

Mouse immunization-Immuocompetent hairless Balb/c mice (20-25g, outbred, males) (8) and Balb/c mice (20-25g, outbred, males) were immunized intradermally with the *E. coli* GI826 cells with the epitopes of TRAP displayed on their surfaceWide type *E. coli* GI826 without displayed peptides on their surface were used as a control. Specifically, each mouse was injected with 200μl of *E. coli* cells (10^9 cells/ml). After 3 and 5 weeks, mice were intradermallly vaccinated with the same number of cells. On the sixth week, sera were colleted and analyzed by ELISA against TRAP protein (1μg/well) on 96 microtiter well plates (Nunc).

Detection of heat-resistant exotoxins screted by

S.aureus- Early exponential wild-type *S.aureus* cells (1ml, 10^8cells/ml) were cultured in CY broth for 6h at 37 ^oC. The cells were removed by centrifugation at 5000 × g, culture supernatant was filtered to remove all remaining cells. The supernatant was boiled for 10 min and centrifuged for 10 min at 14,000 × g. Madin Darby Bovine Kidney (MADK) cells (1x10^5cells/ml, 100µl/well) were seeded into 96 microtiter well plates (Nunc). After six hours, the supernatant (20µl/well) was added into the wells. The plates were incubated at 37 ^oC in a CO_2 incubator for another 24h. Cell viabilities were measured by standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Absorbance at a wavelength of 595 nm was measured to calculate the percentage of live cells (15, 16).

Protection from S. aureus-induced sepsis - Balb/c mice (20-25g, outbred, male) pre-immunized with *E. coli* GI826 expressing the epitopes of TRAP or with control cells, were injected by abdominal cavity with 100µl RN6390B cells (10^9 cells/µl). Mortality time of each mouse was recorded. Each group had 8 mice.

Protection from S. aureus infections in the murine cutaneous cellulites model-Wild type *S. aureus* RN6390B cells (1×10^8 cells/ml) were cultured at 37°C for 90 min in CY culture broth. Cytodex beads (Amersham Pharmacia) were added to the culture to a final concentration of 5mg/ml. The mixture of bacteria and Cytodex in a total volume of 200µl was injected subcutaneously into immunocompetent hairless Balb/c mice (n=8), which were pre-immunized with the *E. coli* GI826 cells expressing the epitopes of TRAP or with control cells. Seventy-two hours after injection, the size of the lesions were measured and calculated [area = $(\pi/2) \cdot (length) \cdot (width)$] (3).

Statistical analysis-Statistical analysis was performed using Student's t-Test by Microsoft Excel (Microsoft, WA). Significance was accepted when the P value was ≤ 0.05.

RESULTS

Anti-TRAP antibodies- The two recombinant TRAP proteins (rTRAP1 and rTRAP2) expressed in *E.coli* were purified by HiTrap™ Chelating column (Amersham) and the His tag was removed by thrombin. Recombinant protein rTRAP1 (accession: AF202641) was used as an antigen to prepare polyclonal antibodies in rabbits. Western blot analysis showed that anti-TRAP antibodies could specifically bind the rTRAP2 (accession: AY248703) (data not shown) and the wild type TRAP protein in different *S. aureus* strains (Fig 1).

Identification of epitopes of TRAP by phage display-The anti-rTRAP1 antibodies were purified by TRAP-affinity column. To select the peptide that can specifically bind to the anti-TRAP antibodies, a completely random peptide phage display library with peptides of twelve amino acids in length was employed. After three rounds of selections, about 100 folds enrichment (input/output) was achieved. Eleven phage clones with specific binding to the antibodies were obtained (Fig 2) and their DNAs were sequenced. Six different sequences were obtained and divided into two groups, group N and group Y. There was strong consensus sequence among each group (Fig 3). TA6 clone in group N and TA21 clone in group Y were chosen for further study.

