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Production of Diagnostic Antibodies against exotoxin A of *Pseudomonas aeruginosa.*, using Phage Display Technique

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Abstract

Patients with compromised immune systems are particularly susceptible to nosocomial infections caused by *Pseudomonas aeruginosa*. Through ADP-ribosylation of eukaryotic elongation factor 2, *Pseudomonas aeruginosa* exotoxin A inhibits protein synthesis in infected cells. Plasmodium infections, even when resistant to antibiotics, can be treated by using antibodies that neutralize the virulent components of the bacteria. *Pseudomonas aeruginosa* produces exotoxin A, which is highly effective in treating *Pseudomonas aeruginosa* infections. For this research. An antibody specific to P. aeruginosa exotoxin A was identified by mining a human scFv phage library. In order to induce protein injection in mice, exotoxin A was produced in Escherichia coli. The scFv repertoires were constructed by combining the coding sequences of the Ig G variable domain. They originated from RNA found in the liver. We prepared a three-phage library using scFv using 1×10^5 plaque forming units. The characteristics of the library demonstrated the production of 31.67-kDa bacterial proteins with good selectivity and affinity scFvs.

Keywords: Pseudomonas aeruginosa, exotoxin A, monoclonal antibodies and scFv.

1. Introduction

Pseudomonas aeruginosa, a Gram-negative opportunistic bacterium, infects individuals with cystic fibrosis, burn wounds, immunodeficiency, COPD, cancer, and serious infections requiring ventilation, such as COVID-19. All biological fields employ *P. aeruginosa* as a model bacterium. In addition to intense research on P. aeruginosa's virulence factors (LPS, quorum sensing, two-component systems, six-type secretion systems, outer membrane vesicles (OMVs), CRISPR-Cas, and their regulation), host-pathogen interaction, particularly host immune networks involving autophagy, inflammasome, non-coding RNAs, cGAS, etc., has advanced rapidly.One common healthcare-associated infection, especially in individuals with weakened immune systems, is *Pseudomonas aeruginosa*. The fact that HPV can spread from patient to patient and through contaminated medical equipment makes it a serious concern **(Wood *et al*., 2023)**. Antibiotic resistance is one of the greatest health risks worldwide. The pathogenicity of bacteria depends on their toxins. *P. aeruginosa* produces

exotoxin A, phosphorylase C, hemolysin, and exoenzyme S, which are crucial for the infection progression and prognosis of the infection.

The single-chain polypeptide exotoxin A weighs 66.583 KDa and has three domains of 613 amino acids in 1985, Smith first introduced phage display Smith integrated foreign DNA into M13 phage and expressed an exogenous polypeptide using its coat protein. Antibodies are polyclonal, monoclonal, or recombinant depending on manufacturing. Despite their inherent limitations, they may thrive in their unique use. Von Behring and Kitasato created the first diphtheria toxin (DT) polyclonal antibody in 1890. Though cheap to make, these antibodies have limited therapeutic, and diagnostic uses due to immunogenicity and low specificity. Köhler and Milstein's hybridoma method produced monoclonal antibodies (mAbs) from merged immortal myeloma cells and mouse spleen B cells. This approach is limited by the instability of aneuploid cell lines, the inability to create human antibodies, the low effectiveness of B lymphocyte fusion with myeloma cells, and the vaccination of experimental animals **(Mitra *et al.,* 2021 and Moraes *et al*., 2021).** Recently, clinical research has relied on phage display technologies. It creates antibodies that attach to complicated antigens, which helps find biomarkers and improve diagnostics and treatments. However, previous assessments generally miss its impact outside cancer research (**Sadraeian *et al.,* 2024)**. This review explains phage display and its uses in diagnosing and treating non-cancerous disorders to address that gap. We emphasize its importance in degenerative, inflammatory, autoimmune, and chronic non-communicable diseases, highlighting how it is transforming diagnosis and treatment**.** Animal scFv phage libraries currently offer a rapid and dependable method for producing mice antibodies against nearly any antigen. Finding a mouse anti-exotoxin A scFv and assessing its specificity to *P. aeruginous* exotoxin A were the goals of this investigation. We want to underline the importance of phage display in current healthcare by showcasing major discoveries and future potential.

2. Materials and Methods

2.1. Production, concentration and precipitation of toxin A from the identified organism

Exotoxin A from the isolated *Pseudomonas aeruginosa* accession number was extracted, concentrated and purified using partial purification with 70% ammonium sulfate and complete purification methods using ion exchange chromatography and gel filtration techniques.

2.2. Immunization of mice with toxin A (mice serum)

The Egyptian Research Center of Ophthalmology sold 10 BALB/c mice. Mice were raised according to NIH protocols. After the Ethics Council approved the study, the Cairo University Institutional Animal Care and Use Committee (CU-IACUC) approved the cytotoxicity of exotoxin A (CUIF7619). Control rats were 4-6 weeks old and 28-30 grams. Each group was housed in a plastic cage in an animal home at (23-25) °C. The rats were well fed and hydrated. Animals received 50 µg crude exotoxin A emulsified in complete Freund's adjuvant and four injections of 100 µg crude toxin A incomplete Freund's adjuvant. After 7 days, the sera and spleens were used for antitoxin A assays and RNA isolation.

3.2. RNA extraction from mice spleen

The mice's spleen tissue was homogenized with liquid nitrogen, placed in a 1.5 ml tube with 300 µl lysis buffer and β-mercapto, vortexed for 10 min, and incubated at 15-25°C for 10 min with 600 µl diluted proteinase K (10 µL stock solution and 990 µL TE buffer). Centrifuge at 12,000 × g for 5 min and transfer supernatant to a fresh Eppendorf tube. The supernatant went to Gene JET RNA. Take 450 µL of 100% ethanol and centrifuge at high speed for 1 minute. Discard the flow-through and transfer the solution to a new GeneJET RNA column. Add 700 µL wash buffer, centrifuge, discard, and add 600 µL wash buffer 2. After adding 250 µL wash buffer 2, centrifuge and discard the run. Finally, transfer the column to a fresh tube, add 100 µL of nuclease-free water, incubate at 15-20 °C, centrifuge at 12000 × g for 1 min, and collect flow-through. Eluted total RNA was kept in 50 µL and 100 µL elution buffer at -80°C.

4.2. cDNA synthesis

5 µL total RNA and 1 µm random primer were incubated at 65°C for 10 min in a R Nase-free tube. The liquid was incubated for 5 min. Combine 4 µL 5x reaction buffer, 1ul Ribo Lock TM R Nase inhibitor (80 U), 1ul Super Script II reverse transcriptase (1,000 U) (Invitrogen), and 2 µL dNTPs (10mU) in 20 µL nuclease-free water. 23°C, 42°C, 70°C PCR. Heavy (VH) and Light (VL) Chain PCR Gene segments vary Reference and target gene primers were generated using (Primer 3) software. All primers were designed to an anneal in gene resigns without secondary structure creation at 55.6 and 52°C.

5.2. First PCR (Isolation of VH and VL)

Changing primer concentrations via the primer matrix optimized qPCR for primer melting temperature changes. 50–60 °C gradient PCR with specific primers was performed. To find the best VL and VH temperature, we used 10 tubes with 10 gene temperatures. The thermal cycler graph displays 50–60 °C temperatures (52.0, 52.1, 52.3, etc.). In the 100-ul PCR

experiment, 5 µl cDNA, 40 µl HF master mix (F531S) with HF buffer, and MWG, India-synthesized degenerate forward and reverse primers for light and heavy variable chains were used. The reaction mixture was cycled 29 times in an Eppendorf master cycler after cooling on ice. Gel electrophoresis verified VL and VH bands in a 10 µL aliquot **(Aavula *et al*., 2011).**

6.2. Second PCR (Assembly PCR reaction (SOE-PCR)

In this PCR, heavy and light chain domains were assembled and amplified using Phusion High Fidelity PCR. Products were analyzed by 1.2% agarose gel electrophoresis at an applied voltage of 12, appeared bands were purified using a GeneJET Gel Extraction Kit (K0691).

7.2. Third PCR (Digestion of PCR product = Scfv)

ScFv and $P^{ADL\text{-}22C}$ phages were cut with restriction enzymes *SfiI* and *BglI* in a PCR protocol under specific conditions, using new primers with these sites. The reaction was analyzed by gel electrophoresis; and the DNA bands were excised for gel purification.

8.2. Gel electrophoresis for PCR products

Digested PCR products were tested for amount and quality by looking for a single 400-bp VL and 450-bp VH band on a 1.2% agarose gel. Cut the band with a sterile blade and observe it with a UV transilluminator after transferring the residual volume to a preparative 0.8% low melting point agarose gel. Measure 0.5 g pure agarose and 50 ml (1 ×) TAE buffer in a 250 ml Erlenmyer flask. Mix thoroughly and heat until dissolved. The liquid was cooled and 10 µL of ethidium bromide was added.

9 Gel electrophoresis extraction

The DNA fragment was removed from the gel portion with a clean razor blade in an Eppendorff tube and weighed using a Thermo Scientific Gel Extraction Kit (K0691). On a hot plate, 1:1 binding buffer (w/v) was added and incubated at 55°C for 10 min. until the gel was fully dissolved. The entire dissolved gel was transferred to a GeneJET purification column and spun at full speed for 1 min. Add 500 µL of wash buffer to the GeneJET purification column, centrifuge for 1 min,

remove the flow-through, and repeat. The GeneJET purification column was transferred to a new Eppendorf tube, 50 µL elution buffer was added, incubated for 5 min. at room temperature, and centrifuged for 2 min. at the highest speed. The 50 ml flow-through was stored at 4°C in a clean Eppendorf tube.

10.2. Transformation into TG1

TG1 competent cells were produced in three days with some modifications:

On Day 1, the TG1 strain was streaked on an ampicillin-free LB plate and cultured overnight at 37°C at 200 rpm to extract colonies.

Day 2: Collect cells from one colony using a sterile loop and inoculate in sterile LB. Cells grew overnight in a shaking incubator at 37°C. Two 250 mL LB flasks were placed in the isolator to control medium temperature.

Day 3: 25mL overnight growth in 250mL LB flask. Equilibrate the medium in LB media flask in the incubator. Cultures grew 0.2–0.3 at O.D 650.

11 Digestion of ScFv insert and P^{ADL22C}

Sfi I digested ScFv and tested on a 1.2% agarose gel. A ThermoScientific Gel Extraction Kit was used to remove the digested vector ~3Kb. Fifteen microliters of DNA plasmid (scFv), 4.5 µL restriction enzyme *SfiI,* 30 µL digestion buffer G, and 250 µL nuclease-free water to process 300 µL the reaction mixture was inactivated at 80°C for 20 min. after 4 h at 50°C. The GeneJET Gel Extraction Kit (#K0691) filtered bands from 1.2% agarose gel electerophoresis at 120 V using a 1Kb ladder (**Kontermann and Dübel, 2010**).

12.2. Digestion of $p^{ADL-22c}$ vector

Fifteen microliters of $p^{ADL-22c}$ vector plasmid (phagmid) **(Figure 1),** 60 µL of nuclease-free water, buffer (O) thermo 30 µL and 3ul *Bgl1* restriction enzyme in a total volume of 300µLthe cells were incubated overnight at 37°C and then inactivated at 70°C for 20 minutes· Products were analyzed by gel electrophoresis then purified using the GeneJET Gel Extraction Kit (K0691).

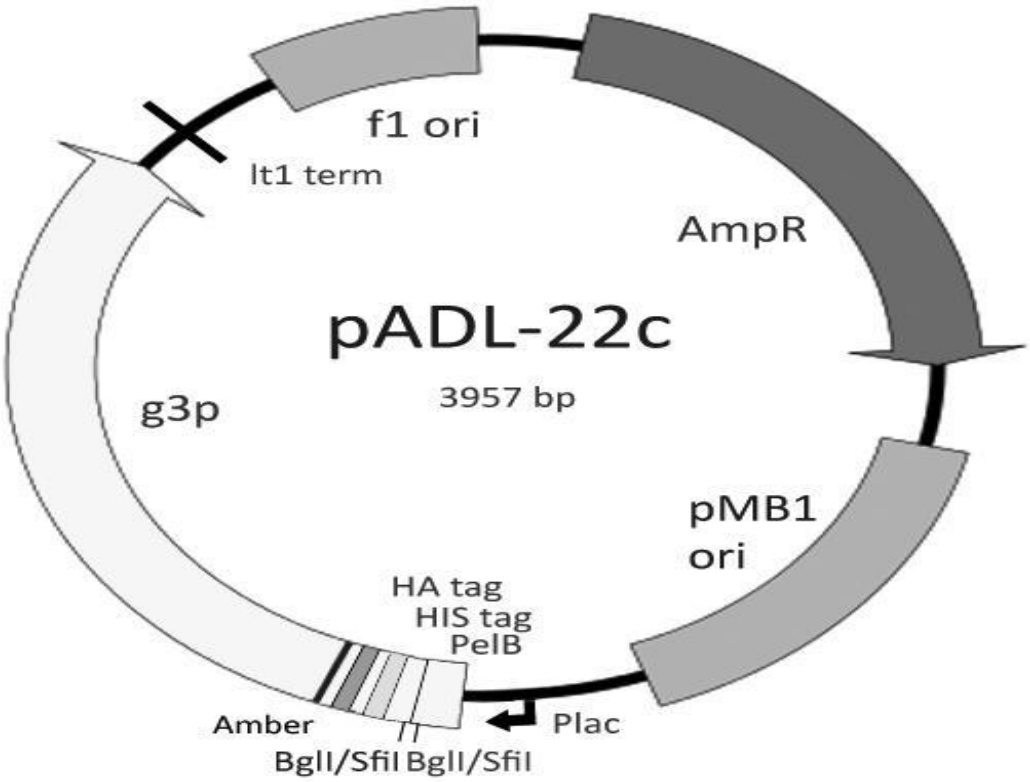


Figure (1): $p^{ADL\text{-}22c}$ vector map

12 Ligation

To optimize the ligation process, the insert-to-vector ratios were tested (1:1, 3:1, 5:1, and 10:1). A UV trans illuminator was used to measure preparations on an agarose gel to identify the vector and insert concentrations. As shown below, adding the right amounts to the test ligation reaction mixture (20 µL) produced the desired insert-to-vector ratio**.** The 37°C overnight ligation reactions [3].

13.2. Purification plasmid DNA (Mini prep)

Double-distilled water was used to make 2TY broth and TYE agar at pH 7.4. A plasmid-strain was cultivated overnight at 37 °C in 2xTY medium, 100 g/ml ampicillin, and 1% glucose. Fill a micro-centrifuge tube with 250 µL of cell slurry to resuspend pelleted cells and recover DNA from one plasmid using ThermoScientific GeneJET Plasmid Miniprep Kit (LOT 00584219). The Lysis solution was mixed thoroughly by inverting 4-6 times and centrifuged for 5 minutes to remove cell debris and chromosomal DNA. After adding 350 µL neutralizing solution, mix immediately. Pipette liquid to the GeneJET spin column. The flow-through was discarded and the column added after 1 min. Centrifuge 500 ml wash

solution in GeneJet spin column for 1 min. Flow-through trash. Repeat washing with 500 ml solution. Remove the flow-through and spin for another minute to remove the wash solution.

14.2. Preparation of helper phage $M_{13}KO_7$

Shake 100 ml of LB medium with one TG1 clone overnight at 37°C. Incubate 200 µL of *E. coli* TG 1 culture (O.D. 0.4) with 10 µL serial dilution (10 plates) of auxiliary phage MI3KO7 from original stock 1.1 $x10^{11}$ pfu/ml at 37°C for 30 min Overnight plaque development on LB agar medium at 37°C was performed on plates. Ten milliliters of residual bacterial culture were kept in 50% glycerol 1-ml aliquots at -80°C. Shake 5 ml of quickly growing E. coli TG1 culture at 37 °C for 2 h with small plaques. TGl infected with phage was diluted with 2xTY and shaken at 37°C for 1 h. After overnight shaking at 30 °C, kanamycin antibiotic was added. For 15 minutes, cells were at 10000g. After 1 h on ice, 100 PEG/NaCl solution precipitated phage particles from the supernatant. After 30 min of 10,000 × g centrifugation, phage particles resuspended in 8 Ml of TE buffer. Centrifuged phages after 20 min in TE buffer on ice with 2 ml PEG/NaCl. The phage powder was resuspended in PBS and centrifuged at 10,000 × g for 10 min to eliminate bacterial cell debris. Phage helper particles were resuspended in PBS with 15% glycerol and kept at 70 and -80 °C.

15.2. Preparation of phagmid and biopanning

Bio-panning for 3 days

Day 1: Incubate 250 ml TG1 library in 100 ml 2xTY at 200 rpm and 37°C for 2-3 hours. Helper phage M13KO7 1 mL was incubated at room temperature without mixing for 30 min. 10 min/4°C/4000 rpm spin. Suspend cells in 300 mL 2xTY (ampicillin/kanamycin). The jar was incubated at 37°C and 200 rpm. TG1 cells incubated at 37 °C. Incubate two ELISA wells with antigen and coating buffer at 4°C.

Day 2: 50minute 4°C/5000 rpm centrifuge. The supernatant and PEG/NaCl were transferred to fresh flasks. Stirred well, the mixture marinated on ice for 1 h. At 4°C, the mixture was spun at 5000 rpm for 50 minutes. The powder was resuspended in 1ml 1× PBS after discarding the supernatant. Phagmid whirled for 5 minutes at 13500 rpm. Post-260 nm optical density measurement, 200 ml phagmid was stored at 4°C. The ELISA dish was blocked with 2% skimmed milk at room temperature for 2 h after rinsing with PBS buffer for 5 min. Five times with PBS, repeat the ELISA dish. Add 200 µL of phagmid and block to each well. The combination is incubated at room temperature for 1 h. After washing wells with PBS and 0.05 % Tween 20, 100 µL Triethylamine) was added to each. Room-temperature incubation for 10 min.

Phage eluted in 200 µL/well with 1M tris pH 7.4. Rest at 4°C. Eluted phage was diluted 10x in PBS. Ten microliters of each dilution were mixed with 90 µm free TG1 (log phase) at 37°C for 45 minutes without shaking. A 37°C overnight culture on LB agar (ampicilin +2% glucose) was done. Eluted phage was incubated at 37°C for 30 min without mixing with free TG1 at 200 rpm for 20 min at 37°C. Add one milliter assistance phage and incubate at room temperature without mixing for 30 min. Reincubate the pellet at 37°C at 200 rpm in 2xTY.

Day3: The mixture was centrifuged overnight at 4°C for 50 min. at 5000 rpm. Inverted supernatant was combined with PEG/NaCl in flasks and incubated on ice for 1 h. Powder was rehydrated in PBS. The sediment was phagmid. This mixture was centrifuged for 5 minutes at 13500 rpm. The optical density at 260 nm was compared to plain PBS **(Ghassabeh *et al.*, 2010).**

16.2. ELISA for screening of individual colonies

Day 1: The colonies from each round were injected into a 1ml deep well plate with comparable colonies from LB agar. Shake 24-well plates at 150 rpm at 37°C until O.D 600 is 0.6. (5-7). The plate shook at 37°C for 1 min after adding 100 µL IPTG. ELISA dish was antigen-coated and incubated at 4°C.

Day 2: Removed coating, washed with 5x PBS for ELISA, added skimmed milk (blocking), and incubated at room temperature for 2 hours. 20-minute 4000 rpm 4°C centrifuge. The supernatant removed and filled each well with 200 ml TES. Mix for 20 mins at 4°C at 250-300 rpm. 30 min. at 4°C, shake 300 µL fourfold TES in H_2O. The mixture was centrifuged at 4000 rpm for 20 minutes at 4°C. A 100 µL supernatant and 100 µL blank well were added to the antigen-coated wells. Following a 1-hour incubation at room temperature, the cells were washed with 5x PBS-Tween and diluted with anti-HA tag antibody alkaline phosphatase conjugate (Sigma) After 15, 30, 45, 60, and 90 min, a 450 nm ELISA reader identified 200 ml of phosphatase substrate solution.

17.2. Touch clone PCR

Cloning PCR was performed in 30 tube PCR with a single colony of each biopanning colony to detect the presence of scFv in each tube.

18.2. Sequencing

One-way R3 primer sequencing was performed on the positive ELISA clones. An integrative database of germ-line variable genes (VBASE2, http://www.vbase2.org/) and Immunoglobulin BLAST (IgBlast, https://www.ncbi.nlm.nih.gov/igblast/) was used to match the scFv sequences produced in mice.

3. Results

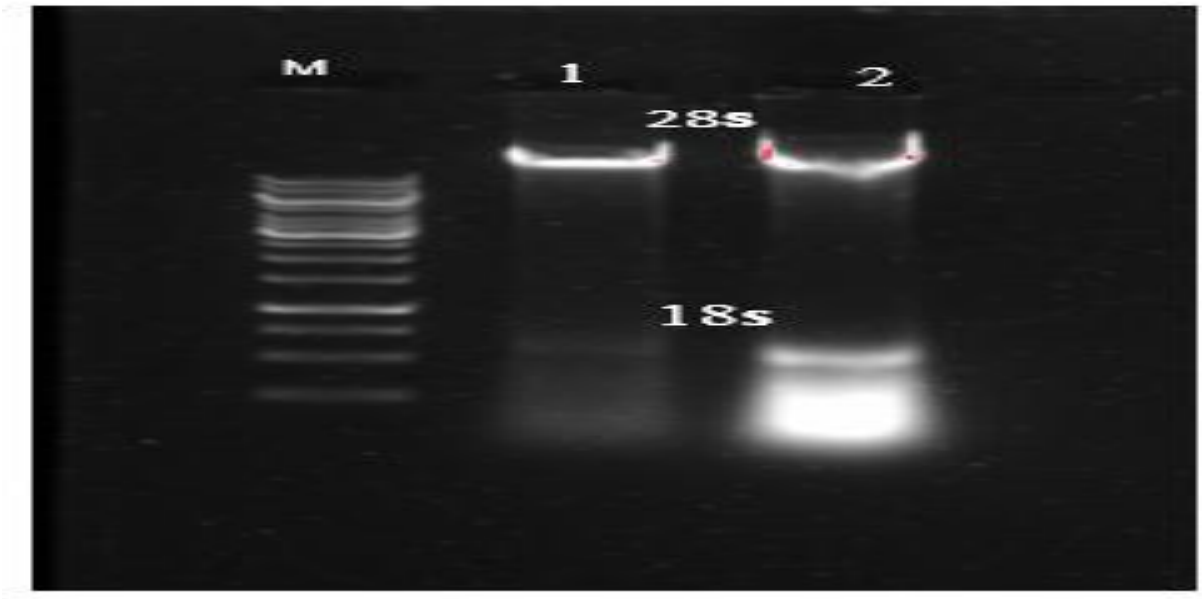
3.1. Immunization of mice

The experimental procedures were approved by the Institutional Animal Care and Use Committee (CU-IACUC) of Cairo University under license number CUIF7619.

3.2 RNA isolation and cDNA synthesis

Total RNA was isolated from immunized mice spleen cells using a Thermo Fischer mi RNA kit, and gel electrophoresis was performed using a 1 kb ladder **(Figure 2)** 28S and 18S rRNA sharp bands appeared, and cDNA was synthesized.

Figure (2): Total RNA isolated from mice1 spleen, lane 1, 50 ul elution and lane 2, 100 ul elusion using 1 kb marker.



3.3. PCR amplification for VL and VH

Gradient PCR selected 52.3 for VH and 55.6 for VL as the optimal temperatures for the best band. Amplifications were repeated several times and the final variable light chain (VL) and variable heavy chain (VH) products were assessed by 1.2% agarose gel electrophoresis at an applied voltage 12 were appeared bands were purified using the GeneJET Gel Extraction Kit (#K0691) and used as a template for PCR amplification of VL and VH using reverse VL and forward VH yielding 400 bp and 450 bp, representable as shown in **(Figure 3).**

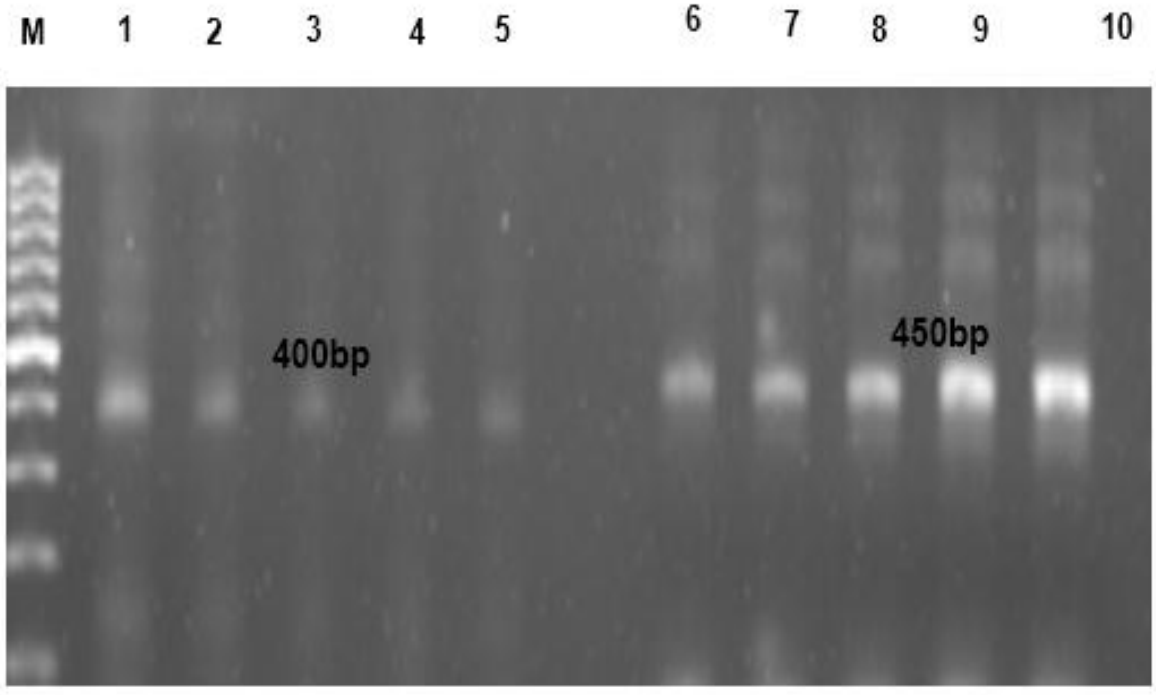


Figure 3. Variable sections of the mouse immunoglobulin gene repertoire were amplified by PCR. The results of five PCR reactions for V_L with a 400 bp size (lanes 1,2,3,4 and 5) and the same number of reactions for VH with a 450 bp size (lanes 6,7,8,9, and 10) the reference DNA was a 100-bp DNA ladder.

