



Expression and purification of *Bartonella henselae* VirB protein

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Abstract

This study aimed to obtain the recombinant 17 kDa or VirB of *Bartonella henselae* or Cat Scratch Disease diagnosis application from these bacteria. Blood samples were taken from two hundred well-care client-owned cats during November 2010–November 2011. Blood collection was performed and cultured in 5% sheep blood agar. *Bartonella* species of positive samples were identified with Polymerase Chain Reaction (PCR) and gene sequencing. DNA of *B. henselae* was used as a DNA template for PCR targeting VirB gene. PCR product of VirB protein gene which is the well-known antigenic protein for *B. henselae* was cloned into pET28a and transformed into *E. coli*. Our VirB specific protein was successfully expressed and purified by affinity chromatography. The purified VirB protein was examined by Western blotting. This protein will be further employed for ELISA assay in order to detect the disease.

Keywords: Expression, *Bartonellahenselae*, Purification, VirB

Introduction

Cat scratch disease is a human disease caused by *Bartonella henselae* which presents worldwide especially in Thailand. The first report of *Bartonella* spp. was done by a surveillance of sero-prevalence in cats (Boonmar et al., 1997). In 2000, prevalence of *B. henselae* in Thailand was confirmed (Maruyama et al., 2000). In 2008, the first human

case was published and it was the first document of *B. henselae* isolation in Thailand (Paitoonpong et al., 2008). Three cases of endocarditis in human caused by *B. tamiae* were reported in KhonKaen Province (Kosoy et al., 2008). Those findings imply that Thailand may be an endemic area of *Bartonella* spp.

Cats are the main reservoirs of many species of *Bartonella* spp. including *B. clarridgeiae* (Kordick et al., 1997), *B. koehlerae* (Droz et al., 1999; Yamamoto et al., 2003) and *B. bovis* (Chomel et al., 2006), formerly *B. weissi* (Regnery et al., 2000). In cats, there have been at least six potential zoonotic *Bartonella* spp., including *B. henselae*, *B. koehlerae*

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(Quorollo et al., 2014), *B. clarridgeia* (Chomel et al., 1999; Jacomo et al., 2002), *B. vinsonii* subsp. *Berkhoffii* (Bvb) (Varanat et al., 2009), *B. Quintana* and *B. bovis*. However *B. henselae* and *B. clarridgeiae* are the major cause of cat scratch disease in human patients. Cats and cat fleas were the major transmission of CSD to humans.

At present, the common clinical diagnostic methods for feline bartonellosis is serological testing. Current serological testing for *B. spp.* antibody includes indirect immunofluorescence assay (IFA) (Pons et al., 2005) and ELISA (Sander et al., 2001). IFA is not practical due to the unavailability of fluorescent microscope outside research laboratory or veterinary schools. Many studies showed the limitation of IFA which is very low in the sensitivity (Bergmans et al., 1997; Zbinden, 1998). IFA is not only very low in the sensitivity but also cross reactivity between *B. spp.* (Iralu et al., 2006) and other bacteria such as *Chlamydia* sp. (Maurin et al., 1997). ELISA can facilitate the diagnosis of this disease as the advantages of the ELISA over IFA are: (1) it is more quantitative and not subjective, (2) more tests can be performed in a given time, (3) it is more sensitive, and (4) it does not need an expensive instrument.

According to the limitation of serological test previously described, many proteins of *B. spp.* were studied for developing and improving diagnosis tools for bartonellosis. Until now, many specific proteins of *B. spp.* from human products were used to develop ELISA test but they showed low sensitivity and specificity (Sander et al., 2001; Schmiederer et al., 2001; Litwin et al., 2004; Loa et al., 2006). The 17-kDa (VirB) (Anderson et al., 1995; Sweger et al., 2000) and outer membrane protein 43 (OMP 43) (Chenoweth et al., 2004) are two of the few characterized proteins of *B. henselae* that induce antibody responses in hosts. There were many reports on serologic detection of bartonellosis by *B. henselae* specific protein (BSP) as 17-kDa (Loa et al., 2006) and OMP 43 (Burgess and Anderson, 1998; Fuhrmann et al., 2001; Riess et al., 2004) but most of the publications are mainly in humans and less study in animals.

