International Journal of Medical Microbiology 306 (2016) 1–9

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Immunoprotective potential of *in silico* predicted *Acinetobacter baumannii* outer membrane nuclease, NucAb

Nisha Garg [a], Ravinder Singh [a], Geeta Shukla [a], Neena Capalash [b], Prince Sharma [a,*]

[a] Department of Microbiology, Basic Medical Sciences Building, South Campus, Panjab University, Chandigarh, India
[b] Department of Biotechnology, Basic Medical Sciences Building, South Campus, Panjab University, Chandigarh, India

ARTICLE INFO

Article history:
Received 20 July 2015
Received in revised form 25 October 2015
Accepted 26 October 2015

Keywords:
Acinetobacter baumannii
Outer membrane nuclease
Reverse vaccinology
Vaccine
Cytokines
Epitopes

ABSTRACT

Acinetobacter baumannii is an emerging multi-drug resistant pathogen causing significant mortality in hospitalized ICU patients which demands developing new methods for prevention and treatment. *A. baumannii* 19606 proteome was analysed *in silico* through the online tool Vaxign for finding potential vaccine candidates. The selected nuclease (NucAb) was predicted to possess all the attributes of a promising vaccine candidate like outer membrane localization, high adhesin probability (0.53), one transmembrane helix only, non-homology to human proteins and presence of B-cell and T-cell epitopes binding with high affinity (percentile rank ≤ 1) to HLA alleles prevalent at high frequency in North Indian populations. *nuc*Ab gene was highly prevalent (100%) among the clinical isolates (40/40) and conserved (>98%) among NCBI sequenced *Acinetobacter* strains. It was cloned in pET28a, purified and its immunoprotective potential was validated in murine pneumonia model. Immunization of BALB/c mice with recombinant NucAb (25 μg) elicited high antibody titre ($1–5 \times 10^5$) which reduced bacterial load by 5 log cycles in lungs of mice challenged with optimized lethal dose (10^8 CFU). Lung histopathology revealed marked suppression of inflammation. Pro-inflammatory cytokines (TNF-α and IL-6) levels were reduced significantly and anti-inflammatory (IL-10) cytokine increased in lungs and serum leading to decreased severity and slow progression of disease. Though active immunization showed low survival rate (20%), passive immunization improved the survival (40%). This is the first study reporting an outer membrane nuclease as a vaccine candidate in Gram negative bacterium, *A. baumannii* through reverse vaccinology approach.

1. Introduction

Acinetobacter baumannii has emerged as one of the most common nosocomial pathogens worldwide (Higgins et al., 2010) and mostly targets the susceptible immunocompromised patients in ICUs where the natural barriers are breached by trauma, surgery or other invasive procedures (Fournier and Richet, 2006). *A. baumannii* is considered as equivalent to methicillin resistant *Staphylococcus aureus* and therefore is called as 'Gram-negative MRSA' (Wachter, 2006). Almost all the antibiotics used clinically like β-lactams, aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline, and rifampicin have been negated by this pathogen (Rosenthal et al., 2010) besides novel classes of antibiotics like tigecycline or colistin (Gordon and Wareham, 2009; Livermore et al., 2010). Its co-existence with other nosocomial pathogens like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in the pneumonia patients has brought this pathogen into limelight (Bhargava et al., 2012; Falagas et al., 2008). An *A. baumannii* strain has emerged from patients with minor underlying disease, showing hypervirulence in mouse models and also very resistant to antimicrobials (Jones et al., 2015). This scenario has initiated clinicians to delve into finding effective vaccine as an alternative or supplement to existing antimicrobials for its control. Several conventional vaccines like outer membrane protein (OmpA) (Luo et al., 2012), trimeric auto-transporter (Ata) (Bentancor et al., 2012), biofilm-associated protein (Bap) (Fattahian et al., 2011), outer membrane complexes (McConnell et al., 2010), outer membrane vesicles (OMVs) (McConnell et al., 2011), K1 capsular polysaccharide (Russo et al., 2013) and poly-N-acetyl-β-(1-6)-glucosamine (PNAG) (Bentancor et al., 2011) and whole-cell based vaccine (McConnell and Pachón, 2010) have been experimented and were providing protection to mice when challenged with pathogenic strains. However, due to the complications of solubility, variability and low prevalence of these antigens, exploration of other vaccine candidates which may provide broad spectrum protection against this evolving pathogen is required. To overcome these difficulties, new approaches like reverse vaccinology that

* Corresponding author.
E-mail address: princess@pu.ac.in (P. Sharma).

http://dx.doi.org/10.1016/j.ijmm.2015.10.005
1438-4221/

facilitate high throughput analysis of pathogens' proteomes and screen them for expression of immunogenicity (Rappuoli, 2001) have shown promising preclinical results (Davies and Flower, 2007; de Groot and Rappuoli, 2004) as in case of *Neisseria meningitidis* serogroup B (MenB) (Pizza et al., 2000), *Streptococcus pneumoniae* (Wizemann et al., 2001), *Mycobacterium tuberculosis* (Betts, 2002), *Bacillus anthracis* (Ariel et al., 2003) and Dengue virus (Baskar et al., 2011).

Nucleases are produced by both Gram positive and Gram negative bacteria either for their survival in harsh environments (Heun et al., 2012; Liechti and Goldberg, 2013) or for virulence in the host (Berends et al., 2010; Hasegawa et al., 2010; Juneau et al., 2015; Uchiyama et al., 2012). Presence of anti-nuclease antibody titres in human serum reflects its expression during infection (Reid et al., 2002) and these antibodies have also been used for diagnostics (Chang et al., 2011). However, the vaccine potential of nuclease is still obscure. In this study, an outer membrane nuclease (NucAb) was identified as a candidate vaccine protein through *in silico* analysis of the proteome of *A. baumannii* and was evaluated for its immunoprophylactic potential in a murine pneumonia model.