3.3.2 Second PCR (ScFv cloning and assembly)

A cDNA template made from spleen RNA of immunized mice produced Scfv. Electrophoresis confirmed the stability of RNA. PCR was used to amplify the VH and VL regions at the expected molecular sizes of 450 and 400 bp, representable. SOE-PCR linked the repertoires of the two domains with a 45-mer polynucleotide linear (GGGGS)3, creating combinatorial scFv repertoires that could be seen on an agarose gel at 850 bp **(Figure 4).**

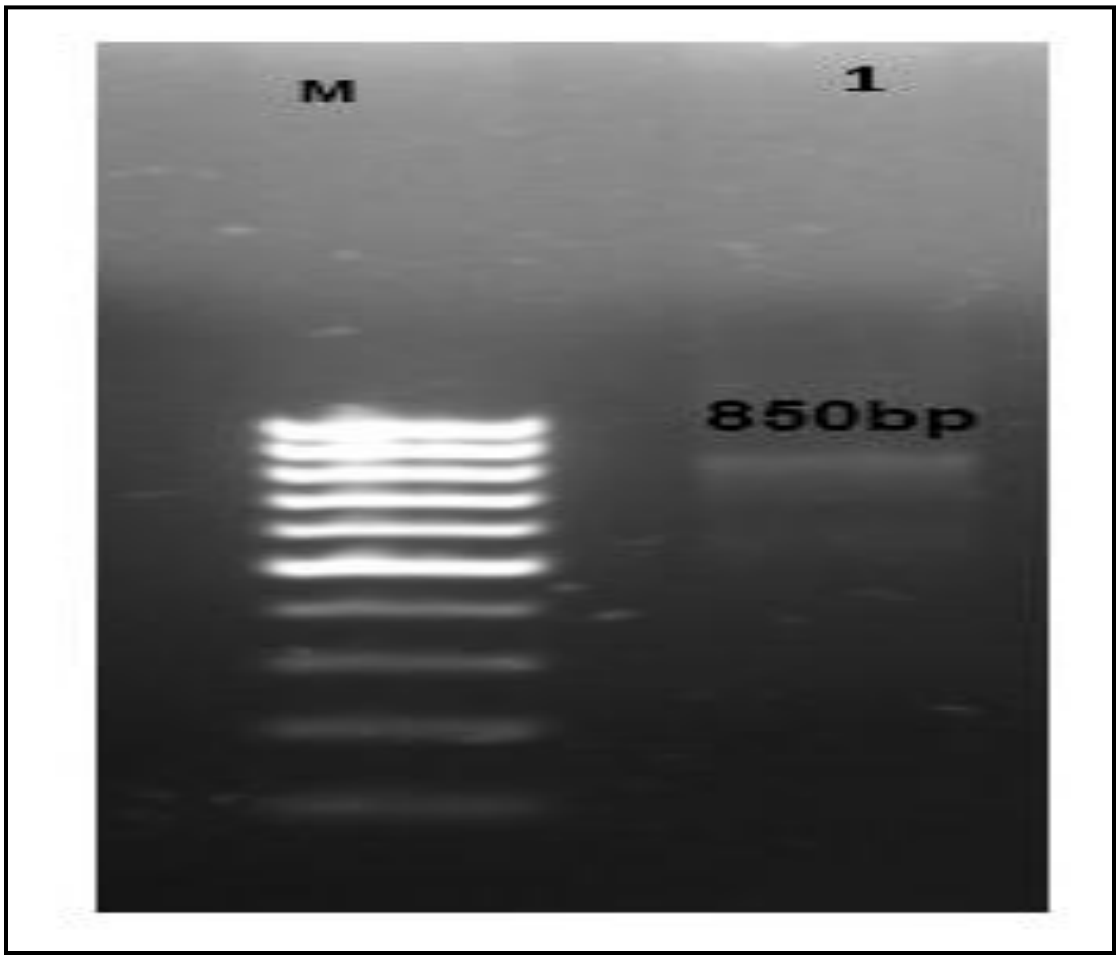


Figure (4): SOE-PCR product (850 bp) with 100 bp marker.

3.3.3 Third PCR

PCR was performed using new primers designed with restriction sites to be adapted with the restriction enzymes cutting the p^{ADL22c} phagmid and the scFv (DNA) template, and the reaction was analyzed by gel electrophoresis, as shown in **(Figure 5).**

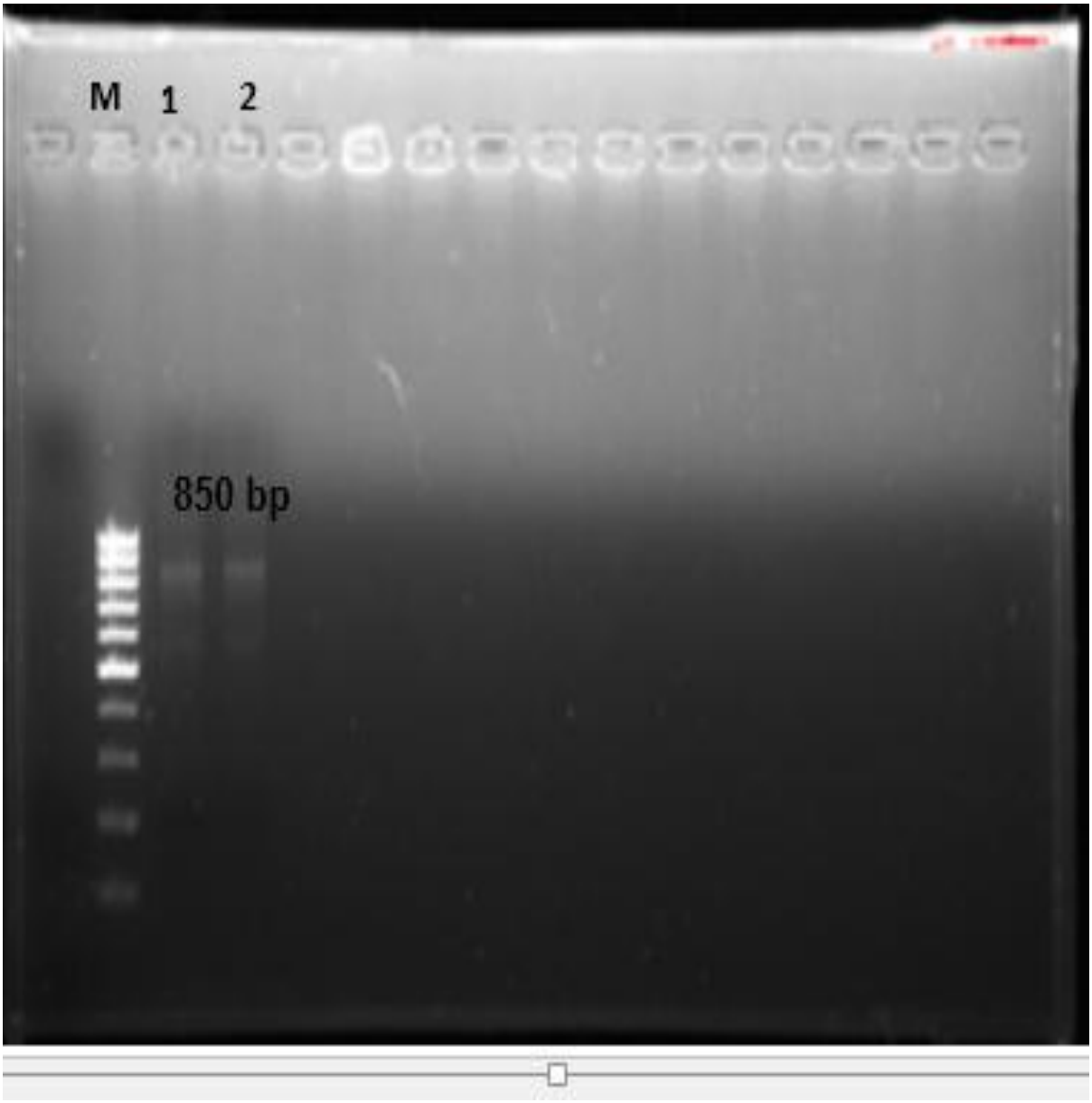


Figure (5): Scfv with restriction sites using new primers at 850 bp.

3.4 Transformation into TG1

3.4.1 TG1 competent cells

The growth of bacteria in an extended culture depended on the cell growth time of separate TG1 colonies. The bacteria were then incubated with an alien plasmid. O.D. 600 also indicates growth. Long incubation times lowered with transformation efficiency. Separate groups grew best in 8-10 h. The highest transformative efficiency was observed at 600 O.D. was 0.53.

