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Investigation on the conA binding properties of *Klebsiella pneumoniae*

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Abstract. *Klebsiella pneumoniae* is a healthcare-associated bacterial pathogen which causes severe diseases in immunocompromised individuals. Concanavalin A (conA), a lectin which recognizes proteins with mannose or glucose residues, has been reported to agglutinate *K. pneumoniae* and hence, is postulated to have therapeutical potential for *K. pneumoniae*-induced liver infection. This study investigated the conA binding properties of a large collection of clinical isolates of *K. pneumoniae*. ConA agglutination reaction was demonstrated by 94 (51.4%) of 183 *K. pneumoniae* isolates using a microtiter plate assay. The conA agglutination reactions were inhibited in the presence of 2.5 mg/ml D-mannose and 2.5 mg/ml glucose, and following pretreatment of the bacterial suspension with protease and heating at 80ºC. Majority of the positive isolates originated from respiratory specimens. Isolation of conA-binding proteins from *K. pneumoniae* ATCC 700603 strain was performed using conA affinity column and the conA binding property of the eluted proteins was confirmed by western blotting analysis using conA-HRP conjugates. Proteins with molecular weights ranging from 35 to 60 kDa were eluted from the conA affinity column, of which four were identified as outer membrane protein precursor A (37 kDa), outer membrane protein precursor C (40 kDa), enolase (45 kDa) and chaperonin (60 kDa) using mass spectrometry analysis. Several conA binding proteins (including 45 and 60 kDa) were found to be immunogenic when reacted with rabbit anti-*Klebsiella* antibody. The function and interplay of the conA binding proteins in bacterium-host cell relationship merits further investigation.

INTRODUCTION

Klebsiella pneumoniae is a healthcare-associated pathogen which can cause severe diseases such as septicemia and pneumonia in immunocompromised individuals (Sydnor & Perl, 2011). The ability of *K. pneumoniae* to agglutinate concanavalin A (conA), a lectin which recognizes proteins with mannose or glucose residues, has been demonstrated recently. ConA has been proposed as a potential therapeutic agent for *K. pneumoniae*-induced liver infection due to its effect in enhancing bactericidal activity of phagocytes and reducing the bacterial burden in the liver (Kuo *et al.*, 2007). The tetrameric metalloprotein of conA possess four binding sites which interact strongly with branched polymer containing non-reducing α-D-glucopyranosyl, α-D-mannopyranosyl or β-D-fructofuranosyl residue (Doyle & Birdsell, 1972). Polyglucosylglygerol phosphate teichoic acid has been identified as the component responsible for conA interaction with *Bacillus subtilis* (Doyle & Birdsell, 1972). It has also been postulated that the interaction between conA with bacterial cell surface could be due to the presence of N-linked glycoprotein (Kang *et al.*, 2010) or O-linked glycoprotein (Castric *et al.*, 2001; Hegge *et al.*, 2004). The glycosylation step introduces the mannose glycans into the protein structure; the core structure is comprised of mannose in N-linked glycans, (Kim & Misek, 2011), while in O-linked glycans, the introduction of N-

acetylglucosamine, mannose, fucose, glucose and N-acetylgalactosamine or xylose (Ohtsubo & Marth, 2006; Rakus & Mahal, 2011) sugar components instigates the conA interaction with the bacterial glycoprotein.

In the past, conA interaction of bacteria was examined by agglutination assay on microtiter plate followed by microscopic examination. Lectin typing or interactions have been reported for Gram-positive bacteria such as *Lactobacillus* (Kim *et al.*, 2006), *Staphylococcus aureus* (Muñoz *et al.*, 1999), *Staphylococcus epidermidis* (Jarløv *et al.*, 1992), and *Listeria* spp. (Facinelli *et al.*, 1994), and Gram-negative bacteria including *Campylobacter concisus* (Aabenhus *et al.*, 2002) and *Helicobacter pylori* (Hynes *et al.*, 1999; Khin *et al.*, 2000).

As limited information on conA binding property of *K. pneumoniae* is available, this study was designed to determine the ability of a large collection of *K. pneumoniae* clinical isolates in conA agglutination. To identify proteins responsible for conA binding, proteins extracted from *K. pneumoniae* ATCC 700603 strain was eluted using conA affinity column and identified by mass spectrometry analysis.

