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Phospholipase d triggers a high antibody response against *acinetobacterbaumanii* infection in murine model

Elaheh Zadeh Hosseingholi[1], Iraj Rasooli[2,3]*, Seyed Latif Mousavi Gargari[2]

[1]P.h.D. (Microbiology student), Department of Biology, Shahed University, Tehran-Qom Express Way, Opposite Imam Khomeini's Shrine, Tehran-3319118651, (IRAN)

[2]Department of Biology, Shahed University, Tehran-Qom Express Way, Opposite Imam Khomeini's Shrine, Tehran-3319118651, (IRAN)

[3]Molecular Microbiology Research Center, Shahed University, Tehran, (IRAN)

E-mail : rasooli@shahed.ac.ir

ABSTRACT

Acinetobacterbaumannii, a major threatfor hospitalized patients, intensive care units (ICUs) residents, military forces, is a public health problem due to increasing rate of antibiotic resistance. Vaccination of these risk groups may lessen occurrence and spread of infections. In the present study efficiency of phospholipase D (PLD), one of previously identiûed virulence factors of *A. baumannii,* was evaluated as a vaccine component. Polymerase chain reaction (PCR) was used to verify the presence of PLD virulence gene in clinical isolates. The *pld* was amplified, cloned and the recombinant protein i.e. rPLD, was expressed. BALB/C mice were immunized with the recombinant PLD followed by intraperitoneal challenge with lethal doses of 10^8 to 10^{11} CFU of *A. baumannii.* Experimental findings showed significantly higher average survival rate of 95% in immunized mice than the control group. Rabbits were immunized with the rPLD and the sera were used to determine its efficiency in protecting unimmunized mice from bacterial challenge.Average survival rate of 80% was achieved in challenged groups with the same lethal doses used in active immunization challenges compared with the control groups receiving naive rabbit serum. rPLD was potentially effective in triggering a high antibody response and protecting mice against *A. baumannii*.

KEYWORDS

Acinetobacterbaumannii;
Phospholipase D;
Vaccine;
Immunity;
Humoral.

INTRODUCTION

A. baumannii, the most important member of genus Acinetobacter, was recognized as a major cause of nosocomial infections since 1970specially in temperate zone[1]. *A. baumannii* habitat in the natural environments like water and soil has not yet been investigated precisely, however rare reports of its isolation from these environmental samples exist[2]. Antibiotic resistant clinical isolates have been reported during the last 15 years as an outcome of abundant application of strong antibiotics in health care institutions. According to World Health Organization (WHO) data, *A. baumannii* has generated a serious alarm as multidrug-resistant (MDR)

pathogen in clinical environments[3]. Non-susceptibility of most isolates to carbapenems, antibiotics of choice in treatment of *A. baumannii*infections, has resulted in introduction of colistin as a newtherapeutic option[4]. Unfortunately, prevalence of pandrug- resistant strains has been reported recently[5-7] and new antibiotic against the gram negative bacilli (GNB) has not been introduced since 2009[8]. This microorganism also has a great potential tosurvival and persistence in dry and disinfectant treated surfaces[2]. These abilities account for outbreaks of *A. baumannii* infections in the battlefields, hospitals especially among ICU patients and immunocompromised individuals. Secondary infections such as meningitis, pneumonia, urinary tract, skin, bloodstream soft tissue, central nervous system, bone and wound infections are some of the *A. baumannii* associated infection[1,2]. It can cause about 28% to 68% mortality in hospitalized severely ill patients[9].

Several virulence factors are associated with a number of pathogenic bacteria to promote its survival and invasion such as extracellular polysaccharides and proteins, outer membrane proteins, toxins, and hydrolytic enzymes[10]. A few virulence factors have been identified in *A. baumannii* such as outer membrane protein A (OmpA), biofilm-associated protein (BAP), AbaIautoinducer synthase and penicillin-binding protein 7/8. Phospholipase D, a protein existing in both eukaryotes and prokaryotes[11,12], has been determined as a key virulence factor contributing to *A. baumannii* development inside the host[13]. Phospholipids refer to heterogeneous class of enzymes which can cleave host membrane glycerophospholipids ester linkages[14]. A, B, C and D letters, used in the nomenclature of phospholipases, determine which phospholipid bond targeted by the enzyme[15]. Phospholipase D have dual activities 1) producing phosphatidic acid and a free polar like choline, ethanolamine and inositol head group by phospholipids substrate cleavage 2) phosphotransferring to different alcohols to yield phospholipids[12]. *A. baumannii* resistance to a variety of antibiotics and their extraordinary potential to survive in hospitals have drawn global attention to finding effective preventive methods of this life-threading microorganism. Vaccination is an approach which can reduce both occurrence of *A. baumannii*infections and antibiotic use in risk groups[16,17]. Efficiency of several single antigens such as outer membrane protein A (OmpA)[18], the biofilm associated protein (Bap)[19] and a surface autotransporter (Ata)[20], the surface polysaccharide poly-N-acetyl-β-(1-6)-glucosamine (PNAG)[21] and the capsular polysaccharide[22] and also multi-component vaccines likewhole bacterial cell[23] and membrane complexes[24], has been investigated in the animal models. Although some of these candidates represented themselves as qualified ones, no vaccine has been licensed for *A. baumanni*[16]. Attempts should be made to arrive at better vaccine candidates with least drawbacks. Importance of PLD for destroying host epithelial cell membranes and *A. baumannii* resistance to host serum has been proven in murine models[13,25]. The work presented here was designed to evaluate *A. baumannii*PLD potential as a protective antigen for vaccine development.

EXPERIMENTAL

Identification of conserved regions in *A. baumannii* phospholipase D

The sequence of *A. baumanii* ATCC19606 phospholipase D with accession number D0CG26 was retrieved from a protein database,UniProt (http://www.uniprot.org/), in FASTA format.

The sequence was analyzed using SMART (Simple Modular Architecture Research Tool) available athttp://smart.embl.de/[26]. This online software identifies and interprets protein domains. The sequence was then subjected to blastp against non redundant protein sequence (nr) Database with *A. baumanii* as a selected organism in NCBI server http://blast.ncbi.nlm.nih.gov/Blast.cgi. The resulted sequence with a coverage>50%, identity> 50% and E-value <10-4 were selected and aligned with multiple sequence alignment tool PRALINE http://ibivu.cs.vu.nl/programs/pralinewww/[27]. The aim was to find conserved sites at the identified domain positions among aligned sequences used in primer design.

Sample collection and processing

In order to recognize *pld* gene in clinical isolates, a total of 88 isolates were obtained from patients of different hospital wards located in Tehran, Iran. Confirmative identification was performed by biochemical tests. The DNA samples from *A. baumannii*isolates

were prepared by heat lysis method. The strains were grown in Brain Heart Infusion (BHI) for 24 h at 37 °C. About six colony forming units were picked and placed into 500 µl sterile DNase/RNase-free water. Subsequently suspensions were held at a temperature of 100 ° for 10 min to thermal lysis of cells and then chilled at 4 °C. After centrifugationat 14000 ×gfor 2 min the supernatant was used as DNA template. PCR was used to check for the presence of *pld* gene with the use of the specificprimers, designed according to their binding to two short stretches of sequence that are relatively well conserved within *A.baumanniipld* genes,(F:GCATAACAAGAGCTTTATTGCTG, R:AAGTTAAATGAACCGATAAAAACC(. The primers for amplification were designed with oligo7 software. The amplification reaction was performed as follows: initial denaturation at94 °C for 30 s, 30 cycles of denaturation at94 °C for 30 s, annealing 56 °C for 40 s and extension at72 °C for 40 s, final extension step at 72 °C for 5 min. PCR products were detected by subjecting a samplefrom each reaction tube to 1% agarose gel electrophoresisstained with ethidium bromide.

Codon usage analysis

Codon usage was measured using the codon adaptation index (CAI). The CAI value is an estimate of optimal codons use in a particular gene toward highly expressed genes in a given genome[28]. The codon usage Table of highly expressed genes of *A. baumannii* ATCC 17978 and *Escherichia coli* K12 obtained through the codon usage database website available at http://www.kazusa.or.jp/codon/[29]. The CAI values for *pld* were calculated using CAIcal server (http://genomes.urv.es/CAIcal/)[30].

Recombinant antigen production

Two primers were designed for *pld* sequence selected by in silico findings. Initially, the forward primer (F: 5'GACAGGATCCGGTTGTAGCACATTAC3', containing a *BamHI* site and backward primer (R:5'AGCTCTCGAGTTACATAAAACCTTCGA3', containing a*XhoI* site) were designed to amplify signalless 1518bp*pld* sequence. The amplification reaction was performed as follows: initial denaturation at94 °C for 2 min, denaturation at94 °C for 30 s, annealing 58 °C for 60 s and extension at72 °C for 2 min, final extension step at 72 °C for 5 min. The 35-cycle PCR products were cut with *Bam*HIand *Xho*I(Feramantas, Denmark) and ligated to the *BamHI* and *XhoI*-digested pET-32a (+) (Novagen Company, USA). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells. Cloning was certified by digestion with the cloning restriction enzymes. A single colony of BL21 (DE3) containing recombinant plasmid was grown in 5mL of Luria Bertani (LB) medium containing Ampicillin until OD_{600} reached 0.5. Cell pellets from the 5 mL culture were resuspended in 1 mL fresh LB medium and used to inoculate 1 L fresh medium. Cultures were grown with shaking (200 rpm) at 37°C to OD_{600}~ 0.6. For high-level expression of PLD, induction by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1mM was performed. The cultures were incubated at 37°C/250 rpm for 5 h. The cells were harvested by centrifugationat 4000 ×g for 15 minutes. The pellet were re-suspended in10 mMTrisHCl containing the 8 M urea as a denaturant and 100 mMNaH_2PO_4 (pH 8.0). The suspension was sonicated for 10 × 30 sec on ice, and the supernatant was separated from cell debris by centrifugation at 14000×gfor 20 min. The supernatant protein profile was monitored by 12% SDS-PAGE to verify recombinant PLD (rPLD) expression and the supernatant used to purifyrPLD using nickel chelation affinity chromatography.The expression of recombinant protein was conûrmed by protein reaction with the anti-His-tag antibody (Qiagen, USA) by Western blotting. For Western blotting, rPLD was electrophoresed on 12% SDS-PAGE and transferred onto nitrocellulose membranes and subsequentsteps were performed as previously described[31]. The purified recombinant *protein concentration was determined*using thestandard*Bradford protein assay.*

Immunization of mice

Antisera to the PLD were raised in test group of 25 male BALB/C mice (~20 g) purchased from Pasteur Institute of Iran. Each mouse in the test group was injected subcutaneously with 10µg of purified rPLD protein with complete Freund's adjuvant (Razi institute, Iran), on days 0. Booster doses were administered on days 15, 30 and 45 with the same manner except for

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incompleteFreund's adjuvant instead of the complete adjuvant. The control group was injected as the preceding protocol except for PBS instead of rPLD. Blood samples were collected from tail-vein nicking from the mice before starting injections and 10 days after the each injection. The antisera were stored at "70 æ%C for enzyme-linked immunosorbent assay (ELISA) analyses. Research was conducted in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The principles stated in the Guide for the Care and Use of Laboratory Animals were followed[32]. The animal care protocol was approved by Shahed University.

Indirect ELISA of sera

An indirect ELISA technique was used to evaluateserum IgG antibody responses to recognize the PLD antigen. 20µg of the purified rPLDwas coated with 100 µl bicarbonate/carbonate coating buffer (100 mM, pH 9.6) in 96 well ELISA microplate and the next steps was carried out according to theprotocolpreviously described[19]except for serial sera dilution which was examined up to 1:12800.

Challenge study

LD_{50} value was determined by infecting 5 groupsof 6 BALB/C mice with 10-fold dilutions of *A. baumannii* 19606 ranging from 10^5 to 10^9 CFU/ml and analyzing survival data by Finney's- maximum likelihood probit technique[33]. A murine model of sepsis was used for bacterial challenge. Two weeks after the last booster, four groups of five immunized mice were intraperitoneally challenged with freshly prepared inocula of *A. baumannii* 19606 at 10^8, 10^9, 10^{10} and 10^{11} concentrations. The fresh inocula were prepared by growing *A. baumannii*strain overnight at 37ºC in *Tryptic Soy Broth* (TSB) broth and passaging it to mid-log-growth; afterward bacterial cells were washed twice with phosphate-buffered saline and resuspended at the appropriate concentration. Unimmunized mice were exposed to the same bacterial treatment as a control groups. The number of deaths for each group was monitored for 7 days.

In vivo neutralization assays

Antisera to the PLD protein were raised in a New Zealand White male rabbits (Razi institute, Iran) as the same procedure applied to mice with 100 µg of rPLD. For neutralization assay[34] groups of five mice were intrapritoneally injected with lethal doses of 10^8, 10^9, 10^{10} and 10^{11} CFU of *A. baumannii*mixed with 200 µl of immune rabbit serum (diluted 1:800 in PBS). The mixture had previously been incubated for 30 minutes at 37°C. In order to verify that natural antibodies in rabbit serum do not offer any resistance to *A.baumannii* in vivo, groups of mice received mixture of lethal doses of *A. baumannii* and normal rabbit serum as control[21]. The animals were monitored for mortality for seven days.

Statistical analysis

All statistical analyses were performed using InStat3 and Prism6 (GraphPad Software). The data are expressed as mean ±standard deviation (SD). One-way ANOVA followed by Bonferroni's post hoc multiple-comparison tests determined statistical signiûcance of ELISA. Log-rank test was used to determine if number of viable and dead mice varied signiûcantly. P values of <0.05 were considered as signiûcant.

RESULTS

Identification of conserved regions of A. baumanii phospholipase genes

Protein domain analysis by SMART revealed that PLDc domain is present between amino acids 435 and 461 and also between amino acids 185 and 212 with E-value of 0.000197 and 0.00832 respectively. Multiple alignments of *A. baumannii* attributed to PLD sequences using PRALINE indicated that two sequences positioned at 189-197 (MHNKSFIAD) and the other positioned at 449-457 (QVFIGSFNF) were well conserved regions.

PLD gene distribution in A.baumannii isolates

The *A. baumannii*strains analysis for *pld* genes led to detection of PCR products on agarose gel with the expected size of 804 bp (Figure 1). The PLD gene was detected in all clinical isolates.

Codon usage analysis

The calculated CAI values for *pld* gene in *A. baumannii*and *Escherichia coli* were 0.75 and 0.704 respectively.