3.4.2 Digestion of ScFv insert and P^{ADL22C}

SfiI digested assembled scFv repertoires, while BglI (Thermo Fisher Scientific, USA) digested P^{ADL22c} phagemid (Antibody Design Labs, San Diego, USA), resulting in similar overhangs (GCCNNNN/NGGC) when the sites opened by each enzyme were related. Gel electrophoresis showed bands of degraded scFv repertoires after batch ligation into the P^{ADL22c} phagemid with T4 DNA ligase (New England Biolabs, UK), as shown in **(Figure 6).**

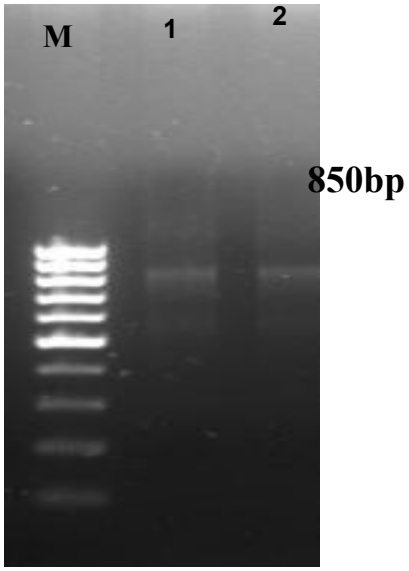
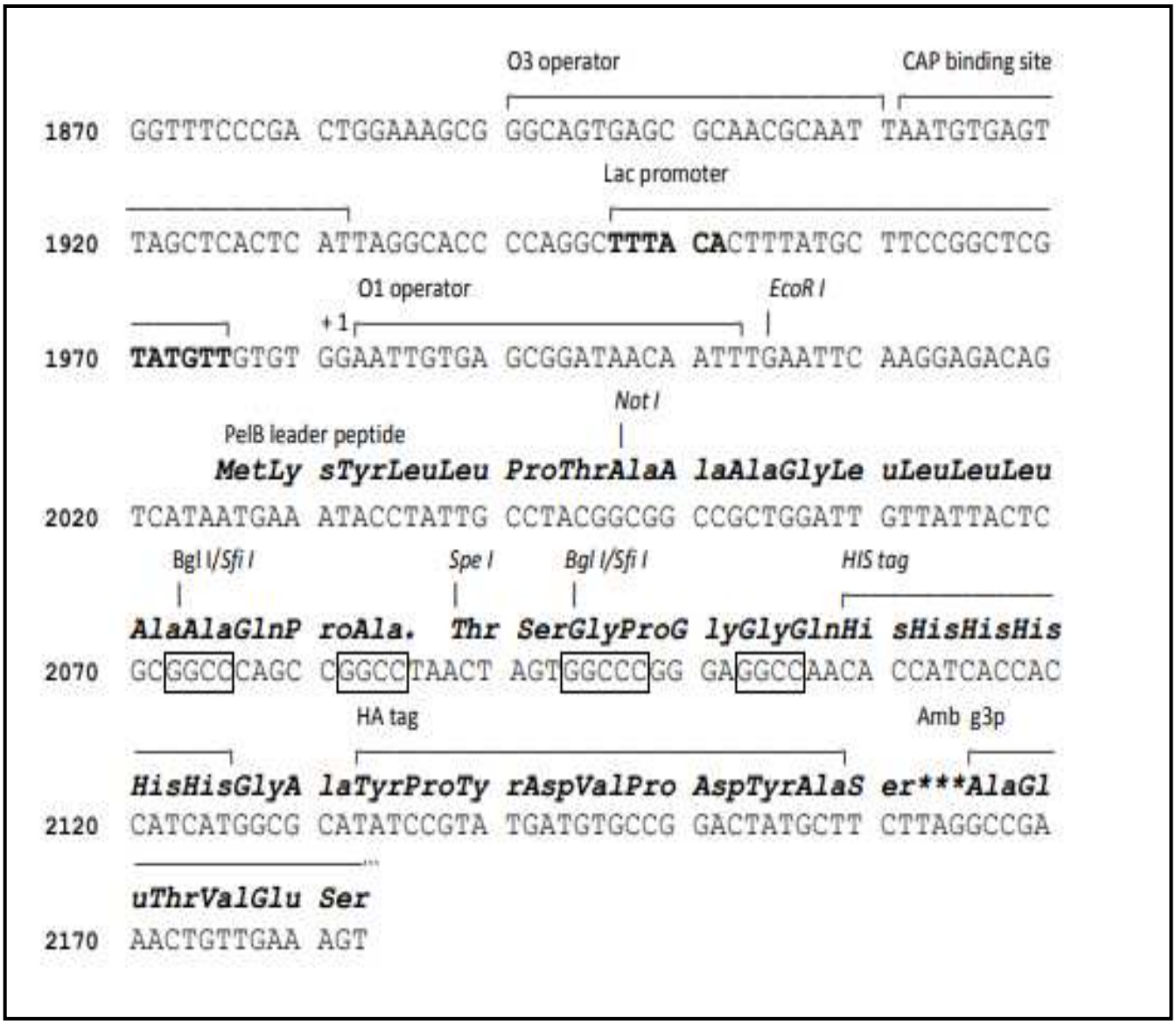


Figure (6): Digestion product at 850 bp for lane 1 and lane 2 using SfiI restriction enzyme with 100 bp marker.

3.4.3 Ligation of ScFv with digested $p^{ADL-22c}$ phagmid

The N-terminus of phage M13 gene III (g3) and the Pe1B peptide leader sequence were inserted into the $p^{ADL-22c}$ phagemid to cause their insertion into the bacterial periplasmic region and display on the phage. The pe1B sequence was interrupted by a stop codon, so the final two amino acid sequences MA had to be reintroduced by PCR to ensusure frame proofreading and cleavage **(Figure 7**). AHis-HA amber codon was used to divide the ScFv and g3 sequences. Sfi1 digestion released an 850 bp scFv-corresponding band from a 4000 bp $p^{ADL-22c}$ fragment, confirming its inclusion.



/

Figure (7): Illustration of $p^{ADL\text{-}22c}$ cloning site.

3.4.4 ScFv library

The scFv phage library against exotoxin A was created by transforming recombinant phagemids (P^{ADL22c}-scFv) into competent *E. coli* TG1, suppressing the amber codon between the scFv and g3 coding sequences that allowed phage M13's g3 protein to display scFv antibodies on the N-terminus (PIII). A large enrichment effect was observed. After M13KO7 helped phage infection of the library, 1.4×10^{8} colonies from the cultured library were exposed. Using touch-clone PCR with S3 and R2 primers, all studied clones had scFv DNA fragments of 850 bp **(Figure 8),** indicating 100% library recombination.

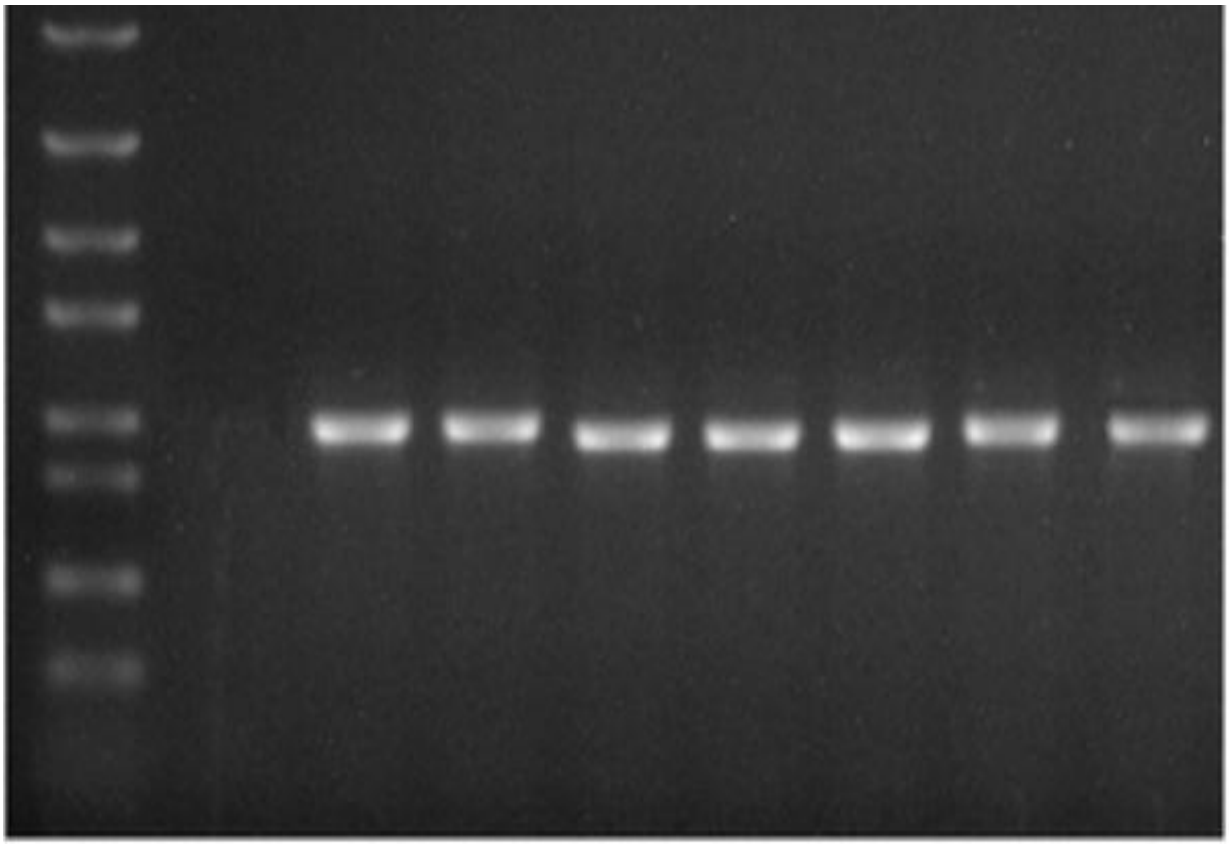


Figure 8. Agarose gel electrophoresis analysis of colony PCR products of the positive clones. Lane M DNA marker; lane 1, 2, 3,4,5,6 and 7 at 850 bp.

