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A new strain of *Acinetobacter baumannii* and characterization of its ghost as a candidate vaccine

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ABSTRACT

Background: Human infection by *Acinetobacter baumannii* has been increased due to its resistance against most of the antibiotics. Therefore, the present study aimed to design a candidate vaccine against *A. baumannii* infection.
Methods: The protein and DNA contents of *A. baumannii* Ali190 were extracted using different critical concentrations of hydrogen peroxide, sodium hydroxide and sodium carbonate leading to the ghost of *A. baumannii* Ali190. Transmission and scanning electron microscope showed that it retained the 3D structure of its cell membrane. The ghost injected to rats via different routes of administrations including oral, subcutaneous, intramuscular, intraperitoneal, subcutaneous with adjuvant, and intramuscular with adjuvant.
Results: β-Lactamase OXA-51 gene, is a predominant gene in all Acinetobacter strains, the gene was partially sequenced. The DNA sequence of OXA-51 gene showed 98% homology with *A. baumannii* isolate 6077/12 and also showed less homology percentage with other strains of Acinetobacter. A new strain of Acinetobacter has been deposited in Gene Bank under accession number MG062776. All routes of ghost administration showed full protection against live bacteria except oral administration showed 67% protection. On the other hand, all non-vaccinated rats did not survive after infection with live bacteria. SDS-gel electrophoresis of protein patterns of both *A. baumannii* and its ghost showed common protein bands with molecular weights 70, 60, and 23 kDa which were detected using western immunoblotting after raising the primary antibodies against *A. baumannii* ghost. The levels of INF-γ were significantly increased in all vaccinated groups, particularly in subcutaneous and subcutaneous with adjuvant compared to the control group.
Conclusion: With the exception of oral administration, all vaccinated rats via different routes of ABG administration showed full protection (100%) against live *A. baumannii*. However, 100% mortality rate was observed in non-vaccinated rats. Therefore, ABG could be useful as a candidate vaccine against *A. baumannii* infection.

Introduction

Infectious diseases are the second leading cause of death worldwide. During the last few decades, *Acinetobacter baumannii* has been increasingly implicated in the outbreak of nosocomial infections as a cause of ventilator-associated pneumonia, bacteremia, and urinary tract infections, especially in intensive care units [1]. Antibiotic resistance in many pathogenic bacteria has spurred interest in generating vaccines to prevent those diseases. The antibiotic resistance caused by *A. baumannii* is on the rise, with a commensurate concern for public health [2]. *A. baumannii* is an opportunistic, Gram-negative, aerobic, non-motile, oxidase-negative, and catalase-positive human pathogen [1]. *A. baumannii* infections are associated with increased morbidity and mortality

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rate (23–71%), as well as prolonged hospital stay [3–6]. In response, many researchers have developed trails for *A. baumannii* vaccines, including inactivated whole cells [5], outer membrane vesicles [6], multiple bacterial antigens [7], rOmpA, and β-(1-6)-poly-*N*-acetyl-D-glucosamine (PNAG) [8]. Previous immunization trials showed promising potentials in inducing protective humoral and cellular immunity; however, they have not been used as a candidate vaccine before since previous trials did not show full protection against *A. baumannii* [8,9].

Bacterial ghosts (BGs) is an approach to develop a safe and potent vaccine against a variety of microbes [9,10]. The main idea of BGs is to get off the cytoplasm and its constituents without damaging the microbes' 3D structure or deforming their cells surface antigens [11]. There are several methods to produce bacterial ghost from Gram-negative species. The first method described was the expression of cloned E lysis gene from bacteriophage PhiX174 and this protocol is restricted only to Gram-negative bacteria. Then sponge-like (SL) protocol was introduced for preparation of BGs from Gram-negative and positive bacteria, as well as yeasts [12]. SL protocol is based on using critical chemical concentrations of NaOH, SDS, H_2O_2, and $CaCO_3$, to evacuate microbes from their cytoplasmic content including proteins and DNA [12,13].

A. baumannii ghosts (ABGs) could reduce the antibiotic resistance image [14]. This study was concerned with the preparation of ABGs from *A. baumannii* using Sponge Like Reduced Protocol (SLRP) with maintaining the 3D structure and the surface protein antigens, then using different immunological experiments aiming to test the use of the ABGs as a vaccine.

Materials and methods

All reagents used in the study were supplied by Sigma–Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated.

Bacterial strains

A. baumannii strain used in this study was kindly provided by the Department of Microbiology, Medical Research Institute, Alexandria University, Egypt. The purity of the strain was confirmed by colonial morphology on nutrient broth (NB), MacConkey agar, and Blood agar. Morphological features of the clinical strain of quest were investigated by Gram stain of non-lactose fermenting colonies on agar media, however, coccobacilli were examined for oxidase test and inoculated on Triple Sugar Iron (TSI) agar plates.

The collected sample was inoculated in 10 ml of nutrient broth and incubated at 37 °C for 24 h, inoculum from the grown culture was spread onto the surface of MacConkey agar plates and incubated at 37 °C for 48 h. Automated microbial identification of the isolated strain was performed by VITEK2 (an automated microbial identification system).

Genotypic detection of A. baumannii *using β-lactamase OXA-51*

Bacterial DNA was extracted using DNA extraction kit (KAPA Express Extract Kits). PCR amplification of the extracted DNA was carried out by Veriti Thermal Cycler (Applied Biosystems, California., USA), according to Mullis [15]. The primers were purchased from (Biosearch Technologies, California, USA). Nucleotide sequence of the primer for OXA-51S was ′5TAATGCTTTGATCGGCCTTG′3 and for OXA-51R was ′5TGGATTGCACTTCATCTTGG′3 [16]. PCR master mix DreamTaq Green was purchased from ThermoScientific, California, USA. Total volume of PCR reaction was 12 μl containing 6 μl master mix (containing DreamTaq DNA polymerase, 2X DreamTaq Green buffer with dNTP and MgCl2), 10 μM forward primer and 10 μM reverse primer each, 3 ng diluted DNA extract and 3 μl PCR grade water. The thermal profile was adjusted as follow; 1 cycle for denaturation at 95 °C for 3 min, 38 cycles for amplification; each cycle included 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min then 1 cycle at 72 °C for 10 min. Amplified target gene was detected by agarose gel electrophoresis.

Molecular identification of strain by 16S rDNA gene

16S rDNA gene was amplified via conventional PCR using the universal 16S rDNA primer set (F-5′-ATGCTTAGTGCTGGTTTAGG-3′ and R-5′ GCCTTCATCATTTCGCTTTC-3′). The forward primer spans from F8 to 27 and the reverse primer spans from 1510 to 1492 from 16S rDNA of *Escherichia coli* [17]. The PCR reaction volume was set to be 25 μl and contained 3 μl (30 ng) genomic DNA, 1.5 μl (15 pmol) of each forward and reverse primer, 12.5 μl 2X Master Mix (Intron Biotechnology, Korea) and 6.5 μl nuclease-free water. PCR reactions were set to be as follow: 95 °C for 5 min for initial denaturation followed by 30 cycles and each cycle was performed at 94 °C for 1 min for denaturation, at 58 °C for 1 min for annealing and at 72 °C for 30 s for extension then a final extension at 72 °C for 10 min. After termination of PCR reactions, the PCR product was detected using 1% agarose along with the DNA ladder (Gene Ruler™ 1000 bp and 50 bp) and visualized under UV-trans illuminator.

