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Production of recombinant OXA-23 carbapenemase: a target for developing antibody-based diagnostics against carbapenem-resistant *Acinetobacter baumannii*

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ABSTRACT

Carbapenems are typically the treatment of choice for drug-resistant *Acinetobacter baumannii*; however, the emergence of carbapenem-resistant strains has elevated this pathogen to the World Health Organization's critical priority pathogen list. Since carbapenem resistance is frequently mediated by carbapenemases, particularly oxacillinases and metallo-beta-lactamases, antibody-based carbapenemase detection tests can be developed for rapid and affordable diagnosis of the infection. However, the development of such tests requires the availability of high-quality target proteins for generating specific antibodies, their characterization, and assay optimization. In this study, we reported a streamlined workflow to obtain a purified preparation of the tagless and functionally active recombinant OXA-23 enzyme, which is one of the major factors responsible for carbapenem resistance in *Acinetobacter baumannii*. The recombinant protein was expressed in the heterologous expression host *Escherichia coli* using auto-induction and purified using a single step of chromatography, followed by the removal of the affinity tag using TEV protease. The simple protocol reported here is expected to facilitate the production of other carbapenemases and similar proteins for the development of diagnostics and therapeutics to support the fight against antimicrobial-resistant bacteria.

1. INTRODUCTION

Acinetobacter baumannii (A. baumannii), a notorious ESKAPE pathogen, is a major cause of nosocomial infections globally [1,2]. *A. baumannii* strains exhibiting resistance against antimicrobial drugs have evolved, presenting a significant challenge. For such multidrug-resistant (MDR) strains, carbapenems are regarded as the preferred therapeutic option. However, carbapenem-resistant *A. baumannii* (CRAB) strains have also emerged over time. This resistance is primarily facilitated by two mechanisms: the production of Ambler class B and/or class D beta-lactamases (also known as oxacillinases), which possess the ability to inactivate carbapenems [3]. Accordingly, the World Health Organization (WHO) has designated this pathogen as a critical priority pathogen for the development of new antibiotics [4]. Strategies to counter CRAB should not only involve the development of newer antibiotics but also include the development of reliable, rapid, and easy-to-use diagnostic reagents that can allow clinicians to prescribe appropriate treatment at the earliest for better outcomes, as well as ensure judicial use of carbapenem drugs and effective management of infection spread in hospital settings.

Currently, the detection of CRAB in a diagnostic laboratory is usually performed using phenotypic antibiotic susceptibility testing, which is time intensive. The sensitivity of culture-based methods can range from 62.5% to 95.3%, depending on the sample collection site on the body [5]. Biochemical tests such as CarbAcineto NP and β-CARBA are also available for the detection of CRAB, but such tests require fresh reagents (e.g., imipenem solution) that have a short shelf-life, or are proprietary, making them less cost-effective [6]. MALDI-TOF/MS-based detection platforms or nucleic acid-based methods such as PCR, qPCR, isothermal amplifications using specific primers for amplification of OXA enzymes or even whole-genome sequencing, etc., can also be used for CRAB detection or surveillance [7-11]. However, implementing these detection techniques often necessitates the use of specialized equipment (such as qPCR machines) and skilled personnel, which can pose significant challenges for settings with limited resources and financial constraints.

Given that the main mechanism conferring carbapenem resistance in *A. baumannii* involves the expression of carbapenemase enzymes, particularly the OXA-23 variant, the development of antibody-based tests specifically designed to detect these carbapenemases could potentially provide an efficient, sensitive, specific, and cost-effective

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approach for identifying CRAB strains. Such antibody-based tests may also offer a more accessible and economical alternative compared to conventional detection methods. Several groups have reported the development of simple and rapid immunochromatographic tests based on the detection of carbapenemases [1,2,12,13].

However, for the development, characterization, and optimization of such antibody-based carbapenemase detection tests, the availability of high-quality functional target proteins is of utmost importance. The target protein should be soluble, pure, and devoid of tags, such as histidine tags commonly used for purification, to avoid unwanted immunogenic responses during the development of specific antibodies. In this work, we present a straightforward protocol for producing functionally active carbapenemase proteins, specifically the OXA-23 enzyme. This approach involves the heterologous expression of the target carbapenemase in an *Escherichia coli (E. coli)* host system. We successfully expressed OXA-23 in *E. coli* and purified it to near homogeneity. The final recombinant OXA-23 protein preparation reported in this study is soluble, easy to produce, and most importantly, devoid of the histidine tag, which was used for its initial purification and subsequently removed using TEV protease. The protein has also been qualitatively characterized using an antibiotic susceptibility test to assess its functionality after production in *E. coli*.

2. MATERIALS AND METHODS

2.1. Cloning of OXA-23 Gene Encoded by *A. baumannii* in pET-28c(+) Vector

A synthetic cassette encoding the sequence for a decahistidine (10 consecutive histidine residues) affinity tag at the N-terminus, the Tobacco Etch Virus (TEV) protease enzyme site, and the mature gene sequence encoding the OXA-23 carbapenemase from *A. baumannii* (19-273 amino acids; Uniprot Q9L4P2) flanked by XbaI and XhoI was obtained from commercial sources (Barcode Biosciences, India). The plasmid encoding gene cassette and pET-28c(+) were digested with XbaI and XhoI restriction enzymes (NEB, USA). The digested pET-28c(+) vector was treated with CIP (NEB, USA), and the digested DNA samples were purified from agarose gel as described before [14]. The OXA-23 was ligated to the linearized pET-28c(+) vector using T4 DNA ligase enzyme (NEB, USA), and the ligation mixture was transformed into *E. coli* TOP10 cells via electroporation, followed by the selection of transformants on $LBKan_{30}Glu_{1\%}$ [14]. Two recombinants were sequenced using Sanger sequencing (HiMedia Laboratories Private Limited, India) to verify the sequence of the cloned insert.

2.2. Small-Scale Expression of OXA-23 Protein in *E. coli* and Localization

E. coli BL21(DE3) cells were transformed with plasmid DNA from recombinant clones via electroporation. The resulting transformants were then evaluated for protein expression levels using an auto-induction approach [14,15]. The clones were cultured in 5 mL of $LBKan_{30}Glu_{1\%}$ at 37°C at 250 rpm for 5 h. Following this initial growth phase, the cultures were centrifuged to harvest the cells. The resulting cell pellets were then resuspended in 15 mL of lactose-containing auto-induction media ZYM5052Kan30 [the media composition was as described by [15], but without trace metals] and grown at 250 rpm, with the temperature being progressively lowered in stages to reach 18°C to facilitate protein expression and folding as described in [14]. Finally, the cells were harvested by centrifugation, and the cell pellet was then resuspended in 3.75 mL Tris Loading Buffer (TLB; 50 mM Tris-HCl, pH 7.5, and 500 mM NaCl) containing 37.5 µL each of 20 mg/ml lysozyme and 10 mM PMSF. The resuspended cells were incubated on ice for 30 min to facilitate cell lysis, followed by sonication (pulse - 4sec on/1 sec off at 30% amplitude) for 30 min. Following sonication, the lysed cell suspension was centrifuged at 10,000*xg* at 4°C for 1 h. The supernatant, containing soluble proteins, was carefully collected after this high-speed centrifugation step. To analyze protein expression and solubility, samples were taken at different stages of the process: the initial total cell culture, the sonicated cell lysate, and the final high-speed supernatant (HSS) fraction. The samples were analyzed using 12.5% SDS-PAGE.

2.3. Large-Scale Expression of OXA-23 Protein in *E. coli*

One clone was selected for large-scale expression and grown in 5 mL $LBKan_{30}Glu_{1\%}$ media at 250 rpm at 30°C for 16 h. The culture was diluted 100-fold in 400 ml lactose-containing auto-induction media as described above. The harvested cell pellet was resuspended in 80 mL TLB containing 800 µL each of 20 mg/ml lysozyme and 10 mM PMSF and processed as described above. The HSS was stored at −80°C, and samples at different stages were analyzed as described above.

2.4. Purification of Tagless OXA-23 Protein

Protein purification was carried out using nickel-affinity chromatography at room temperature (RT). Approximately 60 mL HSS containing H10-T-OXA-23 (equivalent to 300 mL induced culture) was purified in three batches (20 mL per batch x 3). For purification, imidazole (20 mM final concentration) was added to the HSS, and the sample was filtered through a 0.45-micron syringe filter. For every batch, a 20 mL sample was loaded on an equilibrated 2 mL HisTrap NiFF column (2 x 1 mL prepacked HisTrap NiFF columns linked in tandem, Cytiva, USA). Subsequently, the column was washed with 10 mL TLB supplemented with 50 mM imidazole. Finally, the bound target protein was eluted using 8 mL TLB containing 300 mM imidazole. The eluate from 3 batches (~24 mL) was pooled and subjected to a desalting and concentration step using Amicon ultra centrifugal filter units (3 kDa cutoff). During this process, the buffer was exchanged for low salt buffer (20 mM Tris pH 8.0 with 50 mM NaCl). To remove the H10 tag, the purified protein was treated with H6-TEV protease purified in-house from pRK793; it was a gift from David Waugh; Addgene plasmid #8827 [16]. For this, ~ 28.8 mg of H10-T-OXA-23 protein (12 mL) was digested with 150 µg H6-TEV protease (1:200 of protease: protein) at 30°C for 6 h and 0 h and 6 h samples were analyzed using a 15% SDS-PAGE gel. After digestion, sample loading buffer [17] was added to the reaction. The sample was purified using a 2 mL HisTrap NiFF column. A flow-through containing tagless OXA23 protein was collected (~18 mL). The sample was desalted in 1 x PBS (prepared in endotoxin-free water) and concentrated as described above. Approximately 5 mL sample was incubated with an endotoxin removal column (Pierce High-Capacity Endotoxin Removal Spin Column, Thermo Fisher Scientific, USA) as per the manufacturer's instructions, and protein was collected after 1 h. The protein concentration at different stages was estimated using a Bradford protein assay, and samples were analyzed using SDS-PAGE.

2.5. Dot-Blot of Purified OXA-23 Protein

Approximately 1 µg of H10-T-OXA-23 and tagless OXA-23 each was spotted on two nitrocellulose membrane strips (Thermo Fisher Scientific, pore size 0.45 µm, catalog number 88025), and the spots were dried at 37°C for 10 min. The membranes were blocked using 2% BSA-PBST (1 x PBS with 0.05% Tween 20) for 1 h at RT with

constant stirring and washed three times with PBST. Membrane 1 was probed with an anti-His tag antibody (70796, Sigma-Aldrich, Bengaluru, India; 100 ng/mL in 0.2% BSA-PBST), and membrane 2 was probed with an anti-OXA-23 polyclonal antibody (1:10,000-fold in 0.2% BSA-PBST; available in-house) for 1 h at RT with constant stirring and washed three times with PBST. Finally, the membranes were probed with goat anti-mouse IgG (H+L)-HRP (62-6520, Thermo Fisher Scientific, USA) diluted 5000-fold in 0.2% BSA-PBST for 1 h at RT with constant stirring and washed three times with PBST and PBS. The membranes were developed with 1 mg/mL 3,3'-diaminobenzidine (DAB) solution (Sisco Research Laboratories Pvt. Ltd., India) in PBS containing 0.03% hydrogen peroxide.

2.6. Indirect ELISA-Based Analysis of Purified OXA-23 Protein

A 96-well ELISA plate (HiMedia Laboratories, India) was coated with 100 µL of 2 µg/mL H10-T-OXA-23 and tagless OXA-23 protein overnight at 4°C. The next day, the plate was incubated at RT for 1 h and washed three times with PBST. Coated wells were blocked as described before for 1 h at RT and probed with 7 dilutions of anti-histidine tag mouse monoclonal antibody (100 ng/mL-0.137 ng/mL) . After incubation, the plate was washed three times with PBST and incubated with goat anti-mouse IgG (H+L)-HRP diluted 5000-fold in 0.2% BSA-PBST for 1 h at RT. Finally, the plate was washed three times with PBST and PBS, and 100 µL TMB solution (T4444, Sigma-Aldrich, India) was added, and the plate was incubated for 15 min. To terminate the enzymatic reaction, 100 µL of 1 N sulfuric acid solution was added to the mixture. The absorbance of the final reaction product was then quantified at a wavelength of 450 nm using a Multiskan GO microplate reader instrument (Thermo Fisher Scientific, USA).

2.7. Antibiotic Susceptibility Test of *E. coli* DH5α

An antibiotic susceptibility test was performed in solid and liquid media. For both experiments, two concentrations of three antibiotics, namely, ampicillin (10 and 5 mg/mL), oxacillin (30 and 15 mg/mL), and kanamycin (10 and 5 mg/mL) were treated with tagless OXA-23 protein and used to check the antibiotic susceptibility of *E. coli* DH5α. Briefly, 20 µL of each antibiotic concentration was treated with 2 µg tagless OXA-23 at 37°C for 6 h.

To evaluate antibiotic susceptibility using a solid agar-based assay, an overnight culture of *E. coli* DH5α cells grown in Mueller-Hinton broth was first prepared. After the completion of the enzymatic reaction, 150 µL of this overnight grown *E. coli* DH5α cell culture was spread evenly on Mueller Hinton agar plates. After drying, sterile paper discs were placed on the agar plates, and 20 µL aliquots of both the enzyme-treated and untreated antibiotic samples were applied onto separate discs. The plates were incubated at 37°C for 16 h.

To evaluate antibiotic susceptibility in liquid media, 20 µL of treated and untreated antibiotic samples were added to 100 µL of 50-fold diluted overnight grown *E. coli* DH5α culture in a 96 -well plate, and the culture was grown at 37°C, 250 rpm for 16 h. The next day, the absorbance of each culture was measured at 600 nm. This experiment was performed in triplicate, and readings were used to calculate the mean and standard deviation.