However the specific proteins mentioned above should be used for the development of an antibody enzyme-linked immunosorbent assay (ELISA) for the serologic diagnosis of *B. henselae* infections in cats to screen cat scratch disease from cat to their owner in the future

Materials and Methods

Animals

Two hundred healthy client-owned cats presented to the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and other private animal hospitals in Bangkok Metropolitan with the following criteria were included into this study; (1) normal physical examination, (2) being of any age, sex and breed, (3) full vaccination record, (4) no fleas, and (5) no history of previous illness and treatment with antibiotic within three months. Unhealthy cats with antibiotic treatment were excluded from the study. The protocol was approved by the Chulalongkorn University Institutional Animal Care and Use Committee; certificate number 1031068.

Sample collection

Blood samples were collected from 200 healthy client-owned cats from the Small Animal Hospital, Chulalongkorn University and private veterinary hospitals in Bangkok Metropolitan. The amount of 2.5 ml of blood samples was collected by sterile technique from saphenous vein. One ml of blood sample was placed into 2 EDTA tubes (Appendorf, USA), 0.5 ml each. First tube was for complete blood count (CBC) and the second tube was stored at -80°C for bacterial culture (Breitschwerdt et al., 2000). Serum prepared from the remaining blood sample (1.5 ml) was stored at -80 °C for further analysis.

Bartonella spp. culture and identification Bacterial culture (Clarridge et al., 1995)

All blood samples from the second tube was used for *Bartonella* spp. isolation by bacterial culture which was the gold standard method. Thawed blood samples were centrifuged and

the supernatant were drawn off and discarded. Subsequently, the pellet was re-suspended into 125 µl of isolation medium-199 (Koehler et al., 1992). The re-suspended pellets was streaked onto Brain-heart infusion agar plated supplemented with 5% sheep red blood cells and incubated in a humidified chamber at 35- 37 °C with 5% CO2 condition.

Then, the *Bartonella* culture positive samples were tested with polymerase chain reaction (PCR) (Norman et al., 1995). DNA sequencing was employed for the *Bartonella* spp. confirmation

(Sanger et al., 1977).

Production of VirB characterized proteins of *B. henselae*

1. VirB gene construction

The entire coding sequence of the published BSP gene was cloned from *B. henselae* genomic DNA by PCR. The VirB gene specific primers were designed from NCBI database. The oligonucleotide primers were designed and used in the present study.

Primer	Oligonucleotide sequences
Forward Nde I 17 kDa	5'-GGGCATATGAAAAATATAGCTTAGTCA -3'
Reverse Xho I 17 kDa	5'-GGGCTCGAGAAGTCGGACATCAGATTT -3'

VirB gene was amplified by PCR at conditions: 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s then 72 °C for 5 min. The amplified product was digested with Nde I and Xho I and cloned to plasmid pET28a (Novagen, USA). The recombinant plasmid was transformed into competent *Escherichia coli* (*E. coli*) strain BL 21. Plasmids were purified with a QIAquick mini-prep kit (Qiagen, Germany), and the in-frame DNA sequences were submitted for sequencing at AIT biotech, Singapore.

2. Expression of the VirB in *E. coli*

The colonies of *E. coli* BL21 harboring the recombinant plasmid was cultured at 37 °C with shaking 180 rpm in LB broth medium containing 50 µg/ml kanamycin. The empty vector transformed into *E. coli* BL21 cells were used as a control. The 1-3% overnight culture of *E. coli* was used as a starter culture and transferred into a new 200 ml LB broth containing 50 µg/ml kanamycin. The cell cultures were grown at 37 °C with shaking at 180 rpm until the optical density at 600 nm of the culture reached 0.4-0.6.

Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4 °C. The supernatant was discarded and the whole cell lysate was identified using 12% SDS-PAGE gel. The cell suspension was incubated with 1 mg lysozyme at

room temperature for 30 min. After incubation, the cells were lysed by sonication using 40% amplitude, pulse on 10 seconds and off 5 seconds for 20 min. Sample must be on ice. The sample was centrifuged at 12,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was collected and the pellets was re-suspended in 2 ml of SDW as inclusion body fraction. The whole cell lysate, the supernatant and the inclusion body fractions of VirB protein were identified using 12% SDS-PAGE gels.