2. Materials and methods

2.1. Bacterial strains and plasmids

A. baumannii ATCC 19606 was used for the establishment of mouse pneumonia model and for immunoprotective studies. The organism was grown in Luria-Bertani (LB) medium at 37 °C for 24 h. Culture was maintained on Luria agar at 4 °C for routine use and as glycerol stock at −20 °C for long term storage. pET28a plasmid and *Escherichia coli* BL21 cells were from Novagen. Plasmid was maintained in kanamycin (25 μg/ml) containing LB medium. *E. coli* DH10β and BL21 were used as host strains for cloning and protein expression, respectively. Clinical isolates were collected from Government Medical College and Hospital, Chandigarh, India.

2.2. In silico *proteome analysis and selection of nuclease*

Annotated protein sequence data file of *A. baumannii* ATCC 19606 (reference strain) was retrieved from UniProtKB. *In silico* analysis of all the collected proteins was done using VAXIGN to predict the potential vaccine candidates (http://www.violinet.org/vaxign/). Vaxign (Xiang and He, 2009) integrates open source tools including **(i) PSORTb:** Prediction of subcellular localization (Gardy et al., 2005); **(ii) HMMTOP:** Transmembrane helix prediction (Käll et al., 2007); **(iii) SPAAN:** Calculation of adhesin probability (Sachdeva et al., 2005); **(iv) OrthoMCL:** Comparison of sequence similarity between predicted proteins and host (human and/or mouse) proteome (Li et al., 2003); and **(v) Vaxitope:** Prediction of MHC class I and class II binding epitopes (Bailey and Gribskov, 1998; Henikoff et al., 1999).

BLAST analysis was done using both nucleotide and protein sequences of nuclease (NucAb) to search for similar sequences and to build multiple sequence alignments. *A. baumannii* strains and other species of *Acinetobacter* used for bioinformatics analysis are listed in Table S1. Amino acid sequence of nuclease was subjected to prediction of both T-cell and B-cell epitopes through TEPITOPE and BEPITOPE online softwares, respectively at IEDB.

2.3. *Prevalence of* nucAb *in* A. baumannii *clinical isolates*

Using full length gene-specific primers, PCR amplification of entire *nucAb* gene was performed from different clinical isolates of *A. baumannii* to determine the prevalence of nuclease. This was also done to demonstrate homologous nuclease sequences distributed among various clinical isolates using same set of primers as for cloning (given below).

2.4. Cloning and purification of NucAb

The coding sequence of *nucAb* (2439 bp) was amplified from the genomic DNA of *A. baumannii* ATCC 19606 by PCR using the upstream primer (5′-AAAGGATCCATGAAAACTTTTCAATTA-3′) having site for BamHI at 5′ end and downstream primer (5′-TTTCTCGAGACTACGTTTAAAAAGCT-3′) having XhoI site at 5′ end. Double digested PCR products were ligated to the pET28a expression vector cut with same pair of enzymes. Recombinant pET28a-*nucAb* was transformed into *E. coli* BL21 cells by electroporation method and transformants were screened on Luria agar–Kanamycin medium and confirmed by restriction enzyme digestion. Expression of NucAb was achieved by culturing positive *E. coli* clone at 37 °C until optical density reached 0.4–0.6 at 600 nm and then induced with 1 mM of isopropyl β-D-1-thiogalactoside (IPTG) for additional 3 h at 37 °C. Expression was checked by running on 12% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). For purification under native conditions, expressed protein was extracted from the sonicated pellet in extraction buffer (50 mM phosphate buffer, 500 mM NaCl, 20 mM imidazole and 0.2% Tween 20, pH 8.0) and kept at 4 °C overnight. After centrifugation, supernatant was loaded onto the Ni-NTA agarose (Qiagen, Germany) affinity column and purified NucAb was obtained in elution buffer (50 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole, pH 8.0) according to the manufacturer's instructions. Purified protein fractions were dialysed against phosphate buffered saline (PBS). Concentration (280 nm) and purity (12% SDS-PAGE) were checked. The endotoxin level of purified recombinant protein used for immunization was also determined with Limulus Amebocyte Lysate (LAL) assay and was <1 EU/ml.

2.5. Animals and ethical clearance

Six to eight weeks old female BALB/c mice weighing between 20 and 25 g were procured from the central animal house of Panjab University, Chandigarh, India. They were maintained under pathogen-free conditions in clear polypropylene cages and fed a standard antibiotic-free diet (Hindustan Lever Products, Kolkata, India) and water *ad libitum*. The study was conducted after obtaining approval from the Animal Ethics Committee of Panjab University. All experiments were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All efforts were made to minimize the suffering of animals.

2.6. Mouse pneumonia model

Mouse pneumonia model was established according to the protocol of Choi et al. (2008). Briefly, overnight growth of *A. baumannii* ATCC 19606 from fresh glycerol stock in LB broth was passaged to late-logarithmic phase at 37 °C/150 rpm. Cells were washed twice, resuspended in PBS and used immediately. Desired CFU/ml was obtained by appropriate dilutions and the final concentration was quantified by plating serial dilutions onto LB agar plates. The mice were anaesthetized with intraperitoneal (i.p.) injection of xylazine and ketamine, placed in a supine position and their trachea were exposed surgically and inoculated with different doses of bacteria in a total volume of 50 μl. The incised area was sealed with sterile surgical sutures. Group of mice inoculated with only PBS served as control. At specific time intervals, bacterial counts were determined in the lung tissue homogenates of sacrificed mice.