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ORIGINAL ARTICLE

Cloning and expression of *nlpA* gene as DNA vaccine candidate against *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is one of the highly antibiotic-resistant bacteria that cause infections with high rate of death. This bacterium is one the common causes of infection worldwide leading to endemic and epidemic nosocomial infections. Despite many efforts, there is no effective vaccine against *A. baumannii*. As NlpA is one of the important antigenic factors in biogenesis of outer membrane vesicles, and OMV-based reported vaccines in *A. baumannii* stimulated the immune responses, this study was aimed to clone and express *nlpA* gene in eukaryotic HDF cells and evaluate the induced immunization following the administration of resulting construct as DNA vaccine in BALB/c mice. The *nlpA* gene of *A. baumannii* was amplified using PCR. The PCR product was then cloned and subcloned into the pTZ57R/T and pEGFP-C2 vectors respectively. The cloning was confirmed by PCR, restriction enzyme digestion and DNA sequencing. The pEGFP-C2-*nlpA* recombinant plasmid was transferred into the HDF cells using electroporation and the expression of target gene was validated by RT-PCR. The recombinant construct was injected to BALB/c mice through three IM injections and the levels of IgG, IgM, INF-γ, IL-2, IL-4, and IL-12 were determined using ELISA assay. The *A. baumannii nlpA* gene was amplified during PCR as 867 bp band which was successfully cloned in pEGFP-C2-*nlpA* vector. Obtained data from RT-PCR and presence of the 867 bp fragment in transformed HDF cells confirmed the *nlpA* gene expression. Following the injection of pEGFP-C2-nlpA showed the increased level of IgG, IgM, INF-γ, IL-2, IL-4, and IL-12 in serum of immunized mice. Overall, through this study recombinant pEGFP-C2-*nlpA* was generated and successfully expressed the *A. baumannii nlpA* gene in eukaryotic cells. Additionally, our in vivo study confirmed that the recombinant construct capable to induce the immune response in immunized mice. These findings suggest the pEGFP-C2-*nlpA* may be considered as DNA vaccine candidate against *A. baumannii*.

Keywords *Acinetobacter baumannii* · *nlpA* · Gene expression · Immunization · DNA vaccine

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Introduction

The genus *Acinetobacter* has the important position as an opportunistic pathogen in healthcare systems. In recent years, *Acinetobacter baumannii* has emerged as one of the red-alert pathogens on the some parts of the world [1]. This non-motile, gram-negative bacterium can adhere to biotic and abiotic surfaces, though its infection leads to bacteremia, pneumonia, septicemia, urinary and nervous system infections [2]. While some species of *Acinetobacter* are existent in the microbiota of healthy human skin, most isolates obtained from clinical samples show significant pathogenicity. *Acinetobacter baumannii* is one the most important species, since it has been associated with serious infections, especially those acquired in intensive care units (ICU). In addition, since infection with this species has been correlated with increased multiple-drug resistance

(MDR), morbidity, and mortality, also the death associated with this infection is high [3]. Emergence of drug resistance feature in *A. baumannii* against variety FDA-approved antibiotics including carbapenems, aminoglycosides and fluoroquinolones is a major problem making *A. baumannii* difficult to treat [4]. *Acinetobacter baumannii* has poor growth requirements and use a range of nutritional sources, and it is compatible to low and high environment temperature, pH, salinities, and humidity. This resulted in the adaptability of *A. baumannii* to hospital environment, which is the main source of this pathogen [5]. Several resistance mechanisms have been reported in *A. baumannii* among which production of carbapenemases, decreased outer-membrane permeability caused by the loss or reduced expression of some beta-barrel proteins such as porins, alterations in penicillin-binding proteins and efflux pumps are well-understood [6]. As antibiotics resistance has strongly affected the effectiveness of antibiotic treatments, the development of alternative approaches is necessary. Vaccination strategies are emerging as a viable option to prevent and/or treat multi- or pan drug-resistant infections [7]. The use of some antigens as vaccine candidates has made it possible to prevent infections caused by *A. baumannii*, which include the whole cell vaccines, pure protein based vaccines, outer membrane complexes, outer membrane vesicles, OmpA, Ata, Bap and K1 capsular polysaccharide have recently been proven effective at some levels to protect against challenges in experimental animals [8–10].The NlpA is an inner membrane-anchored lipoprotein or periplasmic protein that has a role in methionine import [11]. Some studies have shown that disruption of NlpA decreases the production of outer membrane vesicles [11–14]. The aims of present study were cloning and expression *nlpA* gene of *A. baumannii* in human dermal fibroblast (HDF) cells as a eukaryotic host and evaluating the induced immunization following the administration of recombinant construct as DNA vaccine candidate in vivo.

