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Production and Evaluation of the Immunogenicity of an *Enterobacter aerogenes* Ghost Vaccine in a Mouse Model

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Abstract

Enterobacter aerogenes is a common cause of hospital-acquired infections such as pneumonia and urinary tract infections. Multidrug-resistant strains have emerged as a public health threat. In this study, we used a non-expensive method called the sponge-like reduced protocol (SLRP) to create *E. aerogenes* bacterial ghosts EAGs (deactivated bacteria) and evaluated their safety and immunogenicity in mice. The mice were given three doses of EAGs or EAGs with an adjuvant and then challenged with live *E. aerogenes* bacteria. The results showed that the EAG-immunized mice had higher levels of specific antibodies IgG, IgA, and IgM, lower bacterial load in internal organs after challenge, and improved histopathological examination of the liver and spleen compared to a negative control group. The serum from the EAG-immunized mice also had cross-reactivity with other *Enterobacteriaceae* pathogens using western blotting and enzyme-linked immunosorbent assay (ELISA). These findings suggest that the EAG vaccine may be an effective and safe alternative for the prevention and control of multidrug-resistant infections caused by *E. aerogenes*. This study showed that *E. aerogenes* ghosts could potentially be used as a vaccine.

Keywords:Enterobacter aerogenes, Bacterial ghosts, Vaccine, Immunogenic response, Cross-reactivity, Sponge-like reduced protocol (SLRP);

1. Introduction

The growing problem of multidrug resistance (MDR) in bacterial infections is a global concern [1]. Carbapenems, a beta-lactam antibiotic, are usually effective against most gram-negative bacteria. However, the appearance of carbapenem-resistant *Enterobacteriaceae* (CRE) such as *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Escherichia coli*, has led to increased morbidity and mortality in hospital-acquired and long-term care-related infections [2]. *E. aerogenes*, a gram-negative bacterium from the *Enterobacteriaceae* family, is a common inhabitant of the human gastrointestinal tract and environment [3]. It has been identified as a primary opportunistic pathogen for humans [4] and is resistant to many antibiotics used to treat infections caused by *Enterobacter* [5]. *E. aerogenes* can cause a range of hospital-acquired infections, including bacteremia, pneumonia, surgical site infection, urinary tract infection, and meningitis[3] .

Vaccination has been recommended as the best approach to avoid infections from intracellular infectious pathogens [6]. The bacterial-ghost approach may provide a novel unconventional way to improve such a vaccine [7]. Bacterial ghosts (BGs) have been established against various microbial pathogens [8]. BGs are empty cell envelopes derived from gram-negative

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bacteria [9]. BGs preparation involves causing lysis of the bacterial cells through the controlled expression of the PhiX174 lysis gene E [10,11], or through the use of a minimum inhibitory concentration of certain chemicals [12, 13]. BGs produced using the second method can be used to lyse not only gram-positive and gram-negative bacteria, but also yeast and fungal cells [14, 15]. This method is advantageous as it is a rapid, cost-effective, and simple process with no latent risks [16]. BGs have been explored as vaccine candidates due to their ability to stimulate both humoral and cellular immunity in experimental animals against different infections [17, 18].

Therefore, this study aimed to produce safe bacterial ghosts from *Enterobacter aerogenes* and to evaluate their safety and immunogenicity in the mice.

2. Materials and Methods

2.1. Bacterial Strain and Growth Media

The *Enterobacter aerogenes* reference strain ATCC 13048 was provided by MERCEN at Ain Shams University in Egypt. This strain was regularly cultivated in Nutrient Broth (NB) or on Nutrient Agar (NA) plates at 37°C.

2.2. The minimum inhibitory and minimum growth concentrations of various chemicals

The minimum inhibition concentration (MIC) and minimum growth concentration (MGC) of H_2O_2, SDS, and NaOHwere determined using standard criteria [19]. Stock solutions of 30% H_2O_2 and 10% SDS and NaOH were prepared. 1 ml of each solution was added to 9 ml of medium in a 20 ml test tube, resulting in a final volume of 10 ml and a 1: 10 dilution. 100 µL of an overnight *E. aerogenes* culture was added to each tube in the serial dilution test. For +1 and -1 values of 1.05 µg/mL and -1 value of 0.35 µg/mL respectively, were used. The test tubes were incubated overnight at 37°C in static conditions [20].