3. RESULTS

3.1. Cloning and Expression of Recombinant OXA-23 Protein in *E. coli* and Localization

Gene encoding the mature sequence of OXA-23 protein from *Acinetobacter baumannii* (amino acid 19-273) with N-terminal decahistidine (H10) tag and TEV protease site was cloned in pET-28c(+) vector for T7 promoter-based expression system-based cytosolic expression of OXA-23 protein in *E. coli* BL21(DE3) and purification using nickel affinity chromatography [Figures 1 and 2A]. The TEV protease site was included for the removal of the H10 tag after affinity chromatography. Two clones were selected for expression of the H10-T-OXA-23 protein using lactose-based auto-induction at 18°C and its localization. The low temperature was chosen to enhance the production of soluble proteins. Both clones showed the same expression profile and exhibited soluble expression of the H10-T-OXA-23 protein in the cytosolic fraction [Figure 2B].

3.2. Purification of Tagless OXA-23 Protein

Based on expression and localization results, one clone was selected for production of OXA-23 on a large scale. The soluble cytosolic extract containing H10-T-OXA-23 [Figure 3A, Lane 3] was purified using immobilized nickel ion-based affinity chromatography (IAC), and greater than 80% purity was achieved [Figure 3A, Lane 4]. The eluate was desalted in a buffer compatible with TEV protease treatment for the removal of the H10 tag [Figure 3A, Lane 5]. The purified H10-T-OXA-23 was treated with H6-TEV protease, and after completion of the reaction [Figure 3B, Lanes 1 and 2], the reaction mixture was passed through the IAC column to remove the cleaved H10 tag, uncleaved H10-T-OXA-23, and H6-TEV protease from the purified protein. The flowthrough from the IAC column containing purified tagless OXA-23 protein [Figure 3A, Lane 6] was further desalted in PBS buffer, which is compatible with most of the downstream protein

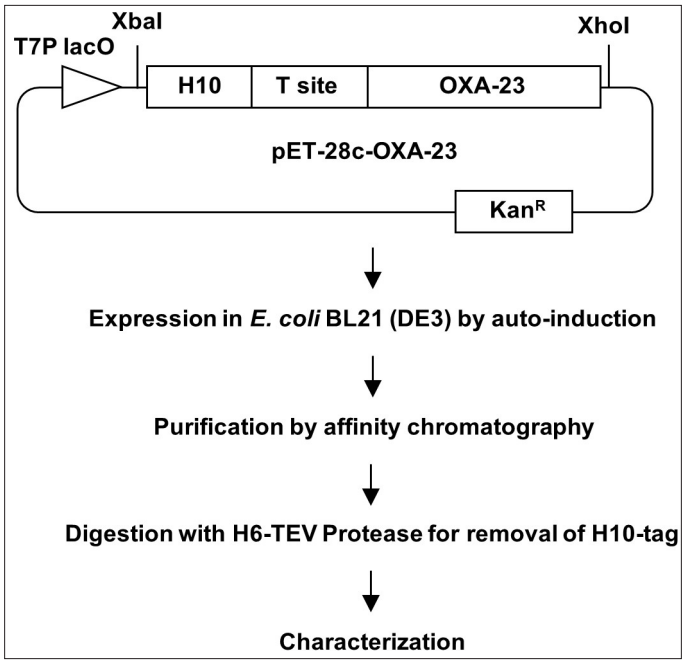

Figure 1: Workflow for expression and purification of recombinant *Acinetobacter baumannii* OXA-23 protein.

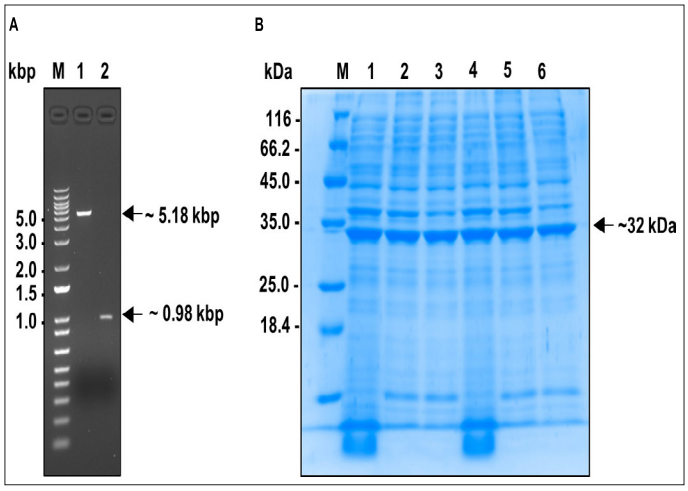

Figure 2: Cloning, expression, and localization of recombinant H10-T-OXA-23 protein. **(A)** Cloning of OXA-23 gene in pET-28c(+) vector. Lane M – 1 kb plus DNA ladder (Thermo Fisher; Cat no. 10787018); Lane 1 – XbaI-XhoI-digested pET-28c(+) vector; Lane 2 - XbaI-XhoI-digested OXA-23 insert. **(B)** SDS-PAGE analysis. Lane M – Protein ladder; Lane 1 – Clone 1-Total Cell (TC) sample, Lane 2 – Clone 1-Total Cell sample after sonication (TCAS), Lane 3 - Clone 1- High-speed supernatant sample (HSS), Lane 4 – Clone 2-TC sample, Lane 5 – Clone 2-TCAS, Lane 6 – Clone 2-HSS sample.