MATERIALS AND METHODS

Bacterial strains

A total of 183 *K. pneumoniae* collected from the Microbiology Diagnostic Laboratory, University of Malaya Medical Centre (UMMC) from 2006-2007 and 2010-2011 were used. The isolates were identified by routine diagnostic procedures and confirmed by polymerase chain reaction (PCR) assay. The isolates were obtained from various clinical specimens including sputa or respiratory secretion (n=43), swab (n=33), blood (n= 31), urine (n=29), pus or body fluids (n=19), tissue (n=19), nasopharyngeal secretion (NPS) (n=6) and cerebrospinal fluid (CSF) (n=3). Several reference strains including *K. pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Burkholderia pseudomallei* ATCC 13178, *Staphylococcus aureus* ATCC 25923 and a clinical isolate of *Streptococcus pyogenes* were used in this study.

ConA agglutination microtiter plate assay

The assay was performed as described by Facinelli *et al.* (1994) and Kim *et al.* (2006) with slight modifications. *Klebsiella pneumoniae* was cultured in 5 ml tryptic soy broth (BD, US) with shaking at 37°C for 24 h, after which, the broth was centrifuged at 3000 X g at 4°C for 10 min. The pellet was washed once in sterile phosphate-buffered saline (PBS), pH 7.2 and resuspended in the same buffer. The cell suspension was adjusted to 75-80% transmittance (%T) (equivalent to 10^8 cfu/ml) at 540 nm using a spectrophotometer (Thermo Scientific GENESYS^{TM} 20 Spectrophotometer, Thermo Scientific, US).

ConA agglutination tests were carried out in sterile U-well microtiter wells (NUNC TC Microwell, Denmark). Conconavalin A lectin (Sigma, Cat #: C2010, St. Loius, Mo., US) was dissolved in PBS (pH 7.2) to a final concentration of 1 mg/ml and stored at -20°C. A volume of 50 µl of conA working solution (ranging from 1 mg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 0 µg/ml) was pipetted into each row of a microtiter plate. This was then followed by the addition of 50 ml of bacterial suspension into each well, thus, making the final conA concentrations in each well half of their original concentrations. The microtiter plate was incubated at 37°C and inspected visually using a reflecting mirror after 24 h of incubation. A positive reaction was indicated by the presence of a carpet of aggregated cells while a negative reaction was indicated by the presence of button or dot formation at the bottom of the well. *Staphylococcus aureus* ATCC 25923 was used as positive control as it demonstrated the strongest conA agglutination reaction. PBS alone was used as the negative control for the assay. The strength of the conA agglutination for each isolate was reflected by minimum lectin concentration (MLC) of each isolate. MLC is defined as the lowest concentration of lectin

(conA) that is required to cause agglutination in the microtiter assay. Gram stain was performed to confirm agglutination reactions.

Carbohydrate inhibition test

Carbohydrate inhibition test was carried out as described above by mixing 2.5 mg/ml of D-mannose (CalbioChem, Merck, Germany) or D-glucose (Amresco, US) in microtiter wells containing 250 µg/ml conA lectin solution, for 94 isolates which demonstrated positive agglutination reactions. Equal volume (50 µl) of conA solution (250 µg/ml) and 2.5 mg/ml sugar solution were pipetted into each microtiter well. After incubation for 10 min, 50 µl of bacterial suspension (adjusted to approximately 10^8 cells/ml) was added to the wells, and incubated at 37ºC for 24 h. The next day the plate was visually inspected using an inverted mirror for mat or button formation. As the presence of mannose and glucose sugar inhibits the interaction of the cells with conA lectin, a positive inhibition test was determined by the observation of button formation, whereas a negative inhibition test was determined by the observation of mat formation in the microtiter well.