Materials and methods

Bacterial strains and cells

Acinetobacter baumannii and *Escherichia coli* strain TOP10F′ were obtained from the Iranian Biological Resource Center (IBRC) and Biotechnology Research Center of Shahrekord, respectively. These strains were grown under optimal conditions in Luria–Bertani (LB) agar (sodium chloride, 5 g/l; yeast extract, 5 g/l; tryptone, 10 g/l; typically combined with agar, 15 g/l) (Difco, USA) at 37 °C overnight. The HDF cells were obtained from the National Cell Bank of Iran, Pasteur Institute and cultured in RPMI 1640 supplemented with 10% inactivated fetal calf serum (FCS) (Gibco, USA).

DNA extraction and gene amplification

Bacterial chromosomal DNA was extracted from *A. baumannii* using the QIAamp® DNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The DNA quality was analyzed by electrophoresis on a 1.0% agarose gel stained with ethidium bromide. DNA concentration and quality were determined using the Nanodrop ND-1000 spectrophotometer (PeqLab, Germany) at a wavelength of 260/280 nm.

Primers were designed by GeneRunner software (version 3.05) for *A. baumannii nlpA* gene (Accession number: CP020598.1). The primers had *Kpn*I and *Sac*II restriction sites in forward (5′-CTC**GGTACC**ATGAAAAAGCTG ATCAGTC-3′) and reverse primer (5′-TAT**CCGCGG**CTA TTTAGCTTGCGTGAG-3′), respectively. PCR reaction was carried out in a final reaction of 25 µl in 0.5 ml microtubes containing 1 µg of template DNA, 2 mM $MgCl_2$, 200 µM dNTP mix, 2.5 µl of 10× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1 unit of Taq DNA polymerase (all Thermo Fisher Scientific, USA), and 1 µM of each primer. 2 µl of sterile ultrapure deionized water instead of template DNA was used as negative control [15]. PCR reaction was performed with initial denaturation at 94 °C for 5 min followed by 32 thermal cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 10 min. Amplified PCR products were analyzed by electrophoresis in 1.5% agarose gels.

Construction of recombinant plasmids

The *nlpA* purified DNA pieces (using Qiagen gel extraction kit) were ligated using PCR cloning kit-Thermo Fisher Scientific (plasmid vector pTZ57R/T) according to the manufacturer's protocol. The ligated products (pTZ57R/T plus *nlpA*) were transformed into the competent cells *E. coli* TOP10F′ by calcium chloride ($CaCl_2$) chemical method. The transformants were selected on LB-ampicillin agar plates and confirmed by PCR. The presence of the *nlpA* DNA insert was determined by screening bacterial colonies using PCR. Plasmid DNA purification was performed with a kit (Bioneer, Korea) and the presence of the *nlpA* gene in pTZ57R/T vector was investigated by restriction enzyme double digestion. The *Kpn*I and *Sac*II digested recombinant pTZ57R/T-*nlpA* and pEGFP-C2 were analyzed by electrophoresis in 1% agarose gels and the *nlpA* gene fragment and linear pEGFP-C2 plasmid were purified using gel extraction kit. Then the *nlpA* gene was ligated to similarly digested pEGFP-C2 and transformed into competent cells *E. coli* TOP10F′. Transformed bacterial cells

were selected on LB-kanamycin agar plates. After plasmid purification, the*Kpn*I/*Sac*II restriction analysis, and PCR technique were used to confirm gene cloning. The recombinant plasmid (pEGFP-C2-*nlpA*) was sequenced by the Sanger sequencing method (Generay, China).