2.3. Production of E. aerogenes ghosts

Sponge-likereduced protocol (SLRP) was used to make EAGs as described by Amara et al. [20].The biomass of the 72 h cultivated *E. aerogenes* culture was collected by centrifuging the bacterial broth at 4000 rpm for 10 min. The cells then washed gently by 0.5% saline and recentrifuged at 4000 rpm for 10 min. The supernatant was then discarded and t*he E. aerogenes*cells were collected as a pellet. The experiment was conducted in three steps. In the first step NaOH, SDS, and $CaCO_3$ were added to the bacterial pellet. After 1 hr incubation the mixture was centrifuged, and the cells were collected as cell pellets using centrifugation at 4000 rpm for 10 min. The supernatant was then transferred to clean and sterile Falcon tubes. The cell pellets were then washed with 0.5% sterile saline and re-centrifuged. The supernatant then discarded, and the cells suspended in H_2O_2 for 30 min. The cells and the supernatant were collected each as above. The cell pellets then washed by saline solution followed by centrifugation (as above). Finally, in the third step, the cell pellets were resuspended in 60% Ethanol and left at room temperature for 30 min with gentle vortex each five min for 30 sec. The cell pellets collection and washing were repeated as above.

2.4. The DNA and protein concentration estimation

The DNA and protein levels were determined in the collected supernatants from the three experimental steps of ghost preparationusing a spectrophotometer following the protocol described in [21].The concentration of the DNA was determined by measuring the absorbance at 260 nm using quartz cuvette. An extinctionE_{260} = 1 corresponds to 50 μg dsDNA mL^{-1}. Whereas the protein analysis was determined using the spectrophotometer at 280 nm using quartz cuvette. The protein different concentration was derived from Bovine Serum Albumin standard curve.

2.5. EAGs assessment by Light Microscope (Optika)

The quality of the EAGs was evaluated using crystal violet staining and examination under a light microscope to determine the 3D shape of the cells as either intact or deformed [22].Smear of cells of ghost and viable *E. aerogenes*were prepared and stained with crystal violet for two minutes and investigated under light microscope.

2.6. Assessment of EAGs by Transmission Electron Microscope

A Transmission Electron Microscope (JEM-1400) was used to assess the correct EAGs 3D empty structure [22].

2.7. Viability Test

The viability of the prepared EAGs was evaluated by incubating a sample on Nutrient agar and MacConkey agar plates for three days at 37°C . The presence of any growing colonies was used to determine the number of still viable cells [22].

2.8. Animals and Experimental design

120 male Swiss-Albino mice, each weighing 25 ± 3 g, were obtained by Faculty of Medicine at Zagazig University and housed in the animal house at the Faculty of Science at Zagazig University in Egypt. The mice were fed on a normal diet and water ad libitum. All mice experiments were performed following the ethical committee – Zagazig University. Number ZU-IACUC/1/F/390/2022.

After 14 days of acclimation, mice were equally divided 4 groups according to the treatment type as follows: -

Group-I: NC (Negative control) non-vaccinated control group not inoculated with bacterial ghosts.

Group-II: PC (Positive control) non-vaccinated group challenged with viable *E. aerogenes* bacteria

Group-III: EAG (EA bacterial ghost) vaccinated group. Each mouse received intraperitoneally 200 µl (10^7 CFU) of EAGs in sterile saline.

Group-IV: EAGA (EA bacterial ghost + adjuvant) vaccinated group. Each mouse received intraperitoneally 100 µl (10^7 CFU) of EAGs in sterile saline + 100 µl Freund's adjuvant. The EAGs were administered in an initial dose on day 0, followed by booster doses on days 14 and 28.

2.9. Challenge test

After 14 days from the final vaccination, all mice "except the NC group" were IP injected with 100 µl (10^8 CFU) viable *E. aerogenes* strain in sterile saline.

