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The detection of antigenic determinants of *Acinetobacter baumannii*

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ABSTRACT

Background: *Acinetobacter baumannii* continues to pose a threat to burdened patients in ICUs all around the world. Lately, infection control techniques are not sufficient to curb *A. baumannii*'s progression and chemotherapeutics are losing their potency against it. Thus, immunization became a key player in providing an ideal solution to the dilemma. None of the vaccines under investigation have reached the market and the search for a tailored vaccine remains a challenge. The notion of unravelling the bacterial antigens to design a novel epitope-based vaccine proved its merits.

Methods: In this work, the propitious polysaccharide and protein antigenic determinants of *A. baumannii* were mapped by mimicking the infection. The immune response was evaluated by western blot, ELISA, and cellular proliferation assay techniques.

Results: The screening showed that OMPs induced the most eminent sustained IgG response. In addition, OMP gave the highest cellular proliferation and a fold increase in ELISA that reached up to 10-fold by week 6. Whilst, the LPS gave a rapid IgM response, that reached 5-fold and the response was visible from week 1 in the western blot. The OMPs had a more pronounced effect in eliciting a cellular immune response.

Conclusion: The results elaborated the valuable role of using pure OMPs and detoxified LPS together; as a major cornerstone in designing an ideal vaccine against *A. baumannii*.

1. Background

Nosocomial infections happen when hospitalized patients acquire an infection from a microbe inhabiting the environment. These microbes usually acquire multi-drug resistance from the gene-pool available through other pathogens in the hospital. Therefore, they pose a great threat, especially to intensive care unit (ICU) patients [1]. International surveillance programs reported the prominence of respiratory tract infections as the major cause of hospital acquired nosocomial infections. Since the late 1990 and until now, the incidence ratio of respiratory infections increased varying from 47 to 65% among different countries [2–4].

A. baumannii became one of the most vicious nosocomial pathogens spreading infections in ICUs around the globe. It has caused numerous outbreaks worldwide [5]. Lately, *A. baumannii* succeeded as a multi-drug resistant (MDR) pathogen even against the last resort antibiotics such as carbapenems also known as "Gorillacillins" [6]. Global mortalities caused by *A. baumannii* reached almost 35% in 2014 [7]. *Acinetobacter* outbreaks were also correlated to wars and disasters with a higher prevalence in immuno-compromised patients [8,9]. These previous facts urge the use of deterrent measures such as infection control and vaccination against *A. baumannii* to protect prone patients [10]. However, infection control can be neglected in times of emergency and is highly dependent on the available resources, the contamination can persist to 25% in rooms post-cleaning [11,12]. Added to that, the antibiotics market is declining and the new pipelines are slithering to nil. This highlights the role of immunization as a fundamental pillar in preventing *A. baumannii*'s outbreaks [4].

Previous immunization trials showed promising potentials in inducing protective immunity both humoral and cellular, but none has thrived to reach the market yet; each having several *pros* and *cons*. In summation, the previous trials can be classified according to their components that is, whole cell vaccines such as Formalin-Inactivated whole cell (FIWC), Outer membrane protein complex (OMC) and outer

Abbreviations: OMP, outer membrane protein; LPS, lipopolysaccharide; ICU, intensive care unit; MDR, multi-drug resistant; FIWC, formalin-inactivated whole cell; OMC, outer membrane complexes; OMV, outer membrane vesicles; AUMH, Alexandria university main hospital; CPS, capsular polysaccharide; BAL, broncho-alveolar lavage; ELISA, enzyme linked immunosorbent assay; SI, stimulation index

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membrane vesicles (OMV). And the second category are pure protein vaccines that chiefly include outer membrane protein A (OmpA) and biofilm associated protein (BAP) and other outer membrane proteins (OMPs) [10]. Pure protein antigens alone can be rendered ineffective due to mutations that occur from antibiotic pressure or by a simple down-regulation in expression such as OmpA, Bap, OmpW, NucAB and Omp22 [13]. While the sole use of pure polysaccharide antigens cannot activate a cellular response to clear the pathogen's invasion such as K1 capsule [10,14]. On the other hand, previous literature revealed a spectrum of virtues offered by the "whole" vaccines, specifically the FIWC, OMV and OMC [10]. Nonetheless, several safety concerns arise due to the presence of lipopolysaccharide (LPS) [13] and unidentified bacterial components in the whole cell vaccines which may induce autoimmune diseases [15]. Recently, it was proved that the use of numerous antigens can produce an antibody repertoire noise which alters both the range and efficacy of immunization [16].

Furthermore, recent vaccination trials used *in silico* computational approaches a type of epitope mapping of antigens to identify the prominent epitopes to be further evaluated in animal models. However, these trials can only identify protein antigens and can have misleading results [10,17]. Simultaneously, another study from infected patients' serum has found that the whole OMPs concoction had a broader spectrum, and are more likely to overcome the bacterial defence-lines [18]. Although the antibodies raised against the bacterial LPS in whole vaccines can prevent the occurrence of a septic shock [19], no epitope mapping experiment have investigated the LPS's potentials. Thus, the use of pure form potential antigens defines and ensures equally, the antigen's quantity and quality [10]. Therefore, the present study aims to determine the potentials of both the protein and polysaccharide antigens of *A. baumannii*, that were revealed by mimicking the infection. This was followed by the assaying and evaluation of the produced immune responses.