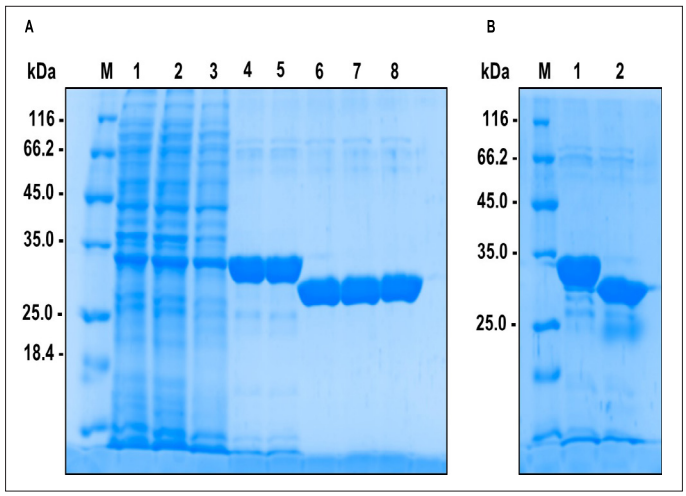

Figure 3: Purification of recombinant tagless OXA-23 protein. **(A)** SDS-PAGE analysis of recombinant OXA-23 protein at different stages of purification. Lane M – Protein ladder, Lane 1 – TC sample before sonication, Lane 2 – TCAS, Lane 3 – HSS sample, Lane 4 – Eluate of Ni-affinity chromatography, Lane 5 – Protein after desalting-1 in 20 mM Tris pH 8.0 (50 mM NaCl), Lane 6 – Protein after TEV protease treatment and its removal, Lane 7 - Protein after desalting-2 in 1 x PBS, Lane 8- Protein after endotoxin removal. **(B)** SDS-PAGE analysis of recombinant OXA-23 protein before and after treatment with TEV Protease. Lane M – Protein ladder, Lane 1 - protein before protease treatment, Lane 2 –protein after protease treatment.

applications [Figure 3A, Lane 7]. The purified tagless OXA-23 protein was also incubated with an endotoxin removal column to reduce the endotoxin load in the purified protein sample and make it suitable for animal immunization experiments [Figure 3A, Lane 8]. The final purified tagless OXA-23 protein preparation was more than 90% pure

Table 1: Recovery of recombinant OXA-23 protein at different stages of purification.

S. No.	Step	Total amount of protein taken	Total amount of protein recovered
1.	Nickel affinity chromatography and desalting-1	~ 48 mg of H10-T-OXA-23 ([a]60 ml HSS)	38.4 mg of H10-T-OXA-23 (16 ml)
2.	TEV protease treatment, protease removal and desalting-2	28.8 mg of H10-T-OXA-23 (12 ml)	14 mg of tagless OXA-23 (10 ml)

[a]1 ml of HSS is equivalent to approximately 5 ml induced culture (explained in methods). The approximate concentration of H10-T-OXA-23 protein in the HSS was estimated by SDS-PAGE. Other estimations shown in the table were performed using the Bradford protein assay.

[Figure 3A, Lanes 6 and 7]. With the purification protocol described in this study, we were able to obtain approximately 14 mg of tagless protein from 300 ml of induced culture [Table 1].

3.3. Characterization of Purified OXA-23 Protein Using Dot-Blot and Indirect ELISA

In this work, the tagless OXA-23 was produced by the removal of the H10 tag using H6-TEV protease. The efficiency of H10 tag removal from the OXA-23 protein was tested using dot-blot and indirect ELISA. For dot-blot, OXA-23 protein before and after H10 tag removal was probed with anti-His antibody to assess the presence of H10 tag and anti-OXA-23 antibody to assess the presence of protein. Blot probed with anti-His antibody revealed a dot only with H10-T-OXA-23 protein [Figure 4A], whereas blot probed with anti-OXA-23 antibody revealed dots for both H10-T-OXA-23 and tagless OXA-23 [Figure 4B], indicating that OXA-23 protein is present in both spots but tagless OXA-23 protein is devoid of the H10 tag.