Sequence alignment- As shown for *S. aureus* and *S. epidermidis* stains, TRAP protein is highly conserved in staphylococci (4). According to the similarity of sequences, TRAP could be divided into three groups named g1, g2 and g3 (Fig 4a). Comparing the sequences of the three groups of TRAP with the peptide sequences we selected, we found

that the peptide TA21 was highly similar to the C-terminal sequence (amino acid 156-167) of all the three groups of TRAP (Fig4b). There was no similarity between the three groups of TRAP and peptide TA6.

TA6 and TA21 displayed on bacterial surface-Since there are only 3-5 copies of PIII protein on phage M13, the titer of antibodies induced by phage-displayed peptides fused with PIII protein is expected to be low. Because it was reported that epitopes exposed on bacterial cell surface induced antibody response in a T-cell-independent manner, genes encoding for peptides TA6 and TA21 were cloned into the thioredoxin active site loop of pFliTrx vectors (Invitrogen). The peptides TA6 and TA21 were displayed separately on the surface of *E. coli* GI826 in a fusion form with the major bacterial flagellar protein (FliC) and thioredoxin (TrxA) induced by tryptophan (Fig 5a). The positive clones were sequenced and named FTA6 and FTA21. Western blot analysis showed that anti-TRAP antibodies could specifically bind both of the flagellin fusion peptides FTA6 and FTA21 (Fig 5b,5c).

Protection from S. aureus infection by Flagellin fusion peptides **-** Balb/c mice were immunized with FTA6 or FTA21 *E. coli* that expressed the peptides on their surfaces or control bacteria without peptide genes. After three vaccinations, the specific binding activity among antisera and TRAP proteins were tested by ELISA and Western blotting. Microtiter plates were coated with rTRAP proteins (1μg/well) and incubated with mice sera in a dilution of 1:500. Bound antibody was detected using goat anti mouse-horseradish peroxidase conjugated antibody was added and detected by using TMB substrate (Sigma).As shown in Fig 6, mice immunized with FTA6 or FTA21 had antibodies against rTRAP1 (accession AF202641) belonging to TRAP group g1 and rTRAP2 (accession: AY248703) belonging to TRAP group g2. The antisera induced by control bacteria could not recognize the two kinds of TRAP proteins. The difference between experimental and control groups were significant ($p<0.01$). Western blot analysis showed that the antisera to the FTA6 and FTA21 could specifically recognize rTRAP1 protein in a dilution of 1:5000 (Fig 7). Mice were challenged with 10^{11} RN6390B cells by abdominal cavity injection and followed for mortality. As shown in Table 1, no mice vaccinated with FTA21 died after 7 days while all the mice died in group vaccinated with FTA6 or control group, indicating 100% protection from mortality by FTA21.

Immunocompetent hairless mice were immunized 3 times with Flagellin fusion peptides FTA21. The animals were challenged with *S. aureus* by a subcutaneous injection together with cytodex beads. Seventy-two hours after injection, the size of lesion was measured. Our results showed that all experimental animals developed a lesion, but the mean size of lesions in the mice vaccinated with FTA21 was about 70% smaller than that in the control group. The difference between experimental and control groups were significant ($p<0.01$) (Fig 8a, 8b).

Antibodies against FTA21 could diminish the hot-resistant exotoxins of S.aureus- Antisera against FTA21 were purified by protein-A affinity (Amersham Pharmacia). RN6390B cells (1ml) were cultured in CY broth for 6h at 37 °C together with antibodies against FTA21 (10mg/ml, 100μl). Normal mouse IgG (10mg/ml, 100μl) or PBS (100μl) buffer were used as negative control or buffer control individually. The supernatant was collected as method described above. The level of hot-resistant exotoxins in supernatant was measured on MDBK cell model (15). Results of MTT assay showed that the antibodies against FTA21 could maintain MDBK cell viability, but the control antibodies could not (Fig 9). It suggested that the antibodies induced

by FTA21 might decrease the level of hot-resistant exotoxins.