2. Materials and methods

2.1. Evaluation of A. baumannii's incidence

A three-successive year survey in Alexandria University Main Hospital (AUMH) was performed in the tertiary university hospital. The hospital serves four geographical governorates in Egypt (Alexandria, Beheira, KafrEl-Sheikh and Matrouh) with a bed capacity of 3497. The retrospective study was done on 2566 broncho-alveolar lavage (BAL) samples from patients residing in the ICU starting from the first of January 2010 till the end of December 2012. Standard methods were applied to collect the appropriate samples [20], to confirm their suitability and nosocomial origin. *A. baumannii* was isolated from BAL samples, and identified by Gram-staining and biochemical tests according to the standard methods [21]. The isolates were subjected to antibiotic susceptibility profiling by disc diffusion method according to the NCCLS (National Committee for Clinical Laboratory Standards) regulations [22].

2.2. A. baumannii preparation and mice vaccination

Healthy 4–5 weeks old C57BL/6 mice were purchased from Misr University of Science and Technology (MUST, Egypt). They were kept at the animal house of the physiology department, Faculty of Medicine, Alexandria University, under the ethical conditions codes of the university. Whole cells of *A. baumannii* were deactivated with 0.05M-formalin for 18 h at room temperature. Then the bacterial cells were washed and re-suspended in (0.9%) normal saline for injection under aseptic conditions, to be finally diluted to 2×10^8 CFU/mL [23] using McFarland standard method [24]. The complete deactivation of the cells and the sterility of the preparation were confirmed by the sterility test on nutrient agar plates incubated at 37 °C for 3 days. The *A. baumannii* FIWC suspension was emulsified with an equal amount of Freund's complete adjuvant (Sigma-Aldrich, USA) for the initial dose, and incomplete Freund's for the booster dose after 21 days [23].

The mice were immunized subcutaneously with an initial dose of FIWC, then with a booster dose 3 weeks later. Concurrently, the control group was injected with sterile normal saline emulsified with the corresponding adjuvant.

2.3. Blood and spleen collection

Starting from day 0, and on weekly intervals, blood was collected via the retro-orbital artery and mice were sacrificed under the ethical codes of Alexandria University. Serum was separated from the blood and stored at −80 °C for further use. The splenocytes were collected aseptically from the spleen and were re-suspended in supplemented RPMI media (Biowest, France) for the proliferation assay to be discussed at a later point.

2.4. SDS-PAGE and western blotting technique

The *A. baumannii* isolate was subjected to electrophoresis on 12% polyacrylamide gels using Laemmli buffer system [25] versus a standard protein marker 15–175 kDa (Nippogenetics, Japan). The process was repeated several times to visualize the isolate with different stains. Protein bands were detected using Coomassie Brilliant Blue R250 stain (Serva, Germany) [26]. Alcian blue stain (Serva, Germany) was used to visualize the acidic capsular polysaccharide (CPS) [27]. Finally, silver stain was used to visualize the polysaccharides(mainly the LPS and CPS) and the protein bands as well [28].

The humoral B-cell immune response was studied by the western technique. It was used specifically as it is sensitive, simple, and able to screen polysaccharide antigens. Moreover, western blot can detect both linear and conformational proteins' antigenicity. In addition, it gives a full overview of the immune response against different antigens, especially when combined with ELISA [29]. Western blot was adopted from the method developed by Towbin et al. [30], and modified by Porsch-Ozcurumez et al. [31]. In addition, the manufacturer's instructions of the apparatus were followed, all at room temperature. Transblotting of the produced bands onto a nitrocellulose membrane (0.2 μm pore size Santa Cruz, USA) was done at 0.8 mA/cm^2 for 45 min in a transblotting unit (Biorad, USA), using Kyhse-Andersen discontinuous buffer system. The membrane was incubated with the immunized murine diluted sera (1:100) overnight. Consecutively, the membrane was washed and incubated with bovine anti-mouse IgG-secondary antibody labelled with horse radish peroxidase (HRP) (Santa Cruz, USA) with a dilution of 1:1000 for 2 h. The membrane was rinsed and bands were detected on X-ray films in a dark room with an enhanced luminol-based detection (ECL) substrate solution kit (Clarity™, Bio-rad USA). The same process was repeated but with goat anti-mouse IgM-HRP secondary antibody (Santa Cruz, USA).

2.5. Extraction of the selected antigens

The bacterial biomass was produced by cultivating the isolate in brain heart infusion broth at 37 °C for 18 h. The collected cells were washed with sterile phosphate buffer saline (pH. 7.4). The OMPs were extracted according to Filip et al. [32]. OMP was detoxified by adding 5% SDS and incubated for 10 min at 4 °C in order to remove the endotoxin contamination [33]. The OMPs were precipitated from the SDS-containing mixture by methanol/chloroform/water protein precipitation method [34].

As for the LPS, the semi-dry biomass pellet was stirred with chloroform/methanol in a ratio of 2:1 overnight at 4 °C to rid the cells of the contaminating phospholipids [35]. Then the biomass was washed with 0.1% cetavlon to remove the remnants of the CPS. Followed by an extraction using the hot-phenol water method based on Westphal and Jann technique [36]. The produced LPS was dialyzed (Visking® Dialysis