Proteolytic degradation and heat pretreatment of bacterial biomass

To examine whether conA agglutination reaction was affected by proteolytic degradation of bacterial cells, the experiment was performed as described by Hynes *et al.* (1999). Bacterial cells were washed and resuspended in 5 ml PBS (pH 4) followed by incubation at room temperature for 30 min to induce cells autolysis and protein release. Treated cells were then washed twice and resuspended in 5 ml PBS containing 0.1 mg/ml of proteinase K (Sigma, US). After incubation at 60ºC for 1 h, the protease was denatured by heating at 100ºC for 5 min. The cell suspension was spun at 5000 x g for 15 min and the pellet was suspended in PBS to an optical density of 0.9 at 550 nm (Hynes *et al.*, 1999). The conA agglutination assay was performed using 250 µg/ml conA lectin solution. To investigate the effect of heating, the cell suspension was heated at 80ºC for 1 h before subjecting to conA agglutination assay, using 250 µg/ml conA lectin. A positive reaction was indicated by the presence of a carpet of aggregated cells while a negative reaction was indicated by the presence of button or dot formation at the bottom of the microtiter well.

Isolation of conA-binding proteins

Klebsiella pneumoniae ATCC 700603 strain was cultured in 500 ml trypstic soy broth with shaking at 37ºC for 2 days before centrifugation at 10 000 rpm for 15 min. The pellet was treated with Bugbuster® HT protein extraction reagent (Novagen, Germany), and incubated at room temperature on a shaking platform for 20 min, in accordance to the manufacturer's protocol. Isolation of conA-binding proteins was performed according to the manufacturer's protocol (GE Healthcare, Uppsala, Sweden). Briefly, 2 ml of conA resin was equilibrated with a binding buffer pH 7.4 (20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$) in a conA sepharose 4B column (GE Healthcare, Uppsala, Sweden). The column was loaded with 1.5 mg of bacterial lysate, and after several washes, the proteins were eluted with 0.4 M methyl-α-D-mannopyranoside, 20 mM Tris-HCL, 0.5 M NaCl. The eluted fractions were pooled and the protein content was quantitated using Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, US).

Detection of conA-binding protein by SDS_PAGE and immunoblotting analysis using conA-HRP peroxidase

Protein sample was separated by SDS-PAGE in a 1-mm thick gel (10 X 7.5 cm, 5% stacking gel and 12% resolving gel) using a Mini-PROTEAN® Tetra cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) (Laemmli, 1970). The gels were stained with Coomassie blue stain and silver stain according to established protocols (Shevchenko *et al.*, 1996).

Immunoblotting was performed in accordance to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, US). Proteins were first separated by SDS-PAGE and transferred to a PVDF membrane (Amersham Bioscience, GE Healthcare, Sweden). Excess binding sites were then

blocked by incubation in PBS (pH 7.4) containing 2% (v/v) TWEEN 20 at 20ºC for 2 min. The membrane was washed twice in PBS and incubated with 20 ml of 3 µg/ml conA-peroxidase (MpBio, US) in PBS containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ at 16ºC for at least 16 h. The membrane was developed in diaminobenzidine (DAB) substrate solution [0.001 g/ml DAB plus 0.0001% (v/v) of 30% H_2O_2] at room temperature for 5-30 min. Control blots were reacted with 0.2 M methyl-D-mannopyranoside in 3 µg/ml of conA-peroxidase in PBS containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ at 16ºC for at least 16 h.

Protein identification by mass spectrometry

SDS-PAGE gel was stained with G-250 Colloidal Coomassie blue (Bio-Rad Laboratories, Hercules, CA, US, Cat #: 161-0406) and proteins of interest were excised for mass spectrometry analysis using Ettan MALDI-Tof pro (Amersham Pharmacia Biotech, US) in reflectron mode in the Proteomic Biotechnology Facility, Faculty of Medicine, University of Malaya. For LC/MS analysis, mass spectrometry analysis of the proteins was performed at the Proteomics International Pty Ltd (Nedlands, Western Australia). The resultant peptides identified were then matched with existing database on LudwigNR, NCBInr and Sprot database (http://www.apcf.edu.au/mascot/home.html).