Transfer of pEGFP-C2-*nlpA* into HDF cell

The pEGFP-C2-*nlpA* recombinant vector was introduced into HDF cells using electroporation. Hundred milligram of the pEGFP-C2-*nlpA* (recombinant plasmid) and 100 µg of pEGFP-C2 (control) were added to two separate microtubes containing 4×10^6 of HDF cells in 200 µl final volume and the mixtures were transferred to a 2-mm sterile electroporation cuvette. Moreover, 2 µl of sterile PBS instead of template plasmid DNA was added to 4×10^6 of HDF cells as negative control. Then the cell/DNA mixtures were placed on ice for 5 min. The cells were electroporated by using Gene Pulser Xcell™ Electroporation Systems (Bio Rad, USA) at 0.174 kV, and 400 µF. The electroporated cells were immediately placed on ice for 2 min and then cultured in selection medium (RPMI containing 10% FBS and 50 µg/ml neomycin) for 48 h at 37 °C and 5% CO_2.

RT-PCR

Total RNA was extracted by the RNA extraction kit (Qiagen, USA) from cultured HDF cells which were transfected with the recombinant pEGFP-C2-*nlpA* and control pEGFP-C2 plasmids. RNA concentration was determined by absorbance readings at 260 nm using the Nanodrop ND-1000 spectrophotometer (PeqLab, Germany). The complementary DNA (cDNA) synthesis was performed using the SuperScript™ First-Strand Synthesis System (Invitrogen, USA) in a total volume of 20 µl according to the manufacturer's instructions. This cDNA was subjected to PCR amplification with specific primers for the *nlpA* gene. The PCR reaction was carried out in a total volume of 25 µl with 1 unit of *Taq* Polymerase, 1 µM of each *nlpA* specific primers, 200 µM dNTPs, 2 mM $MgCl_2$, 2.5 µl 10×PCR buffer, and 2 µl of cDNA. All PCR products were electrophoresed on 1% agarose gel containing 10 µg/ml of ethidium bromide for 40 min at 70 V and bands were visualized under ultraviolet light from gel documentation system (UV Tech, UK).

SDS PAGE analysis

Following 48 h post-transfection, the cells were harvested and centrifuged at 6000 rpm for 20 min at 4 °C. Cell pallet was ultrasonically broken (300 V, 5 s×3). The obtained cell lysate was electrophoresed on SDS-PAGE to detect the recombinant NlpA protein based on its molecular weight.

Immunization study

All stages of experiment were performed in accordance with the guidelines of the Declaration of Helsinki (1964) and its later amendments and animal studies were approved by Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. All efforts were made to minimize animal suffering. Female, BALB/c mice (6–8 weeks-old) were purchased from Animal House of Ahvaz Jundishapur University of Medical Sciences, and cured in specific-pathogen free condition. Mice (10 per group) were immunized by intramuscular injection of 0.33 µg/µl×3 = (100 µg/µl) of vaccine on days 0, 7, 14. Control mice received (0.33 µg/µl×3), empty vector (without target gene) or 100 ml PBS, on the same program. In order to evaluate immune response ELISA assay was performed, following collection of blood samples (Boster Co, USA). In brief, ELISA plates were coated with 100 ml per well of 5 mg/ml of sample. Coated wells were blocked with bovine serum albumin (BSA), incubated with mouse sera, washed, and stained with goat anti-mouse secondary antibody conjugated with horse-radish peroxidase (HRP). Wells were washed again and the optical density was detected at 490 nm. The level of antibodies (IgG and IgM) and cytokines (INFγ, IL-2, IL-4 and IL-12) was determined on days 0, 7, 14 and 30. Challenge with lethal dose of *A. baumannii* (5×10^8 CFU) was done on 42th day and survival of each group was determined for 7 days. Statistics were done using SPSS software, version 17.0 (SPSS Inc, Chicago IL, USA). The measurements were compared with (One way analysis of variance, ANOVA) test and survival monitoring was performed using Kaplan–Meier test. Data were considered significant, at $p<0.05$.