From protein expression, the inclusion body of VirB protein was solubilized in 8 M urea at room temperature for 2 hours. The supernatant was collected by centrifugation at 12,000 rpm for 10 min at room temperature and then transferred into a new tube. The protein solubilized in 8 M urea which was the denatured form of VirB protein was analyzed by 12% SDS-PAGE gels.

Purification of the recombinant BSP

The denatured VirB protein was purified by His-trap-affinity using Protino® Ni-NTA Agarose (Macherey-Nagel, Germany). The column was equilibrated with SDW for 10 column volumes and column buffer (6M Urea) for 10 column volumes. One ml of protein sample (1.364 mg/ml) was loaded into the column and collected the flow through fraction. After that, the column was

washed with column buffer for 5 column volumes. The VirB protein was eluted with 1 column volume of elution buffer (6M Urea which contains 20, 50, 100 and 200 mM imidazole). The flow through, wash and elution fractions were analyzed by 12% SDS-PAGE gels and kept at -20 °C.

Refolding of purified VirB protein

The denatured specific BSP recombinant protein in 8 M urea obtained from purification was refolded using the concentrator, Amicon® Ultra-4 Centrifugal Filter Units (Merck, Germany), with cut off at 5 kDa. Five hundred milliliters of purified protein was transferred into the concentrator and then centrifuged at 6,000 rpm, 4 °C until the supernatant on the top remained at 100 µl. Then, 400 microliters of Sterile Distilled Water (SDW) was added and centrifuged at 6,000 rpm at 4 °C until the supernatant on the top remained at 100 µl. Lastly, 400 microliters of SDW was added and the total supernatant was collected and analyzed by SDS-PAGE.

3. The VirB protein detect by Western immunoblotting

The nitrocellulose membrane, two fiber pads, and two Whatman papers (BioRad Laboratories, USA) were placed in a shallow tray filled with Transfer Buffer for a few min. The stacking gel was cut off from 12% SDS-PAGE gel with a clean razor blade and the gel was soaked in Transfer Buffer for a few min. The transfer apparatus gel cassettes was opened with the black panel lying flat on the bottom of the tray filled with Transfer Buffer, the clear panel should be against the side of the tray. The transfer sandwich was prepared on the black panel in the tray filled with Transfer Buffer: one fiber pad - one Whatman papers - SDS gel - nitrocellulose membrane - one Whatman papers - one fiber pad. The air bubbles were removed by rolling a glass tube on the membrane. The sandwich was covered with the clear panel, fasten with the latch, and the gel cassette was inserted into the electrode module with the black panel facing the black cathode electrode panel. The bio-ice cooling unit was inserted into the

buffer chamber, and filled the buffer chamber with Transfer Buffer, transferred for 70 min at 4 °C, stirred at a constant current of 100V and then the membrane was washed with distilled water on the shaker for 10 min. The membrane was incubated with blocking buffer on a shaker at 37 °C for 30 min. The positive cat serum by culture and PCR was used as primary antibody, diluted to blocking buffer (1:5000) and incubated the membrane with the blocking buffer the diluted primary antibody on a shaker for overnight at 4 °C. The membrane was washed two times with blocking buffer on the shaker for 10 min each time. The anti-cat serum used as secondary antibody (Goat pAb to cat IgG (HRP) (Abcam®, United Kingdom) was diluted with blocking buffer (1:5000) and the membrane was incubated with the diluted secondary antibody on a shaker at 37 °C for 1.30 hours. The membrane was washed two times with distilled water on the shaker for 5 min each time. It was further washed with 0.05% PBS-tween on the shaker for 5 min and incubated in AEC Substrate Reagent (4 ml deionized water, 2 drops Acetate Buffer, 1 drop AEC Chromogen, 1 drop hydrogen peroxide) at 37 °C for 15 min until clear red insoluble signal was obtained for positive control. Finally, it was washed with sterilized distilled water.

Results

VirB protein gene construction

The VirB protein gene was cloned into pET28a; it was composed of 18 bp His tag-coding sequence, 27 bp T7 tag-coding sequence, 51 bp sequence of pET28a expression vector, 6 bp restriction size. Therefore, the total length of VirB protein gene was estimation at 20-23 kDa. The DNA sequence of VirB protein gene recombinant was sequenced by AIT Biotech, Singapore. The result present in Fig 1.