3.4.5 Biopanning

M13KO7, the phage display assembly virus, is highly homologous. Biopanning was performed three times. From the first to the third biopanning cycle, the observed titration values (output/input) show an increase in certain binders. Only tightly bound phages were preserved in the phage libraries containing 10^7–10^6 peptides. Phage display links each peptide to its genome. Thus, amino acid sequences revealed the DNA sequences of phage clones. Randomized peptide libraries can be screened in various target forms.

3.4.6 Sandwich ELISA for screening individual colonies

During the three cycles of biopanning, phage ELISA was used to determine whether antigen-specific phages, such as *Pseudomonas aeruginosa* exotoxin, were enriched in the phage populations. Phages from the original library and later rounds of panning were loaded separately into the exotoxin-coated and BSA-coated (control) wells. Enzyme-coupled anti-M13 secondary antibodies and the substrate yielded 405 nm absorption. The initial phage library had an absorption value of 0.02–0.06 at 405 nm, similar to that of the BSA, plasmid, and helper phage controls **(Table1).** The first-round panned phage had a larger average value than the controls. The best positive results in each pan were plotted against time after 1 hour at 10 minutes interval at 405 nm **(Figure 9)** Screening individual colonies

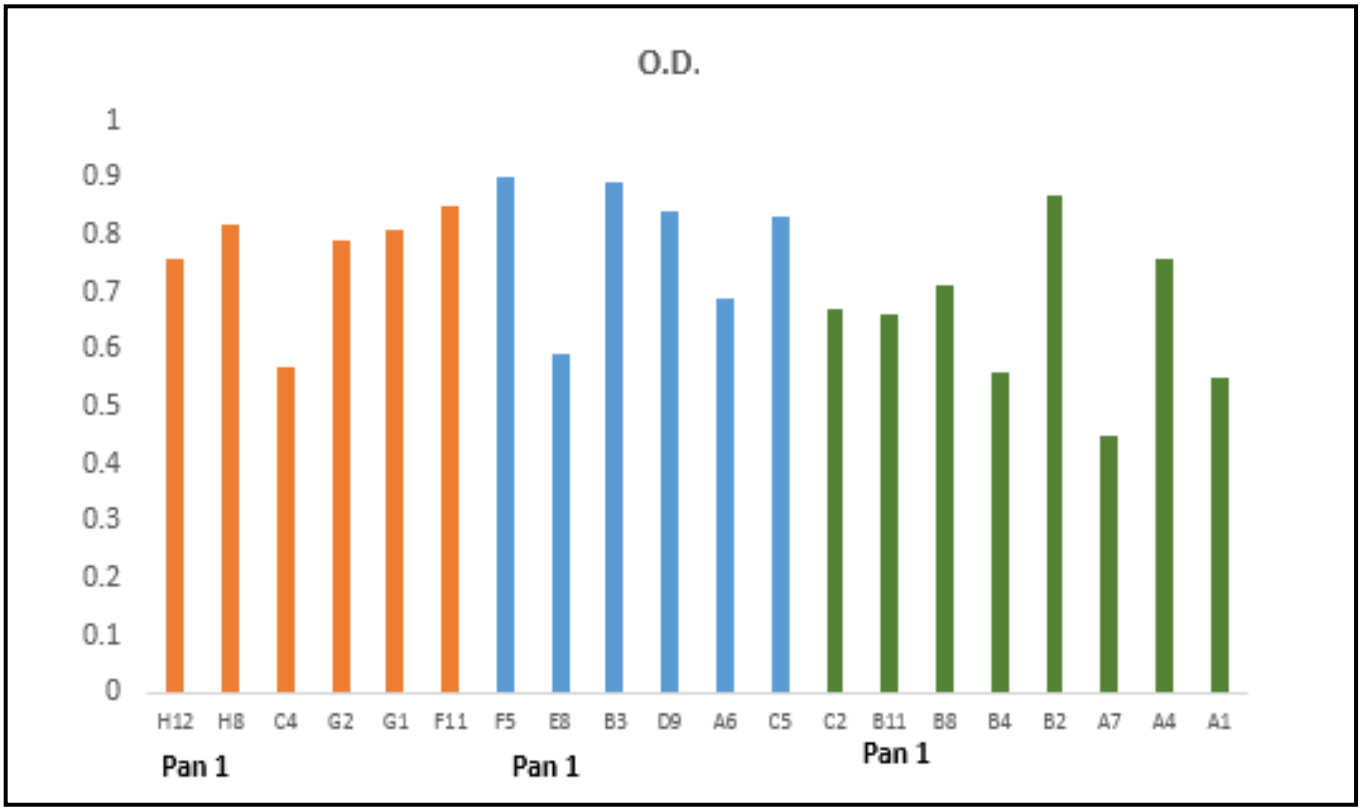


Figure (9): The highest positive results colonies in each pan after 10 minutes interval at 405 nm.

Table (1): Measurement of each sample against O.D. at 405 nm wavelength.

Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	BSA	M13	Helper phage	Original liberary	First round	Second round	Third round
Average	0.024	0.056	0.053	0.096	0.257	0.433	1.68

3.4.7 Touch clone PCR

The ELISA showed five random clones with the greatest signal (17, 28, 69, 81, and 95). Positive clones were cultured overnight in 5 ml LB medium containing 5ul ampicillin at 37 °C and 150 rpm. Touch clone PCR (cloning PCR) for the five positive clones using the S3 and R3 primers showed a sharp band at 850 bp, as shown in **(Figure 10).**

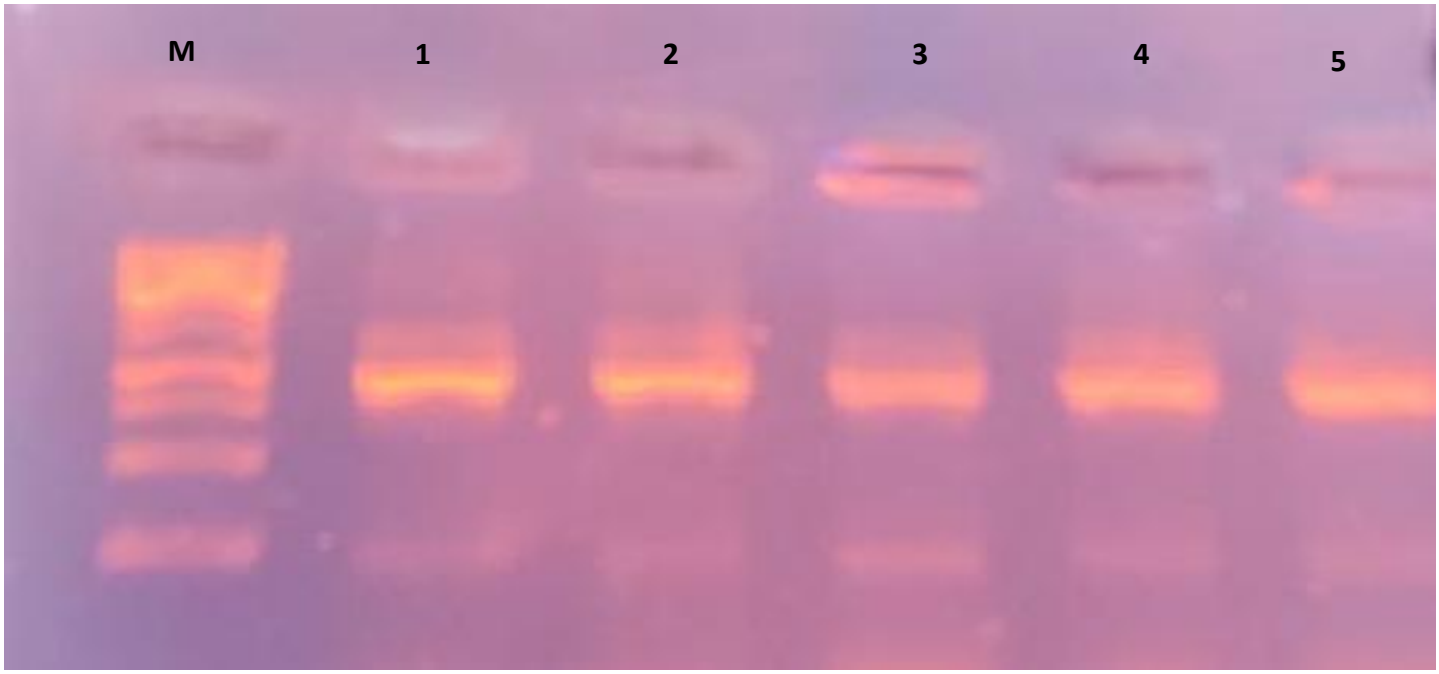


Figure (10): Positive cloning PCR of five random clones using 1Kb marker, lane 17: clone1; lane2: clone 28; lane 3: clone 69; lane 4: clone 81 and lane 5: clone 95 at 850 bp.