2.10. Bacteriological analysis

The organs (liver, kidney, spleen, testes, small intestine) and stool of the mice were collected and homogenized in saline solution after 7 days of the challenge. The homogenates were exposed to serial dilutions and the number of colony-forming units (CFUs) was calculated in triplicate. The mean bacterial loads per gram of each organ were then estimated [23].

2.11. Measurement of serum IgG, IgA, and IgM antibodies

To estimate the levels of the IgG, IgA, and IgM antibodies triggered by the EAGs, mice serum samples collected on days 7, 21, 35, 49 and 56 were tested using a whole-cell ELISA in which *E. aerogenes* bacteria were used as the coating antigen following the protocol described by Ran, Xuhua, et al. [23].

2.12. Detection of antibodies cross-reactivity with other Enterobacteriaceae pathogens 541

Serum collected after the vaccination period was tested against *Enterobacter aerogenes*, *Escherichia coli*, *Enterobacter cloaca*, and *Klebsiella pneumonia* to detect cross-reaction using whole-cell ELISA and western blotting [24].

2.13. Histopathological examination:

The mice tissues of liver and spleen preparation followed Bancroft and Stevens procedure for histological preparations [25].

2.14. Statistical method

Statistical analysis was done using one-way ANOVA with SPSS software (version 14.0). The data were expressed as the mean ± standard error of the mean [26].

3. Results

3.1 Preparation of E. aerogenes ghosts

The main step involved determining the MIC and MGC of the various chemicals used. For NaOH, the MIC and MGC were 1 mg/ml and 0.1mg/ml, respectively. For SDS, the MIC and MGC were 0.2 mg/ml and 0.02 mg/ml ,respectively. For H_2O_2, the MIC and MGC were 0.3 mg/ml and 0.03 mg/ml, respectively. For $CaCO_3$, the used concentration of the +1 value was 1.05 µg/mL and -1 value of 0.35 µg/mL.

3.2 EAGs Quality and Viability check

The results indicated that the DNA and protein were released correctly during the protocol steps (as shown in Table 1).

No growth was observed for the prepared EAG cells when cultured on nutrient agar and MacConkey agar plates for three days at 37°C The quality of the prepared EAGs was evaluated using light and electron microscopy, which showed that the cells maintained their 3D shape (as shown in Figures 1 and 2).

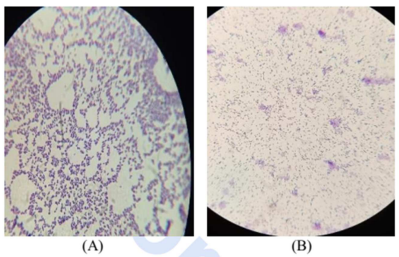
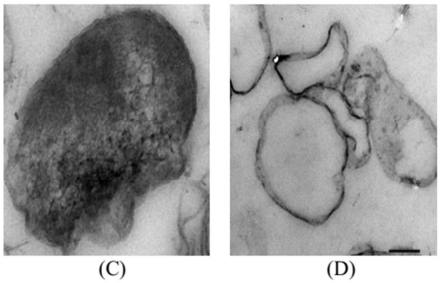


Fig. 1: Crystal violet staining revealed the distinction between lives *E. aerogenes* (A) and *E. aerogenes* ghost (B)



(C) (D)

Fig. 2: Transmission electron micrographs showed live *E. aerogenes* cells before chemical treatments (C) and *E. aerogenes* ghost cells after chemical treatments (D)

Table 1: The amount of Protein and DNA released during various steps in ghost preparation

	NaOH+SDS+$CaCO_3$ Step	H_2O_2 step	Ethanol Step
Protein (µg/ml)	1230	267	34
DNA (µg/ml)	125	23	3.5

3.3 Bacteriological analysis of tissue homogenates

The protective value of the EAGs against viable *E. aerogenes* was established by challenging mice (except the NC group) with the viable *E. aerogenes* strain. After 7 days, the bacterial load was measured in the liver, spleen, kidneys, testes, small intestine, and stool homogenates of all vaccinated and non-vaccinated groups. The results showed a considerable decrease in the two vaccinated groups compared to the non-vaccinated group (Fig.3).