Results

Genomic DNA was purified from *A. baumannii* and PCR amplification with specific primers for the *nlpA* gene amplified a 867 bp fragment of DNA (data not shown). The 867 bp fragment of *nlpA* gene was cloned with T/A cloning technique in a T-vector (pTZ57R/T) and after chemical transformation of *E. coli* competent cells, the pTZ57R/T-*nlpA* recombinant plasmid was generated. Plasmid purification and *Kpn*I/*Sac*II restriction endonuclease digestion of pTZ57R/T-*nlpA* recombinant plasmid, confirmed the correction of *nlpA* gene cloning. So, the pTZ57R/T vector and *nlpA* gene which respectively had 2886 and 867 bp in length, were detected by agarose gel electrophoresis (data not shown).

The *nlpA* fragment was cut out from the pTZ57R/T-*nlpA* and was inserted into the *Kpn*I/*Sac*II restriction sites of the pEGFP-C2 vector. The construction of recombinant eukaryotic expression plasmid pEGFP-C2-*nlpA* was confirmed

through PCR and restriction enzymes digestion. After digestion, the 4735 bp and 867 bp fragments of pEGFP-C2 plasmid and *nlpA* gene were observed on agarose gel. The result of pEGFP-C2-*nlpA* restriction digest using *Kpn*I/*Sac*II enzymes is shown in Fig. 1. The pEGFP-C2-*nlpA* plasmid construct was verified by sequencing. The result of sequencing showed the *nlpA* that was cloned into pEGFP-C2 vector does not have any mutation in its nucleotide residues.

The HDF cells that were transformed with the plasmids containing the gene for neomycin resistance (pTZ57R/T-*nlpA* or pTZ57R/T) were grown in RPMI medium comprised of 10% FBS, and 50 µg/ml neomycin. Whereas, another result for the untransformed HDF cells (negative control) showed that these cells did not grow in the presence of neomycin. RT-PCR results showed that the 867 bp fragment of DNA from *nlpA* was amplified and *A. baumannii nlpA* gene was successfully expressed in eukaryotic HDF cells.

The recombinant construct pEGFP-C2-*nlpA* efficiently expressed NlpA protein in HDF cells

To reveal if the pEGFP-C2-*nlpA* construct can express the NlpA protein, the HDF cells were transfected with recombinant vector for 48 h. The cell extract was then analyzed by SDS-PAGE to find the NlpA protein. As illustrated in Fig. 2, a 31 kDa band was observed on the PAGE which is similar to the predicted molecular weight of NlpA (Fig. 2).