Similarly, H10-T-OXA-23 and tagless OXA-23 proteins were coated on ELISA plates and tested using anti-His tag antibodies in indirect ELISA. Here again, the reactivity was observed only with H10-T-OXA-23 protein [Figure 5], further corroborating the results of dot-blot, and indicating that the removal of the H10 tag using H6-TEV protease was efficient and the tagless OXA-23 protein preparation was devoid of the H10 tag.

3.4. Characterization of Purified Tagless OXA-23 Protein Using an Antibiotic Susceptibility Test

The purified tagless OXA-23 was further characterized functionally using an antibiotic susceptibility test (AST). OXA-23 belongs to Class D beta-lactamases (also known as oxacillinases) and specifically degrades beta-lactam drugs. Accordingly, to assess the activity of the purified tagless OXA-23 protein, two beta-lactam antibiotics, namely, ampicillin and oxacillin, and an aminoglycoside antibiotic, namely, kanamycin, were treated with purified OXA-23, and their growth inhibition activity against *E. coli* DH5α was assessed using AST on solid and liquid media. On solid media, in the absence of OXA-23, both beta-lactam drugs inhibited the growth of DH5α, and a zone of inhibition was observed [Figure 6, a1, a3, b1, b3]. However, treatment with OXA-23 led to their inactivation, and consequently, no zone of inhibition was observed [Figure 6, a2, a4, b2, b4]. On the other hand, treatment of kanamycin with OXA-23 did not affect its ability to inhibit the growth of DH5α [Figure 6, c1-c4].

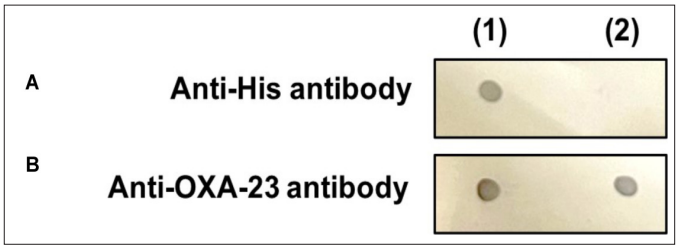

Figure 4: Dot-blot analysis of purified H10-T-OXA-23 protein before and after H6-TEV Protease treatment. To verify the complete removal of histidine tag after treatment with TEV protease, purified protein before (1) and after (2) removal of H10 tag was spotted on the nitrocellulose membrane and probed with **(A)** anti-His tag antibody or **(B)** anti-OXA-23 antibody.

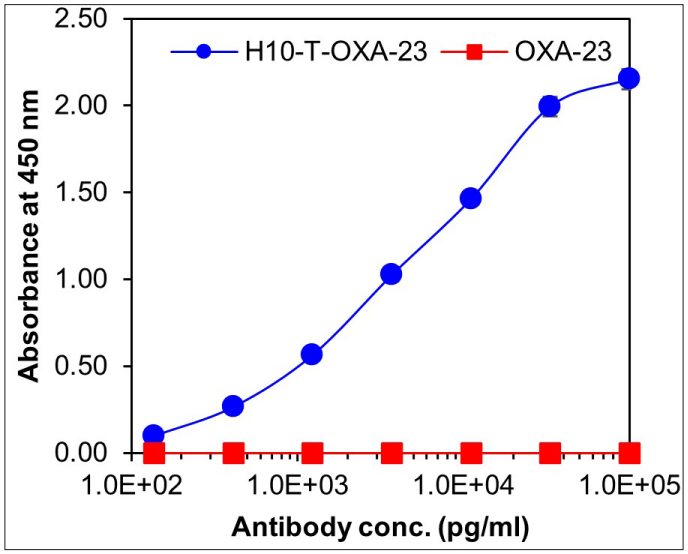

Figure 5: Indirect ELISA to verify the removal of histidine tag after H6-TEV Protease treatment. H10-T-OXA-23 (before tag removal) and OXA-23 (after tag removal) were tested using indirect ELISA with anti-His tag antibody. The experiment was performed in triplicate to calculate the mean and SD.

Similarly, in liquid media, both ampicillin and oxacillin inhibited the growth of DH5α before OXA-23 treatment, whereas treatment with OXA-23 led to their inactivation, and consequently, growth was observed in the overnight culture [Figure 7]. On the other hand, kanamycin remained unaffected by OXA-23 treatment [Figure 7].