Analysing antigenic properties of *K. pneumoniae* enolase preparations using immunized animal serum (anti-*Klebsiella* antibody)

After western blotting, PVDF membrane (with transferred proteins eluted from conA affinity column) was soaked in 2% (w/v) skim milk in PBST at room temperature for 1 h. The membrane was then incubated with biotin-conjugated anti-*Klebsiella* antibody from immunized rabbit (Cat #: Ab 69468, Abcam, UK) at room temperature for 1 h on a rocking platform. The membrane was washed in PBST (0.1% Tween 20 in PBS pH 7.4) three times for 5 min each. Then, the membrane was incubated in horseradish peroxidase conjugated avidin (Cat #: 55898, Cappel, MpBio, USA) (1:10000 dilution in 0.1% PBST) for 30 min. The membrane was then washed three times for 5 min each. Finally, the blot was developed with diaminobenzidine substrate for 5-30 min on a rocking platform.

RESULTS

Figure 1 shows the Gram staining reactions of six bacterial strains upon interaction with conA. Of the Gram-positive bacteria, *S. aureus* and *S. pyogenes* showed the strongest conA interaction (MLC: 31.25 µg/ml). Of the Gram-negative bacteria, *K. pneumoniae* (MLC: 250 µg/ml) demonstrated weak conA agglutination reaction compared to that of *P. aeruginosa* (MLC: 31.25 µg/ml). Minimal/weak agglutination was observed for *E. coli* and *B. pseudomallei* reference strains (MLC: 500 µg/ml), as also observed on the Gram stains (Figure 1).

Table 1 shows the minimum lectin concentrations (MLC) of 183 *K. pneumoniae* isolates investigated in this study. Using 500 µg/ml as a cut off value, a total of 94 (51.4%) isolates were positive, of which 77 (42.1%) isolates had MLC of 250 µg/ml, 12 (6.6%) isolates had MLC of 125 µg/ml and 5 (2.7%) isolates had MLC of 62.5 µg/ml (Table 1).

The conA agglutination capability of all *K. pneumoniae* isolates was inhibited upon carbohydrate, heat and protease pretreatment. Amongst the positive isolates, none (0%) of the isolates agglutinated with conA in the presence of mannose or glucose. Similarly, none of the isolates agglutinated with conA after heat and protease treatment.

ConA binding proteins were extracted from *K. pneumoniae* reference strain ATCC 700603 using conA sepharose 4B column. Specific conA reactive proteins were detected as four faint bands on SDS-PAGE gel with the molecular weights of ~37, ~40, ~45 and ~60 kDa upon silver staining analysis (Figure 2A). Upon blotting analysis with conA-HRP conjugates, most protein fragments within the range of 35 to 60 kDa (~37 kDa, ~40 kDa, ~45 kDa and ~60 kDa) showed reactivity with conA-HRP conjugates (Figure 2B) and the binding was specifically