DISCUSSION

S. aureus is one of the major pathogenic bacteria in humans and animals, and is one of the most common causes of hospital-acquired lethal infection (1). During its proliferation, the bacteria produce a repertoire of different toxins that can cause life-threatening diseases. Regulation of toxin production is a key feature in *S. aureus* pathogenesis.

TRAP protein is a key factor in the regulation of toxin production. It is highly conserved among staphylococcal stains and species (2). Studies showed that the phosphorylation of TRAP leads to the activation of the gene regulatory system *agr*, which regulates more than 20 toxins. So if TRAP is inhibited, toxins are not produced and staphylococcal infections are prevented (3, 4, 10-12).

In this paper, two peptides, TA6 and TA21, were selected from a phage-desplay library using anti TRAP antibodies. 100% of the mice vaccinated with TA21 expressed in *E. coli* (FTA21) were protected of mortality. In a mouse cellulites model, mice vaccinated with FTA21 had 70% reduction in development of lesion. Mice vaccinated with FTA6 were not protected. In MDBK cell model (15), the antibodies induced by FTA21 could diminish the hot-resistant exotoxins of *S.aueus*. So it was reasonable that mice vaccinated by FTA21 could be protected from *S.aureus* infecion.By sequence analysis, it was found that TA21 is highly similar to the C-terminal of TRAP (Amino acids 156-167) which is very conserved among all known strains of *S. aureus* and *S. epidermidis*. It suggests that the C-terminal of TRAPs is an eptitope region that can stimulate the production of protective antibodies against *S. aureus* infections. Because of the high degree of similarity of TRAP among strains, it is suggested that TA21 be used as a powerful vaccine to protect from infections caused by different Staphylococcal strains, including drug resistant ones.

The TA6 has no similarity to the sequence of TRAP, but it can induce mice to produce antibodies to rTRAP. This suggests that TA6 might mimic a conformational epitope of TRAP. Animals vaccinated with the FTA6 were not protected from *S. aureus* infection, suggesting either that this epitope is not an essential part of TRAP or that this epitope is intracellular and is not available for antibody interaction. Future microscopy and directed mutagenesis studies will be carried out to investigate these possibilities.

Curiously, TRAP itself has no predicted transmembrane domain and the mode by which it is bound to the membrane is not yet known. In a further study, it was found that the C terminus of TRAP is an essential domain; perhaps also by accelerating the process of cell lysis via binding of a lysozyme (Yang G *et al* unpublished data). Regardless, identification of a functional domain by phage display should be a useful method in protein research and in this case it clearly indicates that the C terminus of TRAP can be used as a vaccine target site to protect from Staphylococcal infections.

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FOOTNOTES

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Table1: Mice immunized with FTA21 are protected from *S. aureus* infection

Balb/c mice (20-25g, outbred, male) pre-immuned with *E. coli* GI826 which the epitopes of TRAP (FTA6, FTA21) were displayed on their surface, were injected by abdominal cavity with 100µl RN6390B cells (10^9 cells/µl). Mortality time of each mouse was recorded. Each group had 8 mice.

Mice immunized with	Number of died mice			
	Day 1-3	Day 3-5	Day 5-7	Survival
FTA6	5	2	1	0
FTA21	0	0	0	8
Control	3	5	0	0

FIGURE LEGENDS

Figure 1. Rabbit polyclonal anti-recombinant TRAP1 (rTRAP1) antibodies recognize native TRAP in multiple *S. aureus* strains. Cells (1×109) were collected by centrifugation and resuspended in 20μl of 50μg/ml lysostaphin in 10 mM Tris, pH8.0, 1mM EDTA for 15 min at room temperature. Then Laemmli buffer was added into the sample and boiled for 5 min. Total cell homogenate was applied on 12% SDS-PAGE gel and then tested by Western blot with rTRAP1 antibodies (1:1000). Lane1: *S. aureus* strain RN6390B; Lane2: *S. aureus* strain 04018; Lane3: rTRAP1 protein (2μg). Negative control represents the Western blot reaction with normal rabbit serum.