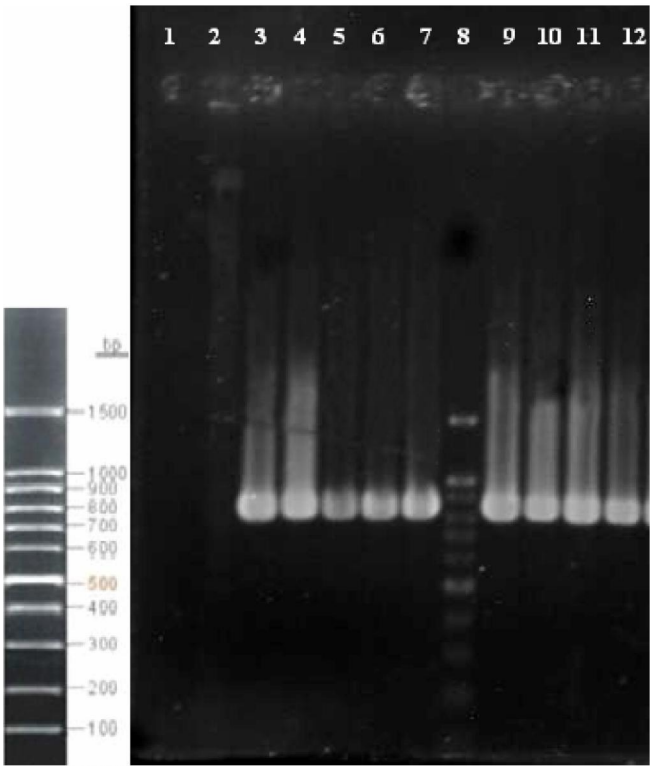

Figure 1 : Gel electrophoresis of PCR products of *pld* conserved region in clinical isolates of *A. baumannii.* Lane1: Negative control (PCR without template), Lane 2: Negative control (*Staphylococcus aureus* genome as a template)Lane 3: positive control (*A. baumannii*ATCC19606genome as a template) Lane 4-7 and 9-12: 804bpPCR products Lane8: 100 bpDNA ladder marker

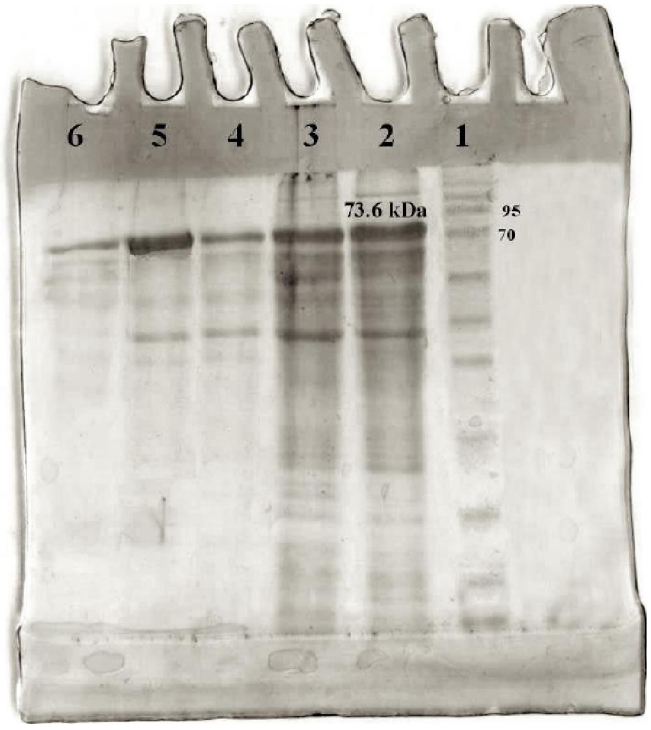

Figure 2 : Expression and Ni-NTA puriûcation of recombinant protein. Lane 1: Protein weight marker. Lane 2: Expression of protein. Lane 3: Fow-through. Lane 4: Column washed with buffer D (pH = 5.9). Lane 5: Column washed with elution buffer (pH = 4.5). Lane 6: Column washed with MES Buffer.

Expression of recombinant protein

The signal-less *pld* amplified by PCR was cloned into pET32a and over expressed in *E. coli* (BL21DE3). The SDS-PAGE analysis showed the presence of a 73.6 kDarPLD protein after induction with 1mM IPTG. Ni-NTA chromatography purification resulted in a single band of rPLD protein (Figure 2). The expression of the recombinant protein was conûrmed by western blotting using anti-His-tag antibodies (Figure 3).

Antibody response after vaccination

An indirect ELISA assay of polyclonal antibody responses against rPLD antigen showed enhanced levels of humoral response after the second booster ($P < 0.01$). No considerable antigen-speciûc antibodies could be detected in control mice sera atany time ordilution (Figure 4A).

Survival of challenged mice

LD_{50} value for *A. baumannii*ATCC19606 was determined as 10^7 CFU. In order to determine vaccination efficiency in protecting mice from lethal infection mice, vaccinated mice were challenged with different lethal doses of *A. baumannii*. All vaccinated mice in experimental groups showed significantly ($p<0.0001$; log-rank test) survival rate. Whereas all mice in control groups died within 24 hours of inoculation (Figure 5).

