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Evaluation of Antigen Preparation Methods for Polyclonal Antibody Production against *Streptomyces spp.*

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Authors' contributions

This work was carried out in collaboration between all authors. KS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. PT isolated and cultured the Streptomyces spp. AS performed the practical laboratory activities managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: To determine the optimum antigen preparation method for producing specific polyclonal antibody specific for *Streptomyces* species.
Study Design: Experimental study.
Place and Duration of Study: Faculty of Science, Mahasarakham University, Mahasarakham Province, Thailand, between May 2011 and December 2011.
Methodology: Two *Streptomyces* isolates were used for antisera production. The sonication method was chosen for antigen preparation. Antigen suspensions were emulsified with incomplete Freund's adjuvant and 1 ml was injected into rabbit thigh muscle for the first, second and third immunization. The fourth and fifth immunizations were injected intravenously. Antibody titer, detection limit and specificity were measured using indirect-ELISA. *Streptomyces* antigen mixed with soil was investigated.
Results: The 5 min sonication method gave a higher protein than other test methods, so this protocol was chosen for all subsequent work. The sonicated *Streptomyces* antigen was

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used for polyclonal antibody production. At week 7, the rabbit anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody gave maximum titers at 1:64000 and 1:32000, respectively. The detection limit of the anti-*Streptomyces* antibodies was 125 ng and 250 ng, respectively. Both anti-*Streptomyces* antibodies were also found to have good specificity. Minimal cross-reactivity was detected with antigens from other test bacteria. Regrettably however, neither antibody was capable of detecting *Streptomyces* spp. STPR78 or STPR84 in inoculated soil.
Conclusion: A specific and high titer of polyclonal antibody was produced using sonicated antigen.

Keywords: Polyclonal antibody; Streptomyces spp.; antigen preparation; sonication method.

1. INTRODUCTION

Streptomyces is a genus of Gram positive bacteria found predominantly in soil and decaying vegetation. These bacteria produce many clinically useful antibiotics (Atta et al., 2009; Deboer and Dietz, 1976; Chater, 2006) and enzymes used in industry (Chellapandi and Jani, 2008; Jayasree et al., 2009; Vishnupriya et al., 2010). *Streptomyces* species are also important in controlling soil-borne pathogens by antibiosis (Shimizu et al., 2009; Rothrock and Gottlieb, 1981; Yuan and Crawford, 1995).

Traditional methods of detecting and monitoring bacteria in their natural habitats include cultural techniques, examination of morphology and pigment production analysis. However, soil samples contain many other microorganisms. The traditional approach therefore involves multiple procedures and is very time-consuming (Williams et al., 1983). Detection of *S. ipomoea* in its natural environment, for example, takes up to 4 weeks (Moyer and Echandi, 1986). In studies seeking to discover new antibiotics produced by soil borne *Streptomyces* spp., isolation of the bacteria requires multiple procedures and takes 25 days or more (Ceylan et al., 2008).

An alternative strategy for detection would be to use immunological methods. Immunodiffusion, indirect-ELISA, immunofluorescence and the immunomagnetic capture technique have all successfully been used for the identification, isolation or tracking of specific microorganisms in the environment (Cross and Spooner, 1963; Kirby and Rybicki, 1986; Schloter et al., 1995). Cross and Spooner (1963) used a serological technique to identify and compare antibiotic producing isolates of *Streptomyces*. Kirby (1986) used indirect-ELISA to determine the relationships between *Streptomyces* spp. Wipat et al. (1994) produced monoclonal antibody against *Streptomyces lividans* spore surface antigens and employed this monoclonal antibody for isolating *Streptomyces* from soil using the immunocapture technique. Also, Bartosch et al. (2002) used a monoclonal antibody specific to a nitrite oxidation enzyme for the detection of *Nitrospira*-like bacteria in various soils. In the present study, we sought to determine the optimum antigen preparation method for producing polyclonal antibody specific for *Streptomyces* spp. and apply these antibodies to monitor the bacteria in soil using indirect-ELISA.