4. DISCUSSION

In this work, we have reported a streamlined protocol to produce the recombinant OXA-23 protein of carbapenem-resistant *A. baumannii* in a heterologous host *E. coli* and its functional characterization using a simple antibiotic susceptibility test. OXA-23 (also known as ARI-1) was first reported in Scotland in *A. baumannii* strain 6B92, which exhibited resistance against drugs like imipenem, penicillin, and other cephalosporins [18,19]. It is categorized under Ambler class D beta-lactamases, commonly referred to as oxacillinases or OXA-type carbapenemases, and utilizes a catalytically active serine residue to deactivate beta-lactam antibiotics, including carbapenems [20,21]. OXA-23 has been detected in various drug-resistant bacteria and is

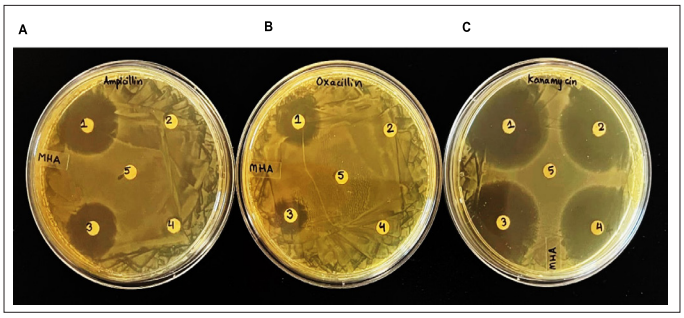

Figure 6: Antibiotic susceptibility test of *E. coli* DH5α with different antibiotics before and after treatment with purified OXA-23 protein (on solid media). Three antibiotics, ampicillin **(A)**, oxacillin **(B)**, and kanamycin **(C)** were treated with 2 µg of purified OXA-23 protein for 6 hr at 37°C and used to check antibiotic susceptibility of *E. coli* DH5α on solid media. (a-1 and a-2) 20 µl of 10 mg/ml Ampicillin before and after treatment, (a-3 and a-4) 20 µl of 5 mg/ml Ampicillin before and after treatment, (b-1 and b-2) 20 µl of 30 mg/ml Oxacillin before and after treatment, (b-3 and b-4) 20 µl of 15 mg/ml Oxacillin before and after treatment, (c-1 and c-2) 20 µl of 10 mg/ml Kanamycin before and after treatment, (c-3 and c-4) 20 µl of 5 mg/ml Kanamycin before and after treatment, (a-5, b-5 and c-5) 20 µl of sterile water.

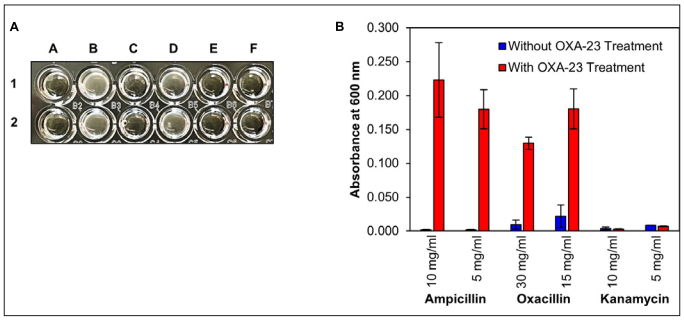

Figure 7: Antibiotic susceptibility test of *E. coli* DH5α with different antibiotics before and after treatment with purified OXA-23 protein (in liquid media). Three antibiotics, ampicillin, oxacillin, and kanamycin were treated with 2 µg of purified OXA-23 protein for 6 hr at 37°C and used to check antibiotic susceptibility of *E. coli* DH5α in liquid media. **(A)** Growth of cells in 96-well plate containing LB Media with - (A-1 and B-1) 20 µl of 10 mg/ml Ampicillin before and after treatment, (A-2 and B-2) 20 µl of 5 mg/ml Ampicillin before and after treatment, (C-1 and D-1) 20 µl of 30 mg/ml Oxacillin before and after treatment, (C-2 and D-2) 20 µl of 15 mg/ml Oxacillin before and after treatment, (E-1 and F-1) 20 µl of 10 mg/ml Kanamycin before and after treatment, (E-2 and F-2) 20 µl of 5 mg/ml Kanamycin before and after treatment **(B)** Analysis of culture density after the growth of cells under different conditions. The experiment was performed in triplicate.

recognized as a significant contributor to carbapenem resistance in *A. baumannii* [3,22].

In this study, OXA-23 protein was expressed using lactose-based auto-induction at low temperatures to promote protein solubility in heterologous host *E. coli*. After single-step purification of the recombinant H10-T-OXA-23 protein using affinity chromatography, it was treated with purified TEV protease to remove the H10 tag. With the method reported in this study, we were able to easily obtain > 90% pure tagless OXA-23 protein with a single step of affinity chromatography.

Further, dot blot and indirect ELISA with an anti-histidine antibody showed that the method for tag removal using TEV protease treatment was efficient, and the final protein preparation was devoid of the H10 tag. Since the protein was produced in a heterologous host, we also determined its enzymatic activity against beta-lactam drugs using an antibiotic susceptibility test, and it was found to be active and highly specific in its action. This indicates that the protein was folded properly and retained its enzymatic activity even when overexpressed in a heterologous host.

