A Thesis

For the Degree of Master of Veterinary Medicine

Development of a Sandwich ELISA Method for Detecting Mycoplasma Contamination in Cell Culture

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Development of a Sandwich ELISA Method for Detecting Mycoplasma Contamination in Cell Culture

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Dedication

I am very grateful to my parents, my brother and sisters for their love, support, patience, and sacrifice.

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Abstract

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ABSTRACTS:

The aim of this study was to develop sandwich ELISA method for the detection of common mycoplasma contaminants in cell culture. We produced the mouse monoclonal antibodies (MAbs) and rabbit antibodies against *Mycoplasma arginini, M. hyorhinis, M. orale* and *Acholeplasma laidlawii*. Binding specificities of MAbs and rabbit antibodies to the immunized antigens were confirmed by indirect ELISA and Western blot analysis. Among the established MAbs and rabbit anti-mycoplasmas, MAb MH03 against *M. hyorhinis*, and rabbit anti-*A. laidlawii* showed high cross reactivities to the four *Mycoplasma* species. We constructed a sandwich ELISA using MH03 as capture, horse radish peroxidase labeled rabbit anti-*A. laidlawii* as detector, and could detect *M.arginini* with the detection limit of 0.5 μg/ml and *M. hyorhinis, M. orale* and *A. laidlawii* with the detection limit of 0.1 μg/ml. These results indicate that the sandwich ELISA developed in this study could be applied for the detection of the four major *Mycoplasma* contaminants in cell culture.

Key words: *Mycoplasma*, *Acholeplasma*, Monoclonal antibody, Rabbit antibody, ELISA,

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Introduction

Mycoplasma species are the most common source among various contaminating agents in cell culture (Salari *et al.*, 2002; Mirjalili, *et al.*, 2005). Since their variable morphology enables them to pass sterilizing filters of (a pore size of 0.22μm), it is very difficult to protect animal cell cultures from mycoplasma contamination.

Many *Mycoplasma* species are able to produce various effects that result in unreliable or false result when contaminated in cell culture (Mc Garrity and Kotany, 1985; Harasawa *et al.*, 1993). For example, abnormal signs of metabolism, cell growth rate and viability, synthesis of DNA, RNA or protein, modulation of immune response and morphology were observed in mycoplasma contaminated cells (Miyazaki *et al.*, 1990; Timenetsky *et al.*, 2006). There, in order to ensure reliable research results, the suitable method for the detection of mycoplasma contamination including microbiological culture, DNA staining, PCR (Tang *et al.*, 2000; Timenetsky *et al.*, 2006; Valley *et al.*, 1995), ELISA (Dvorakova *et al.*, 2005; Dufour-Gesbert *et al.*, 2001), immunobinding assay (Flores-Gutierrez *et al.*, 2004), enzymatic activity test (Valley *et al.*, 1994) were developed.

Since the distribution of species causing multiple infections is still unknown, there needs genus detection method which could detect all kind of mycoplasma contaminants in cell culture. Hence, Tang *et al.* (2000) and Timenetsky *et al.* (2006) reported PCR methods for the detection of multiple mycoplasma infection in cell culture in a single test with specific primers.

Likewise, the purpose of present study was to develop sandwich ELISA method which can detect the most common mycoplasma contamination in cell culture using highly cross reactive monoclonal antibodies and rabbit antibodies against *M. arginini. M,hyorhinis. M,orale* and *A. laidlawii.*

Materials and Methods

Bacterial strains

The reference strains of *Mycoplasma* species in this study were purchased from the American Type Culture Collection (ATCC) include *M. hyorhinis* (ATCC 17981), *M. orale* (ATCC 23714), *M. arginini* (ATCC 2338) and *A. laidlawii* (ATCC 14089).

Bacteria culture

Mycoplasma seeds were activated by culturing in *Mycoplasma* broth containing 200 ml horse serum and 100 ml yeast extract solution (GIBCO BRL, USA). Only for *M. arginini*, 2.1 g of arginini was added before autoclave. *Mycoplasma* was incubated in media at 37℃ for 3 days under the anaerobic condition for *M. orale* and the aerobic condition for others. To confirm cross contamination with each cultured mycoplasmas, ELISA was performed by specify ELISA kit (Roche Diagnostics, GmbH).

Antigen preparation

Cultured mycoplasma suspensions were harvested by centrifugation (20.000x g, 45 min, 4℃) followed by three successive washing steps with phosphate buffer saline, pH 7.4 (PBS). Protein concentrations of harvested mycoplasma antigens were determined using Bradford method (Bio-Rad, USA).

Mouse Immunization

The mouse immunization was performed by the method of Ghadersohi (2005) with some modification. Each *Mycoplasma* species were immunized intraperitoneally (i.p.) to 6 week-old female BALB/c mice (n=2) with whole mycoplasma antigen in PBS. The antigens were emulsified with an equal volume of Freund's complete adjuvant (CFA). For the first immunization, the mice were immunized i.p. with 100 ㎕ of antigen emulsion (200 ㎍ protein/head). On day 14, the mice were injected with the same dose and volume of antigens emulsified with Freund's incomplete adjuvant (IFA). On day 28, 29 and 30, the mice were injected i.p. with 50 ㎕ antigen/head without adjuvant.

Cell fusion for producing hybridoma

The spleens of mouse were removed aseptically and the spleen cells were fused with SP/2 cells in the presence of PEG after 24 hour of the last immunization. SP/2 cells were cultured in growth medium (DMEM, 10% FCS) and checked for growing well before fusion. Blood samples were harvested through heart puncture to check the titer to antigens. Then the spleens were removed aseptically and were broken down using 5 ml-syringe needle with washing medium. The spleen cells were collected into 50 ml conical tube and the cell suspension was washed by centrifugation (150 g, 3 min) for 3 times. Before the fusion work, all the media were warmed to 37℃. The fusion procedure was performed between SP/2 cells and spleen cells in the presence of 50% PEG-1500 (Roche, GmbH). The fused hybridoma cells were cloned by selecting by hypoxanthine, aminopterine and thymidine (HAT) medium.

Rabbit immunization

Polyclonal antibodies against each *Mycoplasma* species were produced by immunization of two male rabbits with 2-3 kg body weight. For the first immunization, 1 ml of antigen was emulsified with 1 ml Freund's complete adjuvant, and was injected subcutaneously with 0.2 ml/site for 10 sites into back of rabbit. With two weeks interval, the rabbits were inoculated 2 ml of antigen with incomplete adjuvant. Blood samples were taken on day 0, 28, 42 and 56 for testing antibodies level in serum. After 10 days of the last immunization the rabbits were exsanguinated and the sera were collected.

Indirect ELISA for the screening of hybridoma and titration of rabbit sera

The indirect ELISA was as described previously by Irina and Karvchenko (2006) with a slight modification. Briefly, the presence of antibodies against mycoplasma antigens were checked in the supernatants from the wells of culture plate containing hybridoma cells after fusion of 7 days. The 96-well ELISA plates (Costar, USA) were coated with mycoplasma whole cell antigen (20 ㎍ protein/ml) diluted in coating buffer (50 mM carbonate, pH 9.6), and incubated at 4℃ for 16 hours. The plates were moved to room temperature, and were washed with PBS containing 0.05% Tween 20 (PBS-T). Serially diluted hybridoma culture supernatants or rabbit serum with PBS-T were added and incubated at 37℃ for 1h. After washing, horseradish peroxidase (HRP) labeled goat anti-mouse IgG or HRP labeled goat anti-rabbit IgG was added. Then the plates were incubated at room temperature for 1 h. The color was developed by adding 100 ㎕ of 1 mM 2,2'-

azio-di-[3-ethylbenzthiazolin sulfate] (ABTS) containing 0.015% H_2O_2 in 0.1M citrate/phosphate buffer, pH 4.2. The optical density (OD) was read at the dual wavelengths of 405 and 492 nm in an ELISA plate reader (SLT, Austria).

Purification of MAbs and Rabbit anti-*Mycoplasma* antibodies

Specific MAbs and Rabbit antibodies against *Mycoplasma* species were purified by affinity chromatography using protein A-Sepharose (Pharmacia-LKB). IgM type MAbs were purified by salting out method with 50% saturated ammonium sulfate. Purified antibodies were dialyzed against PBS for 48 hours.

Labeling HRP to Purified antibodies

Purified antibodies were conjugated with HRP by the method of Wilson and Nakane (1978). Briefly, 0.2 ml of 0.1 M $NaIO_4$ was added to 4 mg of HRP dissolved in sterilized water, and was mixed by gentle stirring at room temperature for 20 min followed by dialysed in 1 mM sodium acetate (pH 4.4) at 4℃ for 20 hours. The pH was raised to 9.5 by addition 0.2 M sodium carbonate-bicarbonate (pH 9.5) and 8 mg of antibody in 1 ml of 0.01 M sodium carbonate-bicarbonate was added immediately. The mixture stirred gently at room temperature for 2 hours. Reaction was quenched by subsequent adding 0.1 ml of freshly made sodium borohydride solution and stand at 4℃ for 2 hours. The HRP labeled antibodies were dialyzed against PBS.

SDS Polyacrylamide Gel Electrophresis (SDS PAGE) and Western Blot Assay (W/B)

SDS-PAGE was carried out as described by Ghadersohi *et al.* (2005). Briefly, about 15 ㎍ of whole cell protein of each *Mycoplasma* species, suspended in PBS were mixed with 4x sample buffer, and boiledin a water bath at 100℃ for 3 minutes. The samples were separated on the 12% separating gel at 100 V constant voltage in a Mini protean II apparatus (Bio-Rad) until the tracking dye reached the bottom of the gel. The gel was immediately transferred to nitrocellulose membrane (Bio-Rad) using Mini trans-blot apparatus (Bio-Rad) with a current of 100 V at 4℃ for 2 hours. After blocking at room temperature for 1 hour, the nitrocellulose membranes were then washed three times with TBS-T and incubated with specific antibodies and shacked gently at room temperature for 1 hour. The membrane was washed three times with PBS-T that was incubated with HRP-conjugated goat anti-mouse IgM for 1 hour at room temperature. After washing, the bands were detected using chemilruminascence detection kit (Bio-Rad).

Sandwich ELISA for the detection of *Mycoplasma*

The optimal dilution factor of coating antibodies and HRP conjugates were determined by applying known concentration of mycoplasma by using MH03 and HRP labeled rabbit anti-*A. laidlawii* with serial dilution.

PCR

The PCR method of Kong *et al.* (2007) was employed in this study with a little modification. Briefly, each 200㎕ of cell culture supernatants was applied to extract DNA by using the DNase tissue kit (Qiagen, USA) according to the manufacture's protocol. The universal PCR primers designed by Kong *et al.* (2007) for amplification of the rpoB gene of Mollicutes on the basis of conserved regions found within sequences of this gene that were available in Gen Bank. The forward primer rpoB-F1-MYC (atgggtgcvaacatgcaacgtcaagc) and a mixture of two reverse primers rpoB-R-MYC (gctcahacttccatttchccaaa) and rpoB-R1-MYC (cgttttgwgctttaccacccattggttgttg) used to amplify the rpoB gene from all Mollicutes species. The reaction condition was as followings, initial activation at 95℃ for 15 minutes, 40 cycles of 94℃ for 1 minute, 55℃ for 1 minute and 72℃ extension for 90 seconds and a final extension at 72℃ for 7 minutes. The PCR products were confirmed by electrophoresis on 1 % agarose gel, and then stained with ethidium bromide.

RESULTS

Preparation of *Mycoplasma* antigen

The morphology of the colonies of each *Mycoplasma* species cultivated in agar plate was translucent with the typical 'fried-egg' shape (Figure 1). Indirect ELISA results of the purified bacterial antigen with detection kit showed that there is no cross contamination with each *Mycoplasma* species subjected in this experiment during the culture and purification (Figure 2).

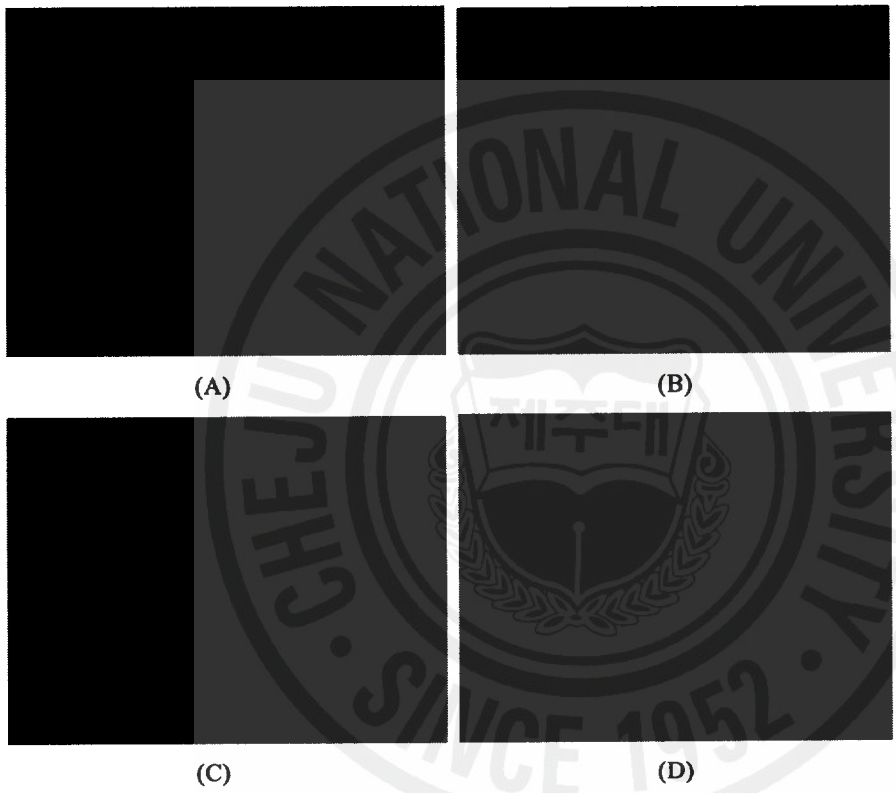

Figure 1. The clonies of *Mycoplasmas* on agar plate. (A): *M. arginini* , (B): *M. hyorhinis*, (C): *M. orale*, (D): *A. laidlawii*

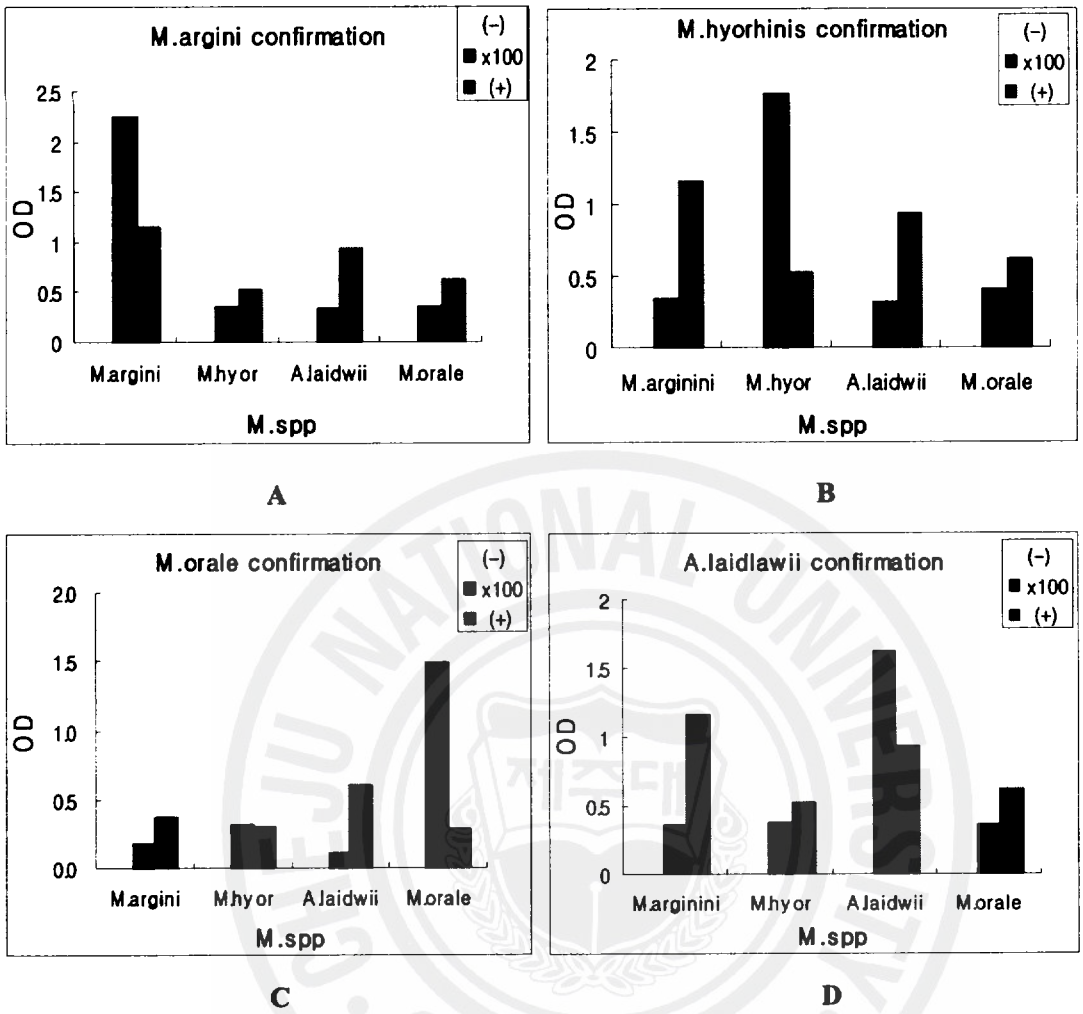

Figure 2. The confirmation of cross contamination of Mycoplasma antigens by ELISA. (A): the confirmation of *M. arginini*; (B): the confirmation of *M. hyorhinis*; (C): the confirmation of *M. orale*; (D): the confirmation of *A. laidlawii*

Monoclonal antibodies against *Mycoplasma* species

Using purified mycoplasma antigen, 6 clones (MA01, MA02, MA03, MA04, MA05, MA06) against *M. arginini*, one clone (MH01) against *M. hyorhinis*, 2 clones (MO01, MO02) against *M. orale* and 4 clones (AL01, AL02, AL03, AL04) against *A. laidlawii* were produced. Among these clones, MH03 and MO01 showed high cross reactivity with the bacterial antigens subjected in this experiment (Figure 3, 4, 5, 6). Since enough quantity of MH03 clone was producd in ascites, MAb of MH03 was selected as capture antibody for developing a sandwich ELISA (Table 1).

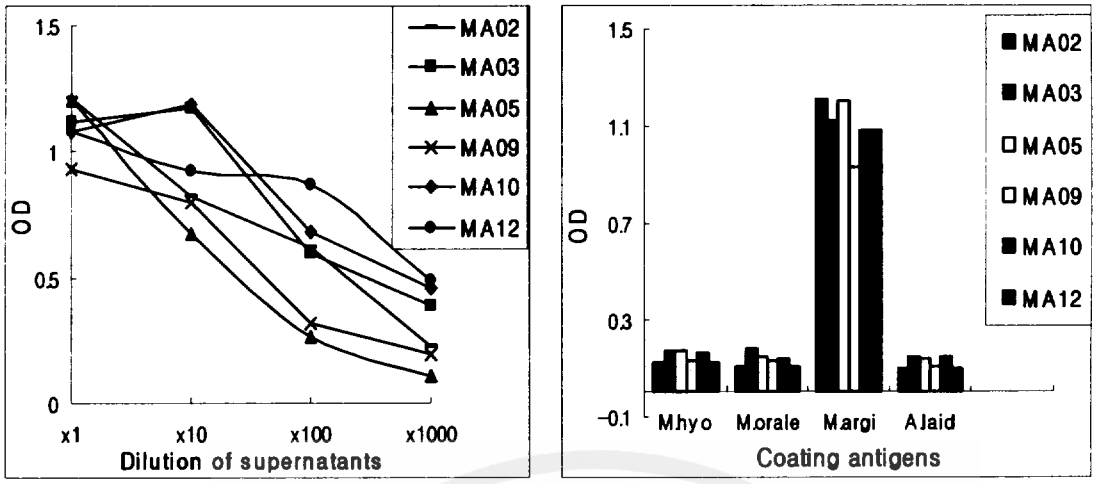

Figure 3. The titration curve and the cross reactivity of MAb against *M. arginini.*

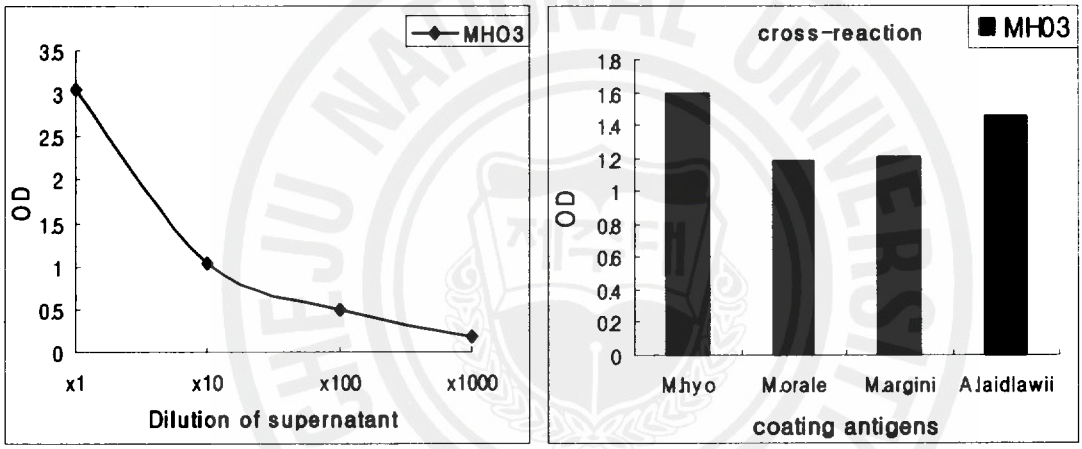

Figure 4. The titration curve and the cross reactivity of MAb against *M. hyorhinis.*

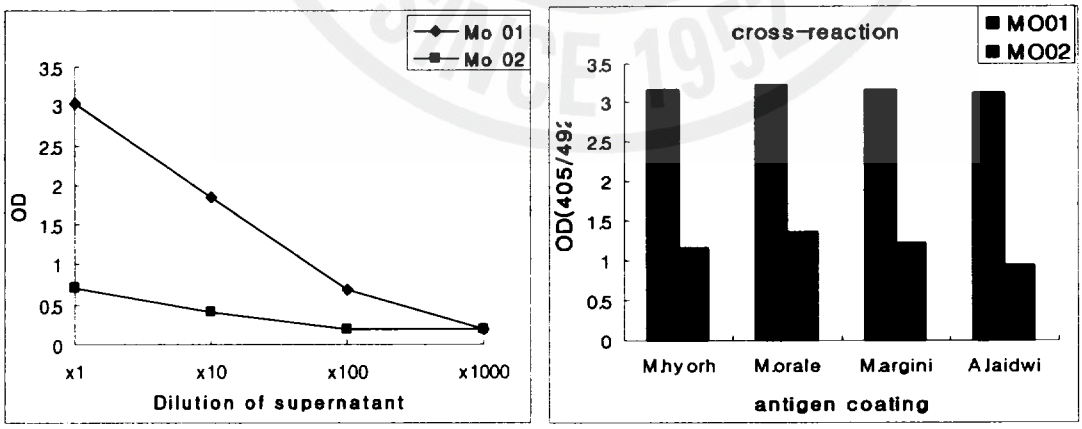

Figure 5. The titration curve and the cross reactivity of MAb against *M. orale.*

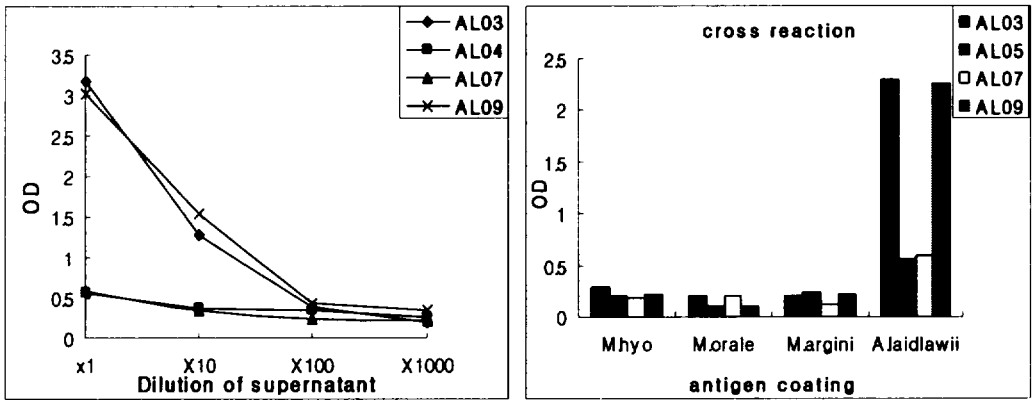

Figure 6. The titration curve and the cross reactivity of MAb against *A. laidlawii.*

Table 1. Specificity analysis of antibodies using indirect ELISA

			Mycoplasma species			
		clones	*M. arginini*	*M. hyorhinis*	*M. orale*	*A. laidlawii*
Anti-*M. arginini*	MAb	MA02	+	-	-	-
		MA03	+	-	-	-
		MA05	+	-	-	-
		MA09	+	-	-	-
		MA10	+	-	-	-
		MA12	+	-	-	-
	Rabbit Ab		+++	+	+	+
Anti-*M. hyorhinis*	MAb	MHO3	++	+++	++	+++
	Rabbit Ab		+	+++	+	++
Anti-*M. orale*	MAb	MO 01	++	++	++	++
		MO 02	+	+	+	+
	Rabbit Ab		+	+	+++	+
Anti-*A. laidlawii*	MAb	AL03	-	-	-	+
		AL05	-	-	-	+
		AL07	-	-	-	+
		AL09	-	-	-	+
	Rabbit Ab		++	++	++	+++

Rabbit antibodies against Mycoplasma species.

Rabbit antibodies immunized with four kinds of *Mycoplasma* species showed the cross reactivity with each others (Figure 7, 8, 9, 10). Among the 4 kinds of rabbit antibodies, anti-*A. laidlawii* showed the highest titer (at least 10^4 against each antigens, Figure 11), and was selected as detector antibody for developing sandwich ELISA.

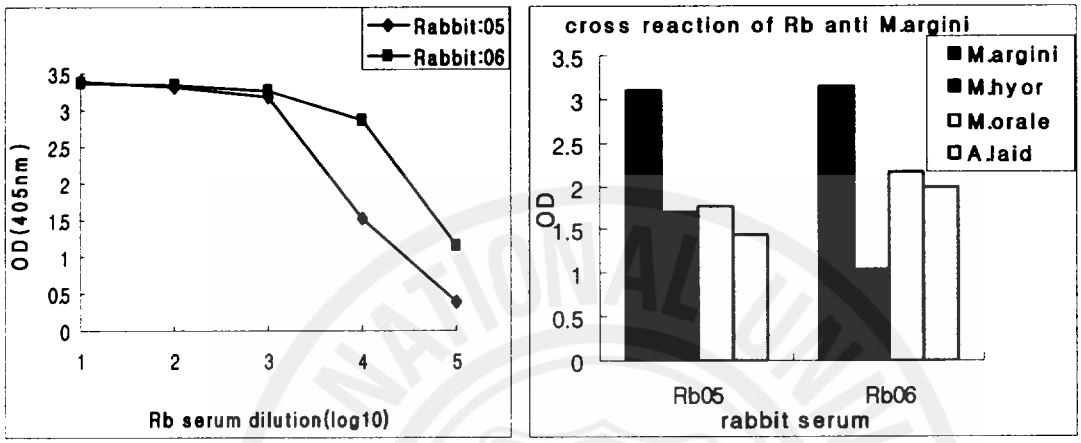

Figure 7. The titration curve of Rabbit Anti *M. arginini* and the cross reactivity.

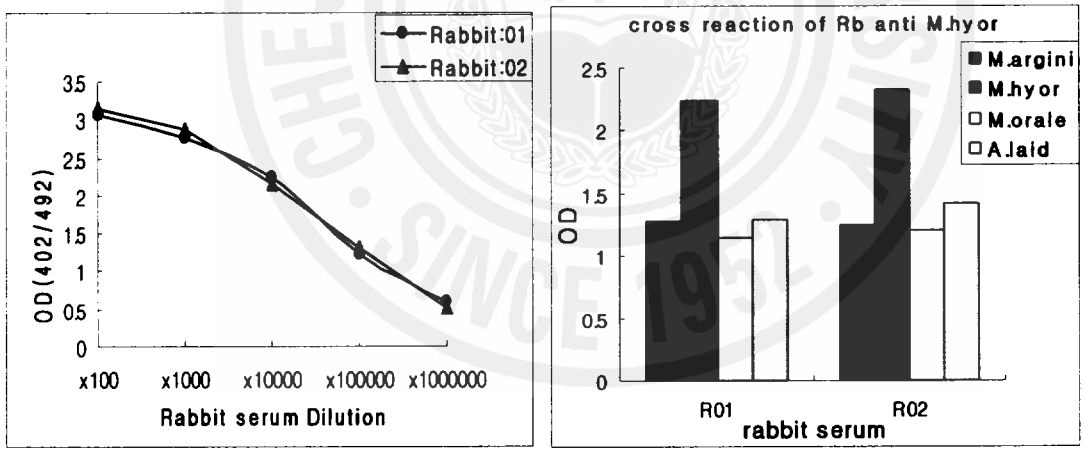

Figure 8. The titration curve of Rabbit Anti *M. hyorhinis* and the cross reactivity.

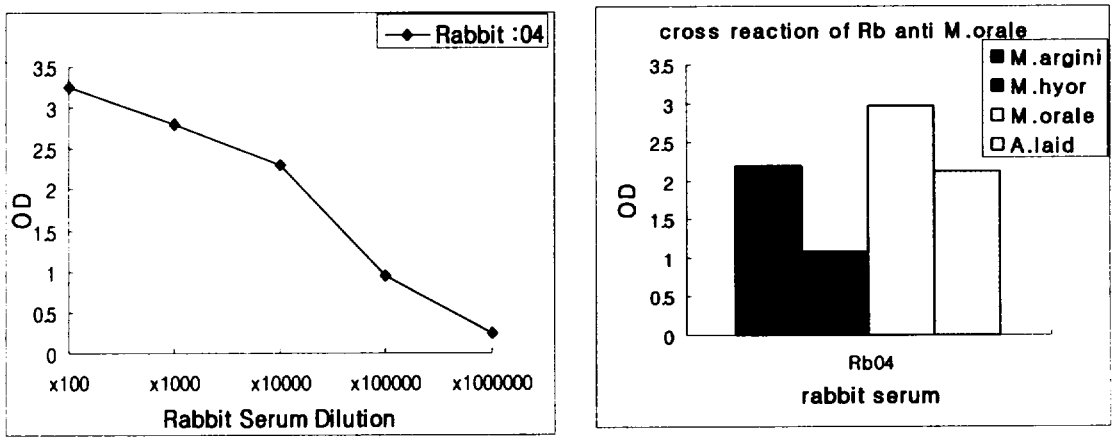

Figure 9. The titration curve of Rabbit Anti-*M. orale* and the cross reactivity.

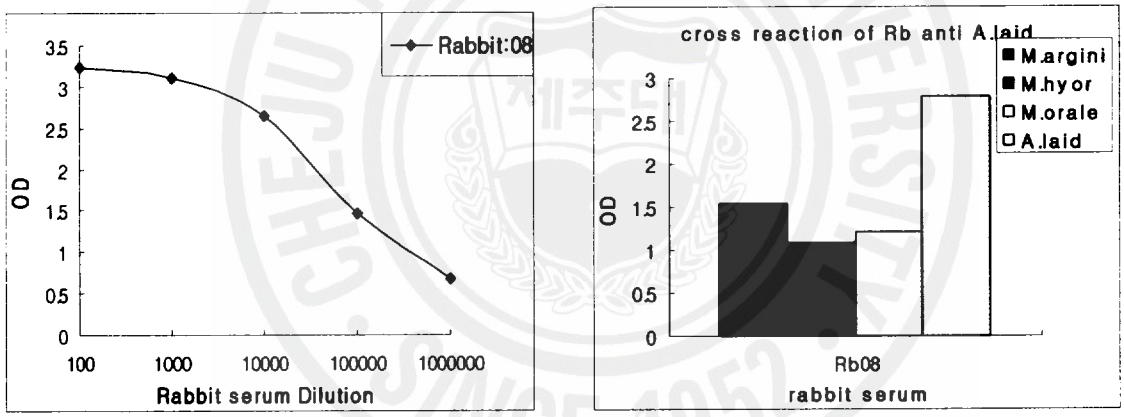

Figure 10. The titration curve of Rabbit Anti-*A. laidlawii* and the cross reactivity.

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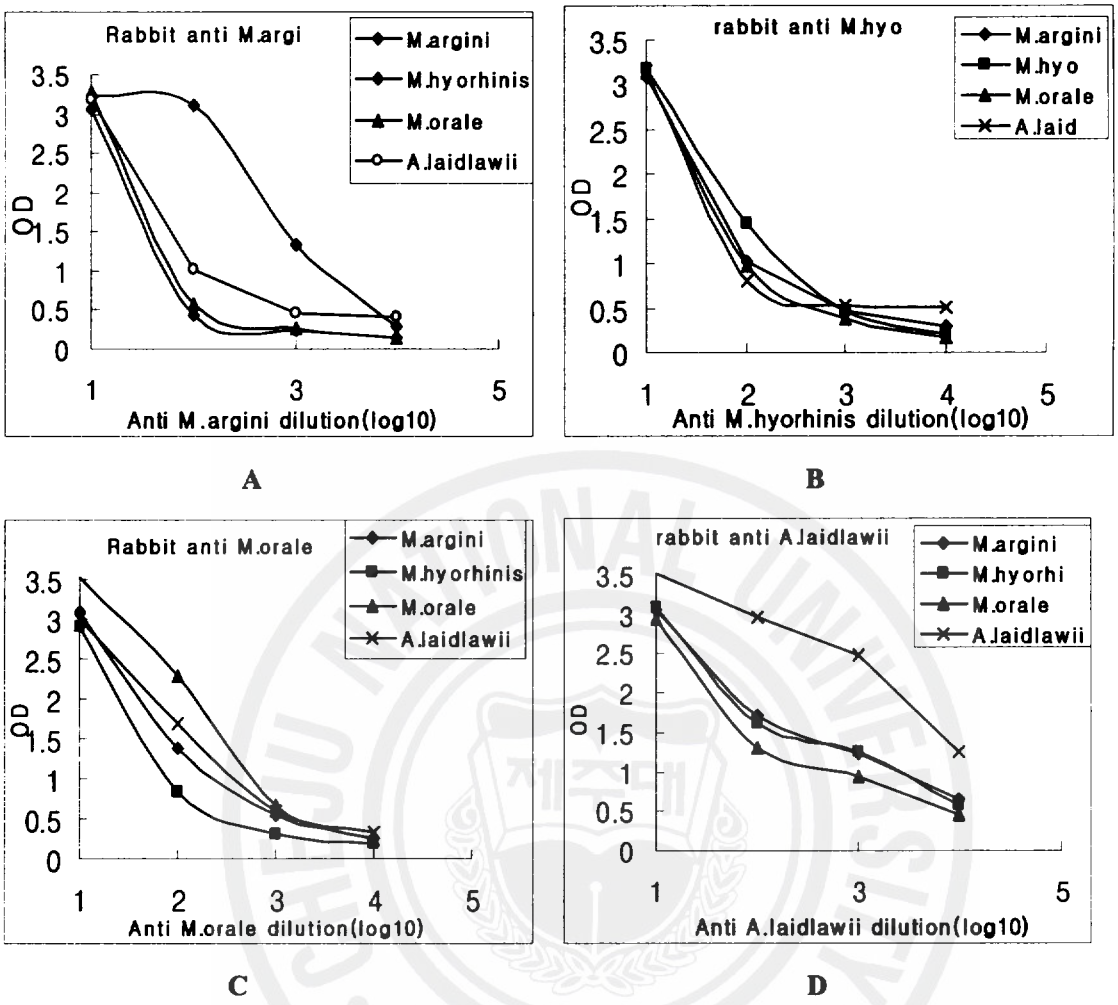

Figure 11. The titer of cross reaction of rabbit IgG against Mycoplasma. (A): anti *M.arginini*; (B): anti *M.hyorhinis*; (C): anti *M.orale*; (D): anti *A.laidlawii*

Purification of MAb MH03

Since MAb of MH03 was revealed as IgM type antibody, it was purified by salting out method using 50% saturated ammonium sulfate. After dialysis with PBS, it was used as capture antibody.

Purification of rabbit specific antibodies

Mycoplasma specific antibodies were purified by affinity chromatography using Protein A-Separose (Figure 12). The fractions eluted by 3 M NaSCN showed high concentration of protein and reactivity with corresponding antigens. After dialysis against PBS, purified antibody was conjugated with HRP.

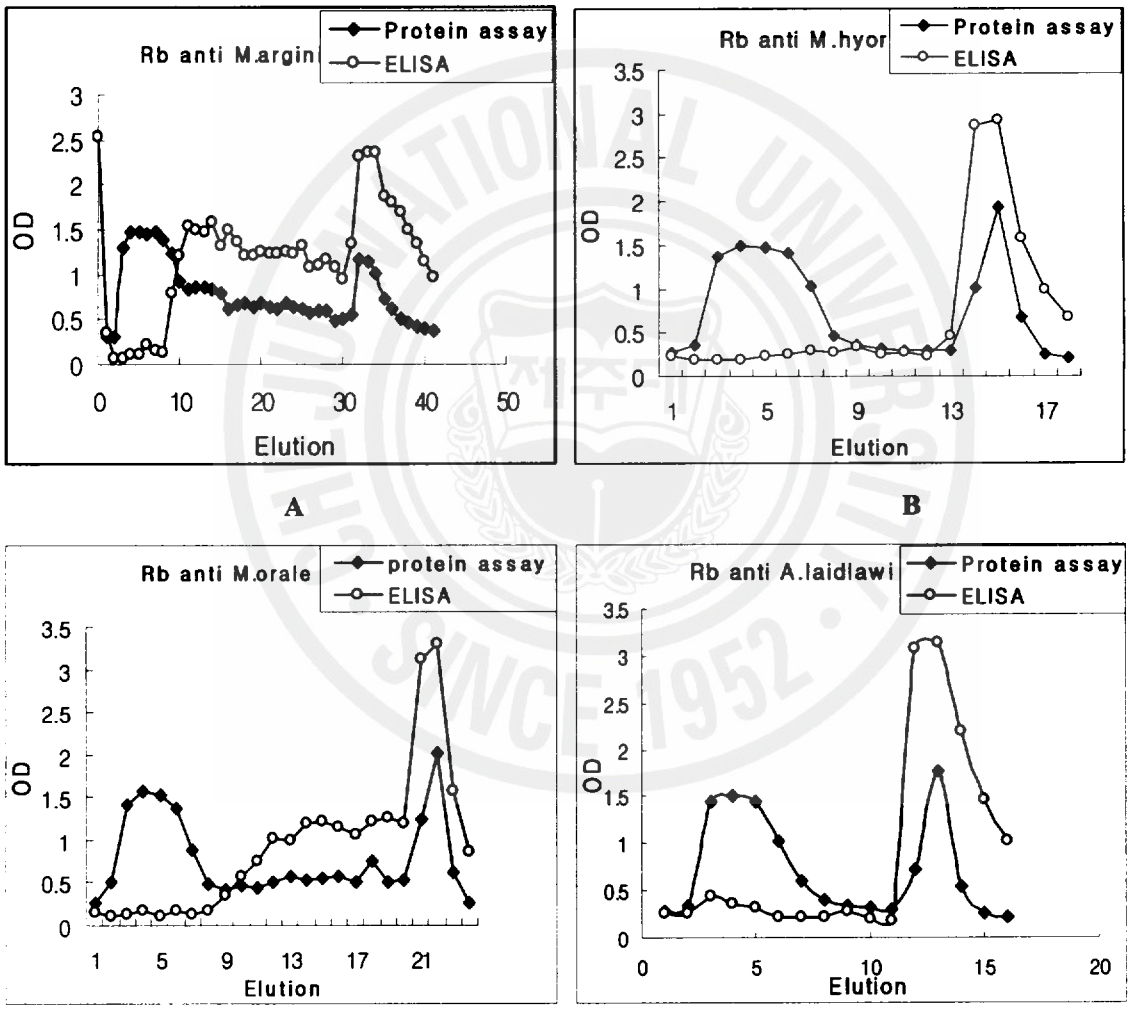

Figure 12. Affinity chromatography purified rabbit anti Mycoplasmas. (A): anti *M.arginini*; (B): anti *M.hyorhinis*; (C): anti *M.orale*; (D): anti *A.laidlaiwii*

Western blot assay

Western blot assay of MAb MH03 showed that there was high reactivity with *M. hyorhinis* antigens at the molecular weight of 80, 75, 65 kDa, showed cross reactivites with other mycoplasma antigens, especially at the molecular weight of 50 kDa. In case of rabbit anti *A. laidlawii*, there were at least 3 distinctly colorized bands at the same site indicating the high cross reactivities among the subjected 4 kind of *Mycoplasma* species.

Figure 13. SDS-PAGE and Western blot. (A): SDS-PAGE of four Mycoplasma species: Lane M, marker; lane1, *M.arginini*; lane2, *M.hyorhinis*; lane3, *M.orale*; lane4, *A.laidlawii*. (B): Western blot of MH03 against four Mycoplasma species: Lane M, marker; lane1, *M.arginini*; lane2, *M.hyorhinis*; lane3, *M.orale*; lane4, *A.laidlawii*

.

Figure 14. Western blot of rabbit anti *A.laidlaiwii* against four mycoplasma species. M, marker; lane1, *M.arginini*; lane2, *M.hyorhinis*; lane3, *M.orale*; lane4, *A.laidlawii*

Optimization of Sandwich ELISA

The optimal dilution factor of capture and detector antibodies were determined by ELISA with serially dilution of capture and detector using mycoplasma antigens of 5 ㎍/ml. Based on the standard curve, the optimal dilution factor of purified MH03 was decided to 400 fold, and the factor of HRP-conjugated rabbit anti- *A. laidlawii* was decided to 500, respectively (Figure 15).

After optimization of sandwich ELISA, the detection limits on each mycoplasma antigen were determined as less than 0.5 ㎍/ml respectively (Figure 16).

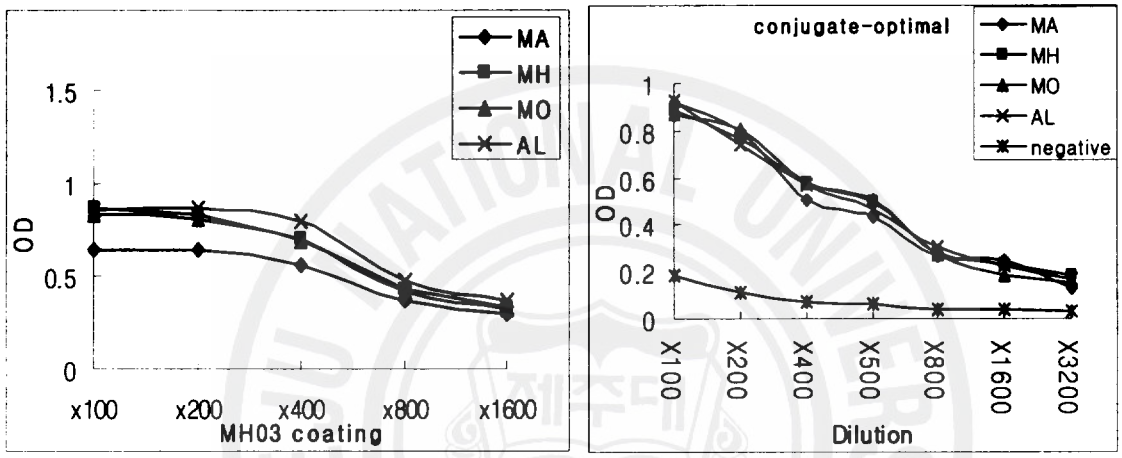

Figure 15. Optimal dilution factor of capture and detector. MA: *M. arginini*; MH: *M. hyorhinis*; MO: *M. orale*; AL: *A. laidlawii*.

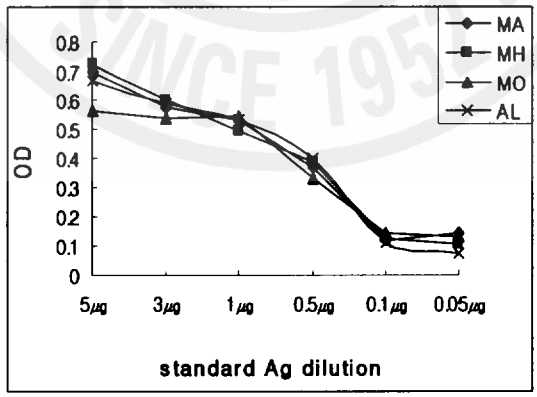

Figure 16. Detection limit of Mycoplasma antigens by sandwich ELISA. MA: *M. arginini*; MH: *M. hyorhinis*; MO: *M. orale*; AL: *A. laidlawii*.

Both sensitivity and specificity of the ELISA in this study were determined as 100% when the standard antigens was compared with the supernatants from cell culture by commercialized ELISA kit (Table 2).

Table 2. Sensitivity and specificity evaluation of the sandwich ELISA developed in this study

Test resuts	Refernce ELISA kit *	
	Positive CS**	Negative CS
Positive	13	0
Negative	0	7

*Commercialized kit: Roche Diagnostics, GmbH
** Cell culture supernatants
Sensitivity: 13/(13+0)*100=100%, specificity: 7/(0+7)*100=100%

PCR

Mycoplasma DNA was amplified by PCR, the products were subjected to 1% gel agarose electrophoresis. The result of PCR revealed that it could detect three out of four kinds of mycoplasma references except *M. hyorhinis* with the size about 1.4kb (Figure 17). Supernatants of cultured cell contaminated with mycoplasmas were determined by commercial ELISA kit, and artificially infected cells with mycoplasma. These cells inoculated with 100 ㎕ of broth medium contained grown mycoplasmas and cultured for four days in aerobic condition with 20 ml of growth medium, and 200 ㎕ of supernatants were extracted DNA by using the DNase tissue kit (Qiagen, USA). The PCR could detect mycoplasmas from artificially infected cell cultures but not in case of natural infected cell culture.

Figure 17. Detection of mycoplasma DNA with PCR method. (A): The detection of mycoplasma DNA from Mycoplasma references. PCR detection was based on amplification of the 16S-23S ITS region. Samples were harvested after three days of inoculation in broth medium, 2㎕ of medium contained grown mycoplasmas applied for PCR. M: marker, lane1, *M.arginini*; lane2 *A.laidlawii*; lane3, *M.orale*; lane4, *M.hyorhinis*. (B) PCR tested mycoplasmas from cell lines M: marker; lane Sup1-2, cell culture supernatants; lane (+), positive control; lane (-), negative control.

Discussion

Many techniques have been developed for the detection of mycoplasma contamination and each method has its own advantage and disadvantages. In this study, we have developed a sandwich ELISA using mouse monoclonal antibodies and rabbit polyclonal anti-Mycoplasma antibodies which could detect four kinds of mycoplsmas. The goal of this study is to screen the infection of main mycoplasma contaminants in cell cultures in one test.

In most cell cultures, there are cross contaminations between mycoplasma species or other organism (Mirjalili *et al.*, 2005). Mycoplasma and Acholeplasma species cultured in anaerobic or aerobic conditions at 37℃ for 2-3 days could yield higher concentration of antigen than a longer culture. Since one of the reason of contamination is that by laboratory personal, a careless handing (Stacey *et al.*, 1997), we could get the bacterial antigens without cross contamination by culturing separately and different times.

Generally, immunogen from live microorganism is inactivated by formalin (Brook *at el.*, 2004). Howerver, in this study, we used a whole-cell antigen for immunization of mice and rabbits without formalin inactivation for convenience sake. No animal showed pathological sign by the infection of inoculated bacteria, which indicates that the four kind of mycoplasma in this study do not have pathogenecity to mouse and rabbit regardless the efficacy of immunity.

All the rabbit antibodies showed cross reactivity with the four mycoplasma antigen. On the other hand, MAbs against *M. arginini* and *A. laidlawii* showed specific reactivity with corresponding antigens. MAbs against *M. hyorhinis* and *M. orale* showed high cross reactivity to other Mycoplasma strains. Though the reason of these phenomena should be further studied, the results suggest that the cross reactive MAbs could be applied for the detection of common Mycoplasma contamination in cell cultures.

Indirect ELISA and Western blot analysis showed that the monoclonal antibody MH03 and Rabbit anti *A. laidlawii* antibody have strong cross reactivities to other mycoplasmasBy using those antibodies as universal antibodies, we have developed a sandwich ELISA.

In this study, MH03 was used as the capture antibody, and HRP labeled rabbit anti-*A. laidlawii* was used as the detector antibody for sandwich ELISA. At this optimal condition, the detection

limit was 0.5 μg/ml for *M. arginini* and 0.1μg/ml of *M. hyorhinis, M. orale* and *A. laidlawii* contamination in cell culture. Here, we propose one of the good serological detection methods for mycoplasma contamination in cell culture.

The universal primer designed by Kong *et al.* (2007), was employed in this study with modification. Without DNA extraction from cultured mycoplasma, the PCR could detect *M. arginini, M.orale* and *A. laidlawii*, except *M. hyorhinis*. Two hundred microliter of cell culture supernatants were used for DNA extraction, and the result revealed that the PCR method detected mycoplasmas from artificial infection cell lines, whereas did not detect mycoplasma from naturally infected cell lines which were detected by ELISA kit and sandwich ELISA. Furthermore, PCR result is different from Kong *et al.* (2007) by the size of products.

In this study, we observed that the sandwich ELISA has more advantages than PCR method. Sandwich ELISA could detect four mycoplasma species whereas PCR detected only three mycoplasma species and with low sensitivity.

In summary, the results confirmed that use a sandwich ELISA for the detection of mycoplasmas contamination is suitable for large-scale test application. The sandwich ELISA quantification could detect 0.5μg/ml of *M. arginini* and 0.1μg/ml of *M. hyorhinis, M. orale and A. laidlawii* contamination in cell culture. Sandwich ELISA should be chose as a new technique and strategy for the detection of mycoplasmas in cell culture. The specific antibodies against Mycoplasmas and Acholeplasma showed be developed continuously for being easier in classifying each mycoplasma and Acholeplasma species.

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