2. MATERIALS AND METHODS

2.1 Test Strains

Streptomyces sp. isolates STPR78 and *Streptomyces* sp. isolate STPR84 were used for antisera production. Twenty two isolates of unknown *Streptomyces* spp., *Pseudomonas aeruginosa* PDMZnCd 2003, *Pseudomonas aeruginosa* (isolated from environment), *Bacillus cereus* ATCC11778 and *Enterobacter* sp. (isolated from papaya wilt) were used for testing antibody specificity.

2.2 *Streptomyces* Protein Preparation

Streptomyces sp. isolate STPR78 and *Streptomyces* sp. isolate STPR84 were inoculated into 100 ml arginine glycerol mineral salt broth and incubated for 3-5 days at 30ºC on a rotary shaker at 150 rpm. The cells were harvested by centrifugation (Tomy MX-301, Japan) at 9,100 g for 15 min. The pellet was washed three times with phosphate-buffer saline (PBS; pH 7.4). Three antigen preparation methods, boiling at 100ºC for 5 and 10 min, autoclaving at 121ºC for 5 and 10 min and sonication for 1 and 5 min using a High Intensity Ultrasonic Processor (Model VCX 750, USA), were used to prepare *Streptomyces* protein antigen. The proteins were adjusted to equal concentrations before measuring on 12% SDS-PAGE.

2.3 Antigen Preparation for Immunization

The 5 min sonication method was chosen for antigen preparation. The *Streptomyces* pellets were resuspended in PBS (pH 7.4), followed by sonication with a microprobe. This was done on ice for a total of 5 min in 10 s bursts with 2 s gaps for cooling. The sonicated solutions were centrifuged at 9,100 g for 5 min and filtered through a 0.2 μm filter. Protein in the sample was measured using a Micro-Volume UV-Vis Spectrophotometer (Nanodrop 2000, USA) at optical density 280 nm. The samples were diluted to 0.5 mg/ml with PBS for antibody production.

2.4 Polyclonal Antibody Production

Antigen suspension (0.5 mg/ml) was emulsified as a 1:1 (v/v) mixture with incomplete Freund's adjuvant (Difco Laboratories, USA), and 1 ml was injected into the thigh muscle of a New Zealand white rabbit on days 0, 7 and 14 respectively. Rabbits were intravenously injected at the marginal ear with 1 ml of antigen suspension (0.5 mg/ml) on days 21 and 28. Blood (10 ml) was taken from the central ear vein one week before immunization and weekly intervals after the fourth immunization. Blood continued to be collected until 5 weeks after the last immunization. Animal experimental procedures were performed with approval of the Animal Ethics Research Committee of Mahasarakham University (MSU-0013/2554).

2.5 Measurement of Rabbit Anti-*Streptomyces* Antibody Titer using Indirect-ELISA

Polystyrene microtitre plates (SPL life sciences, Korea) were coated with twofold dilutions of the antigen samples (ranging from 0 to 5 μg) in coating buffer for 16-18 h. The plates were washed three times using 0.05% (v/v) Tween 20 in PBS. Nonspecific binding was blocked using 3% (w/v) bovine serum albumin in PBS (BSA-PBS) for 2 h at 37ºC. After washing,

twofold dilutions of test serum in BSA-PBS (ranging from 1:1000 to 1:64000) were added in duplicate and incubated for 1 h at 37ºC. Following washing, goat anti-rabbit IgG antibody conjugated with alkaline phosphatase diluted in BSA-PBS (dilution 1: 20000) was added and incubated for 1 h at 37ºC. The substrate *p*-nitrophenyl phosphate (1 mg/ml) in 10% (w/v) diethanolamine, pH 9.8 was added and incubated at room temperature for 45 min to 1 h. The reaction was stopped using 50 μl 3 M KOH. The *p*-nitrophenol released was measured by optical density at 405 nm using an ELISA reader (Opsys MRTM Microplate Reader, USA). The antibody titer was defined as the highest dilution giving an absorbance value higher than 1.5 times the mean of the normal serum negative control plus 2 standard deviations.

2.6 Validation and Reproducibility of the Indirect-ELISA

Validation and reproducibility of the Indirect-ELISA data was expressed as the coefficient of variability (CV). The CV was calculated between plates (inter-assay variation) and within the same plate (intra-assay variation) for both anti-*Streptomyces* antibodies. For inter-assay CV, anti-*Streptomyces* antibodies were tested on five different plates on different occasions and for intra-assay CV, they were measured in duplicate for 15 samples.

2.7 Antibody Detection Limit

The antibody detection limit was evaluated by testing a serial dilution of antigen, ranging from 5 μg to 62.5 ng, using indirect-ELISA. This assay was performed ten times to confirm the reproducibility of results. If the mean value of absorbance was more than that of the negative control, this was defined as positive. Duncan's multiple range tests were performed to check differences in absorbance.

2.8 Antibody Specificity Test

Antibody specificity was evaluated by testing the 22 isolates of *Streptomyces* spp., *Pseudomonas aeruginosa* PDMZnCd 2003, *Pseudomonas aeruginosa*, *Bacillus cereus* ATCC11778 and the *Enterobacter* sp. *Streptomyces* spp. were cultured on half PDA at 30ºC for 5-7 days, while the other test species of bacteria were grown on nutrient agar at 37ºC for 24 h. One loopful of test bacteria was mixed with 500 μl of coating buffer and then sonicated using a High Intensity Ultrasonic Processor (Model VCX 750, USA) fitted with a microprobe. This was done on ice for a total of 5 min in 10 s bursts with 2 s gaps for cooling. The sonicated solution was centrifuged at 9,100 g for 5 min. The supernatant was used as protein antigen for indirect-ELISA detection as described above. Cut off values were determined as 1.5 times the mean absorbance from all tested samples.

2.9 Detection of *Streptomyces* Antigen in Soil by Indirect-ELISA

Antigen of *Streptomyces* sp. isolate STPR 78 and *Streptomyces* sp. isolate STPR 84 were each mixed with a 1:1 (v/v) mixture of soil suspension in coating buffer. These were then serially diluted from 25 to 3.125 μg using twofold dilution intervals. Indirect-ELISA was performed as described above.

3. RESULTS AND DISCUSSION

3.1 *Streptomyces* Protein Preparation

Three *Streptomyces* protein preparation methods were tested to determine the optimum procedure for antibody production. Protein profiles were observed using protein generated from the autoclaving and sonication method, but not with the boiling method. Moreover, the 5 min sonication method gave more protein than either the 1 min sonication or the autoclaving methods (Fig. 1). Therefore, the 5 min sonication method was chosen for *Streptomyces* antigen preparation. This chosen sonication method resembles one previously reported. Kirby and Rybicky (1986) successfully produced specific polyclonal antibodies from sonicated antigen of *Streptomyces*. Moreover, this sonication method is widely used to prepare antigen for antibody production from other microorganisms such as *Mycobacterium bovis* (Wood et al., 1988), *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* (Hampl et al., 1981). In addition, the sonication method is a reliable method of preparing antigens for diagnostic tests. For example, Mallqui et al. (2000) showed that antigen prepared by sonication was more suitable for the immunoblot detection of *Bartonella bacilliformis* than antigen prepared by the glycine extraction method.

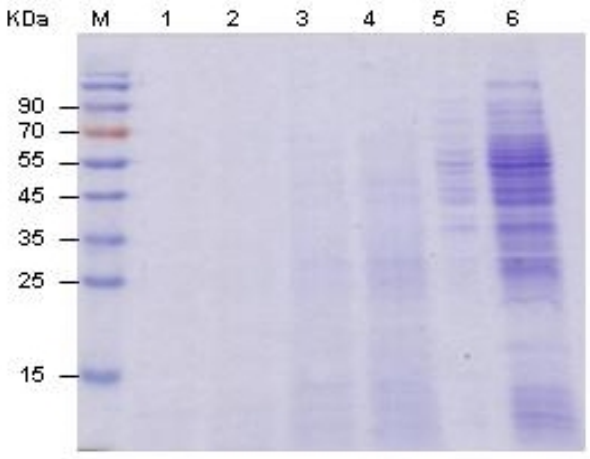

Fig. 1. Antigen protein profile for three antigen preparation methods as determined by 12% polyacrylamide gel electrophoresis; broad range protein marker (M), antigen boiled for 5 min (1) and 10 min (2), antigen autoclaved for 5 min (3) and 10 min (4), and antigen sonicated for 1 min (5) and 5 min (6)