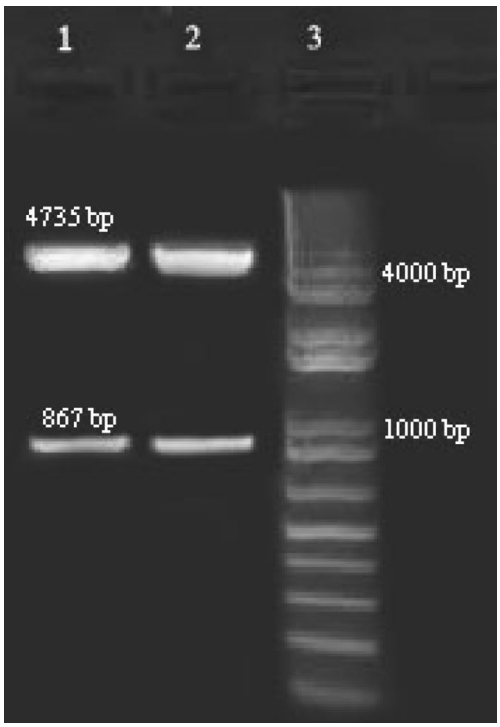

Fig. 1 *Kpn*I/*Sac*II restriction digest on pEGFP-C2-*nlpA* plasmid. Lanes 1 and 2 show the 867 and 4735 bp fragments of *nlpA* and pEGFP-C2, respectively, lane 3 is 1 Kb DNA ladder (Thermo Fisher Cat No. 10816-015)

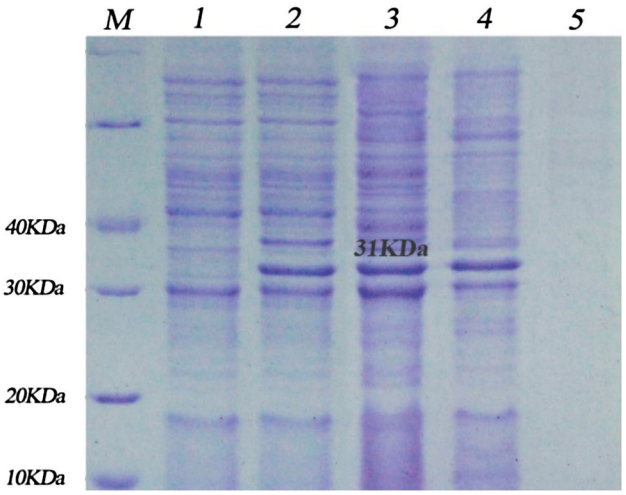

Fig. 2 SDS-PAGE analysis of *A. baumannii* NlpA. SDS-PAGE was used to analyze the expression of rNlpA. Recombinant vectors pEGFP-C2-*nlpA* and pEGFP-C2 were transfected into HDF. All the samples were analyzed by SDS-PAGE, and the protein was stained with Coomassie Blue in the gel. *Lane M* molecular mass marker in 10 kDa, *Lane 1* whole cells lysate of the *HDF* with pEGFP-C2, *Lane 2* the *HDF* including pEGFP-C2-*nlpA*, 6 h after transfection, *Lane 3–4* the whole cells lysate of the *HDF* contained recombinant pEGFP-C2-*nlpA*, after 48 h, *Lane 5* blank control. (Color figure online)

To characterize the effect of immunization with pEGFP-C2-*nlpA* construct on antibody and cytokine levels, specimens were collected from immunized and control mice. As observed in Table 1, vaccination with pEGFP-C2-*nlpA* stimulated high level of antigen-specific IgG and IgM in vaccinated animals after each injection. The IgG and IgM levels in control group were also increased, and their fold of change were significant less than the experimental group. The change of antibody markers was as follows: Level of IgG from 3.58 µg/ml ± 1.36 to 74.53 ± 3.31 (20.81-fold increase) and level of IgM from 1.28 µg/ml ± 0.41 to 41.71 ± 1.15 (36.27-fold increase). Level of INFγ, IL-2, IL-4, and IL-12 was detectably higher in vaccinated group than in control group (Table 2). The change of cytokine markers was as follows: Level of INF-γ from 11.45 pg/ml ± 0.42 to 85.62 ± 3.81 (7.5-fold increase), level of IL-2 from 48.75 pg/ml ± 3.53 to 201.25 ± 6.45 (4.1-fold increase), level of IL-4 from 2.25 pg/ml ± 1.44 to 179.12 ± 13.28 (79.6-fold increase), level of IL-12 from 2.86 pg/ml ± 0.61 to 116.76 ± 5.71 (40.82-fold increase). These data demonstrate that humoral and cellular immune responses are elicited by this DNA vaccine.