In vivo neutralization assays

The antibody level raised against rPLDincreased in the vaccinated rabbits (Figure 4B). Neutralization test was performed to determine if sera from immunized rabbits could protect naive mice against bacterial challenge. As shown in Figure 6, all experimental mice groups were significantly ($p<0.05$; log-rank test) protected. All mice receiving normal rabbit serum succumbed.

DISCUSSION

A. baumannii is an important health challenge in hospitals, especially in individuals treated with broad-spectrum antibiotics, immunocompromised hosts and

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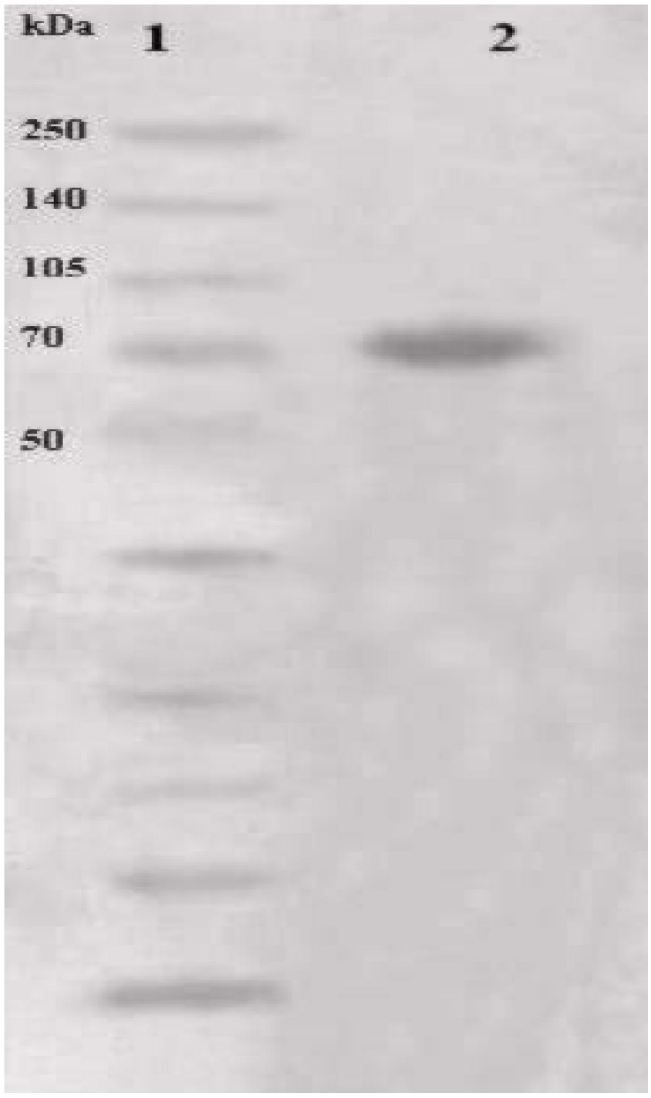
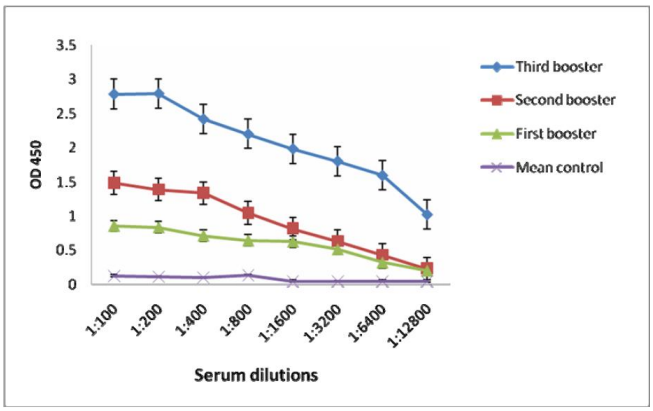
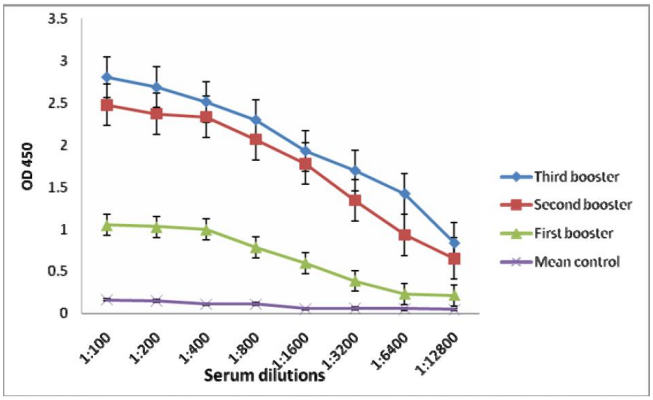