3.2 Measurement of Rabbit Anti-*Streptomyces* Antibody Titer Using Indirect-ELISA

Rabbit anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody titers were measured by reaction with whole cell sonicated proteins of *Streptomyces* spp. antigen at antigen concentrations ranging from 5 to 0 μg. Antibody titers sharply increased (week 5 and 6), both types giving a maximum titer at week 7. The highest titer of anti-*Streptomyces* sp isolate STPR78 antibody was 1:64000, while the highest titer of anti-*Streptomyces* sp. isolate STPR84 antibody was 1:32000 (Table 1). These differences in

antibody titer may simply be due to animal variability (Aref and Saeed, 2012). Antibody titer dropped rapidly 3-5 weeks following the last immunization, possibly due to low levels of circulating antigen in the blood stream (Quan et al., 2009).

Table 1. Antibody titer of anti-*Streptomyces* sp. isolate STPR78 and STPR84 antibody after the fourth immunization

	Antigen concentration (μg)				
	5	**2.5**	**1.25**	**0.625**	**0.3125**
Anti-*Streptomyces* sp. isolate STPR78					
Serum 1 (Week 5)	8,000	4,000	1,000	<1,000	<1,000
Serum 2 (Week 6)	16,000	8,000	4,000	2,000	<1,000
Serum 3 (Week 7)	64,000	32,000	16,000	8,000	4,000
Serum 4 (Week 8)	16,000	8,000	4,000	2,000	<1,000
Serum 5 (Week 9)	ND*	4,000	2,000	1,000	<1,000
Serum 6 (Week 10)	ND*	4,000	2,000	1,000	<1,000
Anti-*Streptomyces* sp. isolate STPR84					
Serum 1 (Week 5)	8,000	2,000	<1,000	<1,000	<1,000
Serum 2 (Week 6)	16,000	8,000	2,000	1,000	<1,000
Serum 3 (Week 7)	32,000	16,000	8,000	4,000	1,000
Serum 4 (Week 8)	16,000	8,000	4,000	2,000	1,000
Serum 5 (Week 9)	ND*	4,000	1,000	<1,000	<1,000
Serum 6 (Week 10)	ND*	4,000	1,000	<1,000	<1,000

** ND = Not determined*

3.3 Validation and Reproducibility of Indirect-ELISA

The inter-assay CV was 1.84% and 2.21%, and the intra-assay CV was 4.07 and 3.34 for anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody.

3.4 Antibody Detection Limit and Specificity Test

The detection limits of anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody were 125 ng and 250 ng, respectively. Both anti-*Streptomeces* antibodies were also found to have good specificity. No cross-reactivity was detected with *Pseudomonas aeruginosa* PDMZnCd 2003, *Pseudomonas aeruginosa*, *Bacillus cereus* ATCC11778, *Enterobacter* sp. or 18 of the 22 *Streptomyces* spp. based on the indirect-ELISA cut off value [1.5 times of mean absorbance value from all tested samples (OD_{405} = 0.10)]. Both anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody cross-reacted with the *Streptomyces* isolates SRF-3, SRF-4, SRF-12 and SRF-13 (Fig. 2). These results show that the cross-reactivity of anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody is 14.29% (4/28). This cross-reaction suggests the environmental *Streptomyces* isolates are closely related to the *Streptomyces* strains used for immunization. This problem could potentially be resolved by cross-absorption of anti-*Streptomyces* antibody with false positive antigen to reduce non-specific signals. For example, the false positive signal of specific antiserum against *S. ipomoea* was reduced by cross-absorbing the antiserum with nonpathogenic *Streptomyces* that frequently found in the environment (Moyer and Echandi, 1986). Moreover, the reduction of cross-reactivity could be resolved by using isolated cell

wall antigens, since different *Streptomyces* strains have different cell wall compositions (Cummins, 1962). Whole cell sonicates, by contrast, contain identical cytoplasmic antigen (Kwapinski, 1963).