The cloning sequence of VirB was blasted in www.ncbi.com. The result of sequence producing alignment with accession number U23447 showed 99% similarity in *Bartonella henselae* VirB protein gene as present in Figs 2 and 3.

ATG AAA AAA TAT AGC TTA GTC ACA TTG TTA TCT TTT TGC ATC CCT CAT GCA AAA GCA CAA ACA GCA
 CCC CTT ACT GAT GAA TAT TAT AAA AAA GCC TTA GAA AAC ACG CAA AAA TTA GAC GTT GCA AAA TCA
 CAA ACA GCT GAG TCT ATT TAT GAA TCT GCA ACA CAA ACT GCA AAA AAA TTC AGG AAT TAA ACG AGA
 AGC TTG AAA AGG CTA AAA CAG CTG AAA AAA CCA AAT CTG AAG AAT TGC AGG CTC TTC AAG TAG AGC
 TCT CTC TTC TCC AAG CAC AGT TGC AAG TGG ATA CTT TAA AAG TTC AGT CCC TTT CTA TGA TTC AAG CAA
 AAG ATA CAA AAA CAA AAG AAG AAC TGC GTG AAG AGC AAA CGC AGC AAA ATC ATA AAA AAA TTG AAG
 AAA AAT TAA AAG AAA AAC TTG GGA AAT CTG ATG TCC GAC TTC TCG AG

Fig 1: Nucleotide sequence cloning alignment of VirB protein gene



Fig 2: Sequence producing alignment

Expression of the VirB protein

The colonies were verified by PCR and DNA sequencing. The DNA sequences of colony mutants are colonies #3,4. The colonies were picked and grown in 10 ml LB containing 50 µg/ml Kanamycin and 0.1 mM IPTG to induce protein expression of each selected colony. Total proteins were separated on 12% SDS-PAGE and visualized by Coomassie blue-R stain. Fig 4 shows the result of protein expression at 23 kDa in both colonies when compared to non-induced colonies. Large scale protein expression was performed for protein purification.

The extraction and Inclusion body solubilization

The protein extraction and solubilization of inclusion body and supernatant were shown in

Fig 5. The product obtained was further performed for protein purification in the next step.

VirB protein purification

After protein expression, the supernatant of VirB protein was purified using His-trap-affinity column. The flow through, wash and elution fractions were analyzed on 12% SDS-PAGE. The result indicated that both inclusion body and supernatant of recombinant BSP protein were successfully purified. The result of purification protein at 23 kDa is presented in Fig 6 and confirmed by Western Immunoblot (Fig 7).

Discussion

There are many diagnostic methods for detection of bartonellosis in cats including blood culture, PCR, immunofluorescent assays (IFA)