Figure 3 : Western blot analysis of rPLD using anti 6X-His-tag antibody, Lane 1 : Protein weight marker, Lane 2 : rPLD



A.



B.

Figure 4 : Speciûc IgG response to immunization with the rPLD in mice (A) (P < 0.01) and rabbit (B)(P < 0.001).

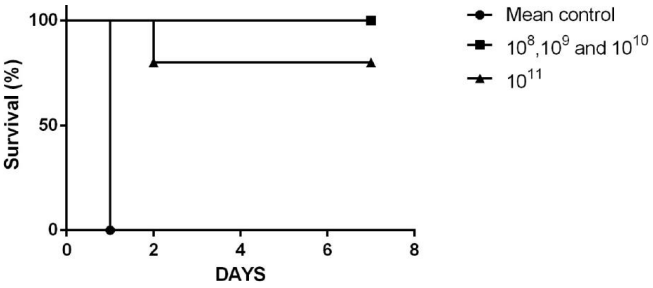


Figure 5 : Active immunization against and challenge with *A. baumannii* using rPLD. Survival rate analysis of vaccinated (rPLD, n =5/group) and control (adjuvant control, n = 5/group) mice after challenge with different doses of the ATCC 19606. Log-rank test analysis showed that the immunized mice were signiûcantly protected against *A. baumannii* challenge compared to the control (p<0.0001).

intensive care unit patients harboring mechanical ventilator tubes and urinary catheters. Antibiotic resistance mechanisms and virulence factors make this microorganism a trouble maker. PLD production is one of *A. baumannii* natural properties that may affect host cells lysis and immune responses[35]. Interest in prevention and treatment of infectious diseases by antibody-based therapies is now being reinvigorated by the wide development of drug-resistant pathogens. Most of the PLDs, CL synthases, poxvirus envelope proteins, PS synthases, tyrosil-DNA phosphodiesterase,Yersinia murine toxin and nucleases are PLD superfamily members which are present in various prokaryotes and eukaryotesand exhibit various functions. Bacterial PLD enzymes usually belong to the PLDc 2 family. Invariant characteristic of this family is double copy existence of HxKxxxxD (HKD) motif[36]. These have been proved that protein functional regions are evolutionarily conserved[37] and the consensus HKD motifs are essential for PLD enzymatic activity[38]. In this study after finding PLD domains in *A. baumanii* ATCC19606 and align-

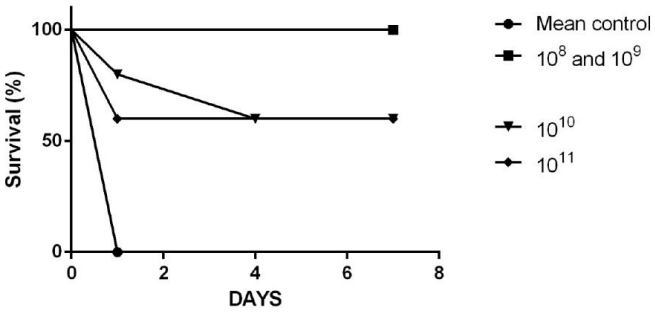


Figure 6 : Passive immunization against *A. baumannii* using rPLD.Survival of mice infected with different doses of *A. baumannii*ATCC 19606 mixed either with rabbit vaccine serum (rPLDserum, n = 5) or rabbit naive serum (n = 5) (1:800 dilution). Log-rank test analysis showedmice receiving immunized rabbit serum were signiûcantly protected against *A. baumannii* challenge compared to the control (p<0.05).