Vaccine performance was examined by infecting vaccinated and control animal with lethal dose of *A. baumannii* (5×10^8 CFU) and then survival was detected. Fifty percent

Table 1 Concentration of antibodies

Immunoglobulin	Day 0	Day 7	Day 14	Day 30	Day 30/Day 0
(pEGFP-C2-*nlpA*) IgM	1.27 ± 0.41	19.59 ± 0.54	29.43 ± 0.93	41.71 ± 1.14	36.27
(pEGFP-C2-*nlpA*) IgG	3.57 ± 1.36	18.51 ± 0.87	43.76 ± 9.00	74.53 ± 3.31	20.81
pEGFP-C2 (IgM)	1.27 ± 0.41	13.67 ± 0.74	18.56 ± 0.79	22.91 ± 0.79	18.04
pEGFP-C2 (IgG)	3.57 ± 1.36	12.42 ± 0.92	20.82 ± 0.87	31.97 ± 5.46	8.95
PBS (IgM)	1.27 ± 0.41	7.69 ± 0.78	10.08 ± 0.55	12.63 ± 1.16	9.94
PBS (IgG)	3.57 ± 1.36	10.43 ± 0.66	13.51 ± 0.66	16.84 ± 1.10	1.31

Female BALB/c mice ($n = 10$ per group) were immunized with pEGFP-C2-*nlpA* (0.33 µg/µl × 3) in days 0, 7 and 14. Data were shown as mean ± SD

Table 2 Concentration of cytokines

Cytokine	Day 0	Day 7	Day 14	Day 30	Day30/Day 0
(pEGFP-C2-*nlpA*) IL-2	48.75 ± 3.53	120 ± 8.53	163.12 ± 6.5	201.25 ± 6.45	4.1
(pEGFP-C2-*nlpA*) IL-4	2.25 ± 1.44	54.12 ± 3.14	82.25 ± 6.61	179.12 ± 13.26	79.6
(pEGFP-C2-*nlpA*) IL-12	2.85 ± 0.61	24.89 ± 2.38	37.70 ± 2.25	116.76 ± 5.71	40.82
(pEGFP-C2-*nlpA*) INFγ	11.46 ± 0.42	36.66 ± 7.42	67.03 ± 7.22	85.62 ± 3.81	7.5
(pEGFP-C2) IL-2	48.75 ± 3.53	108.75 ± 4.08	142.5 ± 6.29	161.25 ± 6.45	3.31
(pEGFP-C2) IL-4	2.25 ± 1.44	15.37 ± 2.39	32.25 ± 4.78	43.5 ± 4.08	19.33
(pEGFP-C2) IL-12	2.85 ± 0.61	18.32 ± 1.58	27.07 ± 1.63	41.45 ± 3.97	14.52
(pEGFP-C2) INF-γ	11.46 ± 0.42	19.79 ± 1.43	26.88 ± 1.25	34.37 ± 2.0	3
(PBS) IL-2	48.75 ± 3.53	79.37 ± 11.25	110 ± 2.5	120 ± 2.5	2.46
(PBS) IL-4	2.25 ± 1.44	9.75 ± 5.20	26 ± 2.88	34.75 ± 2.5	15.44
(PBS) IL-12	2.85 ± 0.61	10.04 ± 3.57	18.64 ± 1.18	28.32 ± 1.63	9.94
(PBS) INF-γ	11.46 ± 0.42	15.62 ± 1.42	23.96 ± 1.96	26.87 ± 1.25	2.34

Female BALB/c mice ($n = 10$ per group) were immunized with pEGFP-C2-*nlpA* (0.33 µg/µl × 3) in days 0, 7 and 14. Data were shown as Mean ± SD

of vaccinated mice with pEGFP-C2-*nlpA* (n = 10) were protected from lethal dose infection. Whereas, all control mice which received PBS or pEGFP-C2 vector (without target gene) were died during the 48 h post infection ($p < 0.05$) (data not shown).