Figure 2. Specific phage clones binding to rTRAP1 antibodies were selected by ELISA and competitive ELISA. The phage clones which could specifically bind rTRAP1 antibodies were selected and sequenced. White bars: negative control. Black bars: screened phage clones (1×10^9) alone Hatched bars: screened phage clones (1×10^9) plus rTRAP protein (5μg)

Figure 3. Sequences of positive bacterial phage clones divided into two groups by sequence similarity to TRAP. TA6 and TA21 were selected for further study. Identical amino acids are in bold.

Figure 4. Sequence alignment. A: Sequence alignment of the three groups of TRAP. Among which, g1 and g2 refer to TRAP in *S. aureus*; g3 refers to TRAP in *S. epidermidis*. B: Sequence similarity between the TA21 and C terminus of TRAP.

Figure 5. Epitopes of TRAP displayed on bacterial surfaces. A, Lane 1 is molecular weight standard in Dalton; Lane 2: the bacteria without fusion peptide before induction; Lane 3: the bacteria without fusion peptide after induction (used as a control in vaccination); Lane 4: the bacteria with TA6 fusion before induction; Lane 5: the bacteria with TA6 fusion after induction (used for vaccination); Lane 6: the bacteria with TA21 fusion before induction; Lane7: the bacteria with TA21 fusion after induction (used for vaccination); B, Western blotting analysis to test binding of the TA6 fusion protein with anti-rTRAP1 antibodies. After induction, the bacteria with FTA6 and the control bacteria without fusion peptide were applied on the 12% SDS-PAGE gel and Western blotted with rTRAP1 antibodies (1:1000). Lane1: the bacteria expressing TA6 fusion after induction; Lane 2: the control bacteria without fusion peptide; C, Western blotting analysis to test binding between the TA21 fusion protein and rTRAP1 antibodies. Lane1: the bacteria expressing TA21 fusion; Lane2: the control bacteria without fusion peptide.

Figure 6. Development of anti TRAP antibodies using fusion peptides: ELISA using sera diluted 1:500. Results are expressed as OD at 450nm. Group1: sera immunized with FTA6; Group2: sera immunized FTA21; Group3: control group without fusion peptide. A, The reaction between antisera with rTRAP1; B, The reaction between antisera with rTRAP2 **: $P<0.01$

Figure 7. Western blot analysis to test the titer of mice antisera immunized with the peptide epitopes. Two micrograms of rTRAP1/lane were applied on 12% SDS-PAGE gel, western blotted and membrane incubated with the antisera in different dilutions. Lane1-4: antisera in a dilution of 1:500 to 1: 5000; A, sera from mice immunized with TA6; B, sera from mice immunized with TA21.

Figure 8. Mice immunized with TA21 were protected from *S. aureus* infection. The murine cutaneous *S. aureus* infection model was used in this assay. Wild type *S. aureus* cells (1×10^8 /ml) were cultured for 90 minutes, Cytodex was added to the culture to a final concentration of 5mg/ml. The mixture of bacteria and Cytodex in a total volume of 200μl was injected subcutaneously into immunocompetent hairless mice. There were eight mice in each group. Seventy-two hours after injection, the size of the lesions were measured. A, S. aureus infected mouse. 1: mice immunized with

E. coli without fusion peptide; 2: mice immunized with FTA21. B, lesion size. 1: mice immunized with *E. coli* without fusion peptide; 2: mice immunized with FTA21. **: $P<0.01$

Figure 9. The level of hot-resistant exotoxins was decreased by antibodies against FTA21. *S.aureus* cells were incubated with antibodies against FTA21 (10mg/ml, 100μl). Normal mouse IgG (10mg/ml, 100μl) or PBS (100μl) buffer were used as negative control or buffer control individually. The supernatant was bolied and collected. The level of hot-resistant exotoxins secreted by *S.aureus* in supernatant was tested in MDBK cell model. 1: normal cell control; 2: antibodies against FTA21; 3: normal mouse IgG; 4: PBS control.

