SURFACE DISPLAY ON *BACILLUS SUBTILIS* SPORES AND VACCINE POTENTIAL OF A TETRASPANIN FROM CARCINOGENIC LIVER FLUKE, *OPISTHORCHIS VIVERRINI*

Wuttipong Phumrattanaprapin[1], Sujittra Chaiyadet[1], Alex Loukas[2], Paul J Brindley[3], Javier Sotillo[2] and Thewarach Laha[1]

[1]Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand; [2]Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, Australia; [3]Department of Microbiology, Immunology and Tropical Medicine, and Research Center for Neglected Diseases of Poverty, School of Medicine and Health Sciences, George Washington University, Washington DC, USA

Abstract. *Opisthorchis viverrini* resides in bile ducts and could be targeted optimally by vaccination, which induces both mucosal and systemic antibodies, reflecting the migratory path of this liver fluke. Tetraspanins are transmembrane proteins essential for tegument formation of *O. viverrini* and are efficacious as vaccine antigens for diseases caused by this and other parasitic flatworms. Tetraspanin-2 of *O. viverrini* (*Ov*-TSP-2) was expressed on the surface of *Bacillus subtilis* spores following transformation of *B. subtilis* strain WB800N with a plasmid encoding a fusion of a large extracellular loop region of *Ov*-TSP-2 (LEL-*Ov*-TSP-2) and *B. subtilis* spore coat protein CotC. Immunogenicity of the recombinant spores was assessed by measuring serum and bile immunoglobulins following oral vaccination of hamsters with the recombinant spores, 2.5×10^8 spores six times over four weeks. Sporulation of recombinant *B. subtilis* expressing fusion proteins was confirmed by western blotting and immunofluorescence using anti-*Ov*-TSP-2 antibodies. Significantly elevated levels of serum- and bile-specific IgG antibodies to LEL-*Ov*-TSP-2 were evident at day 42 following the first oral dosing. The findings indicate oral vaccination of hamster with recombinant *B. subtilis* spores expressing *Ov*-TSP-2 holds promise as a suitable approach for the control of this carcinogenic liver fluke infection.

Keywords: *Bacillus subtilis*, *Opisthorchis viverrini*, oral vaccine, spore, tetraspanin

INTRODUCTION

Opisthorchiasis is endemic in the lower Mekong basin of Southeast Asia including Lao PDR and Thailand (Sithithaworn *et al*, 2012), and is classified by the International Agency for Research on Cancer as a group 1 carcinogen (Bouvard *et al*, 2009). The mechanisms by which *O. viverrini* leads to malignancy are complex

Correspondence: Dr Thewarach Laha, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: +66 (0) 43 348387
E-mail: thewa_la@kku.ac.th,

Dr Javier Sotillo, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, McGregor Rd, Smithfield 4878, QLD, Australia.
E-mail: Javier.sotillo@jcu.edu.au

and include chronic inflammation, mechanical damage of the bile duct epithelium by feeding worms, elevated dietary nitrosamines, and secretion by the parasite of growth factors and other proteins, which stimulate proliferation of biliary epithelium, induction of angiogenesis and interference with homeostatic apoptosis (Sripa *et al*, 2009; Smout *et al*, 2011; Smout *et al*, 2015; Brindley and Loukas, 2017; Jusakul *et al*, 2017). Infection with this fishborne pathogen also is known to modify the intestinal microbiome (Plieskatt *et al*, 2013). In addition, elevated prevalence of *Helicobacter pylori* and *H. bilis* and their associated virulence factors have been reported during opisthorchiasis and associated hepatobiliary disease (Boonyanugomol *et al*, 2012; Deenonpoe *et al*, 2017).

To date, there are no vaccines against human helminth infections despite numerous reports of vaccine candidates in animal models of infection, notably proteins derived from excretory-secretory (ES) products and surface membrane proteins (Acosta *et al*, 2008; Chaiyadet *et al*, 2015; Van Meulder *et al*, 2015).

O. viverrini tegument membrane itself as well as extracellular vesicles (EVs), which are derived from this structure, are enriched in tetraspanins (TSPs), including tetraspanin 2 (*Ov*-TSP-2), belonging to CD63 family (Chaiyadet *et al*, 2017). TSPs are members of a superfamily found on plasma membranes and EVs of virtually all mammalian cells (Rocha-Perugini *et al*, 2016) and are involved in various biological processes, such as cell adhesion, migration, membrane fusion, signaling, protein trafficking as well as tumor metastasis (Boucheix and Rubinstein, 2001; Hemler, 2001; Hemler, 2003; Hemler, 2008). The structure of TSPs is distinctive, containing four transmembrane domains, intracellular N- and C- termini and two extracellular loops, a small extracellular loop 1 (SEL or EC1) and a large extracellular loop 2 (LEL or EC2) (Maecker *et al*, 1997). These extracellular domains are the most variable regions of TSPs. Furthermore, TSPs are candidate molecules in the development of vaccines against numerous platyhelminths (Tran *et al*, 2006; Dang *et al*, 2012; Dakshinamoorthy *et al*, 2013; Merrifield *et al*, 2016), including *O. viverrini* (Chaiyadet *et al*, 2015).

There are increasing evidences that mucosal immune responses might play a key role in controlling liver flukes such as *O. viverrini* and *Clonorchis sinensis* (Chawengkirttikul and Sirisinha, 1988; Jittimanee *et al*, 2007; Zhang *et al*, 2008; Sun *et al*, 2018), suggesting that oral immunization could be an effective strategy to combat this infection. However, one of the major obstacles of orally-delivered vaccines is proteolysis of the antigens in the stomach (Patel *et al*, 2014). One approach that have emerged in the last decade is to deliver vaccine antigens using spores from *Bacillus subtilis*, which has been used as a model vehicle for the delivery of recombinant protein antigens to avoid the extreme environment of the gastrointestinal tract (Duc and Cutting, 2003; Knecht *et al*, 2011; Hinc *et al*, 2013; Rosales-Mendoza and Angulo, 2015). The advantages of using *B. subtilis* instead of other microbial cell-surface display systems are its safety (non-pathogenic) and stability (survival under extreme conditions) (Duc and Cutting, 2003; Wang *et al*, 2017). Despite novel attempts designed to display antigen proteins directly on *B. subtilis* spores without anchor proteins as scaffolds, conventional approaches making use of spore coat proteins (CgeA, CotC, CotE, CotG, CotX, CotZ, and OxdD) as anchoring motifs (Mauriello *et al*, 2004; Wang *et al*, 2017), and CotC has recently

been successfully employed to create a vaccine against *C. sinensis* in animal models (Qu *et al*, 2014; Wang *et al*, 2014; Tang *et al*, 2016; Jiang *et al*, 2017).

The performance of several vaccine candidate antigens against opisthorchiasis has been described (Sirisinha and Wongratanacheewin, 1986; Jittimanee *et al*, 2012; Papatpremsiri *et al*, 2016), but have not included recombinant proteins. Hence, in order to investigate the potential of TSPs for oral vaccination against opisthorchiasis, *Ov*-TSP-2 was employed as a target antigen fused with *B. subtilis* spore coat CotC protein. This study provides a platform from which to deliver mucosal vaccination against human opisthorchiasis and associated cholangiocarcinoma.

MATERIALS AND METHODS

Construction of plasmid pHT01-CotC-LEL-*Ov*-TSP-2 and transfection of *B. subtilis*

The complete coding sequence (CDS) of *B. subtilis* spore coat CotC protein (spanning nt 177-553 of GenBank accession no. X05680.1) was fused to the 5′ end of *Ov*-TSP-2 LEL coding sequence (spanning nt 325-552 of GenBank accession no. JQ678707.1), resulting in CotC-LEL-*Ov*-TSP-2. In brief, a 380 bp fragment from CotC gene (Wang *et al*, 2014) was PCR amplified using specific primers, forward (5′-CATGGATCCTGTAGGATAAATCGTT-3′) and reverse (5′- GCCGAATTCGTAGTGTTTTTATGC-3′), containing restriction site of *Bam*H I and *Eco*R I (underlined), respectively. PCR mixture (25 μl) consisted of 20 ng of *B. subtilis* PY79 strain genomic DNA (kindly provided by the Department of Biochemistry, The Ohio State University, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM forward primer and reverse primers, 1.25 U *Taq* polymerase (Thermo Fisher Scientific, Waltham, MA) and buffer (DNA Amplification Kit; Vivantis, Selangor Darul Ehsan, Malaysia). Thermocycling, conducted in Bio-Rad C1000 Thermocycler (Bio-Rad Lab, Hercules, CA) were as follows: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 40 seconds; and a final step of 72°C for 7 minutes. Amplicon was gel-purified (NucleoSpin® Gel and PCR Clean-up; Macherey-Nagel, Bethlehem, PA) and inserted into pGEM-T easy vector (Promega, Madison, WI) and designated pGEM-CotC. *Ov*-TSP-2 LEL gene fragment (228 bp) was amplified using forward (5′-ACGCGAATTCCGCGATAAGATCCCCGG-3′) and reverse (5′-ACGCTCTAGACTGGATGAACTCTTCGAC-3′) primers, containing restriction site for *Eco*R I and *Xba* I (underlined), respectively, directly from a plasmid carrying *Ov-tsp*-2 LEL gene (Chaiyadet *et al*, 2017). The components of the PCR mixture was as described above except that the above mentioned primers and pGEM-CotC were used, and the thermocycling conditions were as per above. The *Eco*R I/*Xba* I LEL-*Ov*-TSP-2 fragment was inserted into pGEM-CotC at restriction sites for *Eco*R I and *Xba* I using T4 ligation at 22°C for 10 minutes, resulting in pGEM-CotC-LEL-*Ov*-TSP-2 construct. Sequence of fused CotC-LEL-*Ov*-TSP-2 were verified by DNA sequencing (BioBasic, Ontario, Canada). The fusion fragment of CotC-LEL-*Ov*-TSP-2 was subcloned into *B. subtilis* shuttle vector pHT01 (Mobitec, Göttingen, Germany) at *Bam*H I and *Xba* I cloning sites (Nguyen and Schumann, 2014) (resulting in pHT01-CotC-LEL-*Ov*-TSP-2). CotC gene sequence alone was cloned into pHT01 (Mobitec) as control (resulting in pHT01-CotC). The BamH I/Xba I CotC gene sequence was PCR amplified using forward (5′-CATGGATCCTGTAGGATAAATCGTT-3′) and reverse (5′-

GCCTCTAGAGTAGTGTTTTTATGC-3′) primers (underlined are restriction site of *Bam*H I and *Xba* I, respectively). PCR mixture was prepared and subjected to thermocycling as described above.

E. coli DH5α strain (Promega) cells were individually transformed with all plasmid constructs described above (pGEM-CotC, pGEM-CotC-LEL-*Ov*-TSP-2, pHT01-CotC and pHT01-CotC-LEL-*Ov*-TSP-2) by heat shock method (Froger and Hall, 2007). *B. subtilis* WB800N strain (Moabites, Gothingen, Germany) was transformed with pHT01-CotC and pHT01-CotC-LEL-*Ov*-TSP-2 for protein expression according to the manufacturer's instructions with some modification (Ilk *et al*, 2011). In short, one ml aliquot of competent cells was inoculated into 20 ml of LS medium [80 ml of distilled water, 10 ml of 10x S-base (Mobitec), 2.5 ml of 20% (w/v) glucose, 0.5 ml of 0.1% (w/v) L-tryptophan, 0.5 ml of 2% (w/v) casein, 5 ml of 2% (w/v) yeast extract, 0.25 ml of 1 M $MgCl_2$, and 0.05 ml of 1M $CaCl_2$]. Cells were incubated at 30°C for 2 hours with shaking. A 10 μl aliquot of 0.1 M EGTA was added to 1 ml of competent cells, incubated for 5 minutes at ambient temperature, then 10 ng of plasmid were added to the competent cells and incubated for 2 hours at 37°C with shaking. Transformed cells were centrifuged at 1,200*g* at 10°C for 5 minutes, re-suspended with 50 μl of supernatant and grown overnight at 37°C on Luria-Brentani (LB) agar (TM Media, Rajasthan, India) plates containing 5 μg/ml chloramphenicol. Transformed *B. subtilis* cells were assessed for the presence of recombinant plasmids by PCR using specific primers flanking the insert sites as described above.

Calculation of plasmid copy number

Plasmid DNA was extracted from 9 ml of transformed *B. subtilis* cells (2.58×10^8 cells/ml) carrying pHT01-CotC-LEL-*Ov*-TSP-2 using a Minaret plasmid extraction kit (Qiagen, Hilden, Germany). Plasmid concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the corresponding copy number calculated using the equation (Lee *et al*, 2006; Zhong *et al*, 2011): number of copies (molecules) = (DNA amount (ng)$\times 6.0221 \times 10^{23}$)/(DNA length (bp)$\times 660 \times 1 \times 10^9$).

Preparation of *Bacillus subtilis* spores

B. subtilis WB800N was cultured in a sporulation broth (TM Media) as previously described (Wher and Frank, 2004; Wang *et al*, 2014). In brief, a fresh LB agar culture of transformed *B. subtilis* WB800N was washed with normal saline solution (NSS) onto the surface of a Roux bottle (Sigma-Aldrich, Singapore) containing 250 ml of sporulation medium containing 5 μg/ml chloramphenicol and incubated at 35ºC for 5 days with shaking. The sporulation culture was centrifuged at 10,000*g* for 10 minutes, re-suspended in 50 ml of NSS and centrifuged as before. The pellet (spores) was suspended in 50 ml of NSS and purified according to Wang *et al* (2014) as follows. The suspension was treated with 4 mg/ml lysozyme (to break residual sporangial cells) and washed sequentially with 1 M NaCl, 1 M HCl and NSS (twice). Phenylmethylsulfonyl fluoride (1 mM) was included in each solution to inhibit proteolysis. Spores in NSS then were incubated at 65ºC for 1 hour (to destroy residual sporangial cells). Spore numbers were determined by direct counting in a Burker chamber under an optical microscope (40x magnification). Purified spore samples were stored at -20ºC until used. Spores were checked for presence of recombinant plasmids by PCR as described above.

Detection of fusion protein on transformed *B. subtilis* WB800N spore surface

Presence of fusion *Ov*-TSP-2 on the surface of transformed *B. subtilis* WB800N spores harvested on day 5 after sporulation was analyzed by immunoblot analysis. Spore coat proteins were extracted using SDS-DTT extraction buffer (0.5% SDS containing 0.1M DTT and 0.1 M NaCl), separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA). Membrane was washed with PBST [1x phosphate-buffered saline (PBS) containing 0.01% Tween 20], incubated with 5% skim milk in PBST for two hours at ambient temperature, and treated with anti-*Ov*-TSP-2 rabbit serum (Chaiyadet *et al*, 2017) (diluted 1:1,000 in 1% skim milk in PBST) overnight at 4°C followed by goat anti-rabbit HRP-conjugated secondary antibodies (Merck Millipore, Burlington, MA) (1:1,000 in 1% skim milk in PBST) at ambient temperature for 2 hours. Immunoreactive protein bands were visualized using an enhanced chemiluminescence (ECL) method (LuminataTM Forte Western HRP substrate; Merck Millipore).

Semi-quantitative determination of recombinant *Ov*-TSP-2 on transformed *B. subtilis* WB800N spore surface

A 0.4 ml aliquot of 2.5 x 10^8 *Ov*-TSP-2 recombinant *B. subtilis* WB800N spores (equal to single dose vaccination) was used for extraction of coat proteins with SDS-DTT extraction buffer as described above. Protein concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific) and total protein yield per spore was calculated. Semi-quantitative analysis of recombinant *Ov*-TSP-2 on spore surface was determined by a dot-blot analysis as previously described (Isticato *et al*, 2001). In brief, two-fold serial dilutions (200-12.5 μg) of extracted spore coat proteins were dotted onto nitrocellulose membrane and two-fold serial dilutions (50-3.12 ng) of purified *rOv*-TSP-2 (Chaiyadet *et al*, 2017) was used as standard control. Three μl aliquots of extracted *Ov*-TSP-2 spore coat proteins and purified *rOv*-TSP-2 were dotted onto the nitrocellulose membrane and air dried at ambient temperature for 1 hour, then the membrane was incubated with 5% skim milk in PBST for 1 hour at ambient temperature. The membrane was incubated with anti-*Ov*-TSP-2 rabbit serum (1:1,000 in 1% skim milk in PBST) at ambient temperature for 2 hours, followed by goat anti-rabbit HRP-conjugated secondary antibody (1:1,000 in 1% skim milk in PBST) at ambient temperature for 1 hour. Immunoreactive protein spots were visualized using 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich). Semi-quantitative analysis of protein content per dot was performed using Image Studio Lite 5.2 and results were plotted using Microsoft Excel program (Isticato *et al*, 2001).

Immunofluorescence detection of *Ov*-TSP-2 on *B. subtilis* spore surface

In order to confirm expression of *Ov*-TSP-2 fusion protein in *B. subtilis*, spore cultures (250 μl) were treated at day 5 after initiation of sporulation with 30 mM $NaPO_4$ pH 7.5 containing 2.4% paraformaldehyde and 0.04% glutaraldehyde for 10 minutes on ice, then for 50 minutes at ambient temperature (Zhou *et al*, 2008a). The treated bacteria were washed three times in PBS pH 7.4 and re-suspended in 125 μl of GTE solution (50 mM glucose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA, and 2 mg/ml lysozyme). Samples (10 μl) were dispensed onto microscope slides and air-dried. Then slides were immersed in methanol at -20°C for 5 minutes, followed

by acetone at -20°C for 30 seconds, and air-dried. Subsequently, slides were incubated with 2% bovine serum albumin in PBS for two hours at ambient temperature, then with purified IgG from anti-*Ov*-TSP-2 rabbit serum (Chaiyadet *et al*, 2017) (1:200 in PBS) overnight at 4ºC. The corresponding control samples were incubated with PBS. Slides then were treated with Alexa fluor 488-labeled goat anti-rabbit IgG (Invitrogen) (1:400 in PBS) for 1 hour and viewed under fluorescent light at 488 nm excitation (Leica microscope fitted with DFC500 digital camera; Leica, Wetzlar, Germany). Fluorescence intensity of recombinant *Bacillus* spores was examined from 10 areas of each photograph and reported as corrected total cell fluorescence (CTCF) using Image J software version 1.50i (http://imagej.nih.gov/ij/).

Immunization of hamsters with *B. subtilis* spores expressing *Ov*-TSP-2

Fifteen male golden Syrian hamsters *(Mesocricetus auratus)*, reared at the animal facility, Faculty of Medicine, Khon Kaen University, were randomly divided into three equal groups, designated pHT01-CotC-LEL-*Ov*-TSP-2, pHT01-CotC and NSS (negative control). The pHT01-CotC-LEL-*Ov*-TSP-2 group was orally administrated 2.5 x 10^8 *B. subtilis* spores expressing CotC- LEL-*Ov*-TSP-2 (recombinant spores), pHT01-CotC group with 2.5 x 10^8 *B. subtilis* spores expressing CotC and negative control group with NSS on days 0, 1, 14, 15, 28, and 29 using an oro-intragastric feeding tube (Table 1). Blood samples (1 ml) from each hamster in each group were collected one day before the first immunization (pre-immunization serum). Hamsters were euthanized at 14 days after the final oral immunization, and venous blood (5 ml) from heart for serum preparation and bile from gallbladder were collected to determine antibody responses (Fig 3A).

Animal protocols were approved by the Animal Ethics Committee of Khon Kaen University according to the Ethics of Animal Experimentation of the National Research Council of Thailand (approval number ACUC-KKU-41/60).

Detection of hamster anti-LEL-*Ov*-TSP-2 specific IgG by ELISA and western blotting

ELISA for hamster anti-LEL-*Ov*-TSP-2 specific IgG was optimized as previously described (Tran *et al*, 2006; Pearson *et al*, 2012). In short, 96-well microtiter plates (Thermo Fisher Scientific) were coated with 100 µl aliquots of recombinant LEL-*Ov*-TSP-2 (Chaiyadet *et al*, 2017) (1 µg/ml in coating buffer containing 35 mM

Table 1
Corrected total cell fluorescence (CTCF) intensity of *Bacillus subtilis* WB800N spores transformed with pHT01-CotC (CotC) and pHT01-CotC-LEL-*Ov*-TSP-2 (CotC-LEL-*Ov*-TSP-2).

B. subtilis WB800N spores (n = 5)	CTCF (mean ± SD)	Minimum	Maximum
PBS	229 ± 319	-4	1,081
CotC	8 ± 12	0	41
CotC-LEL-*Ov*-TSP-2	4,644 ± 2,241	1,971	8,205

PBS, phosphate-buffered saline used in suspension of wild type *B. subtilis* WB800N control.

$NaHCO_3$ and 14 mM Na_2CO_3 pH 9.6) per well, incubated at 4°C overnight and washed 3 times with washing buffer [9 g (0.154 M) NaCl and 0.5 ml Tween 20 in 1 liter of de-ionized water]. After incubating with 5% skim milk in coating buffer for 2 hours at 37°C, plates were treated with hamster sera or bile [1:50 dilution in 2% skim milk in incubation buffer (0.138 M NaCl, 9 mM $Na_2HPO_4 \cdot 2H_2O$, 0.84 mM $NaH_2PO_4 \cdot 2H_2O$, and 0.5 ml Tween 20)] for 2 hours at 37°C. After washing, plates were incubated with HRP-conjugated rabbit anti-hamster IgG secondary antibodies (Sigma) (1:1,000 in PBS, for 1 hour at 37°C followed by tetramethylbenzidine (TMB) (Thermo Fisher Scientific) stock solution following company instructions for 15 minutes at ambient temperature. The reaction was stopped with 2 M H_2SO_4 and $A_{450\ nm}$ of each well was measured using TMB solution as a blank control.

For western blotting, 2 μg of recombinant LEL-*Ov*-TSP-2 (Chaiyadet *et al*, 2017) was subjected to 15% SDS-PAGE and transblotted onto a nitrocellulose membrane (Mini Trans-Blot Cell, Bio-Rad), washed with PBST and treated with 5% skim milk in PBST for 2 hours at ambient temperature. Membrane was incubated with hamster serum (1:100 in 1% skim milk in PBST) overnight at 4°C, followed by incubation with goat anti-hamster IgG HRP-conjugated secondary antibody (Thermo Fisher Scientific) (1:1,000 in 1% skim milk in PBST) at ambient temperature for 2 hours. Immunoreactive protein was visualized using the ECL method as described above.

Statistical analysis

Experimental values are expressed as mean ± standard deviation (SD) obtained from three independent experiments. Data were analyzed using one-way analysis of variance (ANOVA) and paired *t*-test as post-hoc test using GraphPad Prism software version 7.04 (www.graphpad.com). *P*-value ≤0.05 is considered statistically significant.

RESULTS

Surface expression of *Ov*-TSP-2 on recombinant *B. subtilis* WB800N spores

B. subtilis WB800N strain was transformed with recombinant pHT01-CotC-LEL-*Ov*-TSP-2 plasmid. Mean copy number (± SD) of plasmid pHT01-CotC-LEL-*Ov*-TSP-2 per transformed *B. subtilis* cell was 3.28 ± 0.15, which was in agreement of the manufacturer's claim for pHT01 transfection into *B. subtilis* WB800N of 4-6 copies/cell. Analysis of coat proteins on day 5 of sporulation by SDS-PAGE, a protein of ~ 23 kDa, consistent with that of fusion CotC (14.65 kDa of CotC plus 8.39 kDa of *Ov*-TSP-2-LEL) (Chaiyadet *et al*, 2017), whereas this protein was absent in coat proteins of *B. subtilis* spores transformed with pHT01-CotC (Fig 1A). Western blotting revealed a 23 kDa immunoreactive coat protein from recombinant spores expressing fusion CotC when probed with rabbit anti-*Ov*-TSP-2 IgG (Fig 1B). The quantity of total coat protein per *Bacillus* spore was 3.36 pg. Dot blot analysis showed the amount of CotC-LEL-*Ov*-TSP-2 fusion protein represented 0.024% of the total coat proteins extracted from *B. subtilis* spore coat (Fig 1C). Thus, the approximate amount of CotC-LEL-*Ov*-TSP-2 fusion protein was 0.81 fg/spore. As each hamster was orally administrated with 2.5 x 10^8 spores each time, this corresponds to 0.20 μg of CotC-LEL-*Ov*-TSP-2 per dose for each hamster.

Fluorescence microscopy using purified anti-*Ov*-TSP-2 IgG with Alexa Fluor 488-labeled goat anti-rabbit IgG detected

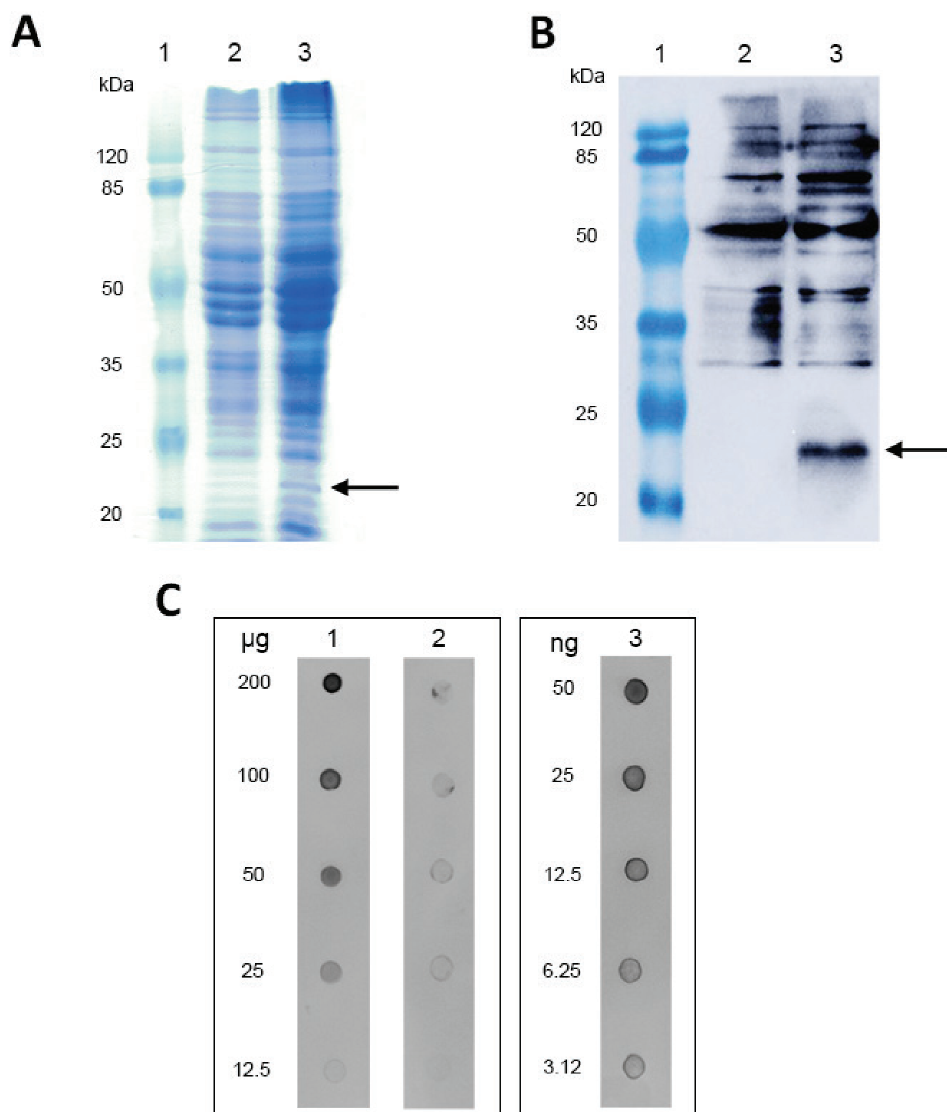

Fig 1 - SDS-PAGE of recombinant *Ov*-TSP-2 expression in transformed *Bacillus subtilis* WB800N 5 days post sporulation (A), western blots of recombinant CotC and CotC-LEL-*Ov*-TSP-2 fusion protein on *B. subtilis* WB800N spore coat (B), and dot blots of proteins extracted from transformed *B. subtilis* WB800N (C). *Bacillus subtilis* WB800N transformed with recombinant expression plasmids were grown in sporulation solution and harvested after 5 days. Panel A. Extracted spore coat proteins were analyzed by 15% SDS-PAGE. Lane 1, protein molecular mass standards; lane 2, *B. subtilis* WB800N spores transformed with pHT01-CotC; lane 3, *B. subtilis* WB800N spores transformed with pHT01-CotC-LEL-*Ov*-TSP-2. Panel B. Western blots of proteins in Panel A immunoreactive to purified rabbit anti-*Ov*-TSP-2 IgG. Panel C. Dot blot analysis of transformed *B. subtilis* WB800N spore coat proteins immunoreactive to purified rabbit anti-*Ov*-TSP-2 IgG. Lane 1, *B. subtilis* WB800N transformed with pHT01-CotC-LEL-*Ov*-TSP-2; lane 2, *B. subtilis* WB800N transformed with pHT01-CotC; lane 3, purified *rOv*-TSP-2. Protein content of each spot is indicated.

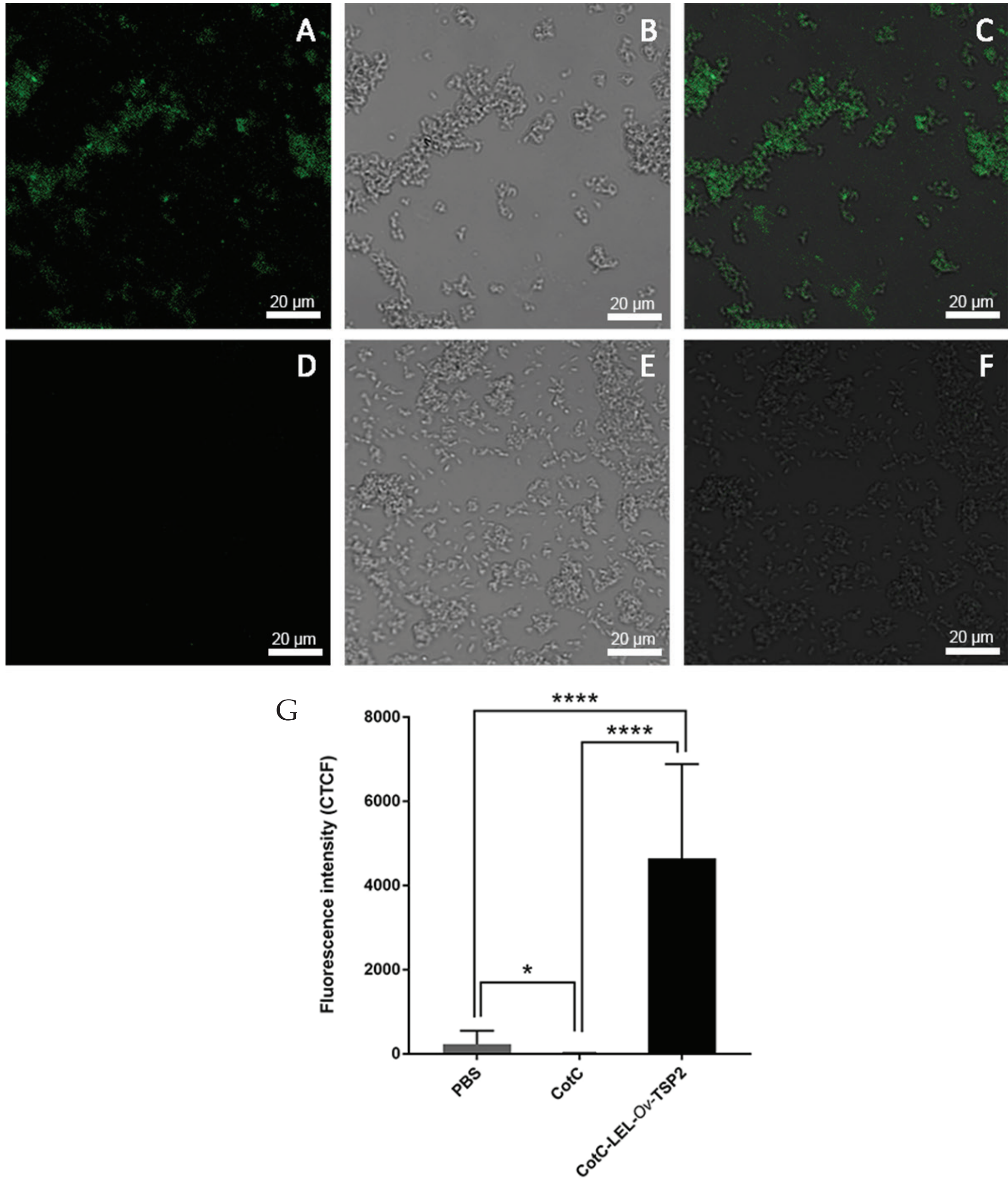

Fig 2 - Immunofluorescence of fusion CotC-LEL-*Ov*-TSP-2 on surface of *Bacillus subtilis* WB800N spores. Panels A-C. Spores transformed with pHT01-CotC-LEL-*Ov*-TSP-2 treated with purified rabbit anti-*Ov*-TSP-2 IgG followed with Alexa fluor 488-conjugated goat anti-rabbit IgG. Panels D-F. Spores transformed with pHT01-CotC treated with purified rabbit anti-*Ov*-TSP-2 IgG followed with Alexa fluor 488-conjugated goat anti-rabbit IgG. Panels A and D are observed under fluorescence microscope, panels B and E under bright field light microscope and panels C and F are merged bright field/fluorescence images. Panel G. Quantitative output of fluorescence micrographs of panels A and D created using an ImageJ software.

Table 2
Specific anti-*Ov*-TSP-2 IgG levels in sera collected from hamsters pre- and post-oral immunization.

Hamster group (n = 5)	A_{450nm} (mean ± SD)	
	Pre-immunization	Post-immunization
NSS[a]	0.091 ± 0.004 (min = 0.086, max = 0.955)	0.103 ± 0.027 (min = 0.081, max = 0.147)
CotC[b]	0.091 ± 0.006 (min = 0.081, max = 0.097)	0.080 ± 0.049 (min = 0.080, max = 0.133)
CotC-LEL-Ov-TSP-2[c]	0.0954 ± 0.0133 (min = 0.082, max = 0.111)	0.133 ± 0.024 (min = 0.102, max = 0.169)

[a]Immunized with normal saline solution. [b]Immunized with *Bacillus subtilis* WB800N spores transformed with pHT01-CotC. [c]Immunized with *B. subtilis* WB800N spores transformed with and pHT01-CotC-LEL-*Ov*-TSP-2. Max, maximum; min, minimum.

expression of *Ov*-TSP-2 on *B. subtilis* spore surface on 5 days of sporulation (Fig 2A, B and C) and on recombinant spores expressing only CotC (Fig 2D, E and F), whereas no specific fluorescence was observed when PBS served as the probe (not shown), indicating that florescence was specific to *Ov*-TSP-2. Fluorescence intensity of *Bacillus* spores expressing CotC-LEL-*Ov*-TSP-2 was significantly higher than control groups (CotC expressing and PBS; $p<0.0001$) (Fig 2G).

Anti- LEL-*Ov*-TSP-2 specific IgG response in orally immunized hamsters determined by ELISA

Sera were collected one day prior to first oral immunization (day-1) and two weeks after the final immunization (day 42 post-immunization). IgG levels specific to LEL-*Ov*-TSP-2 from hamsters orally immunized with recombinant spores containing CotC-*Ov*-TSP-2 were significantly higher than those from pre-immunization sera from the same hamsters ($p<0.05$) (Fig 3A). No significant differences were observed between IgG levels of pre-immunization and post-immunization sera from hamsters orally immunized with recombinant spores containing CotC or from NSS control group (Fig 3B). $A_{450\ nm}$ values of each group are listed in Table 2.

IgG levels in bile from hamsters orally immunized with recombinant spores containing CotC-*Ov*-TSP-2 are significantly higher than those from orally immunized with CotC-expressing spores and NSS group ($p<0.05$) at day 42 post-immunization. No significant differences were observed between control groups (Fig 3C). $A_{450\ nm}$ values of each group are listed in Table 3.

Western blot analysis of recombinant LEL-*Ov*-TSP-2 showed specific immunoreactivity of a 29 kDa protein against antibodies present in hamster vaccinated with recombinant spores containing CotC-*Ov*-TSP-2 (Fig 3D) as previously described (Chaiyadet *et al*, 2017). Sera of hamsters orally administered with *Bacillus* spores

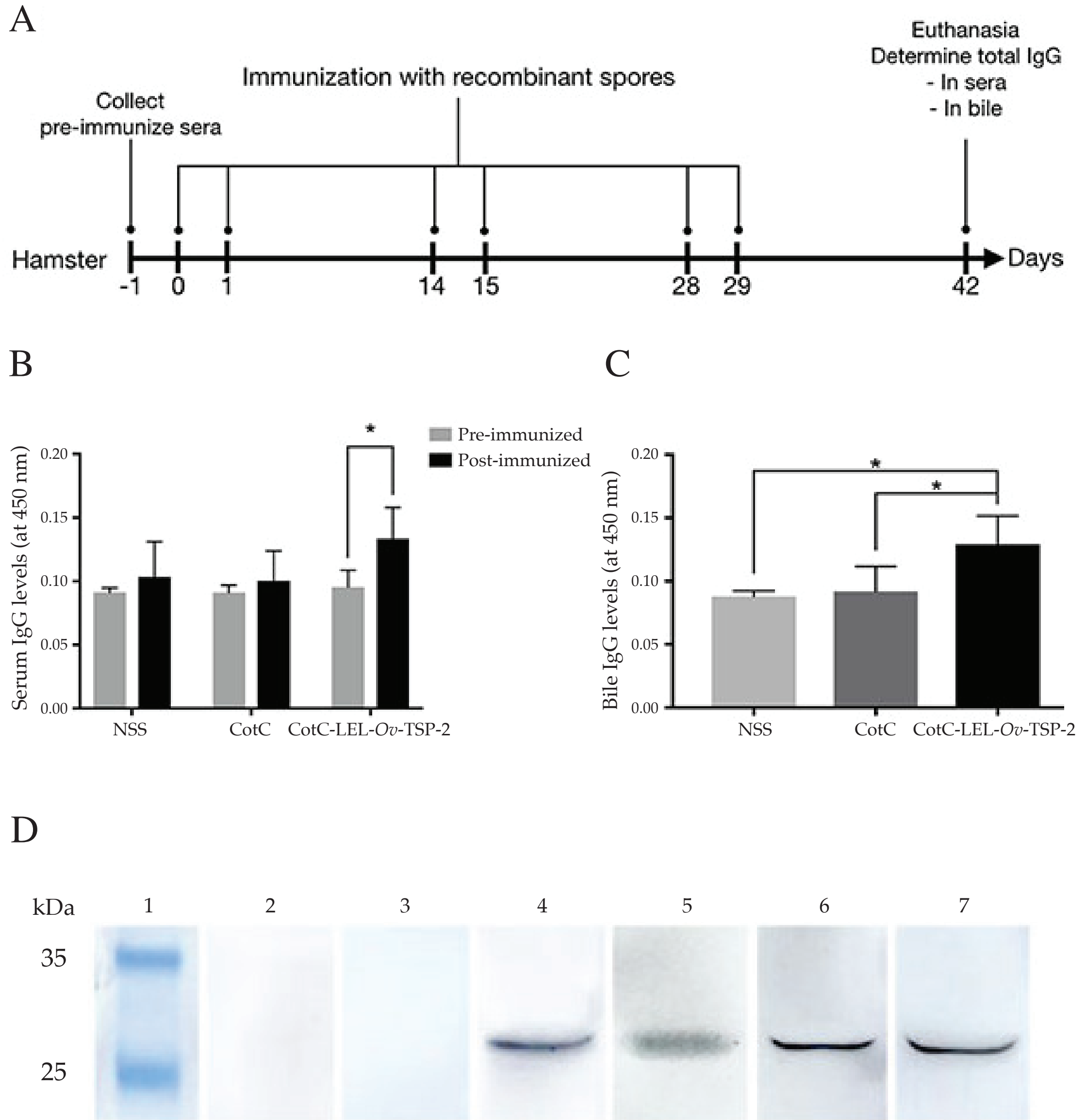

Fig 3 - Levels of immunized hamster serum IgG against fusion LEL-*Ov*-TSP-2 and western blots of hamster sera. A. Protocol and timeline for vaccination of hamsters with transformed *Bacillus subtilis* WB800N spores. B. IgG levels in bile of hamsters immunized orally with spores transformed with pHT01-CotC-LEL-*Ov*-TSP-2. C. IgG levels in bile of hamsters immunized orally with spores transformed with pHT01-CotC. D. Western blots of extracted spore coat proteins of *Bacillus subtilis* WB800N transformed with pHT01-CotC-LEL-*Ov*-TSP-2 separated by 15% SDS-PAGE and treated with hamster immune sera (1:100 dilution), followed by goat HRP-conjugated anti-hamster IgG (1:1,000 dilution and visualized using an enhanced chemiluminescence method. Lane 1, protein molecular mass standards; lane 2, immune serum of hamster immunized with spores transformed with pHT01-CotC; lane 3, serum of control hamster; lanes 4-7, immune serum of hamster immunized with spores transformed with pHT01-CotC-LEL-*Ov*-TSP-2.

Table 3
Specific anti-*Ov*-TSP-2 IgG levels in bile collected from immunized hamsters.

Hamster group (n = 5)	A_{450nm} (mean ± SD)	Minimum	Maximum
NSS[a]	0.088 ± 0.004	0.082	0.093
CotC[b]	0.092 ± 0.197	0.068	0.110
CotC-LEL-*Ov*-TSP-2[c]	0.129 ± 0.022	0.112	0.165

[a]Immunized with normal saline solution. [b]Immunized with *Bacillus subtilis* WB800N spores transformed with pHT01-CotC. [c]Immunized with *B. subtilis* WB800N spores transformed with and pHT01-CotC-LEL-*Ov*-TSP-2.

expressing CotC only and of NSS group did not react to recombinant LEL-*Ov*-TSP-2 (no specific band).

DISCUSSION

B. subtilis is a gram-positive bacterium found in soil and the gastrointestinal tract of ruminants and humans, and its rod-shaped morphology, together with a tough, protective endospore, allows it to tolerate extreme environmental conditions (Knecht *et al*, 2011; Hinc *et al*, 2013). Expression of vaccine immunogens as fusions with spore coat surface proteins of *B. subtilis* confers resistance to degradation within the stomach and gastrointestinal tract (Duc and Cutting, 2003; de Souza *et al*, 2014; Chaiyadet *et al*, 2017), and elicits specific immune responses and protective efficacy in animal models (Zhou *et al*, 2008a; Zhou *et al*, 2008b; Qu *et al*, 2014; Wang *et al*, 2014). In this sense, it has been reported that oral delivery of *B. subtilis* spores expressing specific proteins can induce mucosal and systemic immunity (Belyakov and Ahlers, 2009; Simerska *et al*, 2009).

TSPs are a family of membrane-spanning proteins that display four hydrophobic transmembrane domains interspersed with two extracellular loops and short intracellular amino and carboxyl sequences (Maecker *et al*, 1997; Boucheix and Rubinstein, 2001; Yanez-Mo *et al*, 2009). TSPs are found on the plasma membrane and extracellular vesicles of several helminths including blood and liver flukes (Chaiyadet *et al*, 2015; Rocha-Perugini *et al*, 2016; Sotillo *et al*, 2016), and have been considered as a potential protein target for vaccines against fluke infections (Da'dara *et al*, 2001; Tran *et al*, 2006; Chaiyadet *et al*, 2015; Chaiyadet *et al*, 2017). Herein, we have successfully developed, to the best of our knowledge, the first *B. subtilis* spore coat protein-based immunogen using a protein from the carcinogenic liver fluke *O. viverrini*. SDS-PAGE, western blotting and immunofluorescence reveal that *Ov*-TSP-2 was successfully fused with CotC and expressed on *B. subtilis* spore surface. Although other proteins from the related liver fluke *C. sinensis* have been successfully expressed in *B. subtilis* (Zhou *et al*, 2008a; Zhou *et al*, 2008b; Yu *et al*, 2015; Wu *et al*, 2017; Sun *et al*, 2018), this is the first report of an *O. viverrini* protein fused with a spore coat protein from this bacterium. In addition, this is the first time an extracellular loop of a tetraspanin from any helminth has been expressed in *B. subtilis*.

The recombinant spores were used as an oral immunogen in hamsters, pro-

voking significantly elevated IgG titers in both blood and bile. Control animals immunized with CotC-expressing spores did not show any significant IgG levels, suggesting antibody responses observed were directed against the TSP fragment. Serum IgG levels observed in this study, although low, are significantly greater than the control groups and are consistent with findings in other studies (Zhou *et al*, 2008a; Zhou *et al*, 2008b). IgG levels in the bile of immunized hamsters also are significantly higher than in controls. This is of importance as adult parasites live in bile ducts of the host, and could be a potential site of action for protective antibodies. In this regard, a role in protective immunity has been described for IgG antibodies targeting other antigens of *O. viverrini* (Kaewraemruaen *et al*, 2016; Papatpremsiri *et al*, 2016).

Although a number of studies used a higher number of spores in their immunization studies than the $2.5x10^8$ spores employed in our study (Zhou *et al*, 2008a; Zhou *et al*, 2008b; Yu *et al*, 2015), Qu *et al*, (2014) used only $5x10^5$ spores expressing CotC fused with *C. sinensis* leucine aminopeptidase-2. However, in those studies BALB/c mice are immunized intragastrically via oral administration 9 times during the experimental period, compared to 6 oral immunizations of hamsters in the current study, but IgG levels of immunized mice are higher (Qu *et al*, 2014) than immunized hamsters reported here. These findings suggest higher numbers of spores and more number of times of immunization should be considered in future studies to improve IgG titers in the hamster model.

In conclusion, expression of liver fluke antigens as fusions with *B. subtilis* spore coat proteins represents a promising alternative for oral vaccination against opisthorchiasis and, in turn, liver fluke infection-induced cholangiocarcinoma. This study constitutes the first approach to develop oral vaccines against *O. viverrini*, and further studies comparing different antigens and immunization protocols should be performed. In addition, *B. subtilis* could be engineered to include an array of molecules from the parasite to enhance vaccine efficacy and to facilitate scaling of production of helminth vaccines for the control and prevention of liver fluke infection and other helminth parasites.

ACKNOWLEDGEMENTS

This research was supported by a Postgraduate Scholarship, Faculty of Medicine, Khon Kaen University, a project grant from the National Health and Medical Research Council of Australia (NHMRC), grant number 1085309 and the National Cancer Institute, National Institutes of Health, USA, grant number R01CA164719.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Acosta D, Cancela M, Piacenza L, Roche L, Carmona C, Tort JF. *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. *Mol Biochem Parasitol* 2008; 158: 52-64.

Belyakov IM, Ahlers JD. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? *J Immunol* 2009; 183: 6883-92.

Boonyanugomol W, Chomvarin C, Sripa B, *et al*. *Helicobacter pylori* in Thai patients with cholangiocarcinoma and its association with biliary inflammation and prolif-

eration. *Hep Panc Biliar* 2012; 14: 177-84.

Boucheix C, Rubinstein E. Tetraspanins. *Cell* 2001; 58: 1189-205.

Bouvard V, Baan R, Straif K, *et al*. A review of human carcinogens--Part B: biological agents. *Lancet Oncol* 2009; 10: 321-2.

Brindley PJ, Loukas A. Helminth infection-induced malignancy. *PLOS Pathog* 2017; 13: e1006393.

Chaiyadet S, Krueajampa W, Hipkaeo W, *et al*. Suppression of mRNAs encoding CD63 family tetraspanins from the carcinogenic liver fluke *Opisthorchis viverrini* results in distinct tegument phenotypes. *Sci Rep* 2017; 7: 14342.

Chaiyadet S, Sotillo J, Smout M, *et al*. Carcinogenic liver fluke secretes extracellular vesicles that promote cholangiocytes to Adopt a Tumorigenic Phenotype. *J Infect Dis* 2015; 212: 1636-45.

Chawengkirttikul R, Sirisinha S. Antibodies in serum and bile of hamsters experimentally infected with *Opisthorchis viverrini. Int J Parasitol* 1988; 18: 721-7.

Da'dara AA, Skelly PJ, Wang MM, Harn DA. Immunization with plasmid DNA encoding the integral membrane protein, Sm23, elicits a protective immune response against schistosome infection in mice. *Vaccine* 2001; 20: 359-69.

Dakshinamoorthy G, Munirathinam G, Stoicescu K, Reddy MV, Kalyanasundaram R. Large extracellular loop of tetraspanin as a potential vaccine candidate for filariasis. *PLOS One* 2013; 8: e77394.

Dang Z, Yagi K, Oku Y, *et al*. A pilot study on developing mucosal vaccine against alveolar echinococcosis (AE) using recombinant tetraspanin 3: vaccine efficacy and immunology. *PLOS Negl Trop Dis* 2012; 6: e1570.

de Souza RD, Batista MT, Luiz WB, *et al*. *Bacillus subtilis* spores as vaccine adjuvants: further insights into the mechanisms of action. *PLOS One* 2014; 9: e87454.

Deenonpoe R, Mairiang E, Mairiang P, *et al*. Elevated prevalence of *Helicobacter* species and virulence factors in opisthorchiasis and associated hepatobiliary disease. *Scient Rep* 2017; 7: 42744.

Duc LH, Cutting SM. Bacterial spores as heat stable vaccine vehicles. *Expert Opin Biol Ther* 2003; 3: 1263-70.

Froger A, Hall JE. Transformation of plasmid DNA into *E. coli* using the heat shock method. *J Visual Exp E* 2007: 253.

Hemler ME. Specific tetraspanin functions. *J Cell Biol* 2001; 155: 1103-7.

Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu Rev Cell Dev Biol* 2003; 19: 397-422.

Hemler ME. Targeting of tetraspanin proteins--potential benefits and strategies. *Nat Rev Drug Discov* 2008; 7: 747-58.

Hinc K, Iwanicki A, Obuchowski M. New stable anchor protein and peptide linker suitable for successful spore surface display in *B. subtilis*. *Microb Cell Fact* 2013; 12: 22.

Ilk N, Schumi CT, Bohle B, Egelseer EM, Sleytr UB. Expression of an endotoxin-free S-layer/allergen fusion protein in gram-positive *Bacillus subtilis* 1012 for the potential application as vaccines for immunotherapy of atopic allergy. *Microb Cell Fact* 2011; 10: 6.

Isticato R, Cangiano G, Tran HT *et al*. Surface display of recombinant proteins on *Bacillus* subtilis spores. *J Bacteriol* 2001; 183: 6294-301.

Jiang H, Chen T, Sun H, *et al*. Immune response induced by oral delivery of *Bacillus subtilis* spores expressing enolase of *Clonorchis sinensis* in grass carps *(Ctenopharyngodon idellus)*. *Fish Shellfish Immunol* 2017; 60: 318-25.

Jittimanee J, Sermswan RW, Kaewraemruaen C, *et al*. Protective immunization of hamsters against *Opisthorchis viverrini* infection is associated with the reduction of TGF-beta expression. *Acta Trop* 2012; 122: 189-95.

Jittimanee J, Sermswan RW, Puapairoj A, Malee-

wong W, Wongratanacheewin S. Cytokine expression in hamsters experimentally infected with *Opisthorchis viverrini. Parasite Immunol* 2007; 29: 159-67.

Jusakul A, Cutcutache I, Yong CH, *et al*. Whole-genome and epigenomic landscapes of etiologically distinct subtypes of cholangiocarcinoma. *Cancer Discov* 2017; 7: 1116-35.

Kaewraemruaen C, Sermswan RW, Wongratanacheewin S. CpG oligodeoxynucleotides with crude parasite antigens reduce worm recovery in *Opisthorchis viverrini* infected hamsters. *Acta Trop* 2016; 164: 395-401.

Knecht LD, Pasini P, Daunert S. Bacterial spores as platforms for bioanalytical and biomedical applications. *Anal Bioanal Chem* 2011; 400: 977-89.

Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli. J Biotechnol* 2006; 123: 273-80.

Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. *Fed Am Soc Exp Biol* 1997; 11: 428-42.

Mauriello EM, Duc le H, Isticato R, *et al*. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. *Vaccine* 2004; 22: 1177-87.

Merrifield M, Hotez PJ, Beaumier CM, *et al*. Advancing a vaccine to prevent human schistosomiasis. *Vaccine* 2016; 34: 2988-91.

Nguyen QA, Schumann W. Use of IPTG-inducible promoters for anchoring recombinant proteins on the *Bacillus subtilis* spore surface. *Protein Expr Purif* 2014; 95: 67-76.

Papatpremsiri A, Junpue P, Loukas A, *et al*. Immunization and challenge shown by hamsters infected with *Opisthorchis viverrini* following exposure to gamma-irradiated metacercariae of this carcinogenic liver fluke. *J Helminthol* 2016; 90: 39-47.

Patel H, Yewale C, Rathi MN, Misra A. Mucosal immunization: a review of strategies and challenges. *Crit Rev Ther Drug Car Systs* 2014; 31: 273-303.

Pearson MS, Pickering DA, McSorley HJ, *et al*. Enhanced protective efficacy of a chimeric form of the schistosomiasis vaccine antigen Sm-TSP-2. *PLOS Negl Trop Dis* 2012; 6: e1564.

Plieskatt JL, Deenonpoe R, Mulvenna JP, *et al*. Infection with the carcinogenic liver fluke *Opisthorchis viverrini* modifies intestinal and biliary microbiome. *FASEB J* 2013; 27: 4572-84.

Qu H, Xu Y, Sun H, *et al*. Systemic and local mucosal immune responses induced by orally delivered *Bacillus subtilis* spore expressing leucine aminopeptidase 2 of *Clonorchis sinensis. Parasitol Res* 2014; 113: 3095-103.

Rocha-Perugini V, Sanchez-Madrid F, Martineg Del Hoyo G. Function and dynamics of tetraspanins during antigen recognition and immunological synapse formation. *Front Immunol* 2016; 6: 653.

Rosales-Mendoza S, Angulo C. *Bacillus subtilis* comes of age as a vaccine production host and delivery vehicle. *Expert Rev Vaccines* 2015; 14: 1135-48.

Simerska P, Moyle PM, Olive C, Toth I. Oral vaccine delivery--new strategies and technologies. *Curr Drug Deliv* 2009; 6: 347-58.

Sirisinha S, Wongratanacheewin S. Immunization of hamsters against *Opisthorchis viverrini* infection. *Southeast Asian J Trop Public Health* 1986; 17: 567-73.

Sithithaworn P, Andrews RH, Nguyen VD, *et al*. The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. *Parasitol Int* 2012; 61: 10-6.

Smout MJ, Sotillo J, Laha T, *et al*. Carcinogenic parasite secretes growth factor that accelerates wound healing and potentially promotes neoplasia. *PLOS Pathog* 2015; 11: e1005209.

Smout MJ, Sripa B, Laha T, *et al*. Infection with the carcinogenic human liver fluke,

Opisthorchis viverrini. *Mol Biosyst* 2011; 7: 1367-75.

Sotillo J, Pearson M, Potriquet J, *et al*. Extracellular vesicles secreted by *Schistosoma mansoni* contain protein vaccine candidates. *Int J Parasitol* 2016; 46: 1-5.

Sripa B, Mairiang E, Thinkhamrop B, *et al*. Advanced periductal fibrosis from infection with the carcinogenic human liver fluke *Opisthorchis viverrini* correlates with elevated levels of interleukin-6. *Hepatology* 2009; 50: 1273-81.

Sun H, Lin Z, Zhao L, *et al*. *Bacillus subtilis* spore with surface display of paramyosin from *Clonorchis sinensis* potentializes a promising oral vaccine candidate. *Parasit Vectors* 2018; 11: 156.

Tang Z, Shang M, Chen T, *et al*. The immunological characteristics and probiotic function of recombinant *Bacillus subtilis* spore expressing *Clonorchis sinensis* cysteine protease. *Parasit Vectors* 2016; 9: 648.

Tran MH, Pearson MS, Bethony JM, *et al*. Tetraspanins on the surface of *Schistosoma mansoni* are protective antigens against schistosomiasis. *Nature Med* 2006; 12: 835-40.

Van Meulder F, Ratman D, Van Coppernolle S, *et al*. Analysis of the protective immune response following intramuscular vaccination of calves against the intestinal parasite *Cooperia oncophora*. *Int J Parasitol* 2015; 45: 637-46.

Wang X, Chen W, Tian Y, *et al*. Surface display of *Clonorchis sinensis* enolase on *Bacillus subtilis* spores potentializes an oral vaccine candidate. *Vaccine* 2014; 32: 1338-45.

Wang H, Wang Y, Yang R. Recent progress in *Bacillus subtilis* spore-surface display: concept, progress, and future. *Appl Microbiol Biotechnol* 2017; 101: 933-49.

Wher HM, Frank JH. Standard methods for the microbiological examination of dairy products. 17th ed. Washington, DC: American Public Health Associahion, 2004.

Wu Z, Tang Z, Shang M, *et al*. Comparative analysis of immune effects in mice model: *Clonorchis sinensis* cysteine protease generated from recombinant *Escherichia coli* and *Bacillus subtilis* spores. *Parasitol Res* 2017; 116: 1811-22.

Yanez-Mo M, Barreiro O, Gordon-Alonso M, Sala-Valdes M, Sanchez-Madrid F. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. *Trends Cell Biol* 2009; 19: 434-46.

Yu J, Chen T, Xie Z, *et al*. Oral delivery of *Bacillus subtilis* spore expressing enolase of *Clonorchis sinensis* in rat model: induce systemic and local mucosal immune responses and has no side effect on liver function. *Parasitol Res* 2015; 114: 2499-505.

Zhang H, Chung BS, Li S, Choi MH, Hong ST. Changing patterns of serum and bile antibodies in re-infected rats with *Clonorchis sinensis*. *Korean J Parasitol* 2008; 46: 17-22.

Zhong C, Peng D, Ye W, *et al*. Determination of plasmid copy number reveals the total plasmid DNA amount is greater than the chromosomal DNA amount in *Bacillus thuringiensis* YBT-1520. *PLOS One* 2011; 6: e16025.

Zhou Z, Xia H, Hu X, *et al*. Oral administration of a *Bacillus subtilis* spore-based vaccine expressing *Clonorchis sinensis* tegumental protein 22.3 kDa confers protection against *Clonorchis sinensis*. *Vaccine* 2008a; 26: 1817-25.

Zhou Z, Xia H, Hu X, *et al*. Immunogenicity of recombinant *Bacillus subtilis* spores expressing *Clonorchis sinensis* tegumental protein. *Parasitol Res* 2008b; 102: 293-7.