3.4.8 Miniprep preparation

Mini prep was performed as a pervious protocol for the five positive clones using Gene JET Plasmid Midiprep Kit, the results showed that the five samples gave purified plasmid at 850 bp using a 1Kb marker as shown in **(Figure 11).**

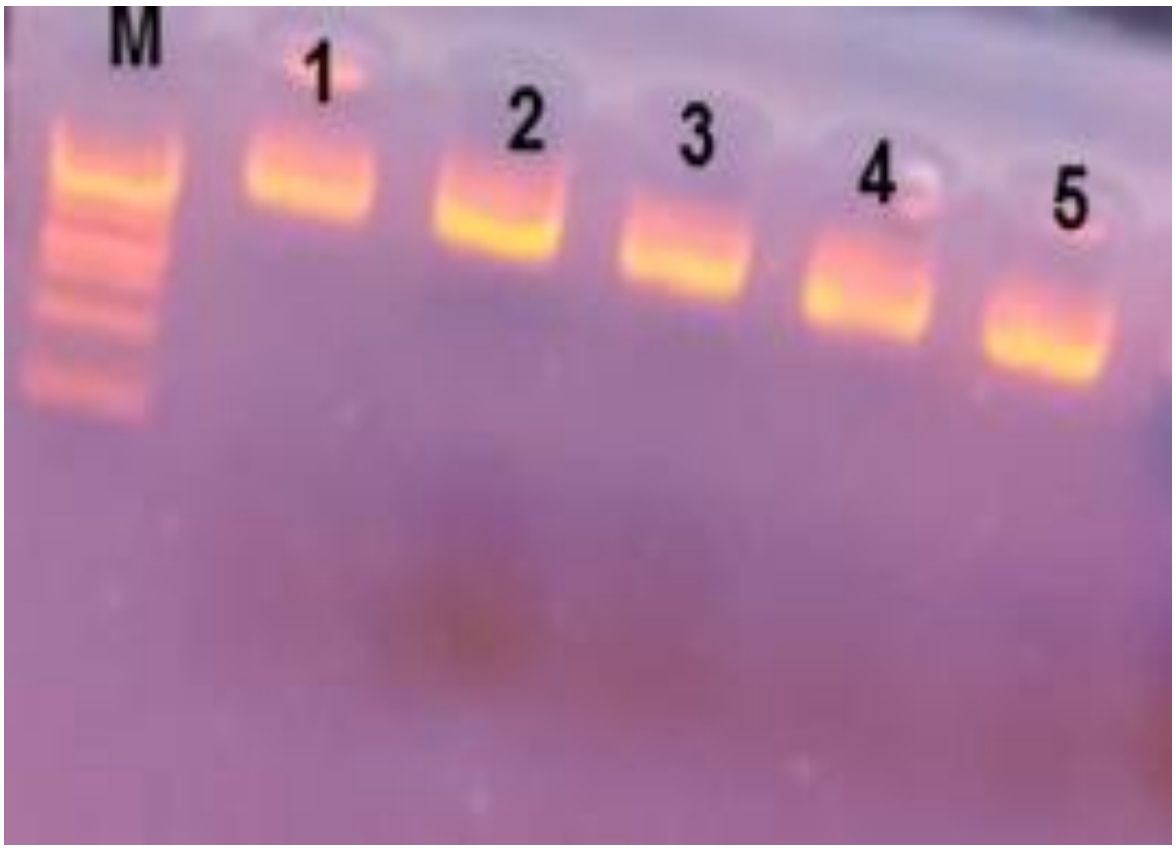


Figure (11): Showed purification of plasmid for five positive clones, Marker: 1Kb; lane 1: clone 1; lane2: clone 2; lane 3: clone 3; lane 4: clone 4 and lane 5: clone.

3.4.9 Sequencing

The polyclonal ELISA-positive clones' recombinant P^{ADL22c} phagemid was sequenced. VBASE2 and IgBlast were used to match the scFv sequences produced in the mice. Using Mega Align lasergene software 4.0, scFv-generated sequences were aligned, four random clones were sequenced, and NCBI accession numbers were assigned **(Table 2)**.

Table (2): Accession numbers for the four random sequenced clones.

Number of clones	Accession on NCBI
Seq 1	OP484949
Seq 2	OP484950
Seq 3	OP484951
Seq 4	OP484952

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4. Discussion

P. aeruginosa infects and illnesses plants and animals. It causes various human diseases, especially in immunocompromised people, and many hospital-acquired infections. *P. aeruginosa* is an opportunistic pathogen, therefore antibiotic resistance makes treatment and eradication challenging. Toxin A, isolated from *P. aeruginosa,* is very virulent. **Liu *et al.* 1974** discovered exotoxin A (PE), a mono-ADP-ribosyltransferase. Toxin A was isolated from P. aeruginosa using Sigma-serum from mice and a universal antibody. Western blotting investigations of serum samples against toxin A components validated its 66 KDa size. Western blotting indicated the normal distribution of all antigens, with pure toxin A at 66 KDa (Drapeau et al. 1972; Taylor and Pollack, 1978 and Saleh et al. 2012). In agreement with **Gong *et al.* (2021),** serological methods for detecting *P. aeruginosa* toxA gene, such as crossed immune electrophoresis (CI), 12 samples yielded positive results, as evidenced by PCR results. Since antibiotic-resistant microorganisms threaten public health, we must immediately identify new antibacterial chemicals. Phage display technology can test peptides or antibodies with significant binding capabilities for several targets. This characteristic is crucial in the urgent search for novel antibacterial drugs to fight bacterial resistance Zhao et al. (2023). The VH and VL genes were isolated and amplified at 400 and 450 bp by PCR using cDNA as a template, respectively, and the scFv gene was generated at 850 bp by SOE-

PCR and finally by third PCR using specific primers SfiI to add restricted sides on both ends of the gene. These results agree with **Zaho *et al.* (2023).** According to (**Ma and O'kennedy, 2017),** antibody libraries contain a wide range of synthetic and semi-synthetic antibodies, making it easier to extract antibodies against specific antigens. Where, scFv phage libraries can reduce monoclonal antibody production without animal testing.

Amplification of VH and VL and reverse transcription of mRNA into cDNA were done in many ways. VH and VL with a short linker (SSRSSGG) generate scFv, which overlap extension PCR ligates. Subcloning incorporates both ends of the scFv into pComb3H via Sfi I. To promote phagemid subcloning, the *Sfi I* restriction site was introduced at VL's 50-end and VH's 30-end. To connect the VH and VL strands, a short linker (GGSSRSS) was added at the 30 ends of the VL primers. Randomly collected PCR findings were purified using 1.5% agarose gel electrophoresis. **Nathan *et al.* (2002)** estimate VH and VL sizes at 340 and 320 bp. Our results showed that the generated scfv was digested with SfiI and ligated to the pADL^{22C} vector, which was digested with BglI, releasing an 850 bp scFv corresponding band from a 4000 bp pADL^{22c} using *E. coli* (TG1) and helper phage M13KO7, as well as PCR analysis for the presence of scFv in biopanning, followed by sandwich ELISA to the phage. The VH fragment repertoire against SARS-CoV-2 was hybridized to each of the four synthetic VLs (four libraries) using overlapping PCR and a GS19 linker peptide, according to **Salazar *et al.* (2022).** After *SfiI* digestion, the scFvs were ligated to the pADLTM 2c phagemid vector. Two scFv libraries were produced from ligated phagemid constructs after 10 rounds of electroporation: one with VH-V scFvs and the other against Covid-19. Each library had over 5×10^{10} colony-forming units (cfu). Randomly sequencing colonies from each library revealed over 80% correct scFv-coding and complete use of all antibody subfamily genes. The EHL Library's efficacy and quality were demonstrated by screening a panel of human targets (mainly tumor- Using S. mRNA. A scFv library was generated from volunteers' peripheral blood mononuclear cells infected with S. aureus. Bacteriophage display helps scientists understand protein-antagonist interactions. Scientists can design new inhibitors, biosensors, cleansers, and immunological modulators that target specific *S. aureus* types by doing this **(Tian et al., 2022**). Transforming the scFv library DNA into *E. coli* TG1 yielded 10^7 distinct clones with full-length insertion. S screened the scFv library three times with the phage display. aureus antigen. For ELISA testing, single clones were randomly selected and scFvs expressed. Approximately 50% of clones were S-positive. Aureus binding is good. E was used to produce and express scFv-fragment crystallizable areas (-Fcs) to stabilize them **(Nian *et al.*2016).** New vaccination platforms and viral therapies have been developed and implemented because to the SARS-CoV-2 COVID-19 pandemic. Using phage display, single-domain antibodies like the camelid variable heavy-chain fragment (VHH) can be used therapeutically. Such therapies can be critical when