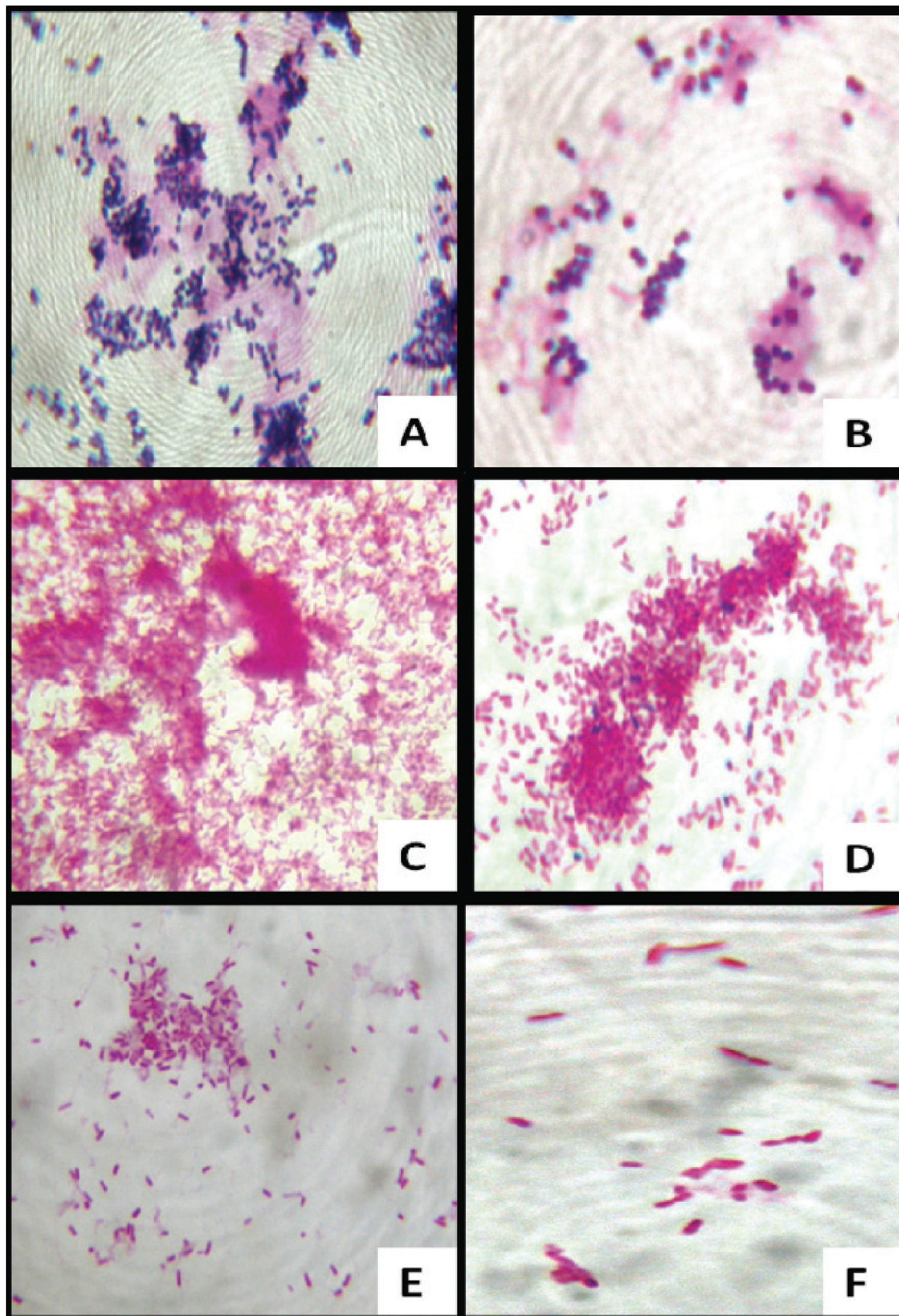

Figure 1. Gram staining reactions of six bacterial strains upon interaction with conA
A) *S. aureus* (MLC: 31.25 µg/ml), B) *S. pyogenes* (MLC: 31.25 µg/ml), C) *P. aeruginosa* (MLC: 31.25 µg/ml), D) *K. pneumoniae* (MLC: 250 µg/ml), E) *E. coli* (MLC: 500 µg/ml), F) *B. pseudomallei* (MLC: 500 µg/ml)

Table 1. Determination of the minimum lectin concentration (MLC) of 183 *K. pneumoniae* isolates investigated in this study

Minimum lectin concentration (MLC), µg/ml	No. (%) *K. pneumoniae* (n=183)					
	Respiratory sample (n=49)	Blood (n=31)	Urine (n=29)	Cerebrospinal fluid (n=3)	Others (n=71)	Total no. (%)
Negative (n=89)						
≥500	25 (13.7)	21 (11.5)	17 (9.3)	0 (0)	26 (14.2)	89 (48.6)
Positive (n=94)						
250 (n=77)	21 (11.5)	7 (3.8)	11 (6.0)	2 (1.1)	36 (19.7)	77 (42.1)
125 (n=12)	2 (1.1)	2 (1.1)	1 (0.5)	1 (0.5)	6 (3.3)	12 (6.6)
≤62.5 (n=5)	1 (0.5)	1 (0.5)	0 (0)	0 (0)	3 (1.6)	5 (2.7)