ment of currently available *A. baumannii* PLD sequences on the NCBI server with abovementioned cut-off values, the most conserved sequences in domain areas were selected. Of single antigen based vaccine candidate disadvantages is their ineffectiveness against all strains[16]. The presence of *pld* in all isolates is in agreement with other studies statingharboring of *pld* by virulent *A. baumannii* strains[25]. The widely used CAI value is to predict gene expression levels, ranging from 0 to 1, correlates with cellular protein levels and translation efficiency[39]. Highly expressed genes with complicated fundamental functions of the cells, possess higher CAI. High CAI level (0.75) of *pld*gene in *A. baumannii*may suggest *pld* asan essential gene for *A. baumannii* survival. Existence of different codon usages in genetic exchange and its absence in our findings implies non genetic exchange origin of this gene[40,41]. *pld*CAI value of 0.704 in *E. coli* verifies existence of a few rare codons and high-throughput gene expression and rules out a need for gene optimization[42]. Existence of some rare codons may even be useful because there is a hypothesis that translational pauses decreasing translation rate prevent protein aggregation and instability[43]. In this study, rPLD lacking the secretion signal peptide was used. The well characterized role of signal sequence is directing proteins to their functional locationsin prokaryotes and eukaryotes. Proteins such as phospholipases and toxins are virulence factors secreted by the type II pathway. Their signal peptides target them to periplasmic and then extracellular environments[44]. In *E. coli*, signal peptide containing recombinant protein can be targeted to membrane and rPLD have a potential to hydrolyze a variety of *E. coli* membrane phospholipids[12]. Moreover the presence of hydrophobic signal sequences can reduces stabilization and increases the aggregation tendency of protein[45,46]. Antigen with intrinsic tertiary structure could elicit strong immunogenic responses. The rPLD was purified with urea denaturants in this study as this process prevents functional toxicity of rPLD in the animal body. Urea decomposes bioactive protein conformational folding, reversibility of which is difficult even after protein dialysis[47,48]. In addition to urea denaturation other methods like site directed mutagenesis in the sequences coding PLD active sites have also been carried out in *Streptomyces chromofuscus* and *Corynebacteriumpseudotuberculosis*prior to its cloning[12,49]. The ELISA results showed high immune response induction in BALB/c mice and rabbits after the 2nd booster dose. In mice, antibody titers were significantly ($p<0.001$) increased after third booster. In the mouse sepsis model used to characterize vaccine efficacy, bacteria disseminate throughout the body shortly after inoculation leading to animal death in 48 h[23]. In this study signiûcant protection was observed in immunized mice against challenge with *A. baumannii*. The immunized mice could tolerate as high as $1000\times LD_{50}$ with no fatality. 80% of the immunized mice survived $10^4\times LD_{50}$ bacterial challenge which was as good as the protection achieved by Bap subunit vaccine under similar challenge conditions[19]. 20% reduction in protection upon ten-fold increased bacterial dose suggests that higher bacterial load may suppress the host immune response[50]. As expected there was no specific antibody response or protection in the control group. 1:800 sera dilution from rabbits vaccinated with PLD protected all mice when mixed with 10^8 ($10\times LD_{50}$) and 10^9 ($100\times LD_{50}$) bacterial load. This protection effect declined to 60% using 10^{10}($1000\times LD_{50}$) and 10^{11} ($10000\times LD_{50}$) bacteria. Antibodies alone can provide relative protective immunity against infection that may partly be related to efficiency of opsonization in deracination of infection[51]. The resultsare good enough toexcludethe use ofantibodiesinantibody-based therapies of *A. baumannii* in future. PLDas a virulence factor of *Neisseria gonorrhoeae*and*Corynebacteriumpseudotuberculosis*, was reported to have successfully been used for pro-

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tection against these bacteria[49,50,52].

CONCLUSION

Inhibition of Phospholipase D could prevent *A. baumannii* infection. This protein has potential to be used in producing vaccine against *A. baumannii*.

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