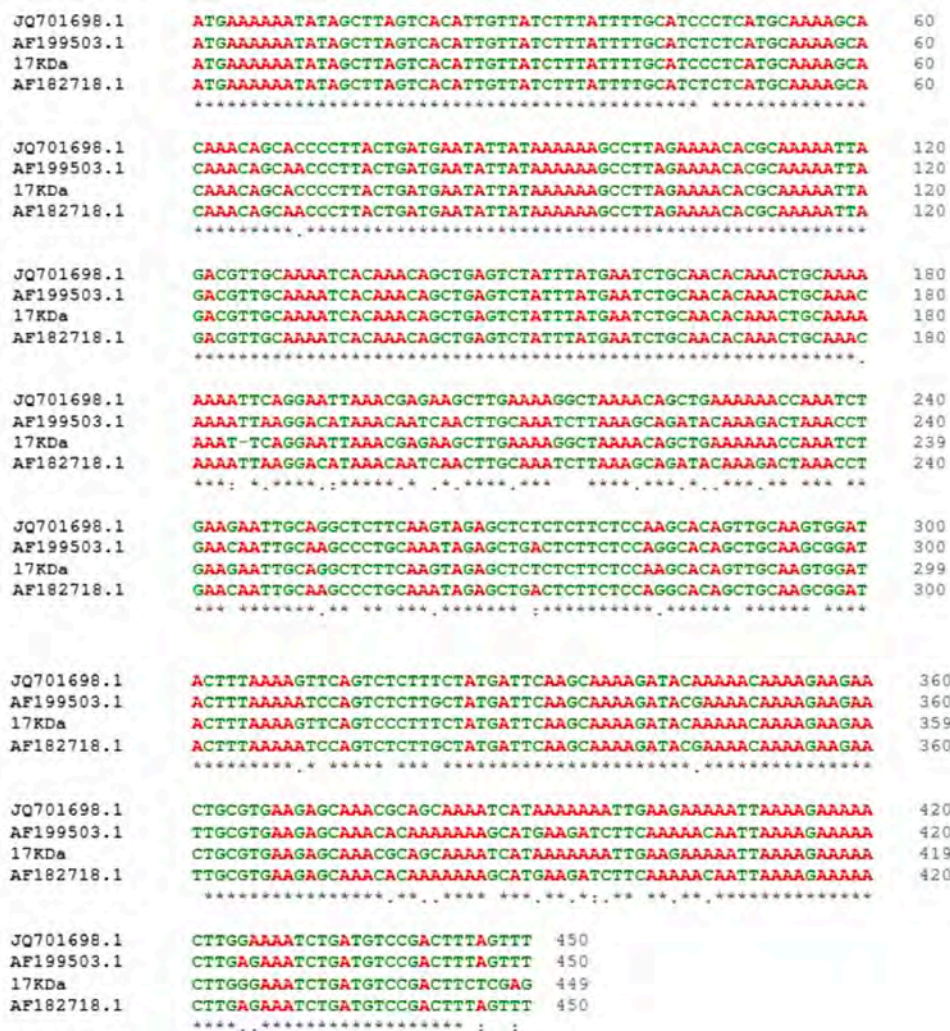


Fig 3 Nucleotide alignment of VirB genes

The accession number 17KDa is *Bartonella henselae* positive sample, AF182718.1 is *Bartonella henselae* virB operon, complete sequence, AF199503.1 is *Bartonella henselae* 17kDa antigen-like protein gene, complete and JQ701698.1 is *Bartonella henselae* VirB2 (virB2), VirB3 (virB3), VirB4 (virB4), VirB5 (virB5), VirB6 (virB6), VirB7 (virB7), VirB8 (virB8), VirB9 (virB9), VirB10 (virB10), VirB11 (virB11), hypothetical protein, BepA (bepA), VirD4 (virD4), BepB (bepB), BepC (bepC), BepD (bepD), BepE (bepE), BepF (bepF), and BepG (bepG) genes, complete cds

and ELISA. For ELISA, many specific proteins of *B. henselae* were used to develop ELISA test including the 17-kDa (VirB), *Bartonella* effector proteins (Beps), outer membrane protein (OMP) (Kempf, 2008), and heat shock protein (GroEL) (Ferrara et al., 2014). The 17 kDa protein (VirB) is one of the most interesting candidates for a good antigenic proteins highly specific for *B.*

henselae infection (Anderson et al., 1995; Loa et al., 2006; Eberhardt et al., 2009; Ferrara et al., 2014) and used for *Bartonella* antibody detection kit in human (Anderson et al., 1995; Sweger et al., 2000). Therefore, the 17 kDa or VirB protein was selected and produced for using in development of indirect ELISA antibody detection for *B. henselae* infection in cats in this present study. This study

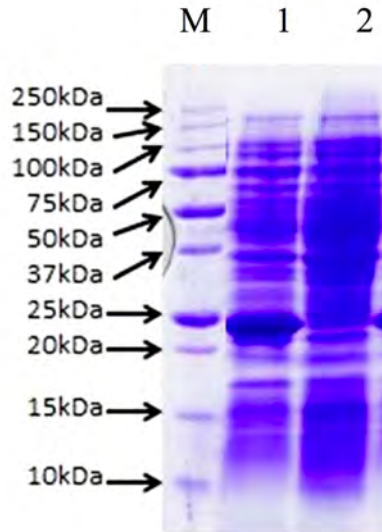


Fig 4: Fraction identification of VirB protein expression with 0.1 mM IPTG in 10 ml LB broth