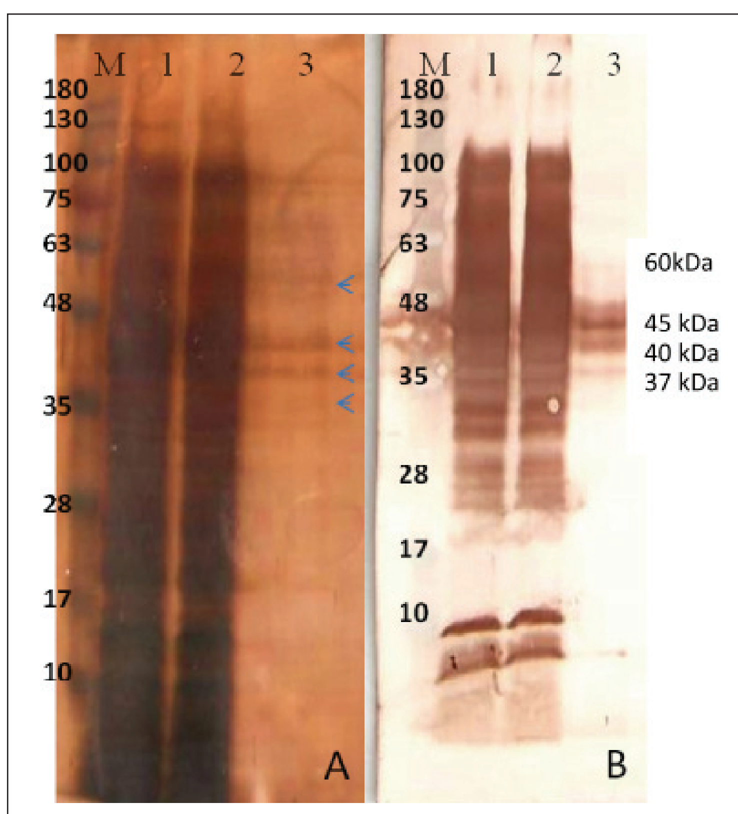

Figure 2. Detection of conA-binding proteins (arrows) of *K. pneumoniae* ATCC 700603 strain (37 kDa, 40 kDa, 45 kDa and 60 kDa) eluted from conA sepharose 4B column

A) Silver staining

B) Western blot analysis using conA-HRP conjugates

Lane M: BlueRay prestained protein ladder (10 to 180 kDa) (GeneDirex, Taiwan)

Lane 1: Whole cell lysate of *K. pneumoniae* ATCC 700603 (prior to loading into column) (32 µg)

Lane 2: Flowthrough sample of *K. pneumoniae* ATCC 700603(32 µg)

Lane 3: Eluted fraction from conA sepharose 4B column (1 µg)

inhibited by methyl-α-D-mannopyranoside (Figure 3).

The identity of four conA binding proteins was obtained by mass spectrometry analysis (Table 2). Enolase (45 kDa) was the most distinct protein eluted from the conA column. The other proteins identified were: chaperonin (57 kDa), outer membrane protein precursor A (37 kDa), and outer membrane protein precursor C (40 kDa).

Figure 4 shows the immunoblotting analysis of *K. pneumoniae* using polyclonal rabbit anti-*Klebsiella* antibody. The anti-*Klebsiella* antibody interacted with seven *Klebsiella* proteins with molecular weights of approximately 20 kDa, 32 kDa, 45 kDa, 60 kDa, 65 kDa, 72 kDa and 80 kDa, of which two proteins, 45 and 60 kDa had been identified as conA-binding proteins in this study.

DISCUSSION

In this study, 94 (51.4%) *K. pneumoniae* isolates demonstrated agglutination with conA. The inhibition of conA agglutination by mannose/glucose by *Klebsiella* isolates is an indication that these sugar residues are responsible for conA binding. In addition, the inhibition of conA agglutination by heat and protease pretreatment implies that the bacterial components involved are most likely protein in nature. Hence, the

