Identification and characterization of antigenic proteins in *Trichomonas vaginalis*

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Identification and characterization of antigenic proteins in *Trichomonas vaginalis*

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The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in Partial fulfillment of requirements for the degree of Master of Science

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To obtain the results of a trial and has undergone numerous. And the result gave me a lot of personal satisfaction. Obtained through the lab was the indescribable happiness. Although many failures, but I am getting these results thanks to those who have worked with. To think about it maybe people work hard because they know that sweet taste of success. I have failed some times in my life but i think time had made me stronger. I have learned over the course of Master of in my life thought it would be helpful to think. Through this lab, as well as to study, even the wisdom I have learned to live life. So rewarding two years and spent more than anyone.

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Identification and characterization of antigenic proteins in *Trichomonas vaginalis*

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(Directed by Professor Soon-Jung Park)

Trichomoniasis is a sexually transmitted disease caused by *Trichomonas vaginalis*, and has serious consequences for women's health. To study the virulence factors of this pathogen, *T. vaginalis* surface proteins were examined using polyclonal antibodies specific to the membrane fractions of *T. vaginalis*. *T. vaginalis* expression library was constructed by cloning the cDNA derived from mRNA of *T. vaginalis* into a phage λUni-ZAP XR vector, and then used for immunoscreening with the anti-membrane proteins of *T. vaginalis* antibodies. The immunoreactive

proteins selected via immunoscreening included adhesion protein AP65-1, α-actinin, kinesin-associated protein, teneurin, and two independent hypothetical proteins. Immunofluorescence assays showed that AP65-1, one of the identified immunogenic proteins, is prevalent in the whole body of *T. vaginalis*. Finally, enzyme-linked immunosorbent assay (ELISA) was performed using trichomoniasis patient's sera showing high reactivity to both AP65-1 and kinesin-associated protein of *T. vaginalis*. These results revealed that AP65-1 and kinesin-associated protein are immunogenic proteins which provide important information for future investigation on *T. vaginalis*-host interaction.

Key words: *Trichomonas vaginalis*, antigenic protein, membrane proteins, immunoscreening, AP65-1

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I. INTRODUCTION

Trichomonas vaginalis causing the most prevalent non-viral sexually transmitted disease worldwide, is an useful pathogen for the study of host-parasite interrelationship[1, 2]. Transmission of this parasite occurs almost exclusively by sexual contact. This protozoan pathogen survives in the vagina of women, an environment that undergoes profound changes during the menstrual cycle. In particular, estrogen and progesterone control the growth and differentiation of

epithelial cells and ultimately lead to the terminal differentiation and exfoliation of vaginal epithelial cells (VECs)[2]. Trichomoniasis is associated with damage to superficial vaginal epithelium and a profuse, acute inflammatory discharge. Infection of the vaginal tract by *T. vaginalis* occurs as multiple steps involving distinct interactions between host macromolecules and protozoan factors. Trichomonal cytadherence to epithelial cells is a highly specific step during initiation of infection and its pathogenesis[3,4]. This parasite possesses several adhesins involved in breaching the mucous layer, the first host surface encountered by trichomonads. Trichomonads interact specifically with mucin, a major proteinaceous constituent of mucus[3], and then contact vaginal epithelial cells at which iron-regulated surface proteins of *T. vaginalis*[5,6], adhesion proteins AP65, AP51, AP33, and AP23, play essential roles. In addition, the trichomonads present higher amoeboid transformation rate and intense phagocytic activity, characteristics of higher virulence behavior when they encountered the host cells. Penetration of the epithelium by *T. vaginalis* also induces a specific interaction with the extracellular matrix basement membrane glycoproteins[5].

T. vaginalis trophozoites colonize the epithelial surface of the human urogenital tract in which they obtain nutrients, multiply, and face a constant challenge from

host immune surveillance. Iron is an essential nutrient for *T. vaginalis* growth. Iron concentration is also a critical factor in the pathogenesis of *T. vaginalis*, and it modulates multiple aspects of *T. vaginalis* including metabolic activity, cytoadherence, and resistance to complement lysis[6,7]. Like other pathogens, *T. vaginalis* has ways of acquiring iron from lactoferrin. Upon binding to lactoferrin via specific receptors of *T. vaginalis*, this protozoan shows increased iron accumulation and pyruvate ferridoxin oxidoreductase activity[8,9]. Iron availability from lactoferrin triggers trichomonads to increase amount of adhesins, thereby enhancing binding to the host epithelial cells. Therefore, iron concentration was shown to regulate the expression of genes encoding for the lactoferrin receptor and immunogens[8]. *T. vaginalis* also binds to erythrocytes from which both lipid and iron can be provided for parasite[9]. Iron acquisition from hemoglobin by *T. vaginalis* occurs via the action of two adhesins, AP51 and AP65[10]. *T. vaginalis* grown in iron-depleted medium shows no pathology whereas trichomonads cultured in the iron-supplemented medium, could cause subcutaneous abscesses in mice[11].

In addition to surface proteins, proteins secreted by *T. vaginalis* were extensively examined with respect to interaction with human VEC[12]. Interestingly, the adhesins AP65, AP33, and AP51 were found to have sequence identity to

metabolic enzymes, which reside within hydrogenosome[13,14]. This parasite possesses numerous cysteine proteases, which are promiscuous in activity and appear to be involved in cytoadherence, hemolysis, and disruption of the host cell membrane[10]. In addition, actin and associated actin-binding proteins (ABPs) play a role in motility and changes in shape of *T. vaginalis*[15]. Coordination between numerous actin isoforms is required for morphological change of *T. vaginalis* when adhering to host cells. The components of *T. vaginalis* secreted proteins such as metabolic enzymes, proteases, and actin, not only affected its adhesion to the host cells but also induced the expression of host components including interleukin-8, COX-2, and fibronectin[14].

Based on a hypothesis that trichomonad's components involved in interaction with the host cells are present on the surface of *T. vaginalis*, we identified the membrane proteins of *T. vaginalis* using immunoscreening. Furthermore, we investigated the antigenicity of candidate proteins using human immune sera.

II. MATERIRS AND METHODS

1. Parasites and cell culture

Strains used in this study are listed Table 1. Korean *T. vaginalis* isolate KT4[4] was used in all experiments. Trichomonads were grown axenically in Diamond's trypticase-yeast extract-iron (TYI-S-33) medium[15] supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; GibcoBRL, Rockville, Maryland, USA). Cultures were incubated at 37ºC in a 5% CO_2 atmosphere, and passaged every two to three days.

Table 1. Strains and plasmids used in this study

Strain	Genotype/source	Reference
Trichomonas vaginalis		
KT4	Isolated from a Korean female with acute vaginitis	This study
Escherichia coli		
DH5α	*supE44, ΔlacU169* (*φ80 lacZ ΔM15*), *hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Invitrogen
BL21 (DE3)	*E. coli strain B F*$^-$*, dcm, ompT, hsdS* (r_B^- m_B^-), *galλ* (*DE3*)	Invitrogen
XL1-Blue MRF'	*Δ*(*mcrA*)*183, Δ*(*mcrCB-hsdSMR-mrr*)*173, endA1, supE44, thi-1, recA1, gryA96, relA1, lac* [*F' proAB, lacl*^{q}*ZΔM15, Tn10* (*Tet*^{R})]	Stratagene
SOLR	*e14*$^-$ (*McrA*$^-$), *Δ*(*mcrCB-hsdSMR-mrr*)*171, sbcC, recB, recJ, uvrC, umuC::Tn5* (*Kan*^{R}), *lac, gyrA96, relA1, thi-1, endA1,* λ^R [*F' proAB, lacl*^{q}*ZΔM15*], *Su*$^-$	Stratagene
pBK-CMV	Expression vector; f1 ori, lacZ', pUC ori; *Kan*^{R}	Agilent Technologies
pET28b	Expression vector, *Kan*^{R}	Novagen
pET21b	Expression vector, Amp^R	Novagen
pBluescript SK (-)	Cloning vector, Amp^R	Stratagene
pZhu115	pET28b, 1,703 bp *T. vaginalis ap65-1* (TVU18346)	This study
pTHSJ2	pET21b, 2,970 bp DNA encoding alpha-actinin of *T. vaginalis* (TVAG_311270)	This study
pTHSJ3	pET21b, 2,796 bp DNA encoding a kinesin associated protein of *T. vaginalis* (TVAG_190450)	This study
pTHSJ4	pET21b, 3,003 bp DNA encoding teneurin of *T. vaginalis* (TVAG_457850)	This study

2. **Preparation of membrane proteins from *T. vaginalis***

Membrane extractions from *T. vaginalis* were carried out as described previously[16]. Briefly, *T. vaginalis* in late logarithmic growth phase was washed three times with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.3), and resuspended in ice-cold PBS containing protease inhibitors (1 mM N-tosyl-L-lysine-chloromethyl ketone hydrochloride, and 0.2 mM leupeptin). Trichomonads were lysed by sonication on ice, and centrifuged for 10 min at 13,000 rpm, 4ºC. Membrane proteins were prepared by resuspending the resultant pellet in PBS containing 1% Triton X-100, and stored at –20ºC until further use.

3. **Formation of polyclonal antibodies specific to membrane proteins of *T. vaginalis***

Membrane proteins prepared from *T. vaginalis* (100 μg) were mixed with 0.5 ml of complete Freund's adjuvant (Sigma, St. Louis, Missouri, USA), and injected intraperitoneally into a specific pathogen-free rat (CrjBgi:CD[SD]IGS, 7-week-old, female). Two additional immunizations were performed with the same amount of

membrane proteins mixed with incomplete Freund's adjuvant (Sigma) at two and four weeks after the primary immunization. A week after the third immunization, serum was obtained from the immunized rat, and used for western blot analysis.

4. **Construction of cDNA expression Library**

Strains and plasmid used in this study are listed Table 1. To prepare a *T. vaginalis* cDNA expression library, total RNA was prepared from freshly grown *T. vaginalis* using TRIzol (Invitrogen, Carlsbad, California, USA), and then processed further to purify mRNA using the PolyATtract mRNA isolation system (Promega, Madison, Wisconsin, USA). *T. vaginalis* cDNA was synthesized from this pool of mRNA as a template using a cDNA synthesis kit (Strategene, Santa Clara, California, USA). The resultant cDNAs were ligated with *EcoR*I and *Xho*I linkers at their 5'- and 3'-ends, respectively, and then cloned into the corresponding sites of *E. coli* phage λ Uni-ZAP XR vector (Stratagene, Santa Clara, California, USA). The resultant ligation mixture of *T. vaginalis* cDNA and Uni-ZAP XR vector was packaged as phage in vitro using Gigapack III gold packaging extract (Stratagene, Santa Clara, California, USA). Twenty plaques were chosen randomly, and their inserts were

amplified by PCR, using primers T3 and T7 annealed at both sides of the insert.

5. Immunoscreening

Polyclonal antibodies specific to membrane proteins of *T. vaginalis* were used for the immunoscreening experiment. The amplified library (with a titer of 3×10^5 plaque forming units/ml) was screened primarily using a 1:2,000 dilution of rat antibodies raised against *T. vaginalis* membrane proteins. Plaques distinct from the background level of antibody binding to the filter were further purified by the second and third screenings. Homogeneous plaques expressing the antigens were selected and excised to a pBK-CMV phagemid, as instructed by the manufacturer (Stratagene). The identities of the phagemid inserts were verified by automatic sequencing of the double-stranded plasmids.

6. Construction of expression plasmids for AP65-1, kinesin-associated protein, alpha-actinin, and teneurin

A 1,703 bp *ap65-1* DNA fragment amplified from the genomic DNA of *T. vaginalis* by PCR using the primers ap65-1F and primer ap65-1R (Table 2), and then cloned into pET28b (Novagen, Darmstadt, Germany) to produce pZhu115. A

2,097 bp DNA fragment encoding alpha-actinin was amplified by PCR using primer HSJ2-F and HSJ2-R (Table 2), and then cloned into pET21b (Novagen, Darmstadt, Germany) to result in pTHSJ2 expressing alpha-actinin of *T. vaginalis*. A 2,097 bp DNA containing the gene for kinesin-associated protein was also made with primers. HSJ3-F and primer HSJ3-R (Table 2), and used to produce in pTHSJ3, an expression plasmid for kinesin-associated protein. A 3,003 bp teneurin DNA fragment amplified using primer HSJ4-F and HSJ4-R (Table 2), was also cloned into the expression vector, pET21b (Novagen) to produce pTHSJ4, which was used to express recombinat teneurin of *T. vaginalis*.

Table 2. Primers used in this study

Name	Nucleotide sequence (5'-3')	Restriction Enzyme site*
AP65-1F	CATGCCATGGGCATGCTTACATCTTCAGTC	*Nco*I
AP65-1R	GTTAGCGGCCGCGTAAAGTGGTTCGTAGTC	*Not*I
HSJ2-F (Kinesin)	CCGGAATTCGATGGAGTACAGTAT	*EcoR*I
HSJ2-R (Kinesin)	CCGCTCGAGCTTTGTAAGTCCCA	*Xho*I
HSJ3-F (Alpha-actinin)	CCGCATATGATGTCTGTTCGTGAA	*Nde*I
HSJ3-R (Alpha-actinin)	CCGGTCGACTTGGTTGTAAAGTGAGTT	*Sal*I
HSJ4-F (Teneurin)	CCGGCTAGCATGTTAACTGCTTTCTTCGC	*Nhe*I
HSJ4-R (Teneurin)	CCGCTCGAGATTTGTTGATTCTGATAAGA	*Xho*I

*Restriction enzyme sites are underlined.

7. Production of anti-AP65-1 polyclonal antibodies and western blotting

Histidine-tagged recombinant AP65 (rAP65) expressed in *E. coli* BL21 (DE3) with an addition of 0.1 mM isopropyl β-D-thiogalactoside (IPTG; Sigma), was purified using a Ni^{2+}-NTA chromatography (Amersham Pharmacia), and then used to immunization of specific pathogen-free rat (CrjBgi:CD[SD]IGS, 7-week-old, female) to make polyclonal antibodies.

E. coli extracts expressing rAP65 were prepared in a phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.3), separated by SDS–PAGE, and transferred to a nitrocellulose filter (Millipore). The membrane was incubated with polyclonal rat anti-rAP65 or rat anti-histidine (Amersham Pharmacia) in a blocking solution (PBS, 5% skim milk, and 0.05% Tween 20), and incubated with alkaline phosphatase (AP)-conjugated anti-rat IgG (Sigma). Cell extracts of *T. vaginalis* were prepared in PBS, and reacted with AP65-specific polyclonal antibodies.

8. Immunofluorescence assay

Trophozoites of *T .vaginalis* were attached to glass slides coated with L-lysine in a humidified chamber. The attached trophozoites were fixed with chilled 100%

methanol at -20°C for 10 min, and permeabilized with PBS/0.5 % Triton X-100 for 10 min. After 1 h-incubation in a blocking buffer (PBS, 5% goat serum, and 1% BSA), the cells were reacted with mouse anti-rAP65 polyclonal antibodies (1:50 dilution with a blocking buffer) at 4°C for overnight. Following three 5 min-washes with PBS, the cells were incubated with FITC-conjugated anti-mouse IgG (1:200 dilution in blocking buffer, Jackson ImmunoResearch Lab) at 37°C for 1 h. The slides were reacted with 1 μg ml^{-1} 4′6-diamidino-2-phenylindole (DAPI; Sigma), rinsed with PBS, and mounted with anti-fade mounting medium (Vectashilde; Vector). They were then observed with a Zeiss LSM 510 laser scanning confocal microscope (Zeiss). The images were collected with serial sections at 0.5 μm intervals, and analyzed by Zeiss LSM image browser software (Zeiss).

9. Enzyme-linked immunosorbent assay (ELISA)

1) Reaction between *T. vaginalis* extracts and human patient's sera

ELISA was performed to find patients' sera showing strong reaction with immobilized *T. vaginalis* extracts. 2.5 microgram of *T. vaginalis* extracts were coated in each well of 96-well plates in 50 mM carbonate buffer, pH 9.6 (3mM Na_2CO_3, 6.9mM $NaHCO_3$ in dH_2O) overnight at 4°C. Following blocking in

phosphate buffered saline (PBS: 1.3mM NaH_2PO_4, 9.2mM Na_2HPO_4, 0.15M NaCl in dH_2O) containing 1% BSA, and three washes with PBS/0.5% Tween 20, immobilized *T. vaginalis* extracts were incubated with sera of trichomoniasis patients (1:100 dilution with PBS/1% BSA). As a control, the extract was also reacted with the polyclonal antibodies specific to membrane proteins of *T. vaginalis*. Human sera uninfected with *T. vaginalis* were also included in ELISA as negative controls. The supernatants were discarded after 1 hr incubation at 37°C. Following three washes with PBS/0.5% Tween 20, alkaline phosphatase (AP)-conjugated anti-human IgG or AP-conjugated anti-rat IgG (Sigma) (1:100 dilution in PBS/0.5% BSA) was added to the each reaction containing human sear or *T. vaginalis* polyclonal antibodies, respectively. After 1 hr incubation at 37°C and three washes with PBS/0.5% Tween 20, 100 μl of substrate solution [1 mg/ml of p-nitrophenyl phosphate in substrate buffer (12% diethanolamine, 3mM NaN_3, 0.5mM $MgCl_2 \cdot 6H_2O$ in dH_2O)] was added to each reaction. After a 15 min-incubation at 37°C, the reaction was stopped with 50 μl stop solution (3M NaOH), and monitored for its absorbance at 490 nm using a Flow Titretek ELISA reader (TECAN, Salzburg, Austria). The cut off value was determined by following the

equation: cut-off = (mean absorbance of negative serum) + 2 x (standard deviations of negative serum).

(2) Reaction between AP65-1 and human patient's sera

(2-1) Antigen preparation: rAP65 expressed in *E. coli* BL21 (DE3) was purified using a Ni^{2+}-NTA chromatography as suggested by the manufacturer (Amersham Pharmacia). Purified rAP65-1 protein was centrifuged using Ambicon Ultra-30 to remove immidazole present in the elution buffer.

(2-2) Test procedure: ELISA plates (Linbro, Flow Laboratories) were coated overnight with 0.05ug/ml of rAP65-1 protein, and then reacted with human sera as described above. Instead of human sera, polyclonal antibodies specific to membrane proteins of *T. vaginalis* and specific to rAP65-1 were included in the reaction as positive controls.

(3) Reaction between kinesin associated protein and human patient's sera

Antigen preparation: Recombinant kinesin-associated protein was expressed in *E. coli* BL21 (DE3) with an addition of 0.5 mM IPTG, and purified using a Ni^{2+}-NTA chromatography. Purified kinesin associated protein was processed by the centrifugal filter devices (Ambicon Ultra-30) to remove imidazole.

Test procedure-Five nanogram of recombinant kinesin associated protein was coated for ELISA and the reaction was performed as described above.

III. RESULTS

1. Identification of *T. vaginalis* membrane proteins reacting with polyclonal antibodies

T. vaginalis is an extracellular parasite that adheres to vaginal epithelia cells to colonize its human host. To identity of surface proteins of *T. vaginalis* involved in interaction with the host cells, Membrane proteins of *T. vaginalis* were prepared, and analyzed them by SDS-PAGE and subsequent silver staining (Fig. 1). In addition, the membrane proteins of *T. vaginalis* were used used to immunize rats. When they were reacted with the polyclonal antibodies against the membrane proteins, diverse proteins of various molecular weights appeared to be immunoreactive (Fig. 2). Therefore, the resultant polyclonal antibodies were used for immunoscreening experiment to find critical factor(s) of *T. vaginalis* involved in interaction with the host cells.

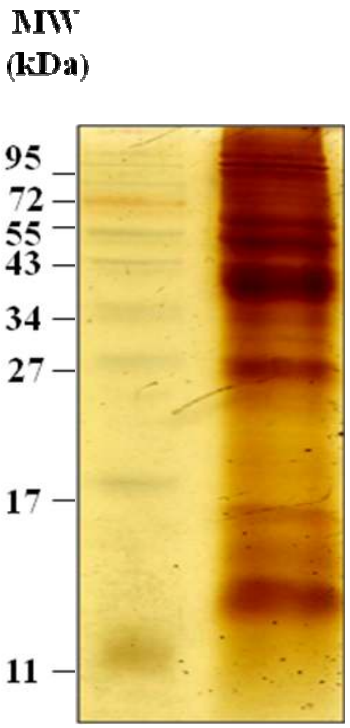


Fig. 1. Preparation of membrane proteins from *T. vaginalis*

Membrane proteins from *T. vaginalis* were prepared in PBS containing 1% Triton X-100, and separated by SDS-PAGE. The proteins were visualized by staining with silver nitrate. lane 1: protein size marker, lane 2: membrane proteins of *T. vaginalis*.

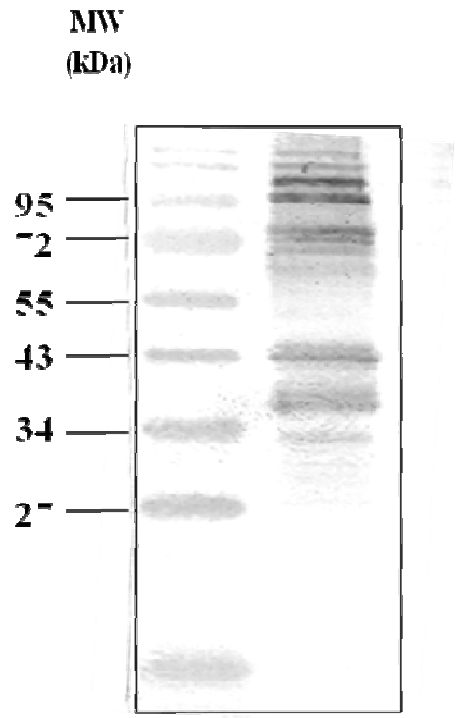


Fig. 2. Western blot analysis of *T. vaginalis* membrane proteins using anti-*T. vaginalis* membrane proteins antibodies

Membrane protein of *T. vaginalis* (20 μg) were separated by 12% SDS-PAGE, and then transferred to a nitrocellulose filter (Millipore, Billerica, Massachusetts, USA). The membrane was incubated with rat antibodies against membrane proteins polyclonal antibodies in a blocking solution (PBS, 5% skim milk, and 0.05% Tween 20), and then incubated with alkaline phosphatase (AP)-conjugated anti-rat IgG (Sigma). The immunoreactive protein was visualized using the nitroblue tetrazolium

(NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) system (Madison, USA).

2. Isolation and identification of *T. vaginalis* cDNA

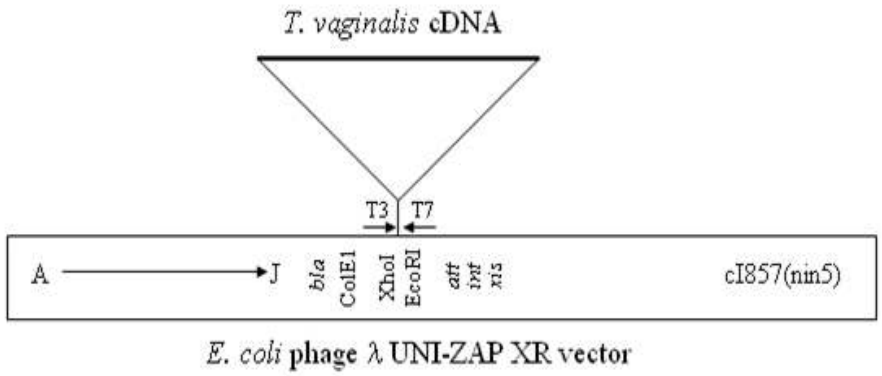
To use in the immunoscreening experiments, *T. vaginalis* cDNA expression library was constructed (Fig. 3A). The quality of the resultant library was evaluated on randomly-selected 20 cDNA clones (Fig. 3B). PCR amplification of the 20 clones using primers T3 and T7 indicated that most clones of the *T. vaginalis* library had inserts of various sizes derived from the diverse mRNA of *T. vaginalis*.

Therefore, 3×10^5 plaques of *T. vaginalis* cDNA expression library were screened using polyclonal antibodies specific to *T. vaginalis* membrane proteins (Fig. 4). Through several screening, nine candidate plaques showing reproducible immune response with the polyclonal antibodies were isolated, and then excised to pBK-CMV phagemid. Identities of the isolated clones were determined by automatic sequencing of double-stranded plasmids (Table 3). Blast searches of nucleotide sequences of isolated clones on the *T. vaginalis* genome database (http://trichdb.org/trichdb/) indicated that nine immunoreactive proteins were identified.

Among nine cDNA clones obtained by immunoscreening of cDNA library with anti-*T.vaginalis* antibodies, the most interesting clone encodes AP65-1, which is one of four iron-regulated surface proteins involved in the cytoadherence of *T.*

vaginalis[16]. The majority of identified clones encode components associated with cytoskeleton, which included α-actinin, and a kinesin-associated protein. Most notably, three of the nine isolated clones turned out to be α-actinin, the actin-binding and Ca^{2+}-binding protein with high immunogenicity in *T. vaginalis*. Two of the identified clones encoded a kinesin-associated protein which functions as a regulator for cellular and intracellular movements via interaction with the motor proteins. One of the clones was also found to encode a putative teneurin, a conserved family of transmembrane proteins involved in intercellular signaling. For the remaining two cDNA clones, I cannot get any information because blast searches for homologous proteins in NCBI databases did not provide meaningful results.

(A)



(B)

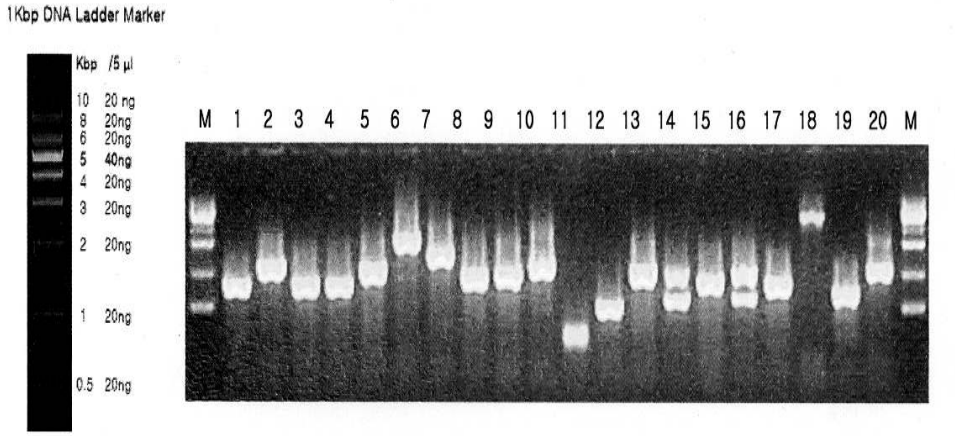
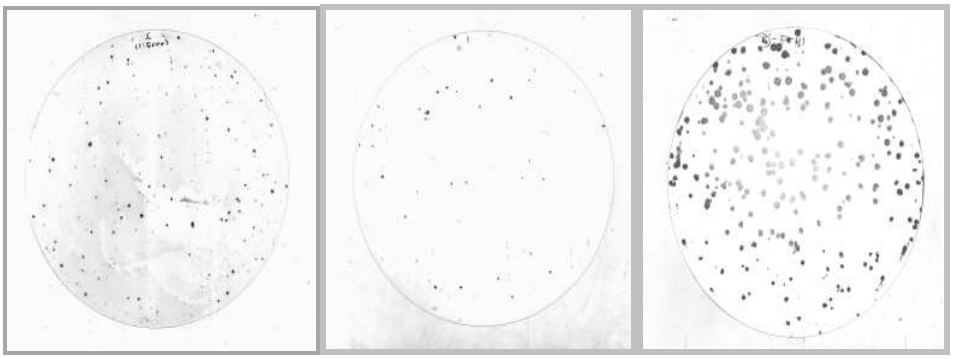


Fig. 3. Construction of cDNA expression library

(A)The resultant ligation mixture of *T. vaginalis* cDNA and Uni-ZAP XR vector was packaged as phage in vitro. (B) Twenty inserts were amplified by PCR, using primers T3 and T7 annealed at both sides of the insert.

(A) (B) (C)



All positive plaques

Fig. 4. Immunscreening of cDNA library of *T. vaginalis* with polyclonal antibodies

Immunoreactive clones were purified three or four times using the polyclonal antibodies specific to *T. vaginalis* membrane proteins. Nine independent clones showing reproducible immune response with polyclonal antibodies were obtained (Table 3).

Table 3. Proteins identified by immuimmunoscreening of *T. vaginalis* cDNA library using anti-*T-vaginalis* antibodies

TVDB ORF#	NCBI accession#	Name
TVAG_311270	XM001303428	kinesin-associated protein
TVAG_340290	U1834	AP65-1 adhesin
TVAG_190450	AF014928	α-actinin: kakapo
TVAG_335250	XM001297646	Hypothetical protein
TVAG_402650	XM001580945	Hypothetical protein
TVAG_457850	XM001312023	Teneurin
TVAG_311270	XM001303428	kinesin-associated protein
TVAG_190450	AF014928	α-actinin: kakapo
TVAG_190450	AF014928	α-actinin: kakapo

3. Production of recombinant antigenic proteins

Among the 9 immunoreactive clones, cDNA clone encoding AP65-1 was investigated further. rAP65-1 was expressed in *E. coli*, and used to make specific antibodies (Fig. 5A). Specificity of the resultant antibodies was confirmed by western blot of the *E. coli* extracts expressing rAP65-1 (Fig. 5B). Cell extracts of *T. vaginalis* were also prepared in PBS, and reacted with AP65-1-specific polyclonal antibodies, clearly indicating an immunoreactive protein of 65 kDa (Fig. 5C).

In addition, a cDNA encoding kinesin associated protein was cloned into pET21b, and recombinant kinesin associated protein was expressed in *E. coli* at a molecular weight of 79 kDa (Fig. 6A). Since the main portion of rkinesin associated protein was present in soluble fraction, I purified this recombinant protein by Ni^{+}-NTA affinity chromatography, which was used for ELISA with human immune sera.

The most frequently found clone, α-actinin cDNA was cloned into an expression plasmid resulting recombinant protein of 106 kDa (Fig. 6B). However, the resultant protein was mainly present as insoluble form. A cDNA clone encoding teneurin was also expressed as a recombinant protein in *E. coli* (Fig.

6C). Despite of optimization experiments to increase the expression of recombinant teneurin, In addition, expression level of recombinant teneurin was too low. Therefore, ELISA was not performed with recombinant α-actinin and teneurin.

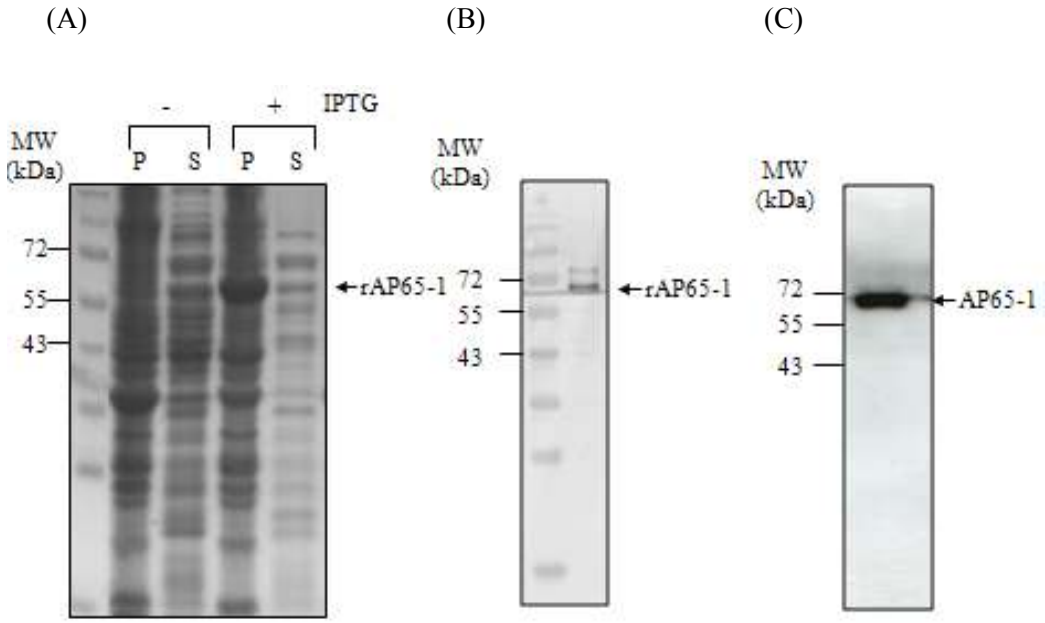


Fig. 5. Expression of rAP65-1 and formation of anti-rAP65-1 antibodies

(A) Expression of rAP65-1 in *E. coli* BL21 (DE3). *E. coli* expressing rAP65-1 (with 1 mM IPTG), was lysed by sonication, fractionated into cytoplasmic (S) and membrane (P) proteins. The resultant proteins were separated by 12% SDS-PAGE. (B) Reactivity of anti-AP65-1 antibodies against *E. coli* extracts expressing rAP65-1. (C) Detection of AP65-1 in *T. vaginalis* extracts by western blot analysis using anti-rAP65-1 antibodies.

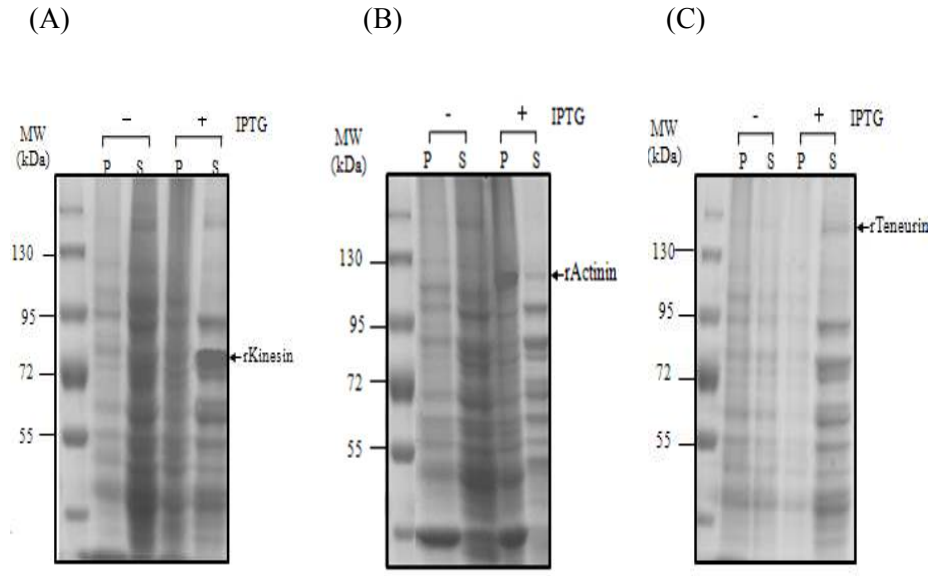


Fig. 6. Expression of recombinant kinesin associated protein (A), α-actinin (B), and teneurin (C)

E. coli carrying the expression plasmid was incubated with IPTG, and then lysed by sonication. The bacterial lysates were then fractionated into cytoplasmic (S) and membrane (P) proteins. Overexpressed recombinant proteins were indicated with arrows.

4. Intracelullar localization of AP65-1 in *T. vaginalis*

Because AP65 is known as the main adhesin for *T. vaginalis* cytoadherence, it was examined whether AP65-1 is located in the membrane of *T. vaginalis* by immunofluorescence assay (IFA) using antibodies specific to AP65-1. Figure 7 showed strong red florescence present in the whole body of *T. vaginalis*, whereas antibodies specific to tubulin stained flagella with green fluorescence, indicating that the intracellular level of AP65-1 is high in cytoplasm and possibly in the membrane of *T. vaginalis*. However, it is difficult to determine whether AP65 is mainly located in the membrane of *T. vaginalis* with this IFA experiment.

(A) (B)

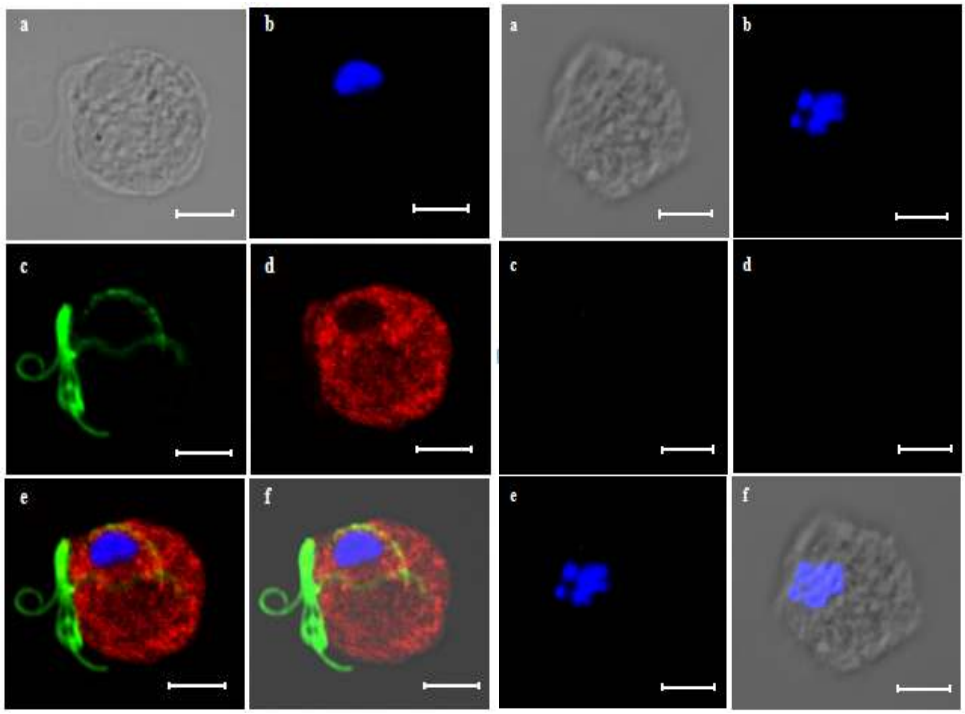


Fig. 7. Immunolocalization of AP65-1adhesin in *T. vaginalis* trophozoites

(A) *T. vaginalis* incubated with rat anti-rAP65-1 antibodies and mouse anti-tubulin antibodies. The slides were then incubated with TRITC-conjugated anti-rat IgG and FITC-conjugated anti-mouse IgG as secondary antibodies. To visualize nuclei, the cells were treated with 1 μg/ml-1 4′6-diamidino-2-phenylindole, mounted with an anti-fade mounting medium (Vectashilde; Vector, Burkingame, California, USA),

and then observed with an immunofluorescence microscope (Zeiss, Thornwood, New York, USA). The bars represent 5μm (a~f). (B) *T. vaginalis* trophozoite incubating with rat preimmune serum. (a) a differential interference contrast (DIC) image, (b) a fluorescence image at a wavelength of 345 nm to detect nuclei, (c) a fluorescence image at a wavelength of 494 nm to detect flagella, (d) a fluorescence image at a wavelength of 547 nm to detect rAP65-1, (e) a combined fluorescence image, and (f) a combined fluorescence and DIC image.

5. The immunogenicity of antigenic proteins

The immunogenicity of the *T. vaginalis* was evaluated using ELISA in which *T. vaginalis* extracts were reacted with *T. vaginalis*-infected human sera (Table 4). Sera from *T. vaginalis*–infected human showed highly specific reaction with *T. vaginalis* extracts as evidenced by high ODs. On the other hand, sera from non-infected human showed a clear negative reaction (OD < 0.4). Therefore, human serum samples showing high reactivity to *T. vaginalis* extracts were randomly chosen and used for ELISA using recombinant AP65-1 or kinesin associated protein as antigens.

rAP65-1 and rkinesin associated protein were used as antigens to evaluated for their antigencity (Fig. 8). Both rAP65-1 and rkinesin showed higher antibody responses than negative controls, indicating that both proteins serve as antigenic protein during *T. vaginalis* infection.

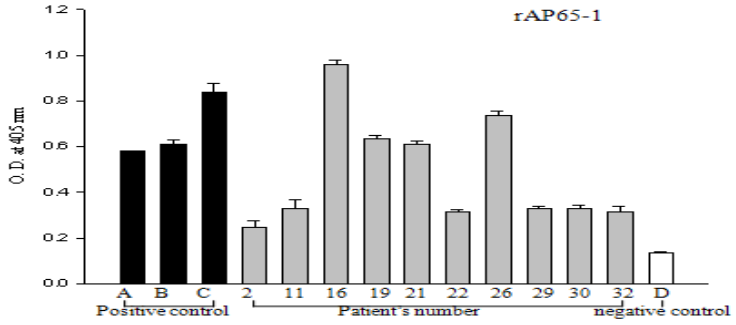
Table 4. The immunogenicity of *T. vaginalis* extracts against human sera

Infected human sera No.	Absorbance at 450 nm[a]	Non-infected human sera No.	Absorbance at 450 nm
1	0.52	1	0.37
2	2.88[b]	2	0.32
3	0.97	3	0.41
4	0.52	4	0.55
5	0.26	5	0.40
6	0.72	6	0.46
7	0.63	7	0.32
8	0.63	8	0.64
9	0.79	9	0.34
10	1.82	10	0.50
11	1.95		
12	1.76		
13	0.31		
14	0.43		
15	0.30		
16	2.85		
17	0.53		
18	0.52		
19	2.50		
20	0.38		
21	2.71		
22	3.99		
23	0.65		
24	0.83		
25	1.51		
26	3.17		
27	0.87		
28	2.51		
29	3.01		
30	3.67		
31	1.06		
32	3.21		
Positive C-pellet	2.94		
Positive C-supernatant	2.96		

[a]ELISA was performed as triplications.

[b]Only this underline human serum samples were used (fig. 8).

(A)



(B)

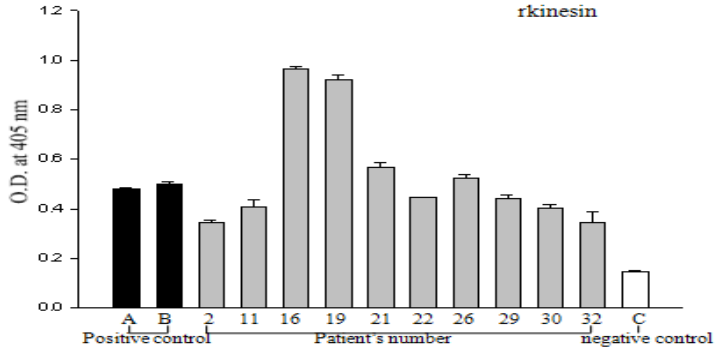


Fig. 8. ELISA using *T. vaginalis*-infected human sera

ELISA plates were coated overnight with 0.05μg/ml of rAP65-1 protein (A) or rkinesin associated protein (B), and then reacted with human sera. The reaction was monitored by measuring its absorbance at 490 nm using a Flow Titretek ELISA reader. Instead of human sera, polyclonal antibodies specific

to membrane proteins of *T. vaginalis* and specific to rAP65-1 were included in the reaction as positive controls. lane A: pellet of anti-membrane protein antibodies, lane B: supernatant of anti-membrane protein antibodies, and lane C: anti-rAP65-1 antibodies.

IV. DISCUSSION

Trichomonas vaginalis, a protozoan parasite which infects the vagina of women and causes vaginitis, is one of the common causes of non-viral sexually transmitted disease (STD) worldwide, especially in women. In women, trichomonal vaginitis is characterized by inflammation of vaginal epithelium, foul-smelling discharge, and tissue cytopathology. Most men are asymptomatic, and the clinical picture of this disease remains controversial[15]. Disease symptoms such as urethritis, prostatitis, balanoposthitis, and others however, have been documented in infected men[16].

Trichomoniasis occurs in the exceedingly complex and constantly changing urogenital tracts of women, as evidenced by the menstrual cycle with fluctuations in pH, iron, and nutrients, the presence of blood components during menses, and the desquamation of the VECs[17]. *T. vaginalis* penetration of the mucous layer, followed by adherence to vaginal epithelial cells (VECs), is preparatory for colonization. VEC adherence by parasites is mediated by numerous distinct trichomonad surface adhesins. Brief contact of *T. vaginalis* with VECs and fibronectin (FN) elicited dramatic changes in parasite morphology, suggesting a host-specific signaling of

parasites[18,19]. Importantly, iron and cell contact by parasites each up-regulated the expression of adhesins in a coordinated fashion via distinct mechanisms. Trichomonal cytotoxicity of vaginal and cervical epithelial cells and the vaginal discharge following infection may be critical obstacles for successful host colonization by the parasite[17,18]. It is known that secretions of patients contain numerous trichomonad proteins, including high-molecular-weight immunogenic proteins[20]. Further, it is known that numerous proteins are readily released during growth, and multiplication without lysis of organisms has been established[21].

In this report, antigenic membrane proteins were isolated and identified by immunoscreening the *T. vaginalis* cDNA expression library with anti-*T. vaginalis* membrane protein antibodies, and whether the immunogenic proteins in understanding host-pathogen interactions.

The study of antigenic protein in protozoa parasites has been focused in AP65-1, which is one of four iron-regulated surface proteins involved in the cytoadherence of *T. vaginalis*[22]. AP65-1 encoding the trichomonad adhesions is coordinately regulated at the transcriptional level by iron. Overall, cytoadherence of *T. vaginalis* via receptor–adhesin interactions is complex, involving proteinase activity, signal transduction, and regulation by environmental factors. Besides representative

adhesins of *T. vaginalis*, AP65, AP51, and AP33, are found to share sequence homology with metabolic enzymes, which include malic enzyme[23], succinyl-CoA synthetase β subunit[24], and succinyl-CoA synthetase α subunit[25], respectively. It is not unusual that some metabolic enzymes of pathogens play an additional function in interaction with the host; for example, streptococcal surface dehydrogenase working as an adhesin for pharyngeal cells[26]. Isolation of AP65 as a membrane protein is contradictory with the result of Garcia and Alderete[22], in which AP65 was found to be secreted and played an important role in *T. vaginalis* binding to the host VECs. And AP65 and α-enolase are found to reassociate with the parasite surface for the expression of adhesion function and binding to plasminogen, respectively[27]. Also, AP65 is encoded by a multigene family, *ap65-1*, *ap65-2*, and *ap65-3*[28].All three proteins are expressed in individual organisms reaffirms the importance of the AP65 proteins to the biology of this parasite and the property of cytoadherence. Therefore, it was examined whether AP65-1 is located in the membrane of *T. vaginalis* in the subsequent immunofluorescence assay (IFA) using antibodies specific to AP65-1 (Fig. 7). The results, a model was suggested in which AP65, a secreted protein, mediated *T. vaginalis*-VEC binding by bridging the two receptors, each of which are derived from parasite and host cells.

T. vaginalis possesses a cytoskeleton and synthesizes structural proteins that can assemble in the cytoplasm to express typical morphologies[29]. These cytoskeleton proteins are considered to be important for formation, localization, and maintenance of specific integral membrane protein complex a barrier restricting the diffusion of both cytoplasmic and membrane proteins to distinct regions or compartments of the cell. The majority of identified clones encode components associated with cytoskeleton, which included actinin, and a kinesin-associated protein (Table 4). Most notably, three of the nine isolated clones turned out to be α-actinin, the actin-binding and Ca^{2+}-binding protein with high immunogenicity in *T. vaginalis*[19]. Two of the identified clones encoded a kinesin-associated protein which functions as a regulator for cellular and intracellular movements via interaction with the motor proteins[30]. One of the clones was also found to encode a putative teneurin, a conserved family of transmembrane proteins involved in intercellular signaling[31,32]. But teneurin, failed to find expression condition (Fig. 6C).

Finally, characterization of immunogenic trichomonal membrane proteins are accomplished by fourty human sera samples by using consisting extracts of *T. vaginalis* as antigen in ELISA assay (Table 5). A screening by ELISA revealed ten serum samples specific to *T.vaginalis* and one serum sample non-specific to

T.vaginalis at least. Performing ELISA assay to know that AP65-1 and kinesin proteins are whether immunogenic proteins or not. The results show positive antibody responses among patients to a major 65kDa protein and kinesin (Fig. 8). In all isolates test, sera 16, 19, and 26 yielded a strongly immunogenic reaction, surprisingly AP65-1 shows high reactive in serum 16 that indicates immunogenic protein.

The significance of antigenic proteins on *T. vaginalis* surfaces remains to be elucidated. I identify that antigenic membrane proteins are immunogenic and the location of these immunogens in the membrane of *T. vaginalis.* Antigenic proteins on their surface would induce infection of a human, and expect diverse functions to host cells in urogenital tissues or during menstruation[32,33]. It may contribute to the antigenic diversity among pathogenic human trichomonads.

Ⅴ. CONCLUSION

1. The immunoscreening reacting anti-*T.vaginalis* membrane proteins antibodies identified five antigenic proteins: AP65-1, kinesin-associated protein, α-actinin, teneurin, two hypothetical proteins.

2. AP65-1, one of the antigenic proteins, is mainly located in the cytoplasm of *T. vaginalis* through immunoflourescence assay.

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ABSTRACTS (IN KOREAN)

질편모충 항원성 표면 단백질의 특성 규명

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형 수 진

질편모충증은 질편모충 감염에 의해 일어나는 비바이러스성 성병으로 여성의 건강을 위협하고 있다. 질편모충증 질환 증상을 일으키는 독력인자를 알아내기 위해 질편모충 막 단백질에 특이적으로 반응하는 다클론 항체를 이용하여 이 원충의 항원성 단백질을 분리하였다. 질편모충 mRNA로부터 cDNA을 얻은 후, expression library를 제작하였고 이를 질편모충 막단백질 특이 항체와 반응하는 immunoscreening을 통해

면역반응을 보이는 클론들을 분리하였다. 그 결과 질편모충의 부착단백질인 AP65-1을 포함하여 α-actinin, kinesin-associated protein, teneurin이 확인되었고 기능을 알 수 없는 두 개의 cDNA를 확인할 수 있었다. AP65-1 의 세포 내 위치를 확인하기 위해서 영양형에 AP65-1 특이 항체를 이용하여 면역형광검사를 수행한 결과, AP65-1 단백질이 질편모충 전체에 분포하는 것을 관찰하였다. 마지막으로 효소면역검사법을 통해 AP65-1을 질편모충에 감염된 환자의 혈청과 강하게 반응하는 것을 알 수 있었다. Immunoscreening 결과 얻어진 kinesin associated protein의 경우도 질편모충증 환자의 혈청과 반응하였다. 이러한 결과는 질편모충 AP65-1와 kinesin associated protein이 항원성단백질로 작용할 가능성을 제시하며 이들에 대한 연구를 통해 질편모충-인체세포 간 상호 작용에 대한 지식을 얻을 수 있다고 본다.

핵심되는 말: 질편모충, 항원성 단백질, 막 단백질, immunoscreeing, AP65-1