vaccinations for new viruses are not yet available **Daly et al. (2023).** A mouse phage display scFv library was utilized to discover recombinant anti-ciprofloxacin single-chain antibodies in animal feeding, according to **Wei and Wang (2021).** After four bio-panning rounds, 25 positive samples were detected. E. The best scFv-22 was made from bacteria BL21. Val160 was needed for scFv to bind to CIP. The parental and mutant scFv were used to make a CIP indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) after cleaning. The results reveal that phage library creation and directional evolution produced a sensitive anti-CIP scFv. The scFv-based IC-ELISA detects CIP residue in animal products. Rounds of biopanning isolate several ExoA-DI reactive clones using polyclonal phage ELISA. One positive clone with high antibody affinity was found by monoclonal phage ELISA. **Shadman *et al.* (2021)** found that purified scFv significantly interacted with recombinant domain I and full-length native exotoxin A. Three rounds of biopanning were repeated to maximize selectivity and affinity. Each round's output/input was doubled and carried over. Biopanning enriches certain binders, as seen by an increase in output/input titration values from the first to the third cycle. The average phage library has 106–108 distinct peptides. Biopanning and phage recombinants with antibody at their tips were used by McCafferty and colleagues to efficiently synthesize recombinant antibodies. McCafferty et al. (1990) replaced hybridoma with in vitro selection of scFv (single chain fragment variable) from vast variable domain libraries. The eluted phages' titer increased significantly after each round of bio-panning and remained stable (about 106 pfu/mL) after three rounds, indicating that more specific antibodies bound to the antigen coated on the plates (Yang et al., 2018). A novel human scFv antibody for P. aeruginosa exotoxin. Biopanning and ELISA screening identified ExoA-DI from a human scFv phage library. The positive colonies were sequenced after three to five cycles **(Shadman *et al*., 2021).** Five randomly selected clones (17, 28, 69, 81, and 95) provided the greatest signal in enzyme-linked immunosorbent tests, therefore R2 and S3 primers were used to perform touch clone PCR, resulting in 850 bp crisp bands. The produced ScFv sequences were compared to other mice scFv using an integrative Immunoglobulin BLAST database (IgBlast, https://www.ncbi.nlm.nih.gov/igblast/) and NCBI-approved accession numbers. One-way primer sequences based on the R2 primer were used for analysis. These results in agreement with **Aavula *et al.* (2011),** who sequenced touch clone PCR positive clones using DNA comparison tools and found that they all had identical variable antibody genes from the IgG1 class. One was chosen for further study. The sequencing scFv was determined by IgBLAST. We uncover many variable and joining genes for VH and VL rearrangements with different frequencies (**Wei *et al*., 2022**).

5. Conclusion

Phage display is a way to identify target-binding proteins from a library of millions of distinct proteins without having to screen each molecule individually. Antibody phage display, a versatile, repeatable, and useful technology, can be used to identify antibody candidates for a wide range of disease indications. Antibody phage display can be used to find antibodies against antigens that aren't readily available in pure form, isolate antibodies that can differentiate between different antigen epitopes, and discover new cell biomarkers via cell-based biopanning. The phage display technique is a quick and low-cost method for studying protein-protein interactions. This technique is used to create monoclonal antibodies, which are useful as therapeutic, diagnostic and investigative tools, as well as epitope mapping, which aids in vaccine development, antigen-antibody interaction, and cell biomarkers.

Authors' contributions Conception/design:

Mary S. Khalil and Reda S. Salem designed and performed the experiments. Doaa R. Zahran and Mary S. Khalil, Amir E. tantawy analyzed the data and wrote the manuscript. All authors have read, agreed and approved of the version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate.

Applicable.

Consent for publication

All authors agree to submit this manuscript for publication.

Competing interests

Authors have no competing interests to declare.

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6. References

Wood, S. J., Kuzel, T. M., and Shafikhani, S. H. (2023). *Pseudomonas aeruginosa*: infections, animal modeling, and therapeutics. *Cells* 12:199. doi: 10.3390/cells12010199.

Mitra S. and Tomar P. C. (2021). Hybridoma technology; advancements, clinical significance and future aspects, Journal of Genetic Engineering and Biotechnology. (2021) **19**, 1–12.

Moraes J. Z., Hamaguchi B., Braggion C., Speciale E. R., Cesar F. B. V., Soares G. F. D. S., Osaki J. H., Pereira T. M., and Aguiar R. B.(2021). Hybridoma technology: is it still useful?, Current Research in Immunology, 2021, **2**, 32–40.

Sadraeian, M.; Malek,R.; Moraghebi,M. and Bahrami,A. (2024). Phage Display Technology in Biomarker Identification with Emphasis on Non-Cancerous Diseases. *Molecules* **2024**, *29*(13), 3002**.**

Aavula, S.; Nimmagadda, S.; Biradhar, N.; Sula, S.; Chandran, D., Lingala, R. and Villuppanoor, S. (2011). Generation and Characterization of scFv Directed against Site II of Rabies Glycoprotein. National library of medicine 2011: 652147.

Kontermann, R. and Dübel, S. (2010). Antibody Engineering Volume 2, chapter 20, Ghassabeh *et al.* 251-266.

Ghassabeh, G.; Muyldermans, S. and Saerens, D. (2010). Nanobodies, Single-Domain Antigen-Binding Fragments of Camelid Heavy-Chain Antibodies. Book, Current Trends in Monoclonal Antibody Development and Manufacturing, pp 29-48.

Liu, V.L. (1974). Exotoxin of *Pseudomonas aeruginosa*, factors that influence the production of toxin A. Journal of infectious diseases 128(4):506-51.

Gong, F.; Wei, H.; Li, Q.; Liu, L. and Li, B. (2021). Evaluation and comparison of serological methods for COVID-19 diagnosis. Front. Mol. Biosci., 23 July 2021 Sec. Structural Biology, 2021 (8): 1-12.

Zaho, H.; Nie, D.; Hu,Y.; Chen, Z.; Hou ,Z.; Li ,M. and Xue, X. (2023). Phage Display-Derived Peptides and Antibodies for Bacterial Infectious Diseases Therapy and Diagnosis. Molecules 28 (2621), 1-17.

Ma, H. and O'kennedy R. (2017). Recombinant antibody fragment production. Methods. 2017; 116:23-33.

Nathan, S.; Li, H.; Mohamed, R. and Embi, N. (2002). Phage Display of Recombinant Antibodies toward Burkholderia pseudomallei Exotoxin. The Journal of Biochemistry Molecular Biology and Biophysics 6(1):45-53.

Salazar K. C., Ma L., Green S. I., Zulk J. J., Trautner B. W., Ramig R. F., et al. (2021). Antiviral resistance and phage counter adaptation to antibiotic-resistant extraintestinal pathogenic *Escherichia coli*. mBio 12:e00211-21. 10.1128/mBio.00211-21.

Tian, L.; Chan,M.; Saif,A.; He,L.; Didar,T. and Hosseinidoust, Z. (2022). Phage display for the detection, analysis, disinfection, and prevention of *Staphylococcus aureus*. Smart medicine 1-16.

Nian, S.; Wu., T.; Ye, Y.; Wang, X.; Xu, W. and Yuan, Q. (2016). Development and identification of fully human scFv-Fcs against Staphylococcus aureus. BMC Immunology 17 (8), 1-12.

Wei, M. and Wang, P. (2021). Progress in molecular biology and translational science- new target for diagnostic and therapeutic development. Progress in Molecular Biology and Translational Science162:25-57.

Shadman, Z.; Farajnia, S.; Pazhang, M.; Tohidkia, M.; Rahbarnia, S.; Najavand, L., and Toraby,S. (2021). Isolation and characterizations of a novel recombinant scFv antibody against exotoxin A of Pseudomonas aeruginosa. National **library of medicine 2021; 21: 300**

Wei, C.; Criner, S.; Sridhar, B.; Shultzaberger, R.; Looney, T.; Fabani, M. and Glezer, E. (2022). Reconstruction of Full-length scFv Libraries with the Extended Range Targeted Sequencing Method. BioRxiv1:8.