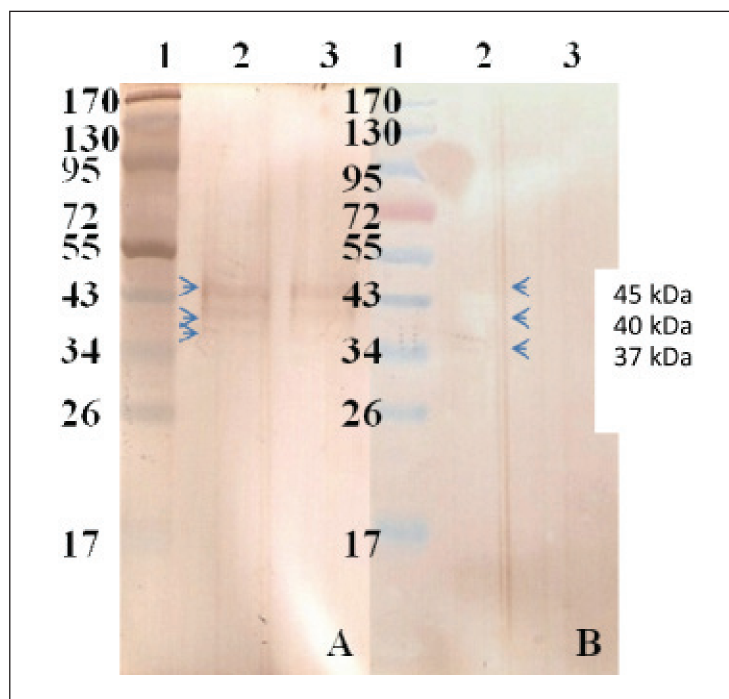

Figure 3. Detection of conA-binding proteins (arrows) of *K. pneumoniae* ATCC 700603 strain (37 kDa, 40 kDa, 45 kDa and 60 kDa) eluted from conA sepharose 4B column

A) conA binding protein was detected at 37, 40 and 45 kDa on the PVDF membrane when reacted with conA-HRP conjugates (arrow) after 16 h of incubation. The 60kDa protein was hardly visible

B) Methyl-α-D-mannopyranoside blocked the binding sites of conA-HRP conjugates, hence, no protein was detected on the PVDF membrane when reacted with conA-HRP conjugates (arrow) after 16 h of incubation

Lane 1: PageRuler prestained protein ladder (10 to 170 kDa) (Fermentas, Lithuania)

Lane 2-3: eluted protein sample from *K. pneumoniae* ATCC 700603 (1µg)

Table 2. Mass spectrometry identification of four *K. pneumoniae* conA-binding proteins

Protein ID	Mass (Daltons)	Individual score	Significant score	Accession	Database
Enolase (*K. pneumoniae*)	45521	1403	>57	ENO_KLEP7 (A6T053)	LudwigNR
Chaperonin (*K. pneumoniae*)	57090	261	>40	CH60_KLEP3 (B5Y368)	LudwigNR/ Sprot
Outer membrane protein C precursor (porin ompC) (*K. pneumoniae)*	39639	115	>64	OMPC_KLEPN (Q48473)	NCBI BLAST/ Sprot
Outer membrane protein A precursor (Outer membrane protein II) (*K. pneumoniae*)	37038	91	>64	OMPA_KLEPN (P24017)	NCBI BLAST/ Sprot

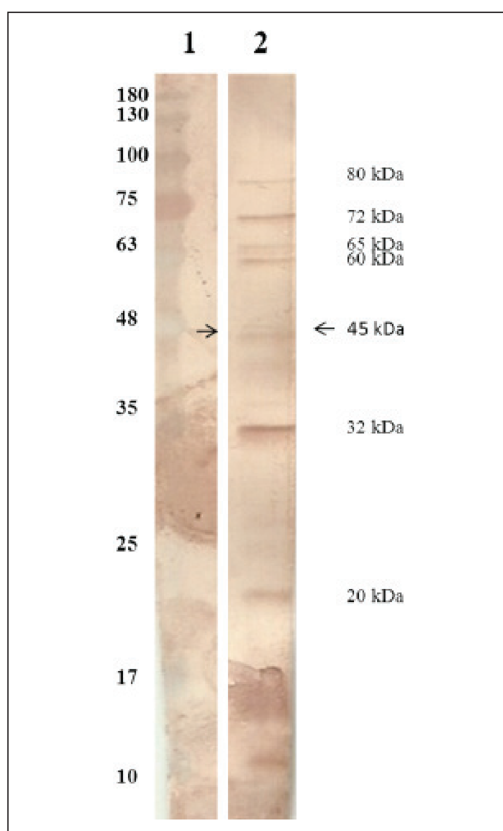

Figure 4. Seroreactivity of *K. pneumoniae* conA binding proteins with rabbit anti-*Klebsiella* antibody. Seven seroreactive bands were detected including two conA-binding proteins (45 and 60 kDa)

Lane 1: BlueRay prestained protein ladder (10 to 180 kDa) (GeneDirex, Taiwan)

Lane 2: Eluted protein sample from conA affinity column (1 μg)

responsible components for conA agglutination are most likely proteins with specific structure possessing mannose/ glucose sugars on the cell surface.