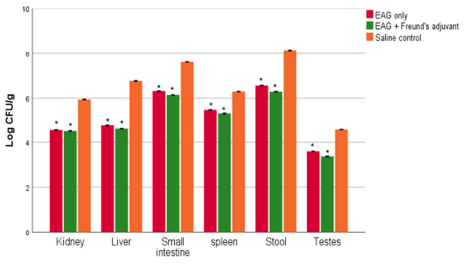


Fig. 3: Bacterial loads in kidney, liver, small intestine, spleen, testes, and stool of mice post challenge

3.4. Serum antibodies analysis

Specific anti-*E. aerogenes* antibodies (IgG, IgA, and IgM) were detected in the sera of mice vaccinated with EAGs or EAGs combined with Freund's adjuvant. In contrast, no specific reactivity was found in the saline controls (Fig. 4,5-6). The titer of IgG, IgA, and IgM antibodies from animals immunized with EAG combined with Freund's adjuvant was slightly higher than those vaccinated with EAG only.

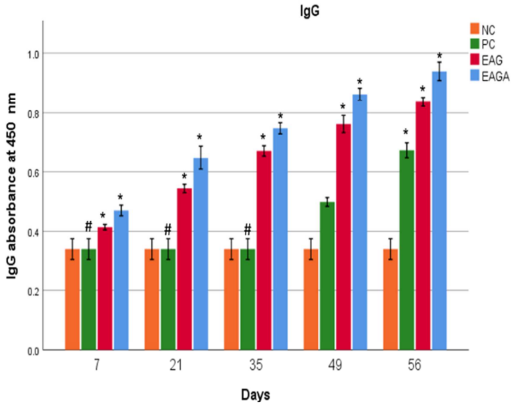


Fig. 4: The levels of IgG antibody responses were determined by whole-cell ELISA

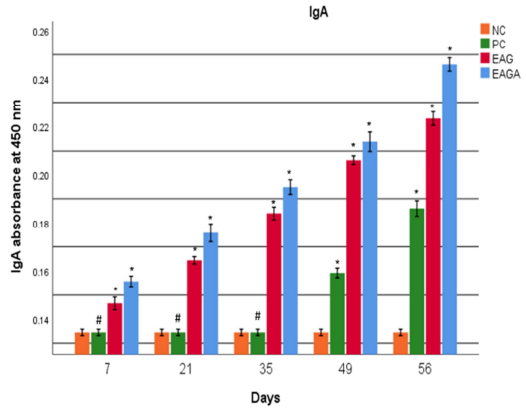


Fig. 5: Whole-cell ELISA was used to determine the levels of IgA antibody responses

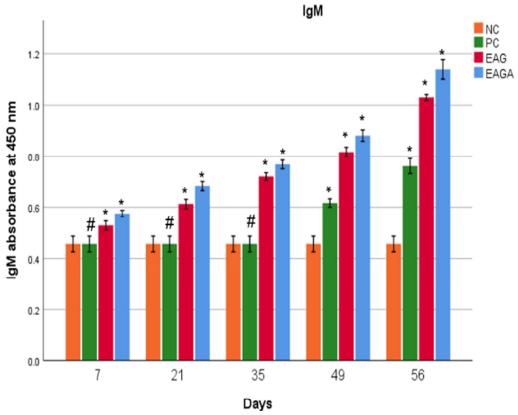


Fig. 6: The levels of IgM antibody responses were determined by whole-cell ELISA

3.5. Cross-reactivity of serum IgG with other Enterobacteriaceae pathogens

Serum collected from EAG-immunized mice after vaccination showed significant reactivity with *E. coli, E. cloaca,* and *K. pneumonia* cells adsorbed on ELISA plates (Fig. 7). Furthermore, a western blot of different bacterial lysates using anti-EAG serum revealed a clear, strong band (Fig. 8).

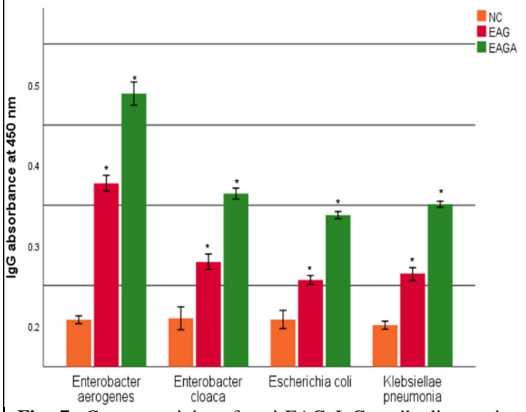


Fig. 7: Cross-reactivity of anti-EAG IgG antibodies against different *Enterobacteriaceae* strains using whole-cell ELISA.

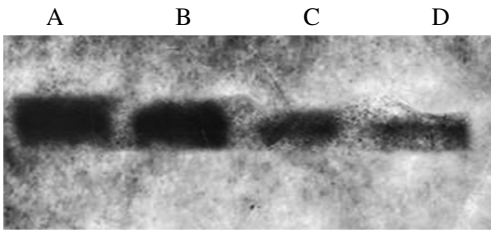


Fig. 8: Western Blot of EAGs antigen detection by X-ray (A) *E. aerogenes*(B)*E. coli*(C)*E. cloaca* (D)*K. pneumonia*

3.6. Histopathological Study

The liver and spleen tissues histopathological modifications were examined 7 days after *E. aerogenes* challenging (Figures 9 and 10). Photomicrographs of liver sections showed the effect of vaccination on the histological structure of hepatic tissue in the different study groups: (A & B) Liver sections from the negative control group (NG) showed a typical central vein (CV) and portal area (PA) enclosing the hepatic artery (HA) and bile duct (BD) surrounding the portal vein. Notice hepatocyte (H) organized in regular cords and sinusoids (Si) between them. (C & D) Histological liver sections from the positive control group (PC) highlighted hepatocytes in different forms; deep basophilic (H), pyknotic (P), and necrotic ones (N), degenerated cords (D), dilated sinusoids (S) homing few inflammatory cells (I), aggregated inflammatory cells (IN) encircling portal area, epithelial desquamation (ED) and congestion of portal vein (Cn). Liver sections from EAG (E & F) and EAGA (G & H) groups exhibited better architecture except[543] for epithelial desquamation of central vein (ED), aggregated (IN), and dispersed (I) inflammatory cells, as well as dilated sinusoids (Si) attaching some lymphocytes (L).

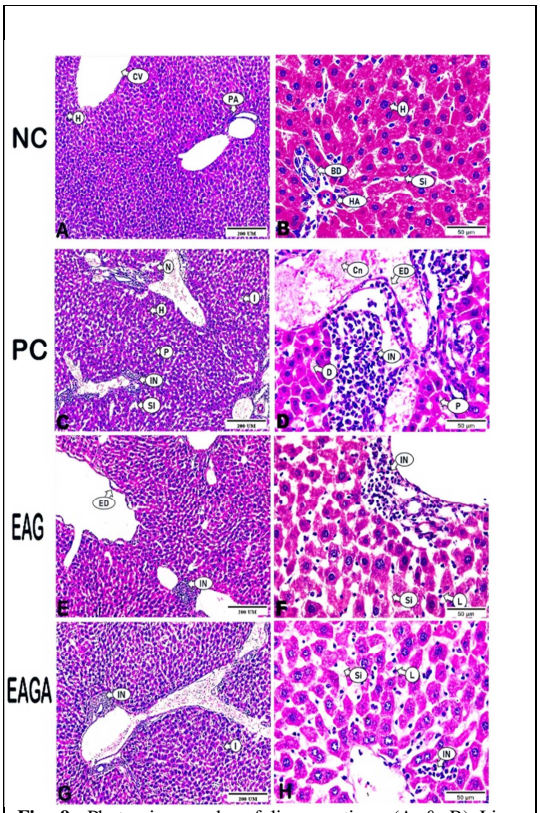


Fig. 9: Photomicrographs of liver sections (A & B) Liver sections from the negative control group, (C & D) liver sections from the positive control group, (E & F) liver sections from the EAG group and (G & H) liver sections from EAGA group

Photomicrographs displayed the outcome of vaccination on the histological structure of splenic tissue among animals of examined groups: (A & B) Spleen sections from the negative control group (NG) revealed the normal structure of white pulp homing aggregated lymphocytes forming lymphoid nodule (arrow). Notice central arteriole closing to white pulp area (A). (C & D) Spleen sections from the positive control group (PC) emphasized degenerated tissue surrounding arteriole (D) and infiltration of inflammatory cells inside capillaries (IN), besides; no distinction was recorded between white and red pulp areas of the spleen (arrows). (E & F) Spleen sections from the EAG group highlighted intense demarcation to

white pulp areas (arrow) by aggregated follicular dendritic cells (FDC) and smooth muscle cells (M). Notice central arteriole inside lymph nodule (arrow). (G & H) Spleen sections from the EAGA group displayed fine demarcation to white pulp area, scattered smooth muscle cells (M), and few infiltrations of inflammatory cells inside capillaries (IN).

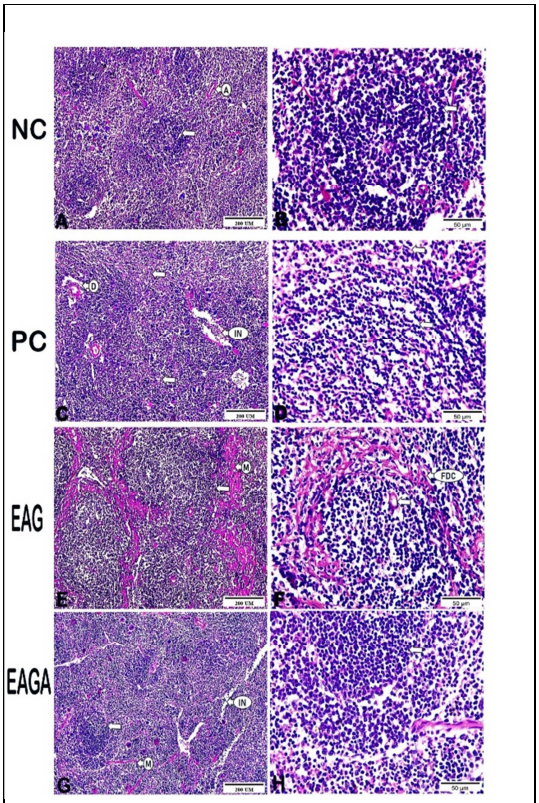


Fig. 10: Photomicrographs of spleen sections (A & B) spleen sections from the negative control group, (C & D) spleen sections from the positive control group, (E & F) spleen sections from the EAG group and (G & H) spleen sections from the EAGA group

4. Discussion

Enterobacter aerogenes is a multidrug-resistant (MDR) pathogen that is frequently causes nosocomial infections, including urinary and respiratory tract, sepsis, bacteremia, meningitis, infant congenital dermal sinus, lumbar spine infection, and post-surgical infections [27,28,29]. Therefore, early vaccination of these patients may be a key to preventing *E. aerogenes* infection.

There is a novel protocol for BG preparation was presented. The protocol is capable of producing ghosts from a variety of microorganisms, such as gram-positive, gram-negative, capsulated, spore-
forming bacteria, viruses, and eukaryotic cells. It has gained widespread acceptance due to its cost-effectiveness, reliability, and versatility with a wide range of microorganisms [30, 31, 32].

In this study, the SLRP protocol was used to prepare chemically induced *Enterobacter aerogenes* ghosts. The preparation involved exposing bacterial cells to specific chemical compounds at certain concentrations, followed by gentle centrifugation and washing steps to evacuate the DNA and cytoplasmic protein while maintaining the correct 3D shape of the bacterial cells. The EAGs was monitored using light and electron microscopy (EM). TEM showed the intact 3D shape of the EAGs. The release of DNA and cytoplasmic proteins was assessed by measuring the content of DNA and protein using a spectrophotometer in the supernatant after centrifugation of chemically treated bacterial cells. The results showed the correct evacuation of cytoplasmic content from bacterial cells. The prepared EAGs inoculated in Nutrient agar, and MacConkey agar plates showed the absence of any still viable cells after incubation for 3 days under suitable conditions. Our results showed the correct preparation of EAGs using the SLRP method. They agreed with [30]which used the same method to prepare bacterial Ghosts of Pseudomonasaeruginosa.

Our study demonstrated that immunization of mice with *E. aerogenes* ghosts might represent an appreciated strategy for vaccination against *E. aerogenes* infection. EAGs produced a strong antibody response and significantly diminished post-infection bacterial loads in mice internal organs. Furthermore, EAGs vaccinated mice showed an enhancement in liver and spleen tissues after histopathological examination. The potential of EAGs to elicit specific cellular and humoral immunity may be the reason for these results.

Three-dose immunization with the EAGs or EAGs combined with Freund's adjuvant protected mice against challenges with live *E. aerogenes* strain. It diminished the bacterial load in organs such as the liver, kidney, spleen, testes, small intestine, and stool. Mice immunized with EAGs combined with Freund's adjuvant showed a more decrease in CFU when compared with mice vaccinated with EAGs only and the control group. This may be due to the adjuvant effect in enhancing immunological response against the EAGs vaccine. The EAGs-immunized group also showed a decrease in bacterial load after the

challenge compared to the control unvaccinated group. This reflects the ability of EAGs to induce the immune response without the need for an adjuvant, but the results in the case of EAGs combined with adjuvants were better than EAGs only. The increase in bacterial load in both immunized groups compared to the control group indicates that EAGs are able to stimulate both cellular and humoral immunity in immunized mice. These resultsagreed with [33]which found that the bacterial load in the internal organs of *Klebsiella pneumoniae* ghost-vaccinated animals was considerably decreased compared to the non-vaccinated group.

This study established that immunization with *E. aerogenes* ghosts induces humoral response since the IgG, IgA, and IgM level in EAG and EAG+adjuvant vaccinated groups is higher than in the non-vaccinated group throughout the vaccination-challenge period. The level of measured antibodies was more elevated in the EAG+adjuvant vaccinated group than EAG vaccinated group. It might be because the combination between EAGs and Freund's adjuvant has a more robust immunological response than EAGs.

The cross-reactivity of produced antibodies in the sera of vaccinated mice was detected against *E. coli, E. cloaca,* and *K. pneumonia* strains by ELISA and western blotting techniques. Surprisingly our results showed the reaction between the produced antibodies in vaccinated mice and these bacterial strains. That indicates the broad effect of EAGs as a suitable vaccine can protect against different bacterial infections. Our data were similar to the results achieved by [24] which proved that Pseudomonas aeruginosa recombinant N-terminal outer membrane porin is an effective vaccine against more than one bacterial strain.

The histopathological results of liver and spleen sections of the four animal groups showed a significant enhancement in EAG and EAG+adjuvant vaccinated groups than the non-vaccinated group after a challenge by live *E. aerogenes*. This noticed an improvement in examined tissues in vaccinated mice might be due to the ability of EAGs to trigger specific cellular and humoral immunity, which subsequently protected the internal organs from the bacterial effect after being challenged by live strain. Our findings are consistent with those of [34] which observed that upon examination, the liver and spleen sections of rats vaccinated with A. baumannii ghosts (ABGs) and subsequently infected with A. baumannii displayed an improvement compared to those of unvaccinated rats. 545

5. Conclusion

In summary, this study designates the production of the *E. aerogenes* ghost vaccine by a non-expensive SLRP chemical method and evaluates its immunogenicity in a mouse model. The data indicated that EAGs could effectively trigger a pathogen-specific antibody response and lessen the bacterial load in internal organs after a challenge with live *E. aerogenes* strain in mice. These data suggested that EAG could confer protection against *E. aerogenes* infection and may be established as a new vaccine candidate against *E. aerogenes* infection. Still, more analysis of the protective efficiency of EAGs against more bacterial strains and the effect of different EAGs doses and *E. aerogenes* challenge doses should be done.

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