Discussion

Acinetobacter baumannii create various types of infections including meningitis, pneumonia, and bloodstream infections. The treatment of infections caused by this pathogen has become a considerable clinical challenge due to the worldwide dissemination of multiple antibiotic resistance strains [16]. Furthermore, the development of new antimicrobial reagents has been challenging. Therefore, a different approach utilizing the immunological characteristics of some antigens of *A. baumannii* might be a viable alternative [6]. In the present study, the *nlpA* gene of *A. baumannii* was cloned into a eukaryotic expression vector and pEGFP-C2-*nlpA* with a strong CMV promoter was generated. The pEGFP-C2-*nlpA* construct was transfected into HDF cells with electroporation and gene expression was evaluated by RT-PCR. DNA vaccines are commonly injected into muscle or skin of the host. The transformed muscle cells at sites of DNA injection clearly express antigen and act as a target for immune effecter cells. Therefore, the study of the eukaryotic expression of the gene that was subjected to a DNA vaccine is very important step on the way to the success of DNA vaccination [17]. All commonly approved antimicrobial drugs such as carbapenems, fluoroquinolones and aminoglycosides have failed to combat *A. baumannii* infections. Colistin, polymyxin and tigecycline, are considered as the last choice of therapy, but emergence of colistin and tigecycline resistant *A. baumannii* strains have been reported. The widespread emergence of MDR–XDR–PDR strains is likely to pose a challenge in the future [18]. Also, antibiotic resistance of clinical isolates in different region of Iran has been reported [6, 19]. Due to high degree of antibiotic resistance among *A. baumannii* isolates, preventive strategies such as vaccination may be a promising alternative. Although there is currently no licensed vaccine against this pathogen, several vaccine candidates, including inactivated whole cell, outer membrane complexes, outer membrane vesicles and some other *A. baumannii* antigens have recently been proven effective at some levels to protect against challenges in animals

from homologue strains and clonally distinct clinical isolates through active or passive immunization strategies. There are not many reports about DNA vaccination study in case of *A. baumannii*. The clinical applications of reported vaccine candidates are limited by the potential regulatory and safety, and these antigens do not directly target antibiotic resistance [7, 20]. *NlpA* plays an important role in the production of vesicles in stationary phase [13]. Vaccine based on outer membrane vesicles had high performance against infections caused by *A. baumannii* [16, 21].Thus cloning of *nlpA* gene as a vaccine target is considered to be a part of vaccination program. Therefore, we constructed a recombinant pEGFP-C2-*nlpA* plasmid as a vaccine candidate. Following amplification of *nlpA* gene, we sub-cloned it into pEGFP-C2 eukaryotic expression plasmid and studied its expression in HDF cells as a eukaryotic host cell. To date this is the first report on amplification, cloning and expression of *A. baumannii nlpA* gene worldwide. Therefore *nlpA* gene was selected for developing a promising vaccine target in order to prevent infections caused by *A. baumannii*.