Several groups have reported the purification of the OXA-23 protein. Toros et al. described the production of the OXA-23 protein in fusion with the maltose binding protein (MBP) [23]. They reported that the removal of the MBP tag from OXA-23 post-expression and purification was difficult, and overnight digestions resulted in an enzymatically inactive protein. In this study, we have demonstrated that the OXA-23 protein can be produced without a large solubility enhancer protein tag such as MBP, and the tag used for affinity purification can be easily removed using TEV protease without loss of its enzymatic activity. Smith et al. reported the purification of OXA-23 from an *E. coli* host using anion-exchange chromatography [24]. However, Torol et al. have reported that using ion exchange chromatography is not suitable for the purification of OXA-23 as its surface contains uncharged amino acid residues [23]. Our study addresses this concern as it only relies on the use of affinity chromatography to produce > 90% pure protein. Lee et al. have reported the use of the enterokinase enzyme for the removal of the purification tag from the OXA-23 protein. However, enterokinases have been reported to be promiscuous and can lead to cleavage at cryptic sites, resulting in the degradation of the target protein using tag removal [25]. Our method involves the use of a highly specific TEV protease that does not exhibit non-specific cleavage during prolonged hours of incubation with the target protein, making it a better choice for the removal of purification tags. Mertins et al. have also reported the purification of OXA-23 with a histidine tag followed by immunization in mice for the production of anti-OXA-23 antibodies [1]. However, this necessitates the elaborate screening of antibodies to identify clones specific to the protein and not the histidine tag. Since our protocol involves the removal of the purification tag, it also reduces the chances of an unwanted immune response against the tag during immunization, making screening strategies easier.

The total time required to purify OXA-23 protein using the method reported in this study is approximately 40 hours (~2-3 working days), which includes the expression of recombinant protein (~22 hours), production of cell lysate (~2 hours), single-step affinity chromatography and desalting (~4 hours), TEV protease treatment (~6 hours), and TEV protease and endotoxin removal (~6 hours). It may be noted that, compared to the existing methods in the literature, the method reported here does not reduce the overall time required to produce a purified protein, but it is optimized to yield functionally active preparation of tagless proteins with reasonably high purity.

The purified tagless OXA-23 protein reported in this study can be used as a target to develop antibody-based diagnostic interventions for the fight against carbapenem-resistant bacteria. Several independent studies have reported the prevalence of different carbapenemases, including OXA-23, in carbapenem-resistant *A. baumannii,* making them an important target for the development of antibody-based antigen detection platforms such as lateral flow assays. Mertins et al. described immuno-chromatographic (IC) kits based on multiple carbapenemases that can allow the identification of CRAB with a quick turnaround time from the culture plate to the result [1,2]. Ji et al. utilized a combination of monoclonal antibodies (6G4/4G6) targeting the OXA-23 enzyme to develop an IC test, achieving an overall sensitivity of 98.36% and a specificity of 97.56% during the detection of 102 *A. baumannii* isolates [12]. Baig et al. reported the development of two antibodies, F241G3sc2 and F241G6sc2, targeting *A. baumannii*, and recommended their use in various assays, including ELISA, Western blot, and co-IP [26]. Tada et al. reported the development of monoclonal antibodies against New Delhi metallo-β-lactamases for the detection of NDM-producing Gram-negative bacteria [13]. Purified carbapenemases like the OXA-23 protein can also be used as a target for *in vitro* screening of new-generation inhibitors against carbapenemase enzymes responsible for carbapenem resistance [27]. In addition, they can also be explored as potential targets to develop neutralizing antibodies against carbapenemases in addition to other bacterial targets [28,29].

5. CONCLUSION

This study presents a streamlined protocol for producing a highly pure, tagless, and functionally active recombinant OXA-23 carbapenemase enzyme from the drug resistant pathogen *Acinetobacter baumannii*. The OXA-23 protein was successfully expressed in *E. coli* with an affinity tag, purified via a single chromatography step, and the tag was efficiently removed using highly specific TEV protease cleavage without compromising protein solubility or enzymatic activity. The entire process, from protein expression to the purified tagless product, can be completed within 2-3 days. The purified tagless OXA-23 enables the generation of specific antibodies against this major carbapenemase without interference from affinity tags. This simple methodology addresses previous challenges and can be readily adopted in standard molecular biology labs to produce other carbapenemases or antibiotic resistance proteins. The purified OXA-23 represents a key reagent for developing much-needed antibody-based diagnostics and therapeutic interventions against the emerging threat of carbapenem-resistant *Acinetobacter baumannii* infections.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. DATA AVAILABILITY

The authors confirm that all the relevant data are included in the article.

9. ETHICAL APPROVALS

This study does not involve experiments on animal and human subjects.

10. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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