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DEVELOPMENT OF RECOMBINANT ANTIBIOTICS AGAINST *ENTEROCOCCUS FAECIUM*

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Ph.D. THESIS

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Hanife SALİH DOĞAN

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ABBREVIATIONS

µg	: Microgram
µl	: Microliter
CDRs	: Complementary determining regions
Dab	: Domain antibody
Fab	: Fragment antigen-binding
Fc	: Fragment crystallizable
g	: Gram
Ig	: Immunoglobulin
IMAC	: Immobilized Metal Affinity Chromatography
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
MCS	: Multiple cloning site
ml	: Milliliter
PBP	: Penicillin binding protein
PCR	: Polymerase chain reaction
rpm	: Revolution per minute
sol-PBP5	: soluble form of PBP5
TSA	: Tryptic Soy Agar
TSB	: Tryptic Soy Broth
VH	: Variable heavy

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ABSTRACT

DEVELOPMENT OF RECOMBINANT ANTIBIOTICS AGAINST *ENTEROCOCCUS FAECİUM*

Salih-Doğan H. Aydın Adnan Menderes University, Health Sciences Institute, Molecular Biotechnology Doctorate Program, Ph.D. Thesis, Aydın, 2024.

Purpose: It was aimed to develop a recombinant antibiotic originating from the VH sequence of antibodies obtained from PBP5-immunized mice

Materials and Methods: The gene encoding soluble PBP5 was cloned into the pET-30a(+) vector, produced recombinantly, and purified by IMAC. CD-1 mice were immunized with sol-PBP5, and the immune response was confirmed by ELISