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Trichomonas vaginalis Adhesion Protein 33: A Useful Target for Diagnosis of *T. vaginalis*

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Trichomoniasis is a sexually transmitted disease induced by *Trichomonas vaginalis*, a parasitic protozoan. The symptoms of trichomoniasis are rarely appeared that the infections are distributed worldwide from underdeveloped to developed countries. The diagnosis of trichomoniasis is mainly taken by wet smear following microscopic examination, of which the diagnostic accuracies are poor and varies with the clinicians' experiences. Therefore, more exact and convenient diagnostic methods for *T. vaginalis* are required. Here, we cloned and expressed recombinant *T. vaginalis* adhesion protein 33 (rTvAP33) using an *E. coli* expression system. rTvAP33 was then immunized to rabbit and BALB/c mice for the production of anti-rTvAP33 antibodies. Sandwich ELISA using these antibodies detected *T. vaginalis* cultured in TYM broth supplemented with ferrous ions. Vagina-parasitizing microorganisms showed low cross-reactivities in this system. These results suggest that TvAP33 is a good diagnostic target for the detection of TvAP33-expressing *T. vaginalis*.

Key Words: *Trichomonas vaginalis*, Adhesion protein 33, Recombinant, ELISA

INTRODUCTION

Trichomonas vaginalis is a parasitic protozoan, which is the causative agent of trichomoniasis, one of sexually transmitted diseases worldwide (Petrin, et al., 1998). It had been reported that 2,500 millions of people are infected with *T. vaginalis* and 170 millions of people are newly infected every year (WHO, 1995). The symptoms of trichomoniasis are rarely appeared, but, if appeared, vulval itching, abnormal vaginal odour, and vaginal discharge are included in women (Bowden and Garnett, 1999). The complications include cervical cancer (Zhang and Begg, 1994), atypical pelvic inflammation (Muller, 1988) infertility (Grodstein, et al., 1993), ectopic pregnancy, preterm delivery and low birth weight (Hardy, et al., 1984; Minkoff, et al., 1984).

Recently, epidemiologic studies give weight to the importance of *T. vaginalis* in HIV infection (Sorvillo and Kerndt, 1998). *T. vaginalis* parasitizing on the vaginal mucosa secret cysteine proteases, which inactivate secretory leukocyte protease inhibitor present in the mucosa, thereby resulting in increased survival rates of infecting HIVs (Draper, et al., 1998). The mechanical injuries on vaginal mucosa by *T. vaginalis* also facilitate HIV infections.

Trichomoniasis is generally diagnosed by microscopic examination of *T. vaginalis* in vaginal swap. However, this conventional method has some disadvantages: organisms of morphological similarity can be present and experienced clinicians are required for exact diagnosis (Singh, 1997). Culture on agar plate (Stary, et al., 2002) or PCR (Ryu, et al. 1999) is sensitive and accurate, but is still not appropriate for local applications.

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It had been reported that *T. vaginalis* express four adhesion proteins on their surfaces (Alderete, et al., 1988; Arroyo, et al., 1992). Utilization of these proteins as the diagnostic markers had been also proposed (Engbring and Alderete, 1998). However, applications of these proteins to sensitive and specific molecular diagnostic systems for *T. vaginalis* had not been attempted yet. In this report, we cloned and expressed adhesion protein 33 (TvAP33) gene and designed sandwich ELISA for the diagnosis of *T. vaginalis*. Usefulness of the recombinant TvAP33 (rTvAP33) was also discussed. Other microorganisms parasitizing in the vaginal mucosa were also examined for the examination of cross-reactivities of this system.

MATERIALS AND METHODS

1. Culture of *T. vaginalis*

T. vaginalis KT-4 isolate (Ryu, et al., 2001) was kindly provided by Professor Ryu of Hanyang University, Korea. One ml of he pre-cultured *T. vaginalis* was inoculated in 5 ml of TYM broth (Table 1) and incubated at 37℃ in 5.0% CO_2 condition. Activities and the number of *T. vaginalis* were examined under microscopy after trypan blue staining. Type B and D Streptococci, *Lactobacillus acidophilus*, *Staphylococcus epidermidis* and *Candida albicans* were kindly provided by Department of Clinical Pathology of Wonju Christian Hospital, Korea.

Table 1. Composition of TYM broth

Component	Weight (g)
Tryptone	20.0 g
Yeast extract	10.0 g
Maltose	5.0 g
L-Cysteine, monohydrochloride	1.0 g
Ascorbic acid (Vit.C)	1.0 g
$FeSO_4, 7H_2O$[1)]	0.1 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
Penicillin	0.06 g
Gentamycin	0.5 g
MEM (Minimal Essential Medium)	0.492 g
Horse serum	100.0 ml
D.W	Up to 1L

[1)] excluded in ferrous-depleted TYM broth

2. Total RNA preparation

1×10^4~1×10^5 *T. vaginalis* were subjected to 1 ml of TRIzol® Reagent (Gibco BRL, USA) to isolate total RNA as described in the user's manual. Isolated total RNA was finally dissolved in RNase-free water and quantified and qualified by the measure of the absorbances at 260 and 280 nm. Total RNA was directly used for next step or stored at -80℃.

3. RT-PCR and cloning

Ten μg of *T. vaginalis* total RNA was used to synthesize the cDNA by reverse transcriptase (Promega, USA) as described in the user's manual. The product was used for the template of PCR by Ex Taq™ (Takara, Japan) to amplify the *T. vaginalis* AP33 (TvAP33) genes. The primers were designed on the base of TvAP33 cDNA sequence information (accession number U87096, GeneBank, NIH, USA) as followed: upstream primer: 5'-GGAATTCATGCTCGCAGGCGACTTC-3' (underlined: *Eco*RI site); downstream primer: 5'-CGCGGCCGCTTAGATCTTGCCCATTCTC-3' (underlined: *Xho*I site). Thermocycling condition was as followed: pretreatment at 94℃ for 3 min, 25 cycles of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec and polymerization at 72℃ for 60 sec, and post-extension at 72℃ for 5 min. One μl of the product was used for the ligation in cloning vector pCR®2.1-TOPO® and transformed TOP10 cells to produce pCR-TvAP33/TOP10 cells using TOPO® TA Cloning Kit (Invitrogen, USA) as described in the user's manual. After confirmation of the DNA sequence of the pCR-TvAP33, the inserted TvAP33 genes were excise out using *Eco*RI and *Xho*I and ligased in expression vector pET32a (Invitrogen, USA) treated with the same restriction enzymes to produce pET-TvAP33. DH5α or BL-21 cells were transformed with pET-TvAP33 for the preservation or expression, respectively.

4. Expression and purification of rTvAP33

Two ml of pre-cultured pET-TvAP33/BL21 cells was inoculated in 200 ml of fresh LB broth (1.0% trypton, 0.5% yeast extract and 1.0% NaCl) and cultured until the absorbance at 600 nm reaches to 0.4. 0.1 mM IPTG was added and cultured with the same condition for 4 hours. The cells were harvested, suspended in 10 volume of lysis buffer (6 M guanidine chloride, 100 mM monosodium phosphate and 10 mM Tris-Cl, pH 8.0) and lysed by ultrasonic homogenizer

(Ultrosonics, USA). The lysate was centrifuged at 12,000 ×g for 20 min and the supernatant was filtered using 0.22 μm filter. Filtered supernatant was loaded on 3 ml of Ni-NTA resin (Quiagen, Germany) equilibrated with the lysis buffer and then washed with 5 bed volume of the lysis buffer under gravity flow. The resin-bound proteins were step-wisely eluted with elution buffers (5, 10 or 20 mM imidazole in lysis buffer) under gravity flow. The elutes were collected and protein concentrations were determined by Bradford method (Bradford, 1976).

5. Immunoblotting

Purified proteins were electrophoresed by 10% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, USA) to perform immunoblotting against His_6 tag of rTvAP33, where mouse anti-His_6 antibody (Serotec, UK) was used for capture antibody and HRP-conjugated rabbit anti-mouse antibody (Serotec, UK) was used for secondary antibody. Overall protocol was followed to the manual of ECL Reagent kit (Amersham Biosciences, UK).

6. Immunization of BALB/c mice and rabbit with rTvAP33

To six-week-old female BALB/c mice or rabbit, 100 μg or 500 μg of rTvAP33 emulsified with Freund's complete adjuvant (Gibco BRL, USA) were i.d. injected on the backs, respectively. The mice and rabbit were subsequently immunized with the same doses of rTvAP33 emulsified with Freund's incomplete adjuvant (Gibco BRL, USA) with 2 week interval for 2 times. The bloods were collected before every injection and 1 week after last injections to prepare immunized sera.

7. Purifications of anti-rTvAP33 antibodies

Forty mg of rTvAP33 was conjugated with Affi-Gel® 10 resin (Bio-Rad, USA) suspended in conjugation buffer (0.1% Sarkosyl in MOPS pH 7.5) at 4℃ for 18 hr. The resin was transferred to the column of 0.6 cm in diameter (Bio-Rad, USA) and equilibrated with 10 mM Tris pH 8.8. Immunized mice or rabbit sera were dialyzed with 10 mM Tris pH 8.8, and then loaded on the rTvAP33-coupled Affi-Gel® 10 column and washed with 20 ml of 10 mM Tris pH 7.5 by gravity flow. The anti-rTvAP33 antibodies were eluted by passing 100 mM glycine pH 2.5 at gravity flow. The elutes were collected by 1 ml in fraction tubes containing appropriate volume of 1 M Tris for the neutralization of the elutes. All fractions were assayed for the protein concentrations by Bradford method (Bradford, 1976) and the peak fractions were pooled and concentrated by Centri-Prep® 10 (Amicon, USA).

8. ELISA

A hundred μl of 1.0 μg/ml rabbit anti-rTvAP33 antibodies in 50 mM carbonate buffer pH 9.8 was dispensed in flat-bottomed 96 well microtiter plate (Nunc, Denmark) and incubated at 4℃ overnight. After removing the solutions, 300 μl of blocking buffer (0.1% casein, 100 mM imidazole and 0.1% Tween 20 in PBS) was added and incubated at 37℃ for 2 hr. After removing the solutions, 100 μl of group B or D streptococci, *Lactobacillus acidophilus*, *Staphylococcus epidermidis*, *Candida albicans* or *Trichomonas vaginalis* each diluted in the blocking buffer were added and incubated at 37℃ for 1 hr. After washing with washing buffer (0.1% Tween 20 and 100 mM imidazole in PBS), mouse ant-rTvAP33 antibodies diluted with the blocking buffer were added and incubated at 37℃ for 1 hr. After washing with the washing buffer, HRP-conjugated anti-mouse IgG antibodies (Serotec, UK) 1:10,000-diluted with the blocking buffer were added and incubated at 37℃ for 1 hr. After washing with the washing buffer, 100 μl of TMB solution (Sigma, USA) was added and incubated at room temperature for 15 min. After addition of 100 μl of 2 N sulfuric acid, the absorbances at 450 nm were measured using microtiter plate reader (Molecular Devices, USA).

RESULTS

1. Construction of expression vector pET-TvAP33

Fresh 1×10^5 *T. vaginalis* were collected and used for the isolation of the total RNA. The size of PCR product on 1.0% agarose gel electrophoresis was similar with that of the full length TvAP33 gene, 938 bp (Fig. 2). According to cloning strategy summarized in Fig. 1, the TvAP33 gene was cloned in *E. coli* expression vector pET32a to construct pET-TvAP33.

2. Expression and purification of rTvAP33

In order to determine the time optimally expressing rTvAP33, pET-TvAP33/BL21 cells were stimulated with

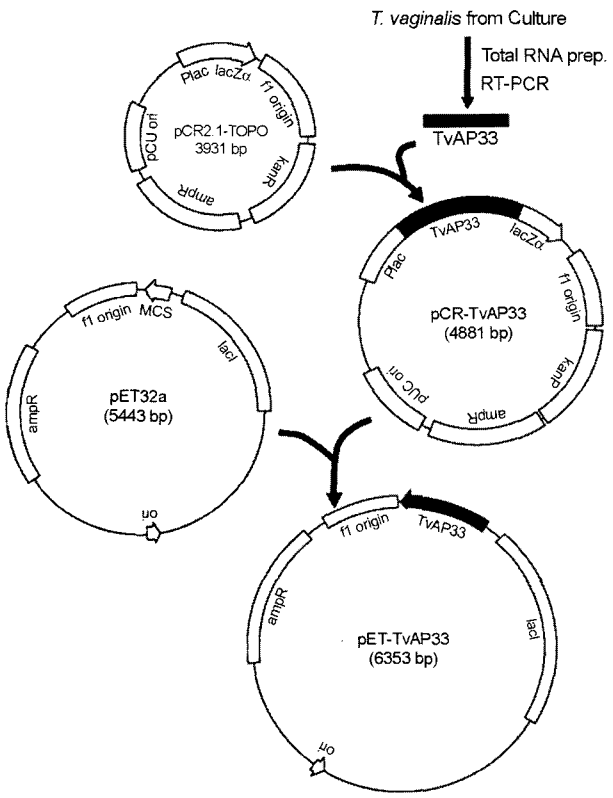

Fig. 1. Scheme of cloning of pET-TvAP33.

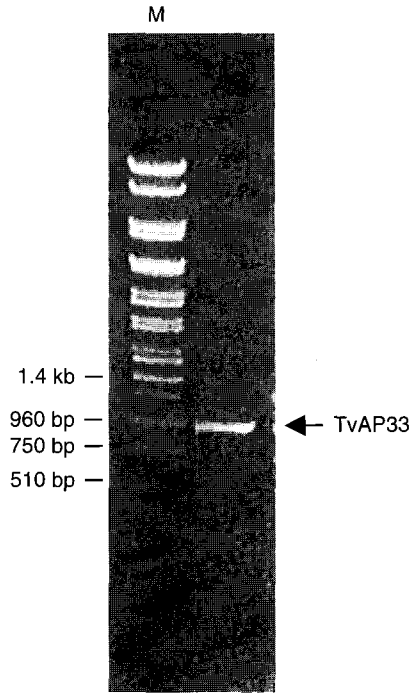

Fig. 2. 1.0% agarose gel electrophoresis of TvAP33 gene.

0.1 mM IPTG for 0 to 5 hr. The intensity of the 55 kDa band on 10% SDS-PAGE reached to maximum at 4 hr after the IPTG induction (Fig. 3A lane 4). This band was confirmed as the rTvAP33 by immunoblot analysis against His_6 tag (Fig. 3B lanes 3~7). The band at 20 kDa position was believed to a His_6-tagged vector proteins, which were expressed by the IPTG induction of the blank vector pET32a (Fig. 3A and 3B lane B).

In accordance to these results, pET-TvAP33/BL21 cells was cultured in 200 ml of LB broth and induced by 0.1 mM IPTG for 4 hr for the large scale preparation of rTvAP33 (Fig. 3C and 3D). The cells were harvested and lysed in the lysis buffer containing 8 M guanidine chloride, by which the inclusion bodies in the cells possibly containing over-expressed rTvAP33 are solubilized. The eluted fractions of metal affinity chromatography were analyzed by 10% SDS-PAGE and revealed that almost of all rTvAP33 were eluted in the condition of between 10~20 mM imidazole in the elution buffer (Fig. 3C lanes 5 and 6). Immunoblot analysis against His_6 tag conformed that the eluted fractions contained rTvAP33 (Fig. 3D lanes 5 and 6).

3. Conjugation of rTvAP33 to Affi-Gel® 10 resin

Theoretical pI of native TvAP33 analyzed by Bjellqvist method (Bjellqvist, et al., 1993) was estimated as 9.48 and experimental isoelectric focusing had shown that native TvAP33 is basic protein of pI = 10 (Engbring and Alderete 1998). However, by the Bjellqvist method, theoretical pI of rTvAP33 was 7.38 because of vector-originated regions flanked at N- and C-terminals. In accordance, the conjugation of rTvAP33 to Affi-Gel® 10 resin was taken at pH 7.5, slightly higher pH than that of theoretical pI of rTvAP33. Unconjugated active sites on the resin were blocked with 10 mM Tris pH 8.8, which is, in this study, used not only for the inactivating solution of unconjugated primary amines on Affi-Gel® 10 resin but also for the sample loading buffer for the purification of anti-rTVAP33 antibodies.

4. Immunization and purification of anti-rTvAP33 anti-bodies

Total three times of immunizations were sufficient for acquiring antisera, of which the titers were not sufficiently increased after the subsequent immunizations (data not shown). Mice antisera from twelve immunized BALB/c mice were pooled for the next step. Five ml of pooled mice

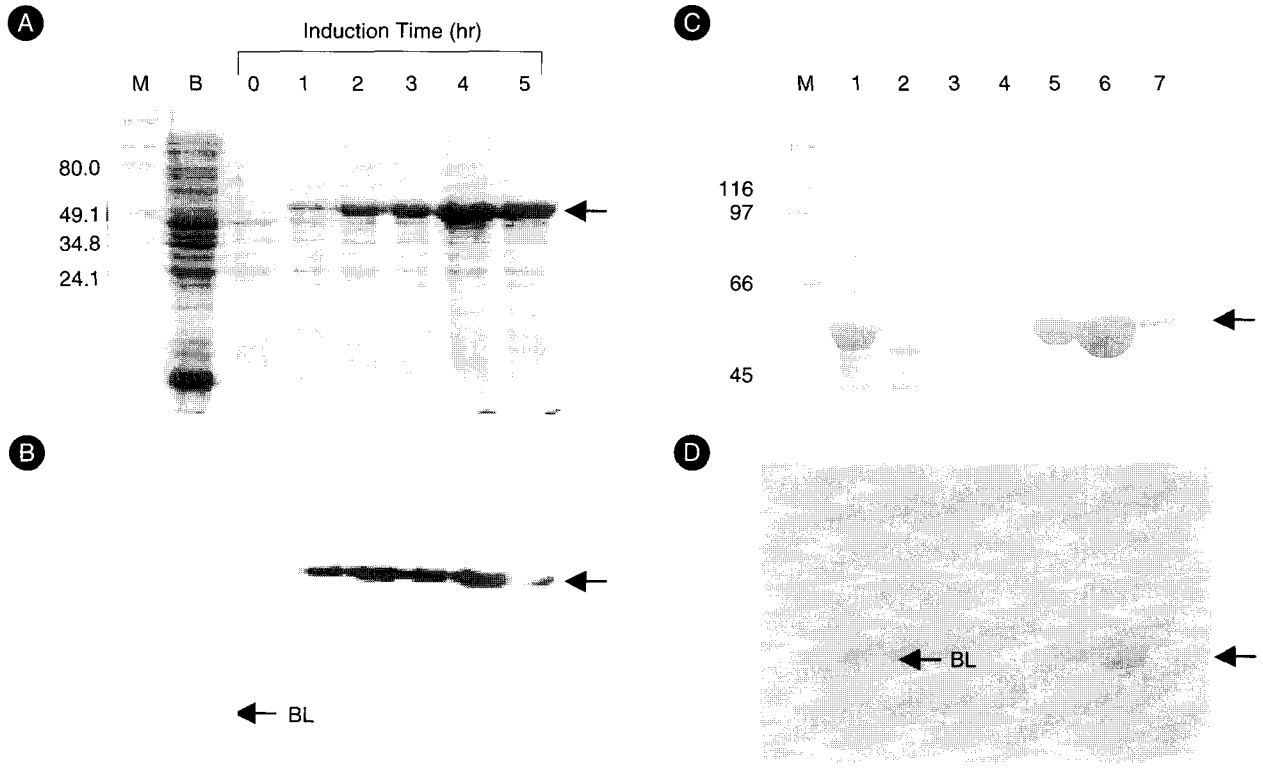

Fig. 3. Expression of rTvAP33. A and B. 10% SDS-PAGE (**A**) or immunoblot (B) analysis of pET-TvAP33/BL21 cell lysates induced by 0.1 mM IPTG for 0, 1, 2, 3, 4 and 5 hr. Arrows indicate rTvAP33. **C** and **D**. 10% SDS-PAGE (**A**) or immunoblot (**B**) analysis of purified rTvAP33. Lane 1: the lysate of pET-TvAP33/BL21 cells; Lane 2: unbound fraction; Lane 3: wash out with the loading buffer; Lane 4~6: the fractions of 5, 10 and 20 mM imidazole in the loading buffer; Lane 7: the fraction of 100 mM EDTA in the loading buffer. Arrows indicate purified 55 kDa rTvAP33. BL indicates vector proteins expressed by blank vector (lane B). Numbers indicates the size of protein markers.

sera or 10 ml of rabbit serum were subjected to rTvAP33-affinity chromatography using prepared rTvAP33 resin. Electrophoresis on 10% SDS-PAGE showed that the anti-rTvAP33 antibodies were purified with a few contaminations with unrelated proteins (Fig. 4A lane 3 for rabbit; Fig. 4B lane 3 for mouse).

5. ELISA

In preliminary experiments, we found that the condition coated with 100 ng of rabbit anti-rTvAP33 antibodies and captured with mouse anti-rTvAP33 antibodies had resulted the absorbances proportional to the concentrations of rTvAP-33, and the minimum detection limit was 62.5 ng/ml (data not shown). Under this condition, live microorganisms parasitizing in vaginal mucosa were applied to examine the specificity and sensitivity of designed ELISA system (Fig. 5). The specimens of type B or D *Staphylococci*, *Lactobacillus acidophilus*, *Staphylococcus epidermidis* or *Candida albicans* showed the absorbances lower than 0.2, while the specimen of ferrous ion-stimulated *T. vaginalis* showed 1.61± 0.05 (P<0.000001), which was 9.3 times higher titer than the specimen of unstimulated *T. vaginalis* (P<0.000001).

DISCUSSION

In immunodiagnosis of infectious diseases, the amount of antigens, differently from the amount of antibodies, in the host is greatly reduced along with the eradication of the pathogens. Therefore, antigen detection is a good diagnostic approach for the determination of infection status. In usual, useful antigens targeted in the immunodiagnosis are the membrane and secretory proteins of the pathogens. In this study, we cloned and expressed rTvAP33 originated from the membrane of *T. vaginalis* by sandwich ELISA.

Electrophoresis of purified rTvAP33 showed that the size of expressed rTvAP33 was 55 kDa: it was postulated that the translation of rTvAP33 was started at the start codon in the expression vector pET-TvAP33, thereby resulting in

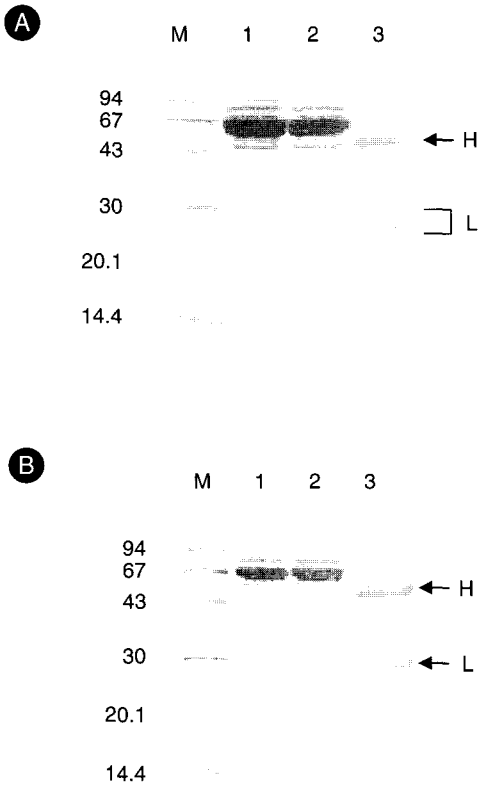

Fig. 4. rTvAP33-affinity purification of anti-rTvAP33. Purified anti-rTvAP33 antibodies from rabbit **(A)** or mouse **(B)** anti-sera were analyzed by 10% SDS-PAGE. Lane 1: anti-serum; Lane 2: the fraction of 100 mM Tris buffer pH 7.5; Lane 3: the fraction of 100 mM glycine buffer pH 2.5. "H" and "L" indicate heavy and light chains of anti-TvAP33 antibodies. Numbers indicates the size of protein markers.

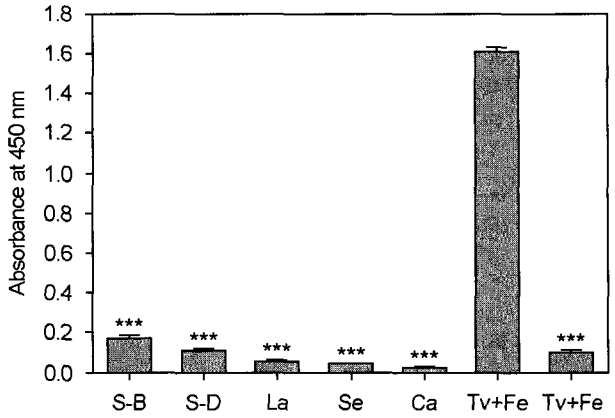

Fig. 5. Sandwich ELISA of vagina-parasitizing microorganisms. 1×10^4 cells of type B (S-B) and D (S-D) *Streptococci*, *Lactobacillus acidophilus* (La), *Staphylococcus epidermidis* (Se), *Candida albicans* (Ca) and Trichomonas vaginalis supplemented with (Tv+Fe) or without (Tv-Fe) ferrous ions were subjected to the sandwich ELISA. Data were expressed as mean ± S.E. (***P<0.000001)

additional 20 kDa cap sequence, and stopped at the stop codon just after C-terminal His_6 tag of the vector, thereby resulting in 2 kDa tail sequence. The rTvAP33 was designed to include enterokinase cleavage site between the cap and insert regions, with which rTvAP33 can be enzymatically separated from the cap sequence. The cap sequence is possibly inhibitory, if any, against the antigenicity of the TvAP33 insert region during immunization. However, because contaminated non-specific antibodies were not believed to significantly influence the results and explanations on the availability of rTvAP33 on the detection of *T. vaginalis* by sandwich ELISA, proteolytic elimination of cap regions were not taken in this study.

One of the important results of this study is the use of live *T. vaginalis* as a specimen. So far, only a little investigations were taken on the detection of a food-borne live *Salmonella* sp. (Brovko, et al., 2004). However, direct detection of live protozoans was not reported yet. Because *T. vaginalis* is larger in size than bacteria and has two flagella for its movement, it is expected that the fixation of live *T. vaginalis* on ELISA plates coated with the anti-rTvAp33 antibodies is troublesome. To solve this problem, we added 100 mM imidazole in the blocking and wash buffers for the sandwich ELISA. In preliminary experiments, it had been proven that 100 mM imidazole sufficiently reduced the movement of *T. vaginalis* under microscopic examinations (data not shown).

It was considered that *T. vaginalis* is possibly killed during the ELISA by the reagents including imidazole, which has similar structure with 5-nitroimidazole, the main frame of *T. vaginalis* drugs such as metronidazole or trinidazole. In hydrogenosome of *T. vaginalis*, 5-nitroimidazoles ($R\text{-}NO_2$) intercept electrons generated by pyruvate :ferredoxin oxidoreductases, and reduce to biologically toxic radicals ($R\text{-}\cdot NO_2^-$) (Kulda, 1999). However, imidazole does not have the 5-nitro group, and, as a result, *T. vaginalis* are not believed to be pharmacologically damaged. In addition, 9.3 times higher absorbance in the sandwich ELISA for the detection of *T. vaginalis* stimulated by ferrous ions than that of unstimulated *T. vaginalis* proved that 100 mM imidazole included in the ELISA reagents has no significant inhibitory effects on the ELISA detection of *T. vaginalis*.

Differential detection of *T. vaginalis* from other microorganisms parasitizing in vaginal mucosa is also important.

In the sandwich ELISA in this study, type B *Streptococci* showed highest cross-reactivity, but the absorbance was 9.3 times lower than that of *T. vaginalis* stimulated with ferrous ions (Fig. 7, $P<0.000001$). This low cross-reactivity was believed to come from low phylogenic relations of *T. vaginalis* with other bacteria.

Worth interesting in this study is the difference of the absorbances of *T. vaginalis* cultured with or without ferrous ions. In previous study, it had been reported that adhesion proteins are upregulated by ferrous supplementation as well as contact to host cells (Engbring and Alderete, 1998). Increased cytoadherence (Alderete, et al., 1995; Lehker, et al., 1991) and the resulting increased cytotoxicity (Ryu, et al., 2001) are believed to be the consequences. Contact to vaginal epithelial cells transforms *T. vaginalis* from globular to amoeboid shapes with filopodia and pseudopodia, which maximize the area of adhesion (Arroyo, et al., 1993). In this study, the absorbances of the sandwich ELISA were totally dependent on the presence of ferrous ions in the medium for *T. vaginalis* culture (Fig. 5). *T. vaginalis* cultured without ferrous ions showed background absorbance, similar with other vagina-parasitizing bacteria. This result means little or no expression of the adhesion proteins and possibly uselessness of TvAP33 as a target antigen for the diagnosis of *T. vaginalis*. However, *T. vaginalis* in vaginal swab are believed to express TvAP33 by the contact to the vaginal epithelial cells, so that no clinical problem remains in the ELISA diagnosis of *T. vaginalis*.

This study suggests the availability of the commercial sandwich ELISA system targeting TvAP33 for the diagnosis of *T. vaginalis* in the specimens. Relatively high background absorbances of this system have to be corrected by the applications of more specific antibodies or by the applications of better diagnostic principles for the reliable diagnostic results. Diagnostic sensitivity and specificity have to be precisely determined after the commercial development, which are the themes out of this study. Development of monoclonal anti-rTvAP33 antibodies has also to be followed for the development of the ELISA kits with improved sensitivity and specificity and the immunochromatographic kits for the rapid diagnosis within short time.

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