The association of conA agglutination to the virulence of *K. pneumoniae* is still under investigation. The presence of mannose (rhamno) biose-containing capsular polysaccharides with Manα2/Man or Rhaα2/3Rha (di Man/Rha) glycoepitope reduces the survivability of *K. pneumoniae* (Sahly *et al.*, 2009). As mannose receptor on macrophage and lung surfactant protein-A (SP-A) could recognize the mannose glycoepitope, this has resulted in enhanced binding and ingestion of the bacterial cells (Ofek *et al.*, 2000; Sahly *et al.*, 2008). Hence, isolates become less virulent toward host due to the increase opsono-stimulation of human polymorphonuclear leukocytes (Sahly *et al.*, 2009). Our study showed that 89 (48.6%) of the *K. pneumoniae* isolates did not react with conA (Table 1). The absence of conA receptor in these isolates may assist evasion of the organisms from phagocytosis and other host immune mechanisms.

Klebsiella pneumoniae conA binding proteins were detected within the range of 37-60 kDa. In a previous study, majority of the conA reactive proteins from *Mycobacterium tuberculosis* were detected in the range 21-70 kDa and they were postulated as potential adhesins and mannose receptors, and proteins important in virulence and

immune response to mycobacteria (González-Zamorano *et al.*, 2009). Mass spectrometry analysis identified four *K. pneumoniae* conA-binding proteins as outer membrane protein A precursor (Outer membrane protein II) (37 kDa), outer membrane protein C precursor (porin ompC) (40 kDa), enolase (45 kDa) and chaperonin (57 kDa). OMPA binds to macrophages and professional antigen presenting cell (APCs) such as dendritic cells to initiate host defense mechanism (Jeannin *et al.*, 2002). OMPC, also known as OMPK36 in *K. pneumoniae* are porins which controls diffusion of a wide variety of molecules including nutrients and antimicrobial agents (Doménech-Sánchez *et al.*, 1999; Nikaido, 1994). Resistance to antibiotic can result from the deficiency or mutation of the porin genes which prevents entry of antibiotics into bacterial cells (Martinez-Martinez *et al.*, 1996).

Enolase (EC 4.2.1.11) is a glycolytic enzyme which plays a vital role in the sugar metabolism (Avilán *et al.*, 2011). It is responsible for carbohydrate degradation by catalysing the conversion of 2-phospho-D-glycerate (2-PGA) to phosphoenolpyruvate (PEP) which is important for glycolysis. Since it plays an important role in regulating the metabolic pathway of fermentation, enolase is present in abundance among most organisms (Wold, 1971). Meanwhile, chaperonins are a set of proteins which assist in the folding, assembly, transport of nascent or new, non-native polypeptides into their native conformational functional state (Martin Vabulas *et al.*, 2010). The proteins are important for survival and expression of virulence-related proteins of bacterial pathogens (Pantzar *et al.*, 2006).

The identification of several immunogenic proteins eluted from conA affinity column suggests that these proteins were exposed on the bacterial cell wall. In this study, the enolase was the most distinct protein eluted from the conA affinity column which demonstrated immunogenic response towards the rabbit anti-*Klebsiella* antibody. The presence of anti-enolase antibodies following *Klebsiella* infections has also been suggested as a useful marker for muscle tissue injury (Witkowska *et al.*, 2005; Pontillo *et al.*, 2011; Zhao *et al.*, 2012).

In conclusion, this study shows that about 50% of *K. pneumoniae* was able to agglutinate with conA using a microtiter plate assay. Four conA binding proteins were identified in this study. It is believed that a larger number of conA binding proteins can be separated and identified by the use of proteomics techniques. Cloning and expression of conA binding proteins will be helpful in demonstrating the function and interplay of these proteins in bacterium-host cell relationship.

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