Purification and sequencing of PCR product

The amplified fragment of 16S rDNA gene was purified using PCR Clean UP-kit (Zymo Research., USA) following manufacturers' instructions. Purified PCR product was sequenced along with primers mentioned above. The sequence of 16S rDNA was determined with a model 373A automated fluorescent-DNA sequencer (Applied Biosystem Co., Ltd., USA), the obtained nucleotide sequence of 16S rDNA gene was processed through BioEdit version 7.2.5 software. Analysis of the PCR product sequence was analyzed using BLAST N, to determine the hits of subjects sequences deposited in the international nucleotide Gene Bank and gave the best matching with the query sequence.

Preparation of the bacterial ghost

ABGs were prepared according to Amara et al. [18]. The minimum inhibitory concentration (MIC) and minimum growth concentration (MGC) for each of NaOH, SDS, H_2O_2, and $CaCO_3$ were determined using twofold broth dilution method in NB medium, according to the standard criteria [10,19]. Tubes were incubated overnight, and both MIC and the MGC for each treatment were determined.

The quality of ABGs preparation was determined using light microscope, transmission electron microscope, and scanning electron microscope. Concentrations of both DNA and protein were determined spectrophotometrically. Samples from each ABGs preparation was checked for the existence of any viable cells according to the method of Amara et al. [12], and by acridine orange stain [20].

Experimental animals

Seventy male Sprague-Dawley rats weighing 70 ± 10 g were purchased from the animal house of the Institute of Graduate Studies and Research, Alexandria University, Egypt. Animals were handled in accordance with the principles of laboratory animal care, National Institute of Health (NIH) guide for laboratory animal welfare. The rats were maintained at a temperature of 25 ± 2 °C, relative humidity of 40–60%, with a 12 h light/dark cycle and free access to a pellet diet and water ad libitum.

After one week of acclimatization, rats were divided into seven groups (10 rats in each group; control, vaccinated rats via oral,

subcutaneous (SC), intramuscular (IM), intraperitoneal (IP), subcutaneous with adjuvant (SCA), and intramuscular with adjuvant (IMA)). For the last two groups, *A. baumannii* ghost suspension (10^8/ml) was emulsified with an equal amount of complete Freund's adjuvant (Sigma–Aldrich, USA) for administration of the initial dose [21]. The initial doses of ABGs were administered at 1st week then followed by two booster doses at 3rd and 5th week. Concurrently, the control group was injected with sterile normal saline emulsified with the corresponding adjuvant. On 6th week, all rats were challenged with 100 μl (10^8 CFU) virulent *A. baumannii* strain. Two days after challenge, all rats under the study were euthanized.

The blood samples were collected, 48 h before the initial dose and 48 h after each dose of ABG (i.e. at day 5, 9, 23 and 37), by aseptic venipuncture from the jugular vein, into plain tubes for serum separation, commercial tubes containing EDTA for complete blood count (CBC), which was performed according to the method of Dacie and Lewis [22], and in heparinized tubes for some immunological assays. Serum was separated by centrifugation of blood samples at 4000 rpm for 15 min, then stored at −20 °C to be used later.

Isolation of peripheral blood mononuclear cells (MNCs)

Peripheral blood mononuclear cells were isolated from blood samples according to the method of Perper et al. [23], by density gradient centrifugation using Ficoll-hypaque 1077 (Sigma, USA) at 1800 g for 30 min.

Preparation of splenocyte suspension

Spleens were collected, immediately after euthanization, then separately into sterile plastic Petri-dishes (7 × 1.5 cm) containing 50 mM phosphate buffer saline (PBS) pH 7.2. splenocytes were isolated from according to the method of Ghazy et al. [24].

Cell viability testing

The viability of peripheral blood mononuclear cells, as well as isolated splenocytes, were tested using 0.02% trypan blue dye exclusion technique [25], based on the impermeability of the viable cells to trypan blue. The percent of viable lymphocytes/ml was calculated according to the following equation: % of viable cells = (number of viable cells/total number counted) × 100 [24].

Assessment of cell proliferation by MTT assay

The immune stimulating effect of ABGs (i.e. cellular immune response) was determined by lymphocyte proliferation assay as described by Ghazy et al. [24] with minor modifications. In brief, isolated splenic lymphocytes were dispensed in 6 wells at a final concentration of 2×10^6 cells/ml supplemented with RPMI-1640 tissue culture medium in 96 wells flat-bottomed microtiter tissue culture plates (Greiner bio-one, Germany). The first triplicates were pulsed with 2 μl of ABG at final concentration of 10^8/ml, while the other triplicates left without the ghost reflecting the basal metabolic activity of the respective splenocyte suspension. Then the plate was incubated in the humidified CO_2 incubator (5% CO_2 and 95% O_2) at 37 °C. After 2 days of incubation, 10 μl of MTT reagent was added to each well and mixed gently and left for 2 h. 100 μl of crystal-dissolving solution was added to each well and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad, USA). The average values of ABG stimulated versus un-stimulated wells were calculated and used to determine the stimulation index (SI) as follow:

SI = mean of absorbance values of each stimulated well/

mean of absorbance values of un-stimulated wells.

Erythrocyte rosetting (E. rosette) test

E. rosette test was used to evaluate the immune response of T-cells against ABG, according to the method of Boyden [29]. 0.25 ml of T-cell suspension, 0.25 ml of sheep red blood cells (sRBCs) and 0.25 ml of antigen were mixed and incubated at 4 °C for 1 h.

Phagocytic activity test

Phagocytic activities of WBCs isolated from vaccinated and control rats were assessed according to the method of Nagl et al. [26]. In brief, 2×10^8 CFU/ml *A. baumanii* colonies (turbidity adjusted to McFarland standard 4.0) were suspended in 250 μl saline then mixed with equal volume of serum. The mixture was incubated for 30 min at 37 °C with occasional shaking. 250 μl of this opsonized bacterial suspension was mixed with 250 μl of neutrophil suspension (2×10^5cells/ml) then incubated for 30 min with occasional mixing. This was followed by centrifugation at 1500 rpm for 10 min at room temperature. After centrifugation, the supernatant was discarded and 50 μl of acridine orange (10 g/l) was added and left for 5 min. The suspension was washed twice with 1 ml normal saline and resuspended in 300 μl saline. Phagocytosis was determined using 40× fluorescent microscope. One hundred cells were counted in each slide. Phagocytic activity was expressed as the percentage of macrophages containing one or more ingested organism from the total number of cells.

$$\text{Percentageofpositivephagocyticcells} = \frac{\text{number of ingesting phagocytic cells}}{\text{total number of cells}} \times 100$$

Opsonophagocytic and killing activity of leukocytes

In a test tube; 100 μl leukocyte suspension (2×10^7 cells per ml), 100 μl of *A. baumannii*, 100 μl serum of normal or ABGs-treated rat, and 100 μl of complement source were mixed and incubated at 37 °C for 90 min. Samples were plated on to blood agar plates. The mean numbers of Colony Forming Unit (CFU) surviving in the various samples were counted [27]. The same procedure was repeated without adding leukocytes.

Measurement of IFN-γ levels

Interferon-γ levels were measured in serum samples 48 h before the initial dose of ABG and 48 h after each dose "i.e. at day 5, 9, 23 and 37"), using ELISA kit according to manufacturers' instructions (BMS62, IFN-gamma Rat ELISA Kit, Thermo Fisher Scientific). A standard curve was constructed by plotting the mean absorbance for each standard against its concentration on semi-logarithmic graph paper with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis. The concentration of INF-γ in each sample was determined and multiplied by the initial dilution factor. Levels of INF-γ were expressed as pg/ml.

Slide agglutination test

It was used to determine the presence of antibodies (humoral immune response) raised against *A. baumannii* in the serum of all studied rats. In brief, bacterial culture was added an to a glass slide and mixed with serum samples from vaccinated and/or unvaccinated rats, then the slide was gently rotated until agglutination was visible under light microscope. Each sample was formed in triplicates and the means of titer were calculated [24].

Delayed-type hypersensitivity test

Skin test was performed on the 18th day after vaccination with ABGs to test the efficacy of ABG vaccinated rats. The procedure was performed according to the method of Hudson and Hay [28].

Passive haemagglutination assay

It was used to evaluate the humoral immune response and was assayed according to the method of Boyden [29].

DNA fragmentation assay

It was performed according to the method of Marczynski et al. [30], to evaluate the apoptosis of WBCs as a result of ABGs administration.

Protein determination

The protein content was determined according to the method of Lowry et al. [31].

SDS-PAGE and western immunoblotting

SDS-PAGE was performed according to the method of Laemmli [32]. Pooled bacteria ghost *A. baumannii* (5 mg/ml) were mixed with sample application buffer and boiled for 3 min, after centrifugation of 2000 × *g* the supernatant was loaded into the wells. The run was started at 15 mA, till the dye passed the staking gel then the current was increased to 30 mA and 120 V until it reached the bottom of the resolving gel. The gel was then removed and ran blotted on to nitrocellulose membrane mini-blots (Hybond®). Following blotting the membrane was blocked skimmed milk. Polyclonal antibodies from rats were used as primary antibody, which binds specifically with the immobilized *A. baumannii* bacterial ghost proteins. The membrane was incubated with anti-rats Streptavidin-Horseradish Peroxidase (HRP) as secondary antibody [33]. Different protein bands were detected and captured on X-ray film [37].

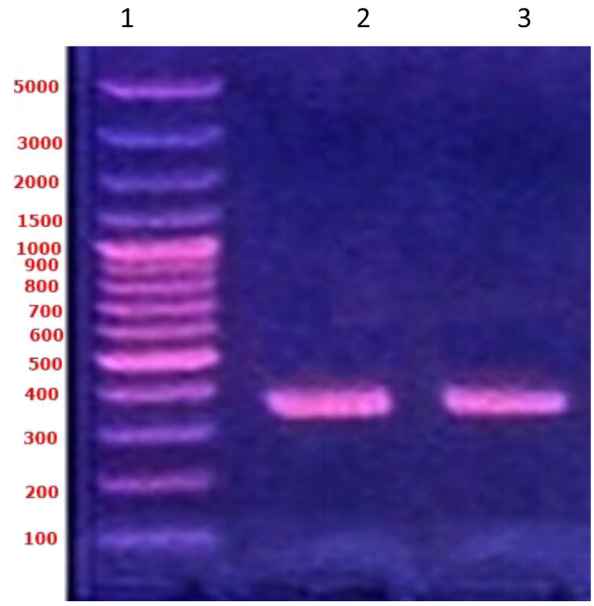


Fig. 1. Agarose gel showed the amplified DNA band of blaOXA-51 gene. Lane 1 represented DNA ladder, and lanes (2–3) showed 353 bp of blaOXA-51 gene of isolated *A. baumannii* using PCR.

Histopathological examinations

Livers and spleens of rats under the study were taken after challenging of the vaccinated rats with live bacteria and fixed in 10% formalin. The specimens were dehydrated in ascending grades of ethanol (70, 80, 90, 90, 100 and 100%), cleared in xylene and embedded in paraffin wax (blocking). Sections of 5–6 μm were sectioned using rotary microtome. The sections were conveyed to water bath of 52 °C, fixed on slides containing glycerin–albumin mixture as a thin film and dried by using hotplate at 40 °C overnight. Sections were stained with hematoxylin and eosin stains [34]. All slides were examined for histopathological changes.

5'ACGGGTGGGTAGCGATGCTCCTAGGAGTTAGAGCTACGTACTTCTGGTGCAACAAACTCTCATGG
TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATTCTGATCTACGATTACTAG
CGATTCCAACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGC
ATGACATCGCTGTGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGG
GGCATGATGACTTGACGTCGTCCCCGCCTTCCTCCGGTTTGTCACTGGCAGTATCCTTAAAGTTCCCA
TCCTAAAGGCGGGCAAGTAACGAAAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCAGGA
CACAAGCTGACAACAGCCAGGCAGCACCGGTATCTAGATTCCCGAAGGGACCAATCCATCTCTGGAA
AGTTTCTAATAAGTCAAGGACAGGTAAGGGTCTTCGCGTTGCATCCAAATAAACCACATGGTCCACC
GCTTGTGCGGGCCCCCGTCAATTCACTTGAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGTCTACT
TATCGCGTTAGCTGCGCCACTAAAGCCTCAAAGGCCCCAACGGCTAGTAGACATCGTTTACGGCATG
GACTACCACGGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCACCGTCAGTATTAAGCCAGAT
GGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTACGCATTTTCCGCTACACCTGGAATTCTACCATC
CTCTCTATACTCTAGCTCACCAGTATCGAATGCGATTCCCAGTTAAGCTCGGGGATTTCCATCCGACTT
ATAAGCGCCTACACACGCTTTACGCCCGTAAATCTGATAACGCTCGCACCTCTGTATACCGCGGCTGC
TGGGACAGAGTTATCCGGTGCTTATTCTGCGAGTAACGCCCCTATCTCTCGGTATTACTAAAGTAGCC
TCCTCCCCCTTAAAGTGCTTTCAACATAAAGGCTTTTTTACAACGGGGATGTGTGGATAAGGGTTCCC
CCTTGTCGAAAATTCCCCCTGCTGCCCCCCCAAAAAAACTGGGGCGGGTTCAATCCCCCGTGGGGCG
AAAACCCCTCAAACCGCTAAAAAAGTGCCCTTTGTGGGCTTTTCCCCCAACAAAGTATCCGGATTGAG
CGTCCTTTAAGGGAAGGGCGGAAAAACCCCCCTTTTTCCTCCGAAGGATGCGGTTTAA-3'

Fig. 2. Reverse sequence of 16S rDNA gene (1274 nucleotides) at FASTA format.

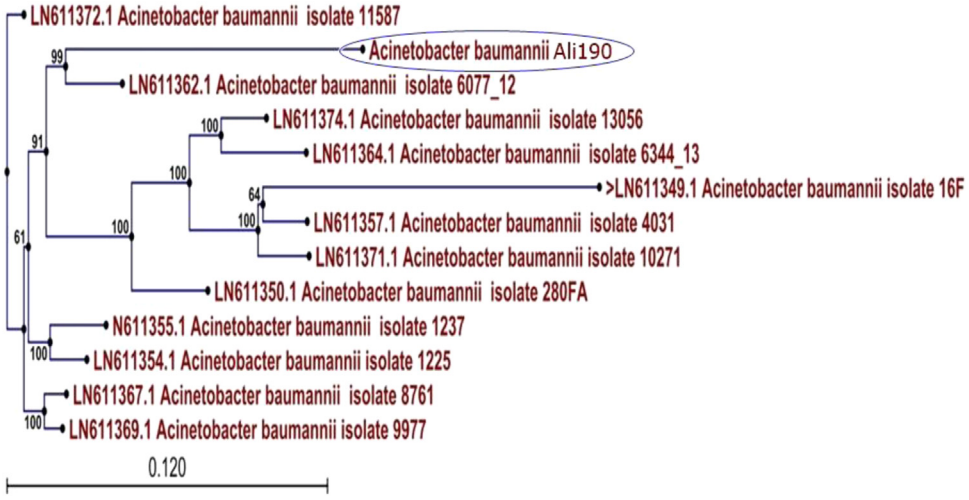


Fig. 3. Neighbor-joining tree showing the phylogenetic relationship between 16S rDNA sequence of the *A. baumannii* isolate and other 16S rDNA sequences belonging to closely related bacteria.

Statistical analysis of the data

All data were expressed as mean ± SE using IBM SPSS (Statistical Package for the Social Sciences) software version 22.0 [35]. For normally distributed data, a comparison between independent variations was performed using F-test (ANOVA) and post hoc test (LSD). The significance of the obtained results judged at the 5% level.

Results

Isolation and identification of A. Baumannii

Phenotypic identification of Acinetobacter isolates included in this study, by BioMerieux VITEK® 2 SYSTEM, was *A. baumannii* complex. Additionally, OXA-51-type carbapenemase, that is intrinsic to all *A. baumannii* and usually not associated with carbapenem resistance unless authentic expression of the carbapenemase, was amplified using PCR and the DNA band was visualized using agarose gel electrophoresis (Fig. 1).

Molecular identification of A. Baumannii *strain by 16S rDNA gene*

However, one of the limitations of a phenotypic method for bacterial identification is the inability to identify the bacterium on a species level in some cases. In the present study, the identification key led to an assignment of the bacterial strain of quest on a genus level only. This, in turn, addressed the indispensable need to identify the bacterial strain of quest on a species level via a molecular approach via 16S rDNA sequencing. After sequencing of 16S rDNA gene, a fragment of 1274 nucleotides was obtained (Fig. 2). The nucleotide sequence was analyzed via BLASTN algorithm of NCBI and showed that it has homology with 16S rDNA deposited in the international nucleotide databases with 93% identity, 98% query coverage with other *A. baumannii* and the e-value was 0.001 as shown in Fig. 3.

A. baumannii Ali190 showed 99% similarity to *A. baumannii* isolate 6077/12 (Fig. 3). Therefore, *A. baumannii Ali190* strain could be considered as a novel strain and has been assigned an accession number in GeneBank (MG062776).

Evaluation of ABGs viability and quality

DNA and protein concentrations were determined spectrophotometrically at 260 and 280 nm, respectively. The results showed the release of DNA contents during preparation steps of bacterial ghost as shown by agarose gel electrophoresis (Fig. 4). The viability of the prepared ABGs was monitored and compared with live *A. baumannii*, by the aid of OLYMPUS U-CMAD3 (JAPAN) and acridine orange staining which showed the green viable *A. baumanniiand* orange stained ABGs (Fig. 5A& B).

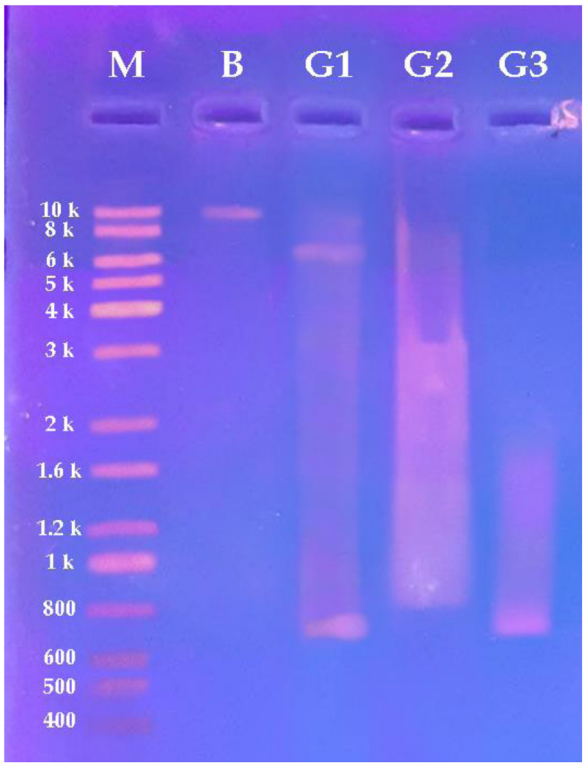


Fig. 4. Agarose gel showed the DNA in both live *A. baumannii* and its ghost after different steps of ghost preparation (M: marker, lane B: live bacteria, lanes 1–3 represent *A. baumannii* ghost).

In addition, smears of cells after each step of bacterial ghost preparation stained with crystal violet compared with live *A. baumannii* under LM (X100). The quality of the ABGs cells, based on the bacterial surface structure, showed the correct surface structure of the ghost (Fig. 6A & B). Moreover, SEM photos for ABG showed the correct 3D structure of cell wall and the pores resulted from treatment with chemical agents. There were no gross changes in cellular morphology (Fig. 7A–C).

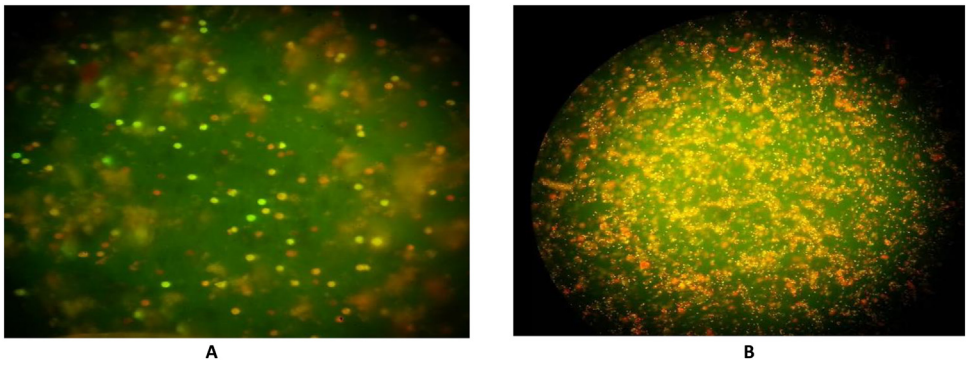


Fig. 5. Fluorescent microscope images showed live *A. baumannii* in green color (A) and its ghost in orange color (B) after staining with acridine orange.

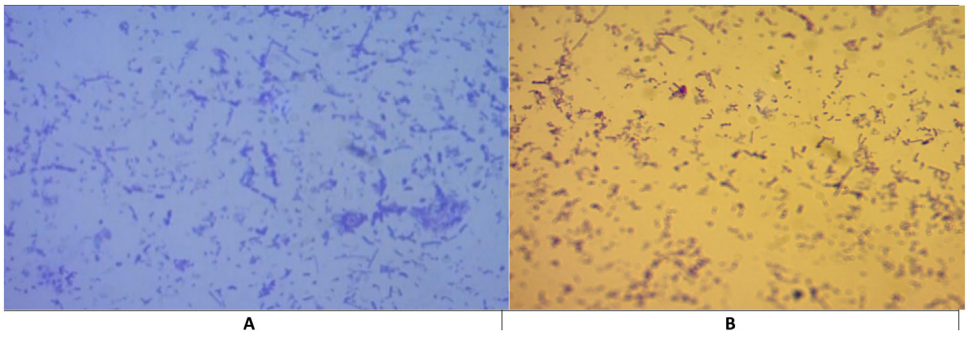


Fig. 6. Crystal violet staining showed the difference between live *A. baumannii* (A) and *A. baumannii* ghost (B) (100×).

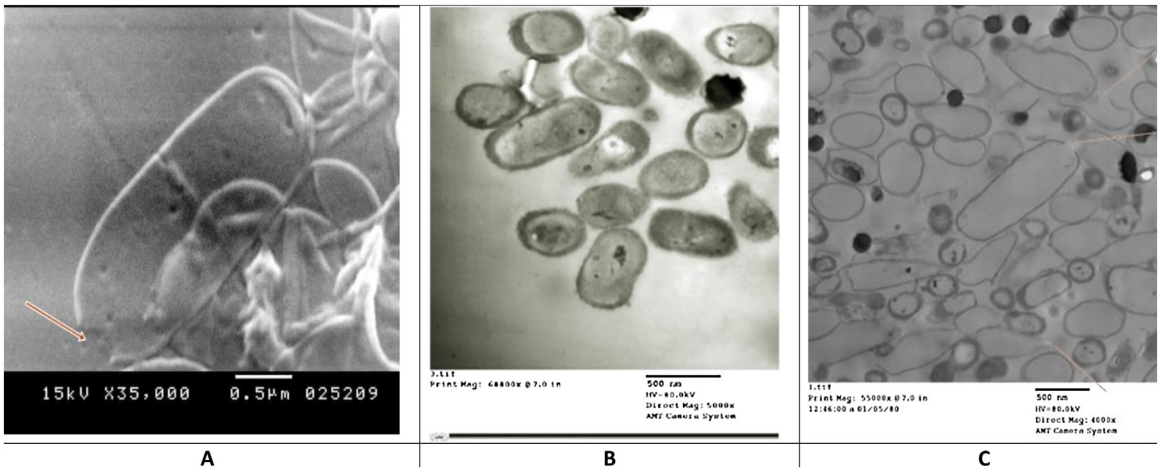


Fig. 7. (A) Scanning electron micrographs showed ABGs cells after chemical treatments. (B) Transmission electron micrographs showed bacterial cell live before chemical treatments. (C) Transmission electron micrographs showed ABGs cells after chemical treatments.

Table 1
Showed the changes in WBC count in vaccinated rats with BG via different routes of administration after booster doses.

Routs of administration							
WBCs* (10^3)	Control	IP	IM	SC	OR	SCA	IMA
1st dose	$6.57^{d}C \pm 0.21$	$7.17^{cd}D \pm 0.21$	$7.73^{bc}C \pm 0.06$	$8.90^{a}C \pm 0.36$	$8.40^{ab}D \pm 0.26$	$5.70^{e}D \pm 0.26$	$3.33^{f}E \pm 0.21$
2nd dose	$8.0^{d}B \pm 0.26$	$8.37^{d}C \pm 0.31$	$12.50^{b}B \pm 0.36$	$14.50^{a}A \pm 0.26$	$12.27^{b}B \pm 0.38$	$10.33^{c}C \pm 0.32$	$14.27^{a}C \pm 0.15$
3rd dose	$9.47^{f}A \pm 0.25$	$11.57^{e}A \pm 0.47$	$16.0^{bc}A \pm 0.72$	$14.80^{cd}A \pm 0.10$	$14.67^{d}A \pm 0.12$	$17.0^{b}A \pm 0.72$	$18.43^{a}A \pm 0.42$

Data were expressed by mean ± SD.
Means in the same column followed by the capital same letter were not significantly different at $P < 0.05$.
Means in the same raw followed by the small same letter were not significantly different at $P < 0.05$.
F: F test (ANOVA) for comparing between the different studied groups.

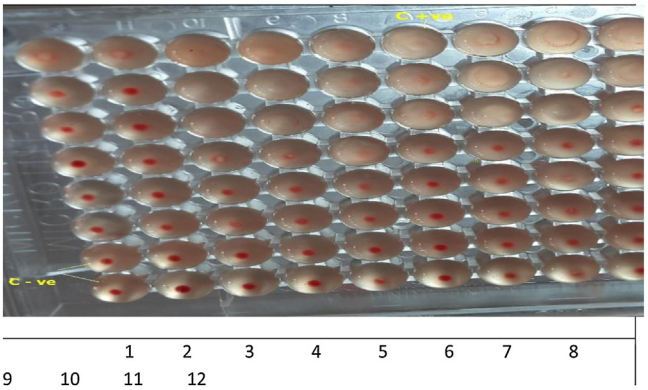


Fig. 8. Microplate of passive heamagglutination test (1, 2: control 3–12: vaccinate groups).

Effect of ABGs on WBC count

WBCs were counted in the seven groups after each vaccination dose. There was a significant increase in all vaccination groups compared to the control group ($P < 0.001^*$) (Table 1).

Lymphocytes viability and effect of ABGs on phagocytic activity of neutrophils

The viability of separated lymphocytes was tested in the all studied groups and did not show any significant difference (Fig. 8). This finding provided a strong evidence that ABGs was safe and did not have any toxic effects on immune cells. Testing of phagocytic activity of neutrophils was carried out against live bacteria. Results showed that phagocytic indices increased significantly among vaccinated groups compared to the control group ($P < 0.001^*$) (Fig. 9, Table 2).

Effect of ABGs on IFN-γ levels

INF-γ levels were measured in all the studied rats after administration of different doses of bacterial ghosts and challenging with live bacteria. The levels of INF-γ were significantly increased in all vaccinated groups, particularly in SC and SCA, in comparison with the control group. This indicates significant activation of T-cells, particularly TH1 cells among vaccinated rates. After challenging of rats with live *A. baumannii*, there was a significant increase in INF-γ level in the control group (Table 3).

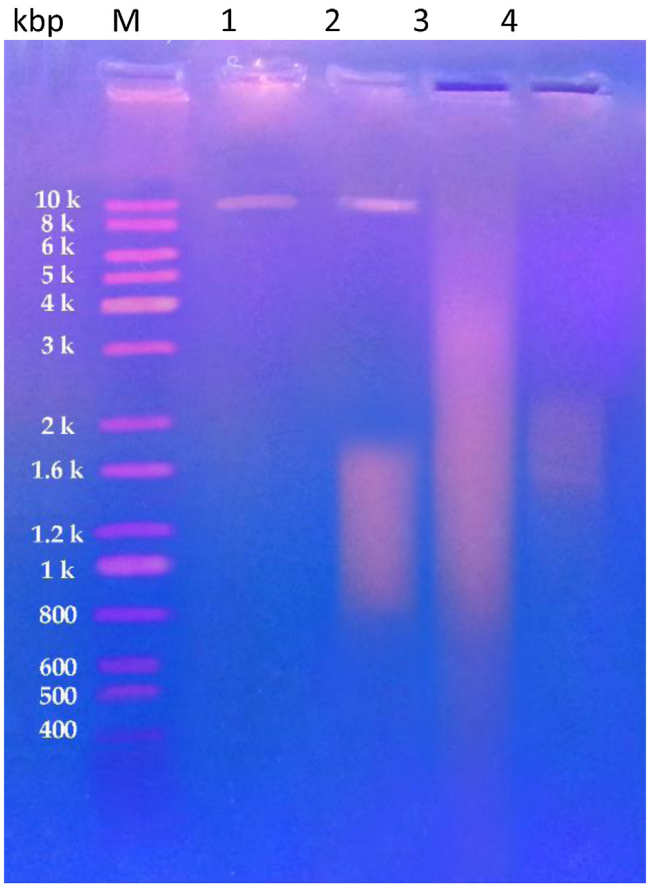


Fig. 10. Agarose gel electrophoresis showed the degree of DNA fragmentation among different treatments. M: marker lane 3: *A. baumannii* infected group, lanes 1, 2, 4: vaccinated groups.

Apoptosis

DNA Fragmentation was used as an indicator for the degree of peripheral blood mononuclear cells (PBMCs) apoptosis among all treated groups with either ABGs or *A. baumannii* (Fig. 10). Smear

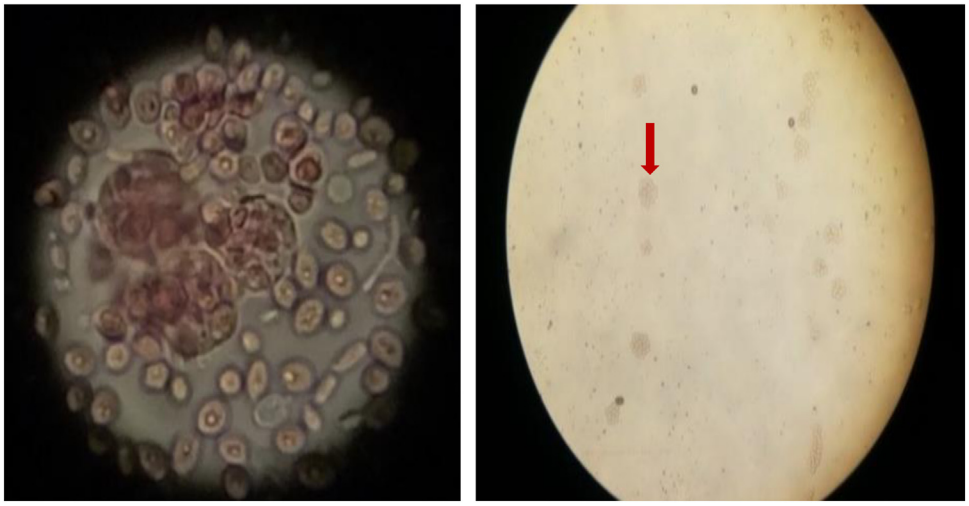


Fig. 9. Showed the phagocytic activity using live *A. baumannii* (LM, 40×).

Table 2
Showed the changes in Phagocytic activity in vaccinated rats with BG via different routes of administration after booster doses and challenging with live bacteria.

Phagocytic activity		Control	IP	IM	SC	OR	SCA	IMA
After vaccination	1st dose	$5.33^{c}B \pm 0.58$	$6.33^{c}D \pm 0.58$	$5.67^{c}D \pm 0.58$	$10.67^{b}C \pm 1.15$	$6.67^{c}C \pm 0.58$	$16.0^{a}C \pm 1.0$	$11.0^{b}C \pm 1.73$
	2nd dose	$7.67^{d}B \pm 0.58$	$21.0^{c}B \pm 1.0$	$31.33^{b}A \pm 1.53$	$10.67^{d}C \pm 0.58$	$10.67^{d}B \pm 0.58$	$22.0^{c}B \pm 2.0$	$41.67^{a}A \pm 1.53$
After challenge		$26.67^{a}A \pm 2.08$	$15.33^{e}C \pm 1.53$	$21.67^{b}C \pm 1.53$	$25.33^{ab}B \pm 0.58$	$15.67^{de}A \pm 1.15$	$19.33^{cd}B \pm 1.15$	$26.0^{a}B \pm 1.0$

Data were expressed by mean ± SD.
Means in the same column followed by the capital same letter were not significantly different at $P < 0.05$.
Means in the same raw followed by the small same letter were not significantly different at $P < 0.05$.

Table 3
Showed the changes in INF-gamma in vaccinated rats with BG via different routes of administration after booster dose and challenging with live bacteria.

INF-gamma Mg/dl		Control	IP	IM	SC	OR	SCA	IMA
After challenge	TC	$2104.3^{c}B \pm 4.5$	$2208.3^{b}A \pm 1.2$	$2317.3^{a}A \pm 8.5$	$2104.3^{c}B \pm 4.9$	$1847.3^{e}C \pm 7.6$	$1880.3^{d}C \pm 7.6$	$1888.0^{d}B \pm 16.5$
	3rd dose	$2612.3^{a}A \pm 4.2$	$1864.7^{f}D \pm 4.5$	$2201.0^{e}B \pm 7.0$	$2503.7^{b}A \pm 3.8$	$2322.7^{d}A \pm 3.8$	$2413.3^{c}A \pm 4.9$	$1781.3^{g}C \pm 8.5$
After vaccination		$1859.3^{d}C \pm 51.4$	$2011.3^{c}B \pm 5.9$	$2073.0^{bc}C \pm 47.6$	$2102.7^{b}B \pm 3.8$	$2195.7^{a}B \pm 4.9$	$2106.3^{b}B \pm 4.0$	$2015.0^{b}A \pm 5.6$

Data were expressed by mean ± SD.
Means in the same column followed by the capital same letter were not significantly different at $P < 0.05$.
Means in the same raw followed by the small same letter were not significantly different at $P < 0.05$.
F: F test (ANOVA) for comparing between the different studied groups.

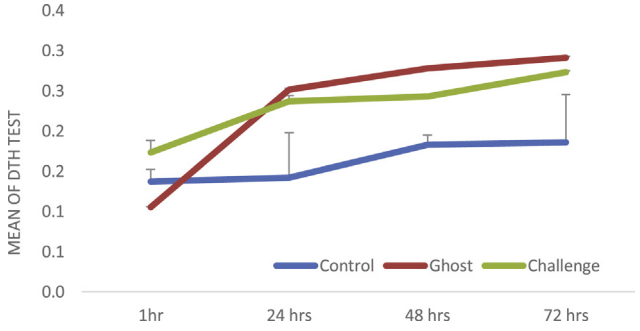


Fig. 11. Means of skin thickness of immunized and control groups in DTH test.

band appeared in the infected control group but not present in the vaccinated groups.

Delayed hypersensitivity skin test (DTH)

Delayed hypersensitivity skin test was recorded in all studied rats. There was an increase in skin thickness of the back of all rats after injection of AB proteins by 24, 48 and 72 h. (Fig. 11). Hypersensitivity was significantly decreased in all immunized groups compared with the control group ($P < 0.001$).

Effect of ABGs on immune response against virulent AB after lethal dose

The protective efficacy of ABGs vaccine against virulent bacteria was determined after challenging of all rats with virulent *A. baumannii* strain. After 5 days from challenging, the bacterial loads were assessed in liver, spleen and kidney homogenates in all treated and untreated groups and showed a significant decrease in all vaccinated groups compared with the control rats (Table 4).

SDS-PAGE and western immunoblotting

SDS-PAGE showed that the presence of multiple protein bands with molecular weights between 43 and 175 kDa in both live and bacterial ghosts (Fig. 12). Western immunoblotting was carried out to determine the molecular weight of the antigenic epitopes that are present on the surface of both live and ghost of *A. baumannii* (Fig. 13). The results confirmed the ability of antibodies raised against ABG to recognize and bind with four protein bands with

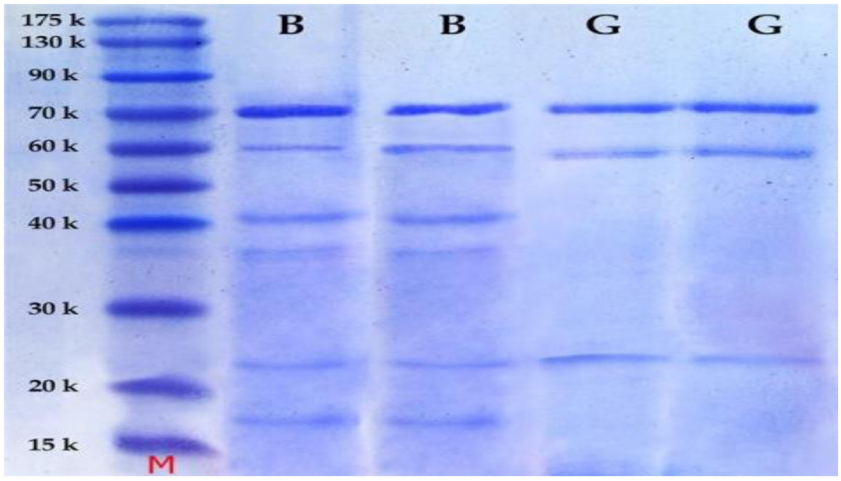


Fig. 12. Gel electrophoresis showed the protein patterns of live bacterial (lanes B) and bacterial ghost (lanes G) compared to the protein marker (lane M).

Table 4
Colony forming unit in different organs of rats after vaccination and challenging with lethal dose of live bacteria. Challenge in rat organs.

$\times 10^4$ (CFU)	Control	IP	IM	SC	OR	SCA	IMA
Liver	$21667^{a} \pm 1528$	$1100^{e} \pm 100$	$1500^{de} \pm 100$	$3167^{bc} \pm 153$	$4167^{b} \pm 153$	$2000^{cde} \pm 100$	$3100^{bcd} \pm 100$
Spleen	$11667^{a} \pm 1528$	$313^{d} \pm 15$	$3133^{c} \pm 153$	$5100^{b} \pm 100$	$4233^{bc} \pm 252$	$5167^{b} \pm 153$	$3133^{c} \pm 153$
Kidney	$18667^{a} \pm 577$	$120^{c} \pm 26$	$2133^{b} \pm 153$	$1100^{bc} \pm 100$	$1067^{c} \pm 58$	$1167^{bc} \pm 58$	$1047^{c} \pm 792$

Data were expressed as mean ± SD.
Means in the same column followed by the small same letter were not significantly different at $P < 0.05$.

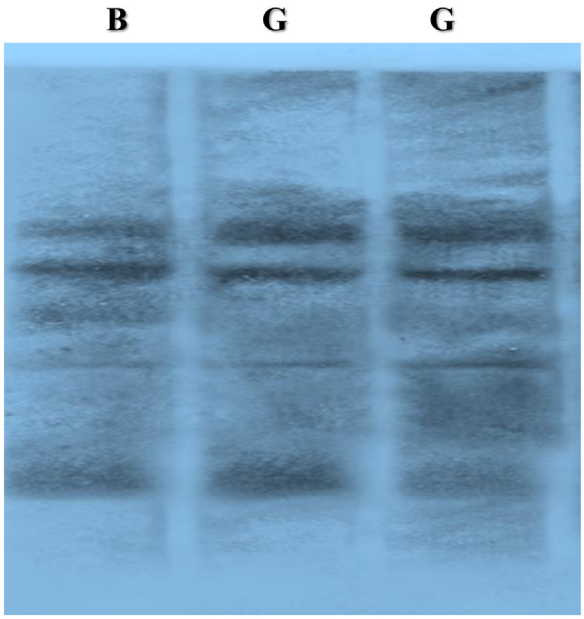


Fig. 13. Western immunoblot showed antigenic proteins in both live and bacterial ghost after using sera of immunized rats as primary antibody.

molecular weight 70, 60 40, and 30 kDa. These proteins may be responsible for the production of immunoglobulins.

Histopathological examination

Histopathological changes in the liver and spleen tissues of ABG-vaccinated rats were examined after challenging with live bacteria by 7 days (Figs. 14–19). Spleen section from positive control received live *A. baumannii* showed a marked depletion of white pulp and the presence of severe hemorrhage in the lymphoid tissue (Fig. 14). No change was noticed in the spleen section of negative control rat (Fig. 16). In the liver tissues, the negative control section showed peri-capsular fibrosis (single arrow), inflammatory cells (double arrow), and hemorrhage (triple arrow) (Fig. 17). The positive control section of liver showed sinusoids aggregation of lymphocytes (single arrow), congested sinusoids (double arrow) (Fig. 18), however, the liver section of rats vaccinated with ABGs then infected with *A. baumannii* showed a minimal peri-portal fibrosis (Fig. 19).

Discussion

A. baumannii infections represent public health problem due to their increased rates of antibiotic resistance. Moreover, it can cause a wide spectrum of diseases among patients receiving mechanical ventilation, residents of long-term care facilities, and military personnel [1,36,37]. Therefore, targeted vaccination of these patients may be the most effective in preventing *A. baumannii* infection. In the present study, we demonstrate that immunization with aluminum-adjuvant *A. baumannii* ghosts may represent a valuable strategy for vaccination against *A. baumannii*. ABGs were able to produce a robust antibody response, and significantly reduce post-infection bacterial loads in livers, spleen, and kidney of rats. This decrease might be due to the ability of BGs to induce both cellular and humoral specific immunity which consequently reduced the bacterial loads in these organs [38], which are in agreement the previous study compatible with other results [39,40]. Importantly, immunization with ABGs was able to protect rats from challenge with virulent *A. baumannii*.

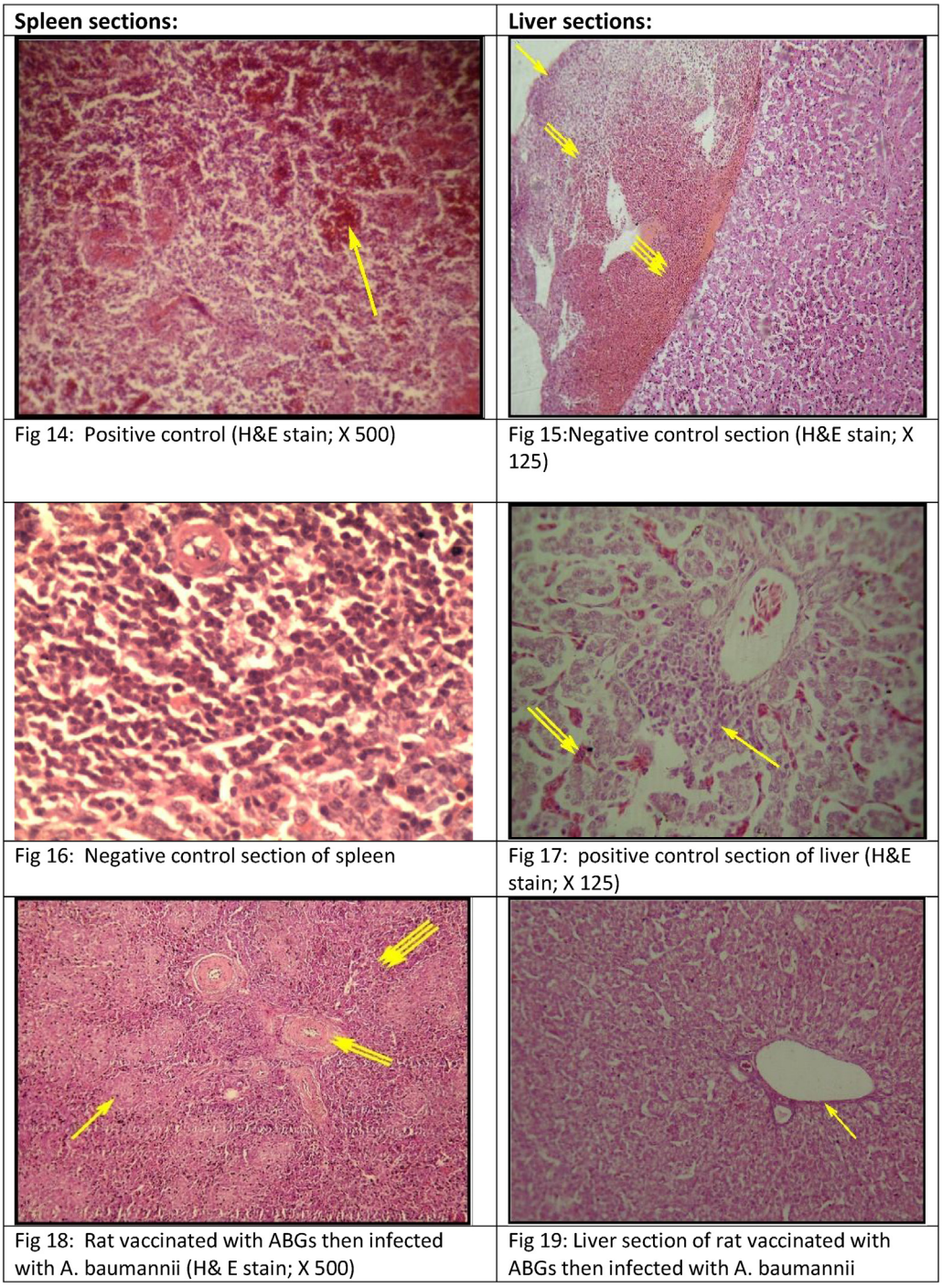
It has previously been shown that the surface expression of certain membrane proteins was down-regulated in *A. baumannii* during the acquisition of antibiotic resistance [13]. This may be problematic if these antigens are targeted by a vaccine composed of a single protein subunit. Conversely, protein subunit vaccines can require complicated and expensive purification processes, and may result in antigens that are distinct from their native form. An additional potential benefit of using ghost as vaccine is that evacuated whole cells with intact outer cellular membrane proteins are relatively easy and inexpensive to manufacture and the production process does not require denaturing of the vaccine antigens.

Recently, BGs drew increasing interest to be used as an immune stimulant [12,18]. The well-adapted method depends on using E-lysis gene, to turn viable cells to ghosts. However, the E-lysis gene method is restricted only to the Gram-ve bacteria. However, in the present study, the chemical method was more efficient in the evacuation of whole cells from DNA and protein contents without changes in the antigenic proteins of the cell membrane. Moreover, the chemical method used in this study could be applied to both Grams positive and/or negative pathogenic bacteria.

In the current study, ABGs displayed strong antigens that caused activation of phagocytic cells with rapid elimination of the bacteria and presentation of foreign antigens to both T and B lymphocyte. Therefore, active E-rosette indices were significantly increased among all ABG-vaccinated groups, especially SC group, when compared to the control group. This increase in E-rosette indices indicates activation of T-lymphocytes to form a rosette with sheep red blood cells. Additionally, ABGs were able to activate B-lymphocytes to produce specific Igs which are responsible for opsonization of the organism to enhance its phagocytosis. This was approved by the marked increase in agglutination between serums of vaccinated rats and live bacteria compared to the control group which did not show any noticeable agglutination. However, there were different degrees of activation of this humoral immune response which were dependent on the route of BGs administration.

Previous studies have indicated that identification of blaOXA-51-gene is a reliable and rapid method to identify *A. baumannii* [41,42] since it is mainly present in *A. baumannii*. Therefore, the blaOXA-51-like gene was detected and sequenced. However, the DNA sequence of blaOXA-51 gene showed 98% homology with *A. baumannii* isolate 6077/12 and fewer percentages of homologies with other strains. This finding confirmed that *A. baumannii* isolate is a new strain and named *A. baumanniiAli190* and registered in Gene-Bank under access number MG062776.

Results of the current study showed that ABGs of the *A. baumanniiAli190* caused activation of WBCs proliferation since there was a marked increase in WBC and neutrophils count. This increment could be related to the capacity of neutrophils for carrying out phagocytosis [43]. The ability of BGs to play an active role



Figs. 14–19. Histopathological evaluation of liver and spleen sections.

in recruiting a variety of immune cells (monocytes, macrophages, neutrophils, natural killer cells, dendritic cells, and memory T-cells) to the site of infection or inflammation was noted. These findings were in agreement with those of Alzubaidi and Alkozai, since they have shown that rats treated with outer membrane proteins (OMP) combined with adjuvant (Chitosa) had a significant increase in the total leukocytes counts compared with OMP alone and/or control groups [44].

After the challenge of the ABG-vaccinated rats with live bacteria; there was a marked decrease in agglutination titer compared to vaccinated group only. A higher dose of vaccine was found to be associated with higher antibody titers especially in SC-vaccinated rats compared to the control group. It has been found that the predominant IgG subtype generated was IgG1 rather than IgG2a or IgG2b, which was consistent with the Type 2 immune response [45]. In addition, in the present study levels of INF-γ were significantly increased among all ABGs vaccinated groups, particularly in SC and SCA groups, in comparison with those of the control groups. This increment might indicate the activation of TH1 cells among vaccinated rates

In the present study, in response to immunization with ABGs, we determined a broad-spectrum effectiveness of antibodies through evaluation of complement activation, opsonophagocytic-dependent killing, passive haemagglutination and determination

of bacterial loads in the liver, spleen, and kidney after infection with virulent strains. The titers of reactive antibodies from anti-ABGs sera were much higher than those of non-vaccinated sera, indicating that IgG antibodies recognized exclusively *A. baumannii* antigens, which was compatible with the finding of Arenas et al. They had reported that antibodies from vaccinated rats possessed significantly stronger and more homogeneous avidities compared to antibodies which recognized whole-cell surface-exposed epitopes [46].

It was reported that apoptosis of epithelial cells may disrupt the mucosal lining and allows for the access of bacteria or bacterial products to deeply invade tissues under mucosal epithelial cells. In this regard, the outcome of the infection with *A. baumannii* may depend on the induction of apoptosis in epithelial cells and OmpA-produced by *A. baumannii* which triggered the activation or apoptosis in dendritic cells [47]. In the present study, DNA fragmentation was used as an indicator for the degree of cell apoptosis among all vaccinated and non-vaccinated after challenging with virulent bacteria. A smear band of DNA was found in the non-vaccinated group compared to the vaccinated groups which confirmed cell apoptosis caused by *A. baumannii*. However DNA-smeared band was decreased in all vaccinated groups which might be due to neutralization and binding of bacterial antigens with specific antibodies produced against ABG. This result was in agreement with the finding of Alzubaidi and Stover [48]. In addition, all vaccinated rats strain via different routes of ABG administration showed marked protection (100%) against virulent *A. baumannii* except the oral administration of vaccine showed 67% protection only. However, 100% mortality rate was observed in non- vaccinated rats. This finding indicated the protective effect of the BG vaccine against the new strain of AB.

From all these findings, the chemical method yielded ABGs, and all assays of both cellular and humoral immune response revealed that ABG could be used as a candidate vaccine against *A. baumannii*. Moreover, the new strain was isolated, characterized and submitted in Gene bank under access number (MG062776).

Competing interest

All authors confirm that there is no known conflict of interest associated with this publication.

Acknowledgment

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