Lane M represents protein molecular weight marker

Lane 1 represents protein induced with IPTG

Lane 2 represents protein non-induced with IPTG

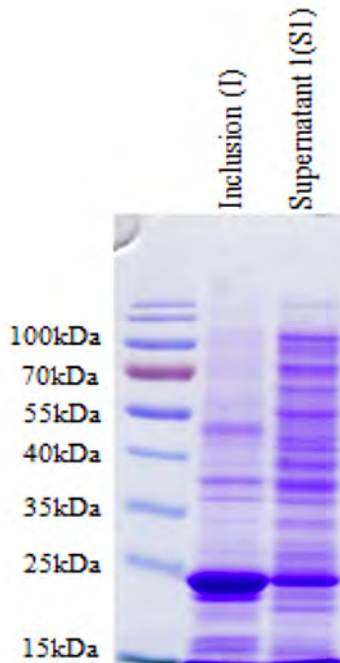


Fig 5: Fraction identification of Inclusion body and supernatant

The first lane: Protein molecular weight marker, Lanes 2 and 3: Inclusion and supernatant after 1st and 2nd re-suspension of bacteria harboring pET28a, respectively.

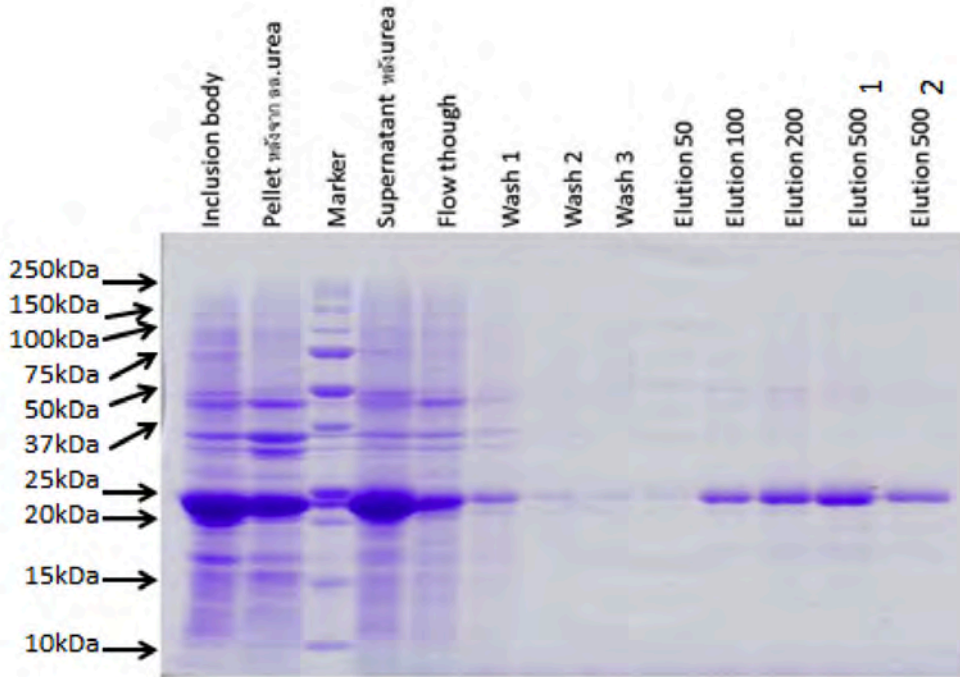


Fig 6: SDS page analysis of supernatant recombination VirB protein after solubilized in 8M urea purification

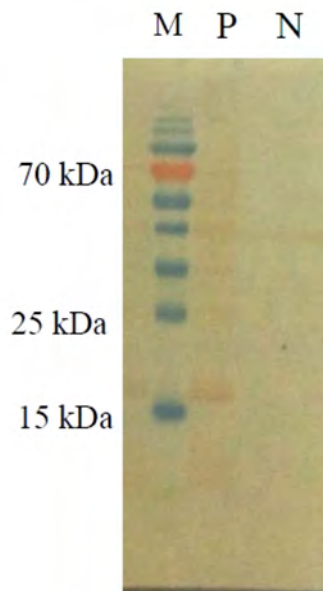


Fig 7: Western immunoblot results of the purified of 0.5 mg VirB protein

Lane M represents molecular weight marker
 Lane P represents positive serum
 Lane N represents to negative serum

has been focused on expression of recombinant protein from the VirB gene by using pET28a *E. coli* expression system which differed from expression vectors and primers in other previous study (Loa et al., 2006; Ferrara et al., 2014). Thus, our study gives the original method for *Bartonella* VirB protein expression by using the expression vector pET28a. The recombinant VirB protein was expressed 0.1 mM IPTG incubation at 37°C for 4 hours. This VirB was expressed both as inclusion body and supernatant. However, it was produced much more as an inclusion body component. The VirB protein is scarcely soluble due to deposition in inclusion bodies, same as experiment of Loa et al. (2006). The result also indicated that the VirB protein found was expressed as inclusion body, because high temperatures during expression and high level expression of recombinant protein in *E. coli* caused aggregated protein formation referred to inclusion body. The inclusion bodies of recombinant protein need to elaborate solubilization, refolding and purification for recover biological activity of protein (Vallejo and Rinas, 2004). Therefore, the inclusion body of VirB protein was solubilized in 8 M urea. The clear supernatant obtained from inclusion body solubilization was successfully purified by affinity chromatography.

Our VirB specific protein gene was successfully expressed in pET28a expression vector and design specific primers for evaluation ELISA in cat blood samples. The difference between the cat and human serum samples was one of the most difficult parameters which give the differences in ELISA results. The other important factor for the differences in results may be due to different methods of antigen preparation as well as the difference in two different of *B. henselae* Houston and Marseille. The study of Zhao et al. (2005) demonstrated the importance of the different virulence proteins and antigenicity on host response. Antigen of *B. henselae* (ATCC 49882) from patients with cat scratch disease yield 86.9% sensitivity and 96% specificity due to the process of protect *Bartonella* antigen from heat degradation (Not et al., 1999).

The evaluation on other specific antigenic protein genes of this bacterial species should be further investigated. The protein expression in different expression vectors and conditions should also be further performed for discovering the soluble proteins which can be a good candidate for development of ELISA assays for detection of cat antibody to *B. henselae*. The development of high sensitivity and specificity of ELISA can also support for the prevention and control strategies of *B. henselae* infection in cats and cat scratch disease (CSD) in humans in the future.

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การผลิตและทำให้มีความบริสุทธิ์ของเชื้อบาโทเนลลา เฮนเซลเลเวอร์บี โปรตีน

บทคัดย่อ

การศึกษานี้ มีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ของการนำเวอร์บีอินจากเชื้อบาโทเนลลา เฮนเซลเล มาใช้ในการวินิจฉัยโรคแมวข่วนที่มีสาเหตุมาจากเชื้อนี้ โดยเก็บตัวอย่างเลือดจากแมวจำนวน 200 ตัวระหว่างเดือนพฤศจิกายน 2553-พฤศจิกายน 2554 เพื่อทำการตรวจหาเชื้อบาโทเนลลา เฮนเซลเล ด้วยวิธีการเพาะเชื้อบนอาหารเลี้ยงเชื้อที่ผสมเลือดแกะ 5% และตรวจยืนยันด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส ตัวอย่างที่ให้ผลเป็นบวกจะถูกส่งตรวจหาลำดับสารพันธุกรรมเพื่อยืนยันชนิดของเชื้อ ดีเอ็นเอของเชื้อบาโทเนลลาเฮนเซลเลถูกนำมาทำปฏิกิริยาลูกโซ่โพลีเมอเรสเพื่อหาเวอร์บีอิน ผลลัพธ์จากปฏิกิริยาลูกโซ่โพลีเมอเรสดังกล่าวถูกนำมาทำการโคลนและกระตุ้นการสร้างเวอร์บีโปรตีนซึ่งเป็นโปรตีนที่มีคุณสมบัติการเป็นแอนติเจนสูงต่อการติดเชื้อบาโทเนลลาเฮนเซลเล การศึกษานี้ประสบความสำเร็จในการกระตุ้นการสร้างเวอร์บีโปรตีนได้โดยการใช้ระบบการสร้างโปรตีน ในพีอีที 28เอของระบบการสกัดโปรตีนจากอีโคโนไลและถูกทำให้บริสุทธิ์ด้วยวิธีแอฟฟินิตีโครมาโตกราฟี ทำการตรวจพิสูจน์เวอร์บีโปรตีนที่บริสุทธิ์แล้วด้วยวิธีเวสเทิร์นบลอตติง และพบว่าโปรตีนนี้อาจสามารถนำไปพัฒนาใช้ในชุดทดสอบอีไลซาเพื่อในการตรวจหาโรคนี้อีกต่อไป

คำสำคัญ: การผลิต, บาโทเนลลา เฮนเซลเล, การทำให้บริสุทธิ์, เวอร์บี