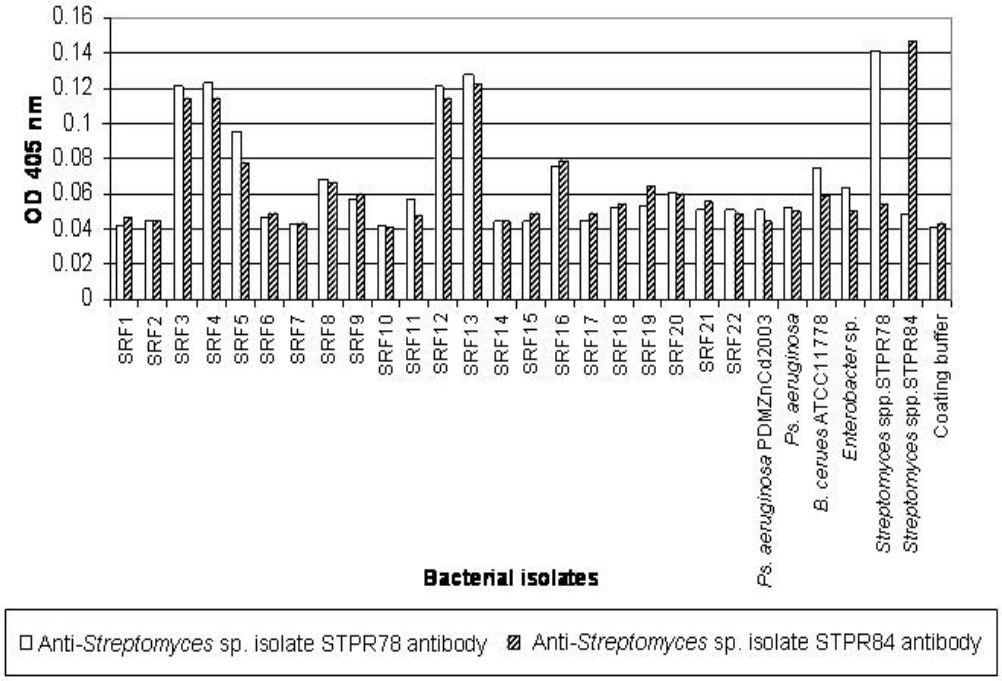

Fig. 2. Antibody specificity of anti-*Streptomyces* sp. isolate STPR78 antibody and anti-*Streptomyces* sp. isolate STPR84 antibody as determined by Indirect-ELISA

3.5 Detection of *Streptomyces* antigen in Soil by Indirect-ELISA

Tests were performed to assess the feasibility of using the rabbit antibodies for monitoring *Streptomyces* spp. from the soil by indirect-ELISA. Whole cell sonicated protein antigens were mixed with soil suspension before coating on microtiter plates. None of the antigen concentrations were detected by either of the rabbit antibodies. Antigen at a concentration of 5 μg was used as a positive control. One possible explanation for this result is the presence of inhibitors in the soil such as humic acid (Le et al., 2003). The chemical composition of humic acid is highly complex and can interact with protein antigen (Otten et al., 1997). Moreover, organic matter in the soil can reduce the quantity of antigen recovered; resulting in loss of sensitivity in immunological assays (Otten et al., 1997). Our results resemble those of Wipat et al. (1994), who found that naturally occurring *Streptomyces* reduce the sensitivity of monoclonal antibody specific for *Streptomyces lividans*. To increase the sensitivity of this detection technique, enrichment of the microorganism may be required. For example, Bartosch et al. (2002) successfully detected *Nitrospira*-like bacteria in soil samples through enrichment of the bacterial population using an immunoblot technique.

4. CONCLUSION

The present study describes a means of generating *Streptomyces* antigen that elicits high titers of specific polycolonal antibody. Our method involves sonicating bacterial cells in 10 s bursts (with 2 s gaps for cooling) for a total of 5 min. The anti-*Streptomyces* antibodies generated can detect protein antigen if it is free from soil contaminants. For the detection of *Streptomyces* in soil samples, optimization of the technique would be required. Soil samples could be cleaned up prior to indirect-ELISA, or immunoanalysis could be performed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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