Figure 1

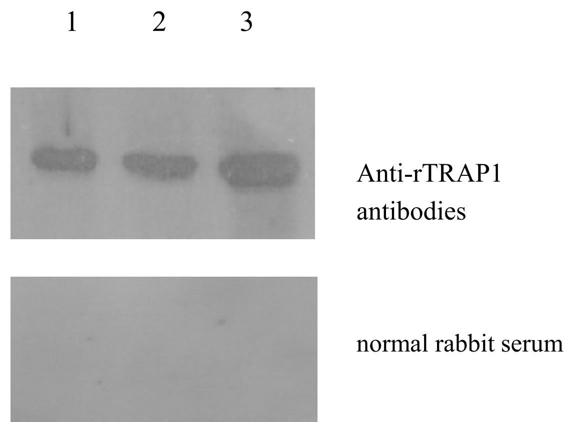

Figure 2

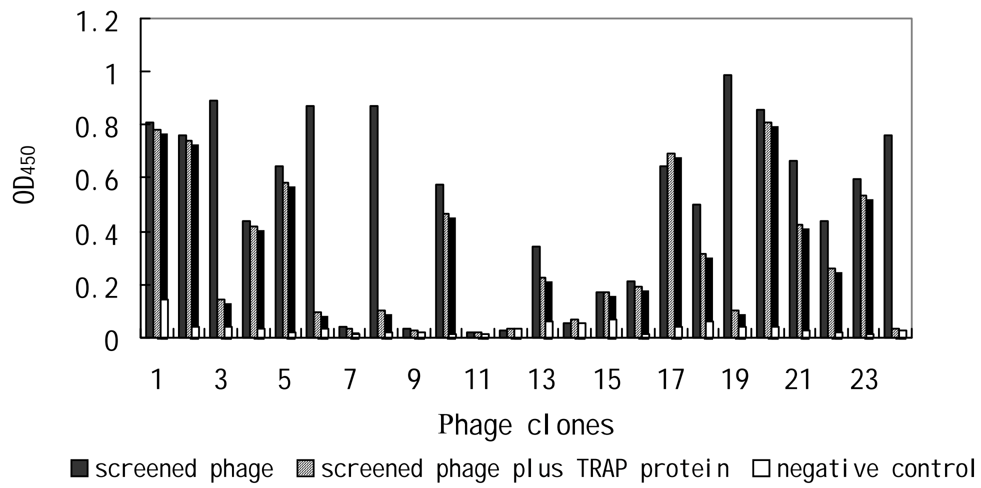

Figure 3

Group	Peptide	AA Sequence	Similarity to TRAP amino acids number
N	TA6,TA8,TA10	T**PFHHQH**S**TGFT**	NONE
	TA24	N**P**L**HHEH**A**TGWT**	
Y	TA3.TA5,TA21	**SWFDN**F**LYP**THD	156-167
	TA22	**SYFD**Y**LYP**PRPA	156-167
	TA13,TA19 TA18	**TYWED**T**LY**AARV	156-167
		Q**TFFD**T**MYP**PPL	156-167

Figure 4

A

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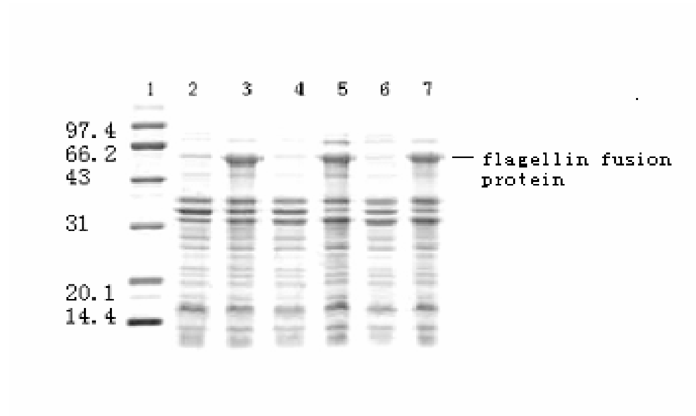
g1           MKKLYTSYGTYGFINQIKINNPSHHLFQFSTADSSVIFEE     40
g2           MKKLYTSYGTYGFIHQIKINNPTHQLFQFSASDISVIFEE     40
g3           .MYLYTSYGTYQFINQIKLNHQERSLFQFSTNDSSIILEE     39
Consensus       lytsygty fl qik n     lfqfs  d s i ee
g1           TEEKTVLKSPSIYEVIKEIGEFNEDHFYCAIFIPSTEDHV     80
g2           TDGETVLKSPSIYEVIKEIGEFSEHHFYCAIFIPSTEDHA     80
g3           SEGKSILKHPSSYQVIDSTGEFNEHHFYSAIFVPTSEDHR     79
Consensus          lk ps y vi   gef e hfy aif p  edh
g1           YQLEKKLISVDDNFKNFGGFKSYRLLRPVKGTTYKIYFGF    120
g2           YQLEKKLISVDDNFRNFGGFKSYRLLRPAKGTTYKIYFGF    120
g3           QQLEKKLLHVDVPLSNFGGFKSYRLLKPTEGSTYKIYFGF    119
Consensus     qlekkl  vd    nfggfksyrll p  g tykiyfgf
g1           ADRQTYEDFKNSDAFKDHFSKEALSHYFGSSGQHSSYFER    160
g2           ADRHAYEDFKQSDAFNDHFSKDALSHYFGSSGQHSSYFER    160
g3           ANRTAYEDFKASDIFNENFSKDALSQYFGASGQHSSYFER    159
Consensus    a r  yedfk sd f   fsk als yfg sgqhssyfer
g1           YLYPIKE                                     167
g2           YLYPIKE                                     167
g3           YLYPIEDH                                    167
Consensus    ylypi
```

B

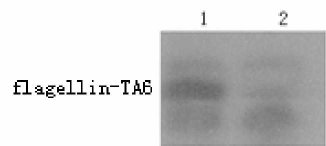
```
g1      SYFERYLYPIKE.     12
g2      SYFERYLYPIKE.     12
93      SYFERYLYPIEDH     13
TA21    SWFDNFLYPTHD.     12
```

Figure 5

A



B



C

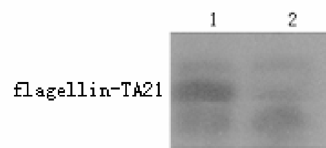
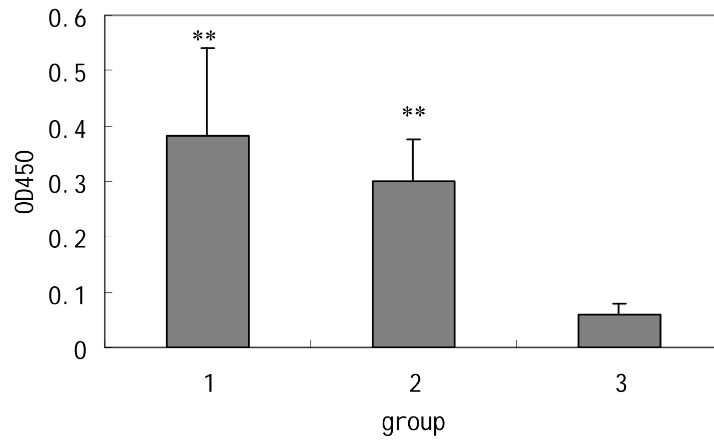


Figure 6

A



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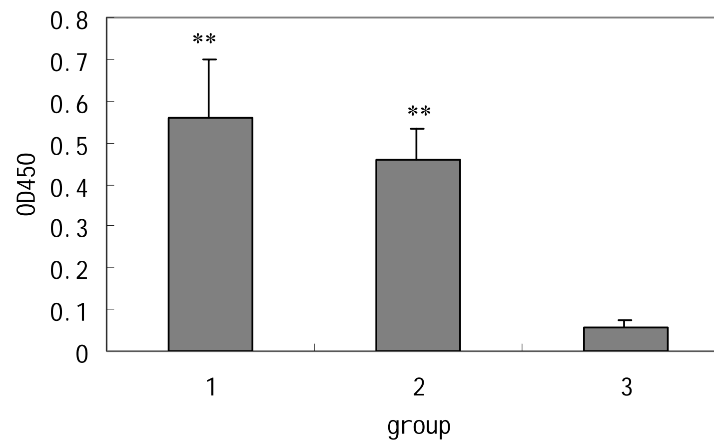
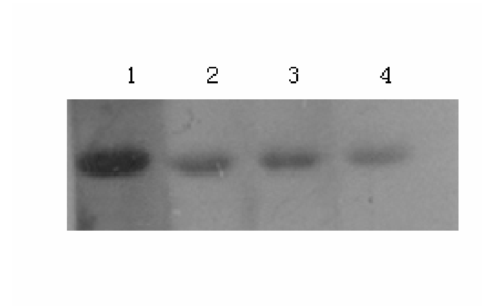


Figure 7

A



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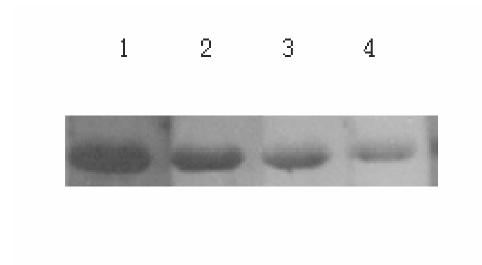
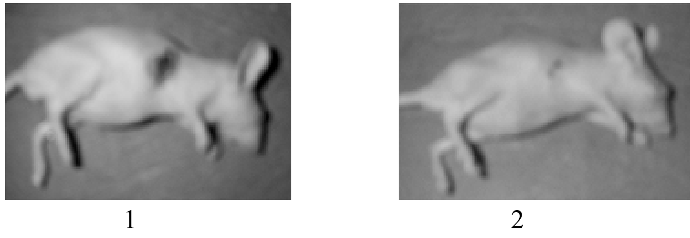


Figure 8

A



B

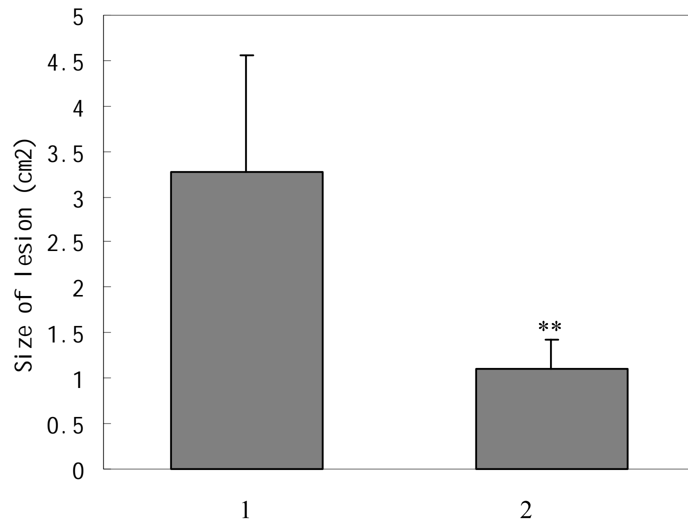


Figure 9