In an investigation that was performed in 2013, researchers cloned *A. baumannii ompA* gene. In this work *ompA* gene was cloned at *Bam*HI and *Pst*I restriction sites into QE-32 expression vector and then *OmpA* recombinant protein was produced and purified. Thereafter, different doses of vaccine were injected along with Alum adjuvant to mice. The findings indicated that different doses of this vaccine have been able to stimulate the host immune system [22]. This work, due to use one of the most immunogenic factors, cloning procedures and suitable stimulation of immune system is similar to our work. However, expression vector, vaccine generation and selected gene were different in two studies. Huang et al., found elevated IgG level with injection of purified outer membrane vesicles (OMV) in mice model. Challenge with lethal dose of bacteria resulted in stimulation of IgM and IgG responses [23]. The results of antibodies stimulation were similar to our works, but we studied pneumonia model of infection which was found to be a promising level of protection. In another study Huang and colleagues, cloned the *ompW* gene of *A. baumannii* into the pThioHisA vector and transfected the resulting construct into the *E. coli* BL21 strain. This construct had high expression in bacterial host [19]. In the mentioned research, prokaryotic vector was used. However in both studies one of the most important antigenic factors was investigated and findings of immunization assays were approximately similar. Also, these researchers reported efficient specific IgG eliciting, increased viability and decreased cytokine levels due to immunization with Omp22 recombinant protein [21]. The results of antibodies elicitation and viability percentages were similar to our research. They produced a recombinant protein and used it as a vaccine candidate, but we injected DNA construct to mice, directly. Singh and coworkers using reverse vaccinology, selected pilus protein (FilF) among 57 proteins. This protein has protected amino acid sequence among different strains. These researchers separated *filF* gene from whole bacterial genome and cloned it into the prokaryotic expression vector, pET-28a. Thereafter, they studied its expression in *E. coli* BL21 strain [9]. The difference between this and our works is the use of prokaryotic vector and also gene expression study in prokaryotic host. However, we used eukaryotic expression system (eukaryotic vector and human dermal fibroblast cells) for the study of gene expression. The obtained results of stimulated antibodies and protection were the same in both researches. In an investigation in Iran, immunization of rBauA recombinant protein was studied in BALB/c mice and rabbit. The high titer of antibody was found and control animals died after 48 h [24]. These findings were similar to our results. However, this group did not assay cytokine levels. Modarresi and colleagues cloned *A. baumannii LuxI* gene into pTG19 T-vector and then sub-cloned it into pET28a expression vector. The expression host was *E. coli* BL21 strain. Four-fold increase of expression was found in comparison with control group [25]. The similarity of this work with our study is the cloning of one the most promising antigens. However, the studied expression vector of those work were suitable for prokaryotic system and they used Real-Time PCR method for gene expression study. Lin and colleagues investigated immunogenicity of *A. baumannii* recombinant protein rOmpA. The titer of IgG, IgM, IFN-γ, IL-4, and IL-17 were higher in vaccinated vs. control group [22]. Our findings are similar to results of their works. Al-zubaidi and coworkers studied immunization of *OmpA* conjugated to chitosan nanoparticle. They observed higher titer of IL-6, IL-2, and IFN-γ in immunized than control mice [26]. In these investigations, the vaccine preparation methods and techniques and utilizing vectors are different from our works. However, marker alteration and subsequent immune response and in vivo protection are similar to our findings. These researchers utilize first and second generation of vaccines. However, we study third generation of vaccine (DNA vaccine). DNA vaccine has several advantages in comparison with other vaccines, including stimulation both arms of immune responses (humoral and cellular), safety, stability in different temperatures, and simplicity of production process.

Conclusion

The pEGFP-C2-*nlpA* construct produced in this study, can express the *A. baumannii nlpA* gene in HDF cells. There is currently no licensed vaccine against *A. baumannii*. The successful expression of *nlpA* gene can be considered as a new recombinant vaccine strategy for use in animal models. The resulting recombinant construct induced the secretion

of IgG, IgM, INF-γ, IL-2, IL-4, and IL-12. Although our findings were promising, further experiments are needed to validate it as a DNA vaccine. This is the first study in which recombinant construct based *A. baumannii nlpA* gene was produced in order to become as DNA vaccine. For future investigation we suggest the immunization study of *nlpA* recombinant protein, the assessment of other important markers (such as IL-6, IL-1β, IL-10, TNF, IgG_1, $IgG_{2a, b, c}$), and the boosting of this vaccine by other reported promising candidates. Also, due to promising result of our prepared construct, we suggest study of DNA vaccines based on *nlpA* gene in other bacteria. Of note, this is the first study in which recombinant construct based *A. baumannii nlpA* gene were produced to become as DNA vaccine.

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Author Contributions RH; literature review, manuscript writing and data collection. AD; literature review and manuscript revision. MK; manuscript revision and MJ; literature review.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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