

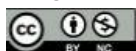
The Role of Antibodies against the COML to Combat *Acinetobacter baumannii* Antibiotic Resistance

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Abstract

Background: When treating infectious diseases, resistance to antimicrobial medications leads to significant challenges. One of the proteins involved in the transformation of a resistant plasmid is COML, a DNA's absorbent protein. *Acinetobacter baumannii*'s outer membrane contains this protein. The immunogenicity of the COML protein and its function in absorbing plasmids resistant to antibiotics were examined in this study.

Methods: The *comL* gene from *A. baumannii* ATCC 19606 was amplified and cloned into the pET28a expression vector, followed by expression in *E. coli* BL21 (DE3). Recombinant ComL protein was purified using Ni-NTA affinity chromatography and administered to BALB/c mice to induce a ComL-specific antibody response. The functional effect of immune serum on transformation and the bacterial burden in challenged mice were subsequently evaluated using selective culture and colony-forming unit (CFU) enumeration.

Results: Recombinant ComL was successfully expressed as an approximately 22-kDa protein and induced a significantly higher ComL-specific antibody response in immunized mice than in control mice ($P < 0.05$). In the in vitro transformation experiment, exposure to immune serum was associated with reduced recovery of antibiotic-resistant colonies under the relevant selective conditions. In the mouse experiments, animals receiving anti-ComL immune responses showed lower recovery of antibiotic-resistant bacteria from the examined tissues than the corresponding control animals.

Conclusion: Immunization with recombinant ComL induces a measurable antibody response and may interfere with ComL-associated natural transformation and the acquisition or recovery of antibiotic-resistant *A. baumannii*. However, the precise molecular mechanism underlying this effect requires further investigation.

Keywords: Recombinant protein, COML protein, *A. baumannii*, Antibiotic resistance, Immunotherapy.

1. Introduction

Acinetobacter baumannii has emerged as a leading cause of healthcare-associated infections worldwide, particularly in intensive care units, where carbapenem-resistant strains (CRAB) predominate and are associated with substantial morbidity and mortality (1, 2). Recent global epidemiological data confirm that CRAB infections carry mortality rates exceeding 40% in bloodstream infections and over 20–50% overall,

depending on infection site and region, with attributable mortality significantly higher than in carbapenem-susceptible cases (3-5). The pathogen's exceptional environmental resilience, coupled with its ability to rapidly acquire and maintain multiple resistance determinants, has rendered most conventional antibiotics ineffective, positioning CRAB as a critical-priority pathogen in the WHO Bacterial Priority Pathogens List and a major driver of the global antimicrobial resistance crisis (6,7).

Horizontal gene transfer, especially natural competence and transformation, is a key mechanism underlying the genomic plasticity and multidrug-resistant phenotype of *A. baumannii*. Clinical and environmental isolates frequently exhibit high transformation efficiencies, enabling the uptake of resistance plasmids and chromosomal determinants directly from the environment (8-10). The competence machinery, including type IV pili and associated proteins such as the outer membrane lipoprotein ComL, plays an essential role in DNA binding and translocation across the peptidoglycan layer, thereby facilitating the acquisition and dissemination of resistance genes even under antibiotic pressure (4, 11, 12).

A. baumannii is the cause of a wide array of infections and contributes to the development of antibiotic resistance (13, 14). In patient populations at risk, the mortality rate from *A. baumannii*-associated illness can reach 68% (15, 16). Resistance to antimicrobials leads to difficulties in treating infectious diseases and facilitates their survival in hospital environments (15, 17, 18). The Capacity of *A. baumannii* for horizontal genetic transfer (HGT) contributes to its genome plasticity and multidrug-resistant phenotype (15, 17, 19, 20). Natural transformation has been studied in a few strains of the genus *Acinetobacter* spp to introduce DNA into the *A. baumannii* genome (16, 21-23). The difficult process of transferring DNA from the external membrane across the peptidoglycan layer requires proteins involved in the construction of type IV pili in secretion systems type II COML (16). Transient modification of the peptidoglycan layer to permit DNA passage may be one of its functions in transformation. COML is a 22-kda peptidoglycan-linked lipoprotein which binds DNA in the membrane of a variety of pathogenic and non-pathogenic bacteria (21). COML is a DNA receptor that increases DNA uptake. This phenomenon facilitates the transfer of traits in bacteria and antibiotic resistance. *A. baumannii*'s outer membrane contains this protein. Given the rapid global spread of extensively drug-resistant *A. baumannii*, the scarcity of effective therapeutic options, and the critical role of natural transformation in the ongoing evolution of resistance, novel strategies that prevent the acquisition—rather than merely target existing resistance mechanisms—are urgently needed. Immunotherapeutic approaches directed against conserved competence proteins such as ComL represent a promising and innovative avenue to impair resistance gene uptake and restore the efficacy of existing antibiotics. The present study investigated the immunogenicity of the ComL protein and its potential as a target for antibody-mediated prevention of antibiotic resistance acquisition in *A. baumannii*.

2. Methods

Finding signature patterns and doing bioinformatics analyses

Alpha-helical element predominance and the COML secondary structure primary prediction were performed using the J PSIPRED service (17, 24), whereas the presence and positioning of transmembrane helices were predicted using MEMSAT3 (25). The ExPASy site was first used to assess the presence of DNA-binding motifs (26), while the charging software from the EMBOSS package (27) was used to calculate the electrostatic charge. In order to identify structure-dependent DNA binding motifs, the COML sequence was first uploaded to the DP-bind service (Hwang et al., 2007) for sequence-based DNA binding prediction. The sequence-based fold recognition and model creation were then performed using the PHYRE (Protein Homology/analogy Recognition Engine) service (Bennett-Lovsey et al., 2008).

Expression, purification, and cloning of COML

The following primers were used to amplify the COML gene: Forward primer: 5'- F (TGCA GAATTCATGGTAAATCAGGAAATGGCCAATA TTC)-3' and Reverse primer: 5' R (TTTTCTCGAGTTAGGGTGCAGGCCCAATCAA GAC)-3'. EcoRI and XhoI were the endonucleases located at the 5' and 3' ends of the amplicon, respectively. Following the extraction of *A. baumannii* ATCC 19606 DNA, a PCR reaction was carried out in a 25 µl volume. It included 0.5 µl of taq DNA polymerase, 250 µm of each dntp, 2 µl of each primer (20 pm), 1 µl of DNA (50 ng/ml), and 2 µl of mgso4 (50 mm). The program was amplified using 30 PCR-cycles, including an initial denaturation at 95°C for 5 minutes, followed by 1 minute at 94°C, 1 minute at 57°C, 45 seconds at 72°C, and an additional 7 minutes at 72°C. The PCR products were digested with EcoRI and XhoI, purified using PCR preparation kits, and then ligated into the pet28a (+) vector (Novagen, Darmstadt, Germany). The new construct was named pET28a-COML. *E. coli* BL21 cells that were chemically competent were created from the ligated product. The clones were chosen by screening on lb plates with 70 µg/ml kanamycin. The pET28a-COML was purified using a plasmid extraction kit and then sequenced to verify the integrity of the cloned insert. The selected clones were inoculated into 200 milliliters of lb medium to an optical density of 0.6 at 600 nm. To stimulate expression, the culture was exposed to 1 mm isopropyl β-d-thiogalactoside (IPTG) for 5 hours at 37°C. Centrifugation was then used to extract the cells at 14000 ×g for 10 minutes at 4°C. After being reconstituted in lysis buffer (8 m urea, 10 mm tris-cl, and 100 mm NaH2PO4), the cell

pellets were sonicated five times for one minute at one-minute intervals. Ni²⁺-nta agarose (Qiagen, Hilden, Germany) was used for affinity chromatography to separate the protein from the supernatant. Next, an elution solution (pH 4.5, 10 mM Tris, 100 mM NaH₂PO₄) was used to elute the protein from the Ni-NTA matrix. Each fraction was collected separately and analyzed using 15% SDS-PAGE. The Bradford method was used to estimate the amount of densely purified protein (15). Western blotting using an anti-His-tag antibody was used to assess the specificity of recombinant protein (COML). Mice were purchased from the Animal Center of Shahed University with informed consent. In the present study, for blood collection and liver and spleen tissue isolation, the animals were first anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) (Alfasan, Netherlands). After this procedure, euthanasia was performed by placing the animals in a CO₂ chamber. A total of 40 BALB/c mice were randomly divided into two groups of twenty animals each: (1) The recombinant COML was administered subcutaneously to the first group at doses of 20 µg, and (2) the control group was administered a mixture of Freund's adjuvant and PBS ten days after the second blood dosage. After the ten-day treatment period, the animals were first anaesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) (Alfasan, Netherlands). Blood samples (~2 mL) were collected in sterile centrifuge tubes and clotted at room temperature for 20 minutes. The samples were centrifuged at 3000 rpm for 15 minutes to separate the serum. The infra-orbital plexus was used to collect blood samples ten days after the second dosage. Following this, the animals were subjected to necropsy. After the initial weight and volume measurements, the liver and spleen were placed in a 10% buffered formalin solution for stereological studies. Finally, euthanasia was performed by placing the animals in a CO₂ chamber. ELISA was used to quantify the antibodies (22). All animal experiments were conducted in compliance with accepted standards. Serum from BALB/c-immunized mice were subjected to ELISA assays in order to assess anti-COML titers.

Evaluation transformation of *A. baumannii*

A. baumannii ABI055, which was sensitive to ampicillin, was inoculated into LB medium and incubated for 18 hours. Then, 20 µl was transferred to fresh LB medium to reach an OD of 0.3. It was then centrifuged at 13000 rpm for 5 minutes and the precipitate was dissolved in 900 µl of PBS. *A. baumannii* ABI044, which was resistant to ampicillin, was incubated into LB medium after 18 hours. After reaching an OD of 0.3 and centrifugation at 13000 rpm

for 7 minutes, the precipitate was dissolved in 900 µl of PBS. The resistant bacterium was diluted to 10⁶ CFU / ml, and then 1cc of it was sonicated 8 times. Subsequently, 100 µl was cultured on LB agar containing ampicillin and incubated to ensure that the resistant bacterium was lysed. 1. A 2-cc suspensions containing *A. baumannii* ABI055 and 1–10 µl of diluted (1/250) serum were incubated in a shaker for two hours at room temperature, centrifuged at 13000 rpm for five minutes, and then cultured for eighteen hours on LB agar supplemented with ampicillin at 100 µg/ml. 2. A 2-cc suspension containing *A. baumannii* ABI044, 10 µl, of diluted serum, and 10 µl of lysed *A. baumannii* ABI055 was placed on a shaker. After 2 hours, it was centrifuged at 13,000 rpm for 5 minutes and was cultured for eighteen hours on LB agar supplemented with 100 µg/ml ampicillin. 3. A 2-cc suspension containing *A. baumannii* ABI044, 10 µl of the antibiotic-resistant bacteria, and 10 µl of PBS was incubated at room temperature on the shaker for 2 hours, centrifuged at 13000 rpm for 5 minutes, and cultured for eighteen hours on LB agar supplemented with 100 µg/ml ampicillin.

Study of immunogenicity in mice

A. baumannii ATCC19606, resistant to ampicillin and expressing COML, was inoculated into LB medium and incubated until reaching an OD of 0.2. Then, centrifugation was performed at 13000 rpm for 7 minutes, and the precipitate was dissolved in 100 ml of LB medium. For each immune mice, 1 µl of 40 ng pET28a, 9 µl of *A. baumannii* ATCC19606, and 90 µl of three immune serum were injected peritoneally. For non-immunized mice serving as negative controls, 1 µl of 40 ng pET28a, 9 µl of *A. baumannii* ATCC19606, and 91 µl PBS were injected peritoneally. The mice were monitored for 48 hours, their spleen and liver were removed, and sterile PBS solution was added to the liver and spleen. 100 µl of the samples was cultured on plates containing 100 µg /ml ampicillin and 70 µg / ml Kanamycin.

Evaluation of the protective effect of anti-ComL immune serum in mice

One µl of 40 ng pET28a, 9 µl of *A. baumannii* ATCC19606, and 90 µl of inactivated mouse serum (dilution of 1/250), were incubated in a vial at room temperature on a shaker for 1 hour. Then, 100 µl of the suspension was injected into three non-immune mice peritoneally. The three male mice were surgically treated after 48 hours, and their spleens and corneas were removed. Dilution was performed and each was cultured in triplicate. The number of colonies in each group was then counted.

Blotting

Nitrocellulose membranes were blotted with COML-pET28a. The membrane strips were cleaned with phosphate-buffered saline (PBS) following blocking with a 5% nonfat dry milk. The membranes were again washed with PBST after an hour of incubation with recombinant COML protein. They were then treated for 90 minutes with a rabbit anti-6X-His-tag antibody (Abcam). The goat anti-rabbit IgG antibody (Sigma) conjugated with horseradish peroxidase (HRP) was then incubated at a dilution of 1:10000. Detection was performed using HRP staining solution.

Analysis of statistics

Continuous data were summarized using descriptive statistics, including mean and standard deviation (SD), when replicate-level measurements were available. For comparisons between two independent groups, the independent-samples Student's *t*-test was used when the assumptions required for parametric analysis were considered appropriate. All statistical tests were two-sided, and a *P* value < 0.05 was considered statistically significant. For in vivo experiments, each mouse was considered an independent experimental unit, whereas repeated measurements obtained from the same biological sample were not treated as independent observations. The statistical analysis was performed using the available experimental data. Because complete replicate-level raw data were not available for all experimental procedures, statistical parameters that could not be reliably reconstructed from the available records, including standard deviations, exact test statistics, degrees of freedom, and exact *P* values, were not retrospectively estimated. Results for which replicate-level data were unavailable are therefore presented descriptively and interpreted cautiously.

3. Results

Bioinformatics study of blotting and DNA binding components

The amino acid sequences of the DNA-binding proteins were analyzed to match recognized DNA-binding motifs, such as helix-turn-helix, helix-hairpin-helix, zinc finger, leucine zipper, and helix-loop-helix. A DNA-binding motif was identified in the sequence through bioinformatics analysis. Due to the consistent negative sequence-based DNA binding predictions for COML, the threading model structure was used as a basis to investigate the likely surface distribution of positive charges, which resulted in a plausible explanation for the observed DNA-binding.

Expression, purification, and cloning of COML

Following the successful amplification of the 528-bp DNA fragment encoding COML, the PCR product was ligated into the pEt-28a (+) expression vector.

EcoRI and XhoI restriction sites were introduced into the forward and reverse primers, respectively. A 22-kDa protein was detected in the induced recombinant COML by Western blotting using an anti-His-tag antibody.

In vitro transformation assay

The effect of immune serum obtained from ComL-immunized mice on the recovery of antibiotic-resistant *A. baumannii* was evaluated using the experimental bacterial strains described in the Methods section. Under the selective culture conditions, 98 CFU were recovered from the immune-serum condition, compared with 341 CFU in the corresponding control condition. These findings indicate a lower recovery of antibiotic-resistant colonies in the presence of immune serum. However, because the available experimental records did not include replicate-level measurements, the corresponding standard deviation, exact statistical significance, and transformation frequency could not be determined. Therefore, the reported CFU values are presented as observed colony counts rather than as mean $\pm$ SD values. These findings should be interpreted as preliminary evidence suggesting that anti-ComL immune serum may reduce the recovery of antibiotic-resistant bacteria under the experimental conditions used in this study. Further experiments with independent biological replicates and predefined transformation-frequency measurements are required to confirm the magnitude and statistical significance of this effect.

Study of immunogenicity in mice

The mice were surgically treated and the liver and spleen were cultured on a plate. In the negative-control group, no colonies were observed on plates containing ampicillin and kanamycin; however, growth was observed in the ampicillin-containing plates. No growth was observed in the lung samples. There was a significant difference between the test group and the control group. After 48 hours, mice that received the recombinant protein vaccination had 100-fold fewer resistant germs in their spleens than unvaccinated mice.

The Evolution of passive immunity in mice

The mice were surgically treated and the liver and spleen were cultured. The bacterial loads in the liver and spleen were shown in Fig 2. The quantity of resistant bacteria found in the spleen of ampicillin plate was 7600 CFU (70%), and on the plate containing kanamycin and ampicillin, it was 3200 CFU (30%). Regarding the liver, 8700 CFU (85%) were found on the ampicillin plate and 4700 CFU (45%) on the kanamycin-ampicillin plate. In the positive control group, in the spleen, the number of ampicillin-resistant bacteria was 9300 CFU (90%), on the plate containing kanamycin and ampicillin, it was 7600 CFU (75%). In the liver, 9400 (95%) CFU were found on the ampicillin plate and 7400 CFU (77%) on the kanamycin-ampicillin plate. In the negative control group, 14300 CFU (100%) were found on the ampicillin plate, and no growth was found on the ampicillin-kanamycin plates.

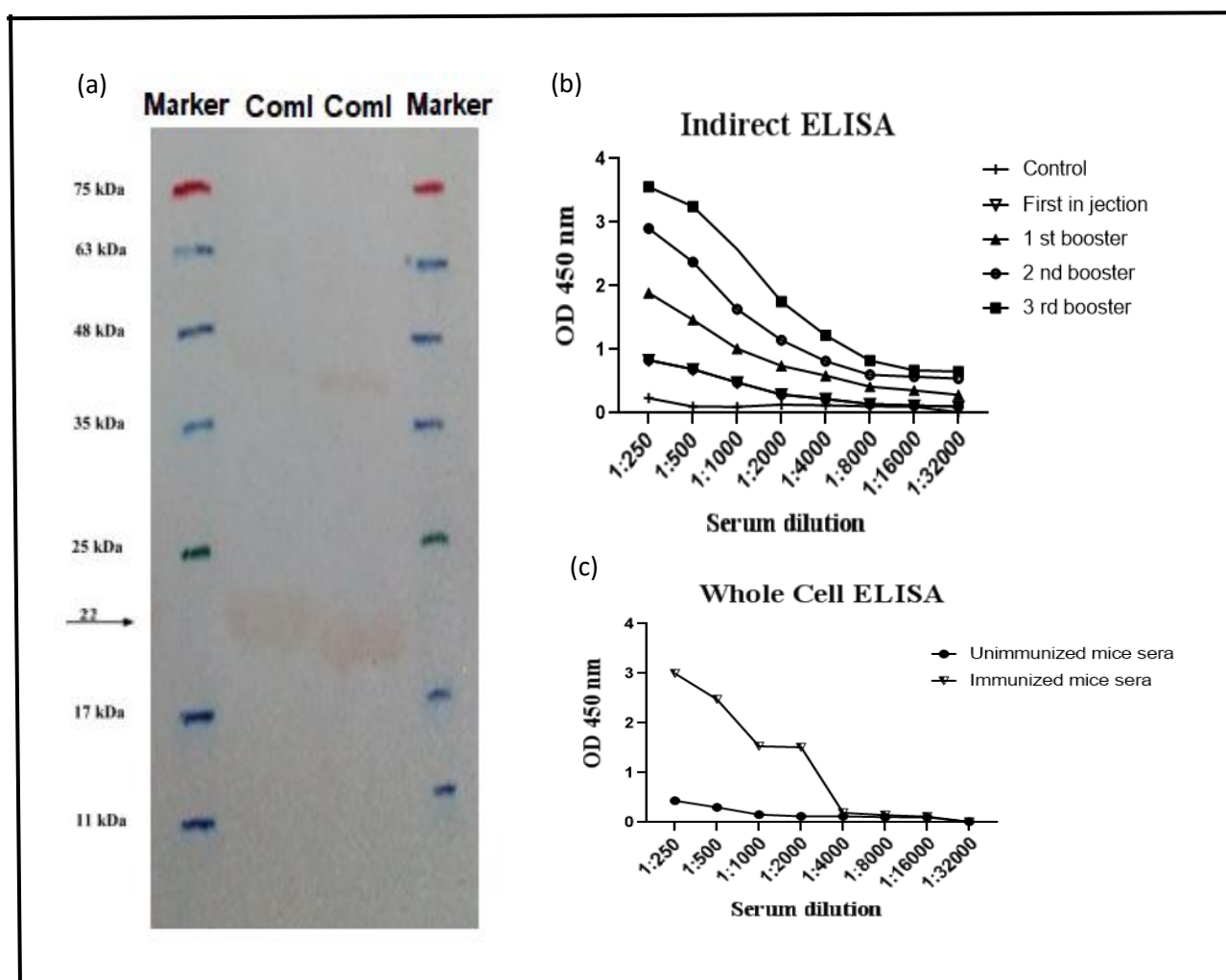


Figure 1. (a): Western blot analysis of the recombinant protein from cells harboring COML *A.baumannii*. Column1: A 22 kDa band exoprotein *A.baumannii*. The recombinant proteins were then verified by western blotting using an anti-his-tag antibody. (b): Increase in titer-antibody after the first blood donation to fourth blood sample. The test group's antibody titer was significantly higher than the control group's ($P < 0.05$). Antibodies were much higher in mice immunized with COML proteins than in the control group. (c): There is a significant difference between immunized and unimmunized mice ($P < 0.05$).

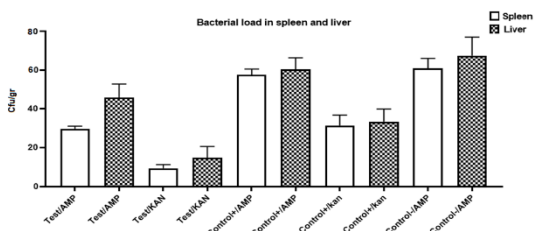


Figure 2. The graph shows the bacterial load in the liver and spleen

4. Discussion

ComL is a competence-associated outer membrane

lipoprotein that has been implicated in natural transformation and DNA uptake in *Neisseria* and other Gram-negative bacteria. Previous studies have demonstrated an important role for ComL in natural transformation and have characterized its association with the competence machinery (21, 22). More broadly, DNA uptake during bacterial transformation requires a specialized competence apparatus that mediates the binding, processing, and translocation of extracellular DNA across the bacterial cell envelope (16, 23). In the present study, recombinant ComL was successfully expressed and purified, and immunization with the recombinant protein generated a measurable ComL-specific antibody response in

mice. In the in vitro experiment, 98 CFU were recovered under the immune-serum condition, compared with 341 CFU in the corresponding control condition. Although these observations suggest a possible inhibitory effect of the immune serum on the recovery of antibiotic-resistant bacteria under the experimental conditions, the available data do not allow us to determine a formal transformation frequency or establish the statistical significance of this difference.

The potential mechanism by which anti-ComL antibodies could interfere with natural transformation remains to be established. Because ComL is associated with the bacterial cell envelope and competence machinery, antibody binding to surface-exposed regions of the protein could potentially interfere with the accessibility or function of competence-associated structures (21, 22). One possible mechanism is steric interference with the interaction of extracellular DNA with the competence machinery. Another possibility is that antibody binding could alter the conformation or functional interactions of ComL with other components of the transformation apparatus, thereby reducing the efficiency of DNA uptake or subsequent translocation across the cell envelope. The involvement of multiple competence-associated components in DNA uptake and translocation supports the biological plausibility of such interactions (16, 21, 23). However, these mechanisms were not directly investigated in the present study.

Importantly, the present study did not identify specific antibody-binding epitopes on ComL, directly measure ComL–DNA binding, or demonstrate that the antibodies block a defined DNA-binding site. Therefore, it would be inappropriate to conclude that the antibodies directly prevent DNA uptake through a specific molecular interaction. Instead, the current findings provide preliminary evidence supporting the potential of ComL-directed immunity as a strategy for interfering with processes associated with antibiotic-resistance acquisition. Direct transformation-frequency assays, DNA-uptake measurements, ComL mutagenesis, and antibody-binding or epitope-mapping studies will be necessary to determine the precise mechanism.

The in vivo findings provide additional preliminary support for this concept. Mice immunized with recombinant ComL showed differences in the recovery of antibiotic-resistant bacteria from the examined tissues compared with the control groups. In particular, bacterial recovery on kanamycin-containing selective medium was lower in the immunized animals. However, because the current study does not directly distinguish between reduced bacterial survival, altered bacterial burden, and inhibition of horizontal gene transfer in vivo, these

findings should not be interpreted as direct evidence that anti-ComL antibodies prevented DNA uptake. Rather, they indicate that ComL immunization may influence the recovery of antibiotic-resistant bacteria under the experimental conditions used in this study. Several limitations should be considered when interpreting these findings. First, the study did not directly quantify natural transformation frequency or DNA uptake using a defined genetic reporter or transformation-frequency assay. Second, the specific epitopes recognized by the antibodies were not determined, and direct interactions between anti-ComL antibodies and ComL were not characterized. Third, the experiments were performed using a limited number of bacterial strains and an experimental mouse model; therefore, the findings cannot yet be generalized to genetically diverse clinical isolates or to human infection. Finally, the available experimental records for the in vitro transformation assay did not contain sufficient replicate-level information to calculate standard deviations or exact statistical parameters retrospectively. Further experiments using independent biological replicates, appropriate statistical analysis, ComL-deficient and complemented bacterial strains, direct measurements of DNA uptake, and a broader collection of clinical isolates are therefore warranted.

5. Conclusions

Recombinant ComL was successfully expressed and purified and induced a measurable ComL-specific antibody response in BALB/c mice. The experimental findings provide preliminary evidence that anti-ComL immune responses may be associated with reduced recovery of antibiotic-resistant *A. baumannii* under the conditions evaluated in this study. These findings support further investigation of the bacterial competence machinery as a potential target for limiting the acquisition or dissemination of antibiotic-resistance determinants. However, the present study does not directly demonstrate inhibition of DNA uptake or establish the precise molecular mechanism of anti-ComL antibodies. Further studies using direct transformation-frequency assays, DNA-uptake measurements, ComL-deficient and complemented strains, antibody-binding or epitope-mapping approaches, and diverse clinical isolates are required to validate these findings.

Abbreviations

COML: DNA absorbent protein involved in plasmid transformation.

A. baumannii: *Acinetobacter baumannii*, a pathogenic bacterium.

ATCC: strain identifier.

E. coli: *Escherichia coli* is a model organism for

genetic studies.

BL21 (DE3): A strain of *E. coli* used for protein expression.

Ni-NTA: Nickel-nitrilotriacetic acid, used for protein purification.

BALB/C: A strain of laboratory mice.

CFUs: Colony Forming Units, a measure of viable bacteria.

LD50: Lethal Dose 50, the dose required to kill 50% of a test population.

HGT: Horizontal Gene Transfer, the movement of genetic material between organisms.

kDa: Kilodalton, a unit of molecular mass.

PCR: Polymerase Chain Reaction, a technique for amplifying DNA.

IPTG: Isopropyl β -D-1-thiogalactopyranoside, used to induce protein expression.

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, a technique for protein separation.

ELISA: Enzyme-Linked Immunosorbent Assay, a method for detecting antibodies.

PBS: Phosphate Buffered Saline, a buffer solution.

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Ethical Considerations and Compliance with Ethical Guidelines

I certify that this study was reported in accordance with the ARRIVE principles. The guidelines provided in the guide for the Care and Use of Laboratory Animals were followed. The animal care protocol was approved by the Shahed University ethics committee (number: 947584500). Mice were purchased from the Animal Center of Shahed University with informed consent.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

R.R., E.A., and N.H. came up with the original concept, planned the project, carried out the experiments, and composed the manuscript.

AI Using Declaration

Chat GPT was used for language and grammar corrections. The final output was read and modified by all authors.

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