Construction of a trivalent candidate vaccine against *Shigella* species with DNA recombination

WANG Hengliang (王恒樑), FENG Erling (冯尔玲), LIN Yun (林　云),
LIAO Xiang (廖　翔), JIN Ming (金　明), HUANG Liuyu (黄留玉),
SU Guofu (苏国富) & HUANG Cuifen (黄翠芬)

Beijing Institute of Biotechnology, Beijing 100071, China
Correspondence should be addressed to Wang Hengliang (email: wanghl@nic.bmi.ac.cn)

Abstract In this work *asd* gene of *Shigella flexneri* 2a strain T32 was replaced by *Vibrio cholerae* toxin B subunit (*ctxB*) gene with DNA recombination *in vivo* and *in vitro*. The resulting derivative of T32, designed as FWL01, could stably express CtxB, but its growth in LB medium depended on the presence of diaminopimelic acid (DAP). Then form I plasmid of *Shigella sonnei* strain S7 was labeled with strain T32 *asd* gene and mobilized into FWL01. Thus a trivalent candidate oral vaccine strain, designed as FSW01, was constructed. In this candidate strain, a balanced-lethal system was constituted between the host strain and the form I plasmid expressing *S. sonnei* O antigen. Therefore the candidate strain can express stably not only its own O antigen but also CtxB and O antigen of *S. sonnei* in the absence of any antibiotic. Experiments showed that FSW01 did not invade HeLa cells or cause keratoconjunctivitis in guinea pigs. However, rabbits immunized FSW01 can elicit significant immune responses. In mice and rhesus monkey models, vaccinated animals were protected against the challenges of wild *S. flexneri* 2a strain 2457T and *S. sonnei* strain S9.

Keywords: ***Shigella* spp., *ctxB* gene, balanced-lethal system, vaccine.**

Shigella spp. are enteric pathogens that result in bacillary dysentery. The *Shigella* genus includes four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. As the infective dose of *Shigella* spp. is very low (e.g. 10—150 germs), it is difficult to control the prevalence of *Shigella* only by improving the sanitary conditions, particularly in the developing countries[1]. In recent years, it has become more difficult to treat shigellosis with the emergence of strains resistant to multiple antibiotics. At the end of 1996, a meeting was organized at WHO, Geneva by the Steering Committee on Diarrehoeal Disease Vaccine (Global Programme for Vaccines and Immunization), which has placed *Shigella* vaccine development first in line of its priorities[2].

Although the research on *Shigella* vaccines has lasted for half a century, no satisfied product can be put into practice up to the present. The main problem is that expression plasmids either contain the antibiotic gene or are not stable. Recently the vector/host balanced-lethal system was applied in construction of multivalent vaccine, which could solve the above problem[3, 4]. In our lab a multivalent candidate *Shigella* vaccine FSD01 was constructed once before with this system[4—6]. However, the expression level of *S. sonnei* form I O antigen was low, and the protective

effect was not satisfied. It is possible that the gene, encoding form I O antigen from form I plasmid of *S. sonnei*, was not entire. In this work we tried to raise the expression level of form I O antigen by using form I plasmid instead of the cloned gene. Consequently a new vector/host balanced-lethal system was constructed. In this system *asd* gene of *S. flexneri* 2a strain T32 was replaced by *ctxB* gene in chromosome and used to label form I plasmid of *S. sonnei* strain S7. Results show that the constructed strain can express stably three antigens, O antigens of *S. flexneri* 2a and *S. sonnei*, and CtxB, in the absence of any antibiotic, and elicit perfect protective effects. These suggest that the trivalent vaccine may be a promising candidate strain.

1 Materials and methods

1.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in table 1. Bacteria were grown in Luria Broth (LB) medium. Sucrose selection media, consisting of 10% sucrose, 1% Bacto-tryptone, 0.5% Bactoyeast extract, pH 7.2, were used to enrich the cointegrants. For solid media, agar was added to 1.2%, when required the media were supplemented with chloramphenicol (Cm 15 mg/L), streptomycin (Sm 100 mg/L), ampicillin (Ap 100 mg/L), nalidixic acid (Nal 30 mg/L) and diaminopimelic acid (DAP 50 mg/L).

1.2 Antisera

Shigella serotypic antisera were the products of the Institute of Biological Product Identification and of Lanzhou Institute of Biological Product. Rabbit antiserum against CtxB was prepared in this laboratory.

1.3 Primers

P1: 5′-CCGGAATTCCCCATCAGGCGTTCA-3′; P2: 5′-CCGGTCGACCAGGGATAAA-CAGT- TC-3′; P3: 5′-CTGGCACACTTCAGGA-3′; P4: 5′-GATTTCCCGATCGTTC-3′; P5: 5′-GGGGTACCTCAACATAATGCCG-3′; P6: 5′-CGCGGATCCGGTGACTCTATTACCG-3′; P7: 5′-GCTCTAGACATCGTGTTCATTGCT-3′; P8: 5′-TGCATCTAGAACTAAGC-3′; P9: 5′-CCTGTAGTAGGATCAAC-3′. Primers were synthesized by the Center of Shanghai Biotechnology of Chinese Academy of Sciences. Their locations in chromosome and plasmid are shown in figs. 1 and 2.

1.4 Animals

Rabbits, Kunming (KM) mice, guinea pigs and rhesus monkeys were all provided by Center of Experimental Animals, Academy of Military Medical Sciences. Their certification numbers are, respectively, BDW95014, BDW95001, 01-3061, and BDW95002.

1.5 Recombinant DNA techniques

Endonucleases, T4 DNA ligase and DNA polymerase I klenow fragment were purchased from SABC, Promega, and Biolabs, respectively, and used according to manufacturer's recom-

mendation. Molecular genetic procedures and recombinant DNA techniques (plasmid isolation, agarose gel electrophoresis, isolation of restriction fragment, ligation and transformation of plasmid DNA) were carried out according to the standard protocols[14].

Table 1　　Bacterial strains and plasmids

Strains and plasmids	Description	References or sources
E. coli		
X6097	ara Δ(pro-lac)rpsL Δ asd-4thi φ 80lacZ Δ M15	[3]
DH5α	endA1 hsdR17($r_k^-$$m_k^+$) supE44 thi-1 recA1 gyrA(Nal^r) RelA1 Δ (lacIZYA-argF] U169 deoR(φ80dlac Δ(lacZ)M15)	GIBCO/BRL
CC118λpir	Δ(ara-leu) araD Δ lac X74 galK phoA20 thi-1 rpsE rpoB argE(Am) recA1,λpir	[7]
SM10λpir	thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu,Km^r,λpir	[8]
MC1061	F$^-$ araD139 Δ (ara-leu)7696 galE15 galK16 Δ lac X74 rpsL(str^r) hsdR2($r_k^-$$m_k^+$)mcrA mcrB1	[9]
S. sonnei		
S7	avirulent, Nal^r	[10]
S9	wild type, virulent, Sm^r	this lab
S. flexneri		
T32	avirulent Δ(ipa-virG), Sm^r	[11]
FWL01	T32 *asd*::ctxB,Sm^r	this study
FSW01	FWL01 Form I virG::*asd*, Sm^r	this study
2457T	wild type, virulent	this lab
V. cholerae		
Wujiang-2	wild type, virulent	this lab
Plasmids		
pUC18	Ap^r;α-lac/MCS	SABC
pCT332	Tc^r;pAT153 inserted by *ctxB*	[12]
pUASD2	Ap^r; pUC18 inserted by *asd* and its 5′, 3′-flanks	This study
pASD21	Ap^r; pUC18 inserted by T32 *asd*	This study
pUACT8	Ap^r;pUASD2 *asd* replaced by *ctxB*	This study
pXL275	Cm^r; ori R6K,mob RK2,sacBR	[5]
pKNAC6	Cm^r;pXL275 inserted by *ctxB* and 5′, 3′-flank of asd	This study
pVIRG6	Ap^r;pUC18 inserted by virG*	this study
pUVAG4	Ap^r;pVIRG6 virG*::asd	this study
pKNVA1	Cm^r;pXL275 inserted by virG*::*asd*	this study
pTH10	Km^r,Tc^r,Ap^r;temperature-sensitive mutant of RP4	[13]

1.6　Chloramphenicol-sensitive enrichment procedure

The method for enrichment of chloramphenicol-sensitive strains was previously described[15]. Strains carrying cointegrants with chloramphenicol resistance were grown in LB medium containing chloramphenicol at 37℃ overnight. 100 μL overnight culture was transferred to 5 mL fresh LB medium. After 1—2 h growth, chloramphenicol was added to incubate for an additional 1—2 h. Then D-cycloserine was added to a final concentration of 1 g/L. After another 1—2 h, cells were harvested and spread onto selection solid medium containing 10% sucrose. After incubation for 16 h at 37℃, individual colonies were picked and screened for their chloramphenicol-sensitive phenotype.
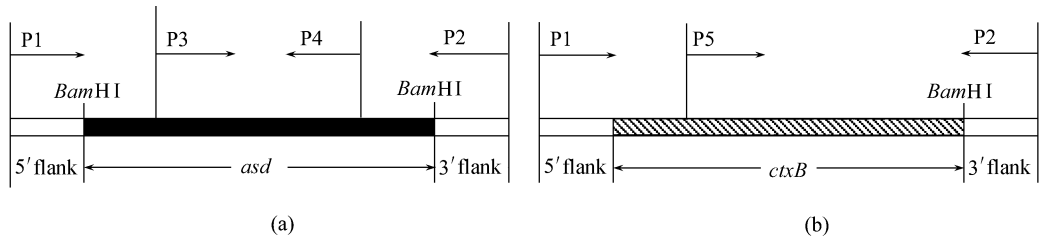
Fig. 1. The location of primers in chromosome. (a) T32; (b) FWL01.

1.7 G$_{M1}$-ELISA

Preparations of bacterial protein samples containing CtxB and G$_{M1}$- ELISA were carried out as described by Sack et al.[16].

1.8 LPS silver-staining and Western-blot

Small scale LPS preparations from different bacterial strains, SDS- PAGE, silver-staining and Western- blot were carried out as described by Rui et al.[4] and Sambrook et al.[14].

1.9 Analysis of virulence

Sereny test (Keratoconjunctivitis test in Guinea pigs' eyes) was used as a reference assay for virulence determination[17]. Approximately 5×10^{10} cells were inoculated into the conjunctival sac of a guinea pig. HeLa cell invasive assay was carried out by microscopic examination of Giemsa-stained cells after 2 h of inoculation with tested strains at 37℃[18].

Fig. 2. The locations of primers in plasmid. (a) S7 form I plasmid; (b) FSW01 form I plasmid.

1.10 Immunogenic test

The strains tested were harvested from plates and suspended in physiological saline. Rabbits were immunized intravenously for six times at interval of 5 d. The doses were $(5, 7.5, 10, 15, 20, 20) \times 10^8$ CFU, respectively. Eight days after the last immunization, bloods were collected from the tested animals and the sera were separated. Then microagglutination test and ELISA were used to measure the antibody titers against O antigens and CtxB, respectively.

1.11 Protective tests in mice

Strains tested were grown in 5 mL LB medium at 37℃ for 16 h, then 2 mL culture was transferred to 100 mL LB medium and inoculated again at 37℃. When OD$_{600}$ value reached 0.9, the culture was harvested by centrifugation. The pellet was washed once with physiological saline,
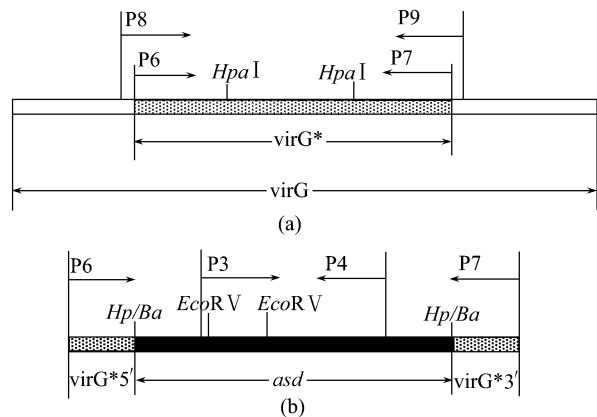
and resuspended in physiological saline to the density of 2.5×10^9 CFU/mL. Ninety mice were randomly divided into two groups. One group was injected with culture sample for three times at 3-d interval as test group, and the other with physiological saline (control group). Ten days after the last administration, animals of control group and test group were challenged with virulent *S. sonnei* strain S9 ($10 \times LD_{50}$), *S. flexneri* 2a strain 2457T ($2 \times LD_{50}$), and *V. cholera* ($5 \times LD_{50}$), respectively. The number of deaths was recorded over 7 d.

1.12　Protective test in monkeys

Test strain was grown on solid LB medium, flushed with physiological saline and diluted in NaHCO_3 buffer to culture suspension of 5×10^9 CFU/mL. Rhesus monkeys for test group were orally administered with 10 mL of the above sample, then flushed with 10 mL NaHCO_3 buffer for three times at one week interval. Monkeys for control group were orally administered with 20 mL NaHCO_3 buffer instead. Two weeks after the last immunization, the monkeys were challenged with virulent *S. flexneri* 2a strain 2457T or *S. sonnei* strain S9 at a dose of 5×10^{10} CFU. After challenge, the animals were examined for their health and stool.

2　Results

2.1　Cloning of *asd* gene from *S. flexneri* 2a strain T32 and construction of its Δ*asd* mutant

Sequence of *asd* gene of *Shigella* spp. cannot be found in GenBank yet. As the genomes of *E. coli* and *Shigellae* are highly homologous, a pair of primers, located about 500 bp of *asd* gene downstream and upstream respectively, were designed according to the sequence of *E. coli* strain K12. Restriction sites of *Eco*R I and *Sal* I were introduced into the 5′ ends of the primers, respectively (See sec. 1.3). An about 2.7-kb product was obtained by PCR from the overnight culture of T32. Then the PCR product was inserted into plasmid pUC18 and transformed into X6097, resulting in X6097 (pUASD2). Sequence analysis of pUASD2 indicated that the cloned flanking sequences of strain T32 *asd* gene share 99.2% homology with those of *E. coli* strain K12 (PCR artefacts not considered). Furthermore, there is a *Bam*H I restriction site at both flanks of T32 *asd* gene, like *E. coli* K12. Thus, the strain T32 *asd* gene was cloned from its chromosome digested by *Bam*H I in order to avoid mutations induced by PCR, and sequencing result indicated that the sequence of T32 *asd* gene was the same as that of *E. coli* K12.

The *asd* gene of pUASD2 was substituted by the *ctxB* gene of pCT322. Then the sandwich containing the flanking sequences of *asd* gene and *ctxB* gene was ligated into *Bam*H I- and *Sal* I-digested plasmid pXL275, which is a mobilizable suicide plasmid. The resulting recombinant pKNAC8 was firstly transformed into CC118λpir, a host with high transforming rate, and then transformed into the mobilizable host SM10λpir. Next, solid-phase mating on an LB plate between SM10λpir (pKNAC8) and T32 was carried out. The cointegrants, in which the first homologous recombination event had taken place, were selected on LB plates containing chloramphenicol and streptmycin. The final integrant, designated as FWL01, in which a second homolo-

gous recombination event had finished, was screened by counterselection of sucrose and chloramphenicol-sensitive enrichment. FWL01 was further analyzed by PCR and digested with *Bam*H I (fig. 1). Results indicated that *asd* gene in FWL01 chromosome was surely replaced by *ctxB* gene so that FWL01 could not grow in LB medium without the presence of DAP. To detect whether FWL01 can express CtxB or not, clear lysats of FWL01 and T32 were prepared with freezing and melting procedure and measured with G_{M1}-ELISA and there was CtxB in lysate of FSW01. The P/N value between FWL01 and T32 was 4.27.

2.2   Construction of trivalent candidate *Shigella* vaccine strain FSW01

The *virG* gene located in *S. sonnei* form I plasmid is associated with virulence of *Shigella*. A part of *virG* gene of attenuated *S. sonnei* strain S7, designated as *virG**, was obtained by whole-cell PCR with primers 6 and 7. Then the middle part of *Hpa* I-restricted fragment of *virG** was replaced by T32 *asd* gene. Herein both ends of *asd* gene were ligated with an about 600-bp *virG** fragment. Next the sandwich (*virG*-asd-virG**) was cloned into the mobilizable suicide plasmid pXL275, resulting in recombinant plasmid pKNVA1. The latter was first transformed into CC118λpir and then into SM10λpir. SM10λpir (pKNVA1) and *S. sonnei* S7 were mated on LB plates. By the first homologous recombination event, pKNVA1 was integrated into form I plasmid of strain S7. The resulting cointegrant S7 (Form I::pKNVA1) was selected on plates with nalidixic acid and chloramphenicol. Furthermore, the temperature-sensitive suicide plasmid pTH10 was introduced into the cointegrant, resulting in S7 (Form I::pKNVA1, pTH10). Then the latter was mated with FWL01 on plates. By in-trans acting of mobilizable proteins encoded by pTH10, the cointegrated plasmid (Form I::pKNVA1) was mobilized into FWL01. Finally, the trivalent candidate vaccine FSW01, in which the second homologous recombination event had occurred, was picked by chloramphenicol- sensitive enrichment and counterselection of sucrose. As the above (sec. 2.1), FSW01 was determined by PCR with primers shown in fig. 2, and the PCR products were digested with *Eco*R V. The results suggested that T32 *asd* gene had been integrated into *virG* gene locus of form I plasmid so that FSW01 could grow in LB medium without DAP. In slide ag-glutination test FSW01 was a strongly positive response to standard antisera against *S. flexneri* 2a and *S. sonnei* O antigens, and its agglutination reaction to the latter was much stronger than that of FSD01 (table 2). Data from G_{M1}-ELISA showed that the expression level of *ctxB* in FSW01 was the same as in FWL01, which suggested that the introduction of form I plasmid did not interfere with the expression of *ctxB*.

To further demonstrate that form I large plasmid of *S. sonnei* had been mobilized into FWL01, the large plasmid species were extracted from strains S7, T32 and FSW01 and analyzed by 0.7% agarose gel electrophoresis. As shown in fig. 3, strain S7 contained a large plasmid. However, both strains T32 and FSW01 harbored two large plasmid species. FSW01 should harbor three large plasmids after introduction of form I plasmid. The reason why there were only two large plasmids in FSW01 is that the invasive plasmid of T32 was lost in FSW01 because form I
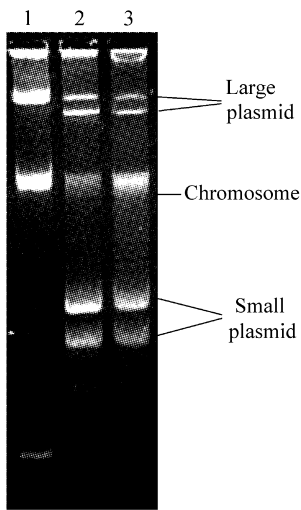
Fig. 3.　Agarose gel electrophoresis of plasmid DNA. 1, S7; 2, FSW01; 3, T32.

plasmid of *S. sonnei* and invasive plasmid of T32 belong to the same plasmid incompatibility group, IncFI group[19]. These data are consistent with those reported by Wang et al. and Mu et al[20,21].

The LPS samples of S7, T32 and FSW01 were extracted and separated by sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining. As shown in fig. 4(a), *S. flexneri* T32 LPS formed a typical ladder pattern. However, bands of *S. sonnei* S7 LPS were a smear-like pattern. LPS profile of strain FSW01 harbored characteristics of both *S. flexneri* 2a and *S. sonnei*, which suggested FSW01 expressed both the O antigens above. From Western-blot (fig. 4(b)) with *S. sonnei* O-antigen-specific antibodies, the results showed that reaction with T32 was not observed, but that with S7 was strong,

and the Western-blot band of FSW01 was similar to its silver-staining form, further demonstrating that LPS of *S. sonnei* was expressed in FSW01. However, the major blot bands of S7 and FSW01

were not at the same position probably because *S. sonnei* O antigen in FSW01 is valently bound to the core polysaccharide of *S. flexneri*, leading to the change of its electrophoretic mobility.

To test the stability of form I large plasmid in FSW01, the latter was successively inoculated and cultured in LB medium for 30 generations. Then slide agglutination test with *S. sonnei* O-antien-specific antis-
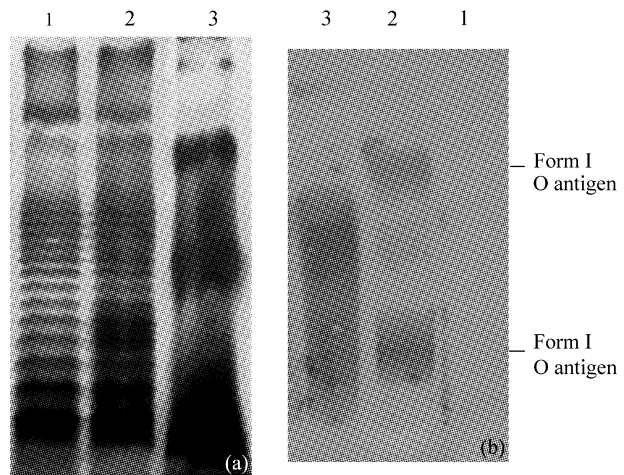

Fig. 4.　SDS-PAGE and Western-blot analysis of LPS. (a) Silver-stained LPS; (b) Western-blot with anti-*S. sonnei* O-PS-specific antibody. 1, T32; 2, FSW01; 3, S7.

era and assay of agarose gel electrophoretic profiles of large plasmid were performed. All data indicated that stability of *S. sonnei* form I large plasmid in FSW01 reached 100% without the presence of any antibiotic.

2.3　Safety and immune protective effect of the trivalent candidate vaccine FSW01

2.3.1　Safety test of FSW01.　Inocula of FSW01 and 2457T were, respectively, introduced into the conjunctival sacs of guinea pigs. One day after inoculation, a slight keratoconjunctivitis was

provoked in the guinea pig eye inoculated by 2457T. The symptom became severe after two days. On the fourth day, the upper and lower eyelids were closing, and lots of purulent exudates were visible. However, the guinea pig eye inoculated by FSW01 elicited no keratoconjunctivitis. These data suggested that FSW01 was avirulent.

According to sec. 1.9, HeLa cells were invaded by strains FSW01 and 2457T overnight cultured, then stained and inspected under microscope. The results showed that FSW01 could not invade HeLa cells but 2457T could.

Rhesus monkeys were orally administered with three doses of 5×10^{10} CFU, and no side effects were found.

2.3.2   Immunogenicity of FSW01.   According to sec. 1.10, five Japanese rabbits were immunized. Eight days after the last immunization, rabbit bloods were gathered and sera were separated. Specific antibodies against strains T32 and S7 were measured by micro-agglutination assay. Results showed that the titer of antibody against *S. sonnei* S7 and *S. flexneri* 2a T32 reached 640 and 1280, respectively. When the antisera were tenfold diluted with physiological saline and determined with ELISA, the titer of antibody against CtxB was still positive (P/N>2), suggesting that the immunized rabbits elicited antibodies against CtxB. Moreover, it was also found that antibody titers peaked on the eighth day after the last immunization, then fell after ten days. After one month, antibodies rose again after the boost.

2.3.3.   Immune protective test in mice.   KM mice were immunized three times subcutaneously with FSW01 or physiological saline, and challenged intraperitoneally with virulent *Shigella* strains. The experimental results summarized in table 2 indicated that the mice immunized by FSW01 elicited strong protective responses against challenges of the virulent *S. flexneri* 2a and *S. sonnei* strains, as well as *V. cholera* Inaba serotype strain.

Table 2   Immune protective test of FSW01 in mice

Immunogens	Challenge strains	Survival/Total	Survival efficacy (%)
FSW01	S9	14/15	93.3
	2457T	14/15	93.3
	Wujiang-2	12/15	75.0
Saline	S9	0/15	0.0
	2457T	1/15	6.7
	Wujiang-2	0/15	0.0

2.3.4   Immune protective test in monkeys.   Twenty rhesus monkeys, 3—7 years old, weighing from 4.0 to 5.5 kg, were used in this study. They were divided into two groups (test and control), each consisting of six females and four males. The test and control groups were orally administrated with FSW01 and placebo, respectively. Two weeks after the last immunization, both test and control groups were again subgrouped into two (three female and two male monkeys for each), respectively. Then each group of five monkeys was challenged with *S. flexneri* 2a strain 2457T and *S. sonnei* strain S9. Results indicated the protective rates against *S. sonnei* and *S. flexneri* 2a

were 80% and 70%, respectively. Compared with that of the previous vaccine candidate FSD01, the protective rate of FSW01 against *S. sonnei* is largely increasing (table 3). In summary, compared with test group the clinical symptoms in the control monkeys were severer, and occurred earlier and recovered slower.

Table 3   Comparison of expression level, immunogenicity and immune effect of *S. sonnei* O antigen between FSW01 and FSD01

Candidate vaccine	Slide agglutination (expression level)	Titer of antisera in rabbits (immunogenicity)	Protective rate in monkeys (immune effect)
FSW01	+++	1:640	80%
FSD01	+	1:320	30%

After vaccination, shedding duration of vaccine strain FSW01 was detected by rectal swab test. It lasted two days on average, which is concurrent with the reported results. Furthermore, agarose gel electrophoretic profiles of large plasmid and slide agglutination assay indicated that all of the shedded vaccine strains harbored *S. sonnei* form I large plasmid, and expressed both O antigens of *S. sonnei* and *S. flexneri* 2a. These data further proved that the trivalent candidate vaccine strain FSW01, constructed with a balanced-lethal system, could stably express *S. sonnei* O antigen *in vivo* without the presence of any antibiotic.

3   Discussion

At present, development of *Shigella* vaccines is a worldwide urgent subject due to extensiveness and harmfulness of *Shigella* epidemic. As far as construction of *Shigella* vaccines is concerned, developing an oral live vaccine is given the highest priority in the world[1]. In addition, construction of subunit vaccines is in some degree concerned[2]. Because *S. flexneri* 2a and *S. sonnei* are the two most extensive-spread strains among *Shigella* spp., construction of a bivalent *Shigella* vaccine is a hot spot. *S. flexneri* 2a strain T32 is a spontaneous mutant. The published results of field trials in Romania and China indicate that T32 is safe and effective. Its attenuation is based on the deletion of *ipaBCDA*, *invA* and *virG* genes of the invasive large plasmid[11]. And these genes are related to virulence of *Shigella*. *S. sonnei* strain S7 is also a spontaneous mutant. Research results indicate that its invasive form I large plasmid has lost *ipaBCDA*, *virF* and a 17 kb *Eco*R I-digested fragment which is present in all of the invasive plasmids of *Shigella* spp. The loss leads to the negative results of strain S7 in Sereny test, invasion of HeLa cells and Congo red binding test. However, colony morphology and seratype of strain S7 are identical with those of wild *S. sonnei*, suggesting that strain S7 expresses the entire form I O antigen[10]. Accordingly, strains T32 and S7 were used to construct bivalent vaccines against *S. flexneri* 2a and *S. sonnei*. The method previously used to construct bivalent *Shigella* vaccines is as follows: form I large plasmid of strain S7 was, firstly, randomly labeled by transposons with a drug-resistant gene and then mobilized into the acceptor strain T32. However, vaccines constructed with this method have several disadvantages: firstly, in these vaccines stability of form I large plasmid is dependent on the outside selective pressure. Furthermore, if the resistant genes carried by transposons were an-

tibiotic genes, it does not meet WHO's claim for oral live vaccines, for that could result in the spread of antibiotic genes. Secondly, transposon in vaccine may interfere with stability of the vaccine to some degree.

In this work the above disadvantages were overcome as the vector/host balanced-lethal system, based on labeling *S. sonnei* form I large plasmid with non-antibiotic *asd* gene, was used to construct the multivalent candidate vaccine, FSW01, against *Shigella*. Furthermore, owing to mobilizing *S. sonnei* large plasmid, which expresses form I O antigen, into the derivative of T32, the expression level of form I O antigen in FSW01 increases largely, compared with that in FSD01. Tests in rabbits, mice and monkeys demonstrated that FSW01 could raise specific antibodies against O antigens of *S. flexneri* 2a and *S. sonnei*, and elicit strong protection against the challenges from the corresponding virulent strains. Nevertheless, the expression of *ctxB* was not high in FSW01 because *ctxB* gene was integrated into the chromosome of strain T32 with single copy. However, the results on rabbits and mice illustrated that CtxB expressed in FSW01 could still stimulate immune response and produce some effective protection against the challenge from *V. cholera* Inaba serotype. Consequently, FSW01 is a promising trivalent candidate vaccine strain and deserves further test in human.

References

1. Linderberg, A. A., Pal, T., Strategies for development of potential candidate *Shigella* vaccine, Vaccine, 1993, 11: 168—179.
2. WHO, Vaccine research and development: new strategies for accelerating vaccine development, Wkly. Epidemiol. Rec., 1997, 72: 73—79.
3. Nakayama, K., Kelly, S. M., Curtiss III, R., Construction of an asd+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a Salmonella vaccine strain, Bio/technology, 1988, 6: 693—697.
4. Rui, X. L., Xu, Y. Q., Wu, X. D. et al., Construction of a trivalent candidate *Shigella* vaccine strain with host-vector balanced-lethal system, Science in China, Ser. C, 1997, 40(1): 52—59.
5. Rui, X. L., Xu, Y. Q., Wang, H. et al., Construction of stable and non-resistant bivalent vaccine candidate strain against *Shigella flexneri* 2a and *Shigella sonnei*, Chin. J. Biotechnol (in Chinese), 1996, 12: 134—139.
6. Qian, F., Rui, X. L., Su, G. F. et al., Expression of *Shigella sonnei* form I O antigen and *Vibrio cholera* toxin B subunit gene in *S. flexneri* 2a strain and investigation of their immunoprotective response in mice, Chinese Journal of Genetics (Acta Genetica Sinica) (in Chinese), 1996, 23(4): 322—328.
7. Herrero, M., Lorenzo, V., Timmis, K., Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria, J. Bacteriol., 1990, 172(11): 6557—6567.
8. Miller, V. L., Mekalanos, J. J., A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibro cholerae* requires toxR, J. Bacteriol., 1988, 170(6): 2575—2583.
9. Wertman, K. F., Wyman, A. R., Botstein, D., Host/vector interactions which affect the viability of recombinant phage lambda clones, Gene, 1986, 49: 253—262.
10. Liu, W., Wang, B. R., Studies on the 120 Md plasmid and its derivative plasmid in *Shigella sonnei*, Chin. Microbiol. Immunol. (in Chinese), 1990, 10: 205—208.
11. Venkatesan, M., Fernandez-Prada, C., Buysse, J. M. et al., Virulence phenotype and genetic characteristic of the T32-ISTRATI *Shigella flexneri* 2a vaccine strain, Vaccine, 1991, 9(5): 358—363.

12. Gennaro, M. L., Greenaway, P. J., Broadbent, D. A., The expression of biologically active cholera toxin in *Escherichia coli*, Nucl. Acids. Res., 1982, 10(16): 4883—4890.

13. Harayama, S., Tsuda, M., Iino, T., High frequency mobilization of the chromosome of *Escherichia coli* by a mutant of plasmid RP4 temperature-sensitive for maintenance, Mol. Gen. Genet., 1980, 180: 47—56.

14. Sambrook, J., Fritsch, E. F., Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd ed., New York: New York Cold Spring Habor Press, 1989.

15. Hone, D., Harris, A. M., Chatfield, S. et al., Construction of genetically defined double aro mutants of *Salmonella typhi,* Vaccine, 1991, 9(11): 810—816.

16. Sack, D. A., Huda, S., Neogi, P. K. B. et al., Microtiter ganglioside enzyme-linked immunosorbent assay for *Vibro* and *Escherichia coli* heat-labile enterotoxins and antitoxin, J. Clin. Microbiol., 1980, 11(1): 35—40.

17. Maurelli, A. T., Black, B., Curtiss III, R., Temperature-dependent expression of virulence genes in *Shigella* species, Infect. Immun., 1984, 43(1): 195—201.

18. Nassif, X., Mazert, M. C., Mounier, J. et al., Evaluation with an iuc::Tn10 mutant of the role of aerobactin production in the virulence of *Shigella flexneri*, Infect. Immun., 1987, 55(9): 1963—1969.

19. Makino, S., Sasakawa, C., Yoshikawa, M., Genetic relatedness of the basic replicon of the virulence plasmid in *Shigellae* and enteroinvasive *Escherichia coli*, Microb. Pathog., 1988, 5: 267—274.

20. Wang, B. R., Song, S. Z., Chen, J. R. et al., A bivalent strain with the characteristics of *Shigella flexneri* 2a and *sonnei,* Chin. Microbiol. Immunol. (in Chinese), 1987, 7: 373—377.

21. Mu, Z. Q., Xing, L., Mao, P. J., Use of transponson Tn501 in construction of bivalent strain of dysentery bacillus, Chin. Microbiol. Immunol. (in Chinese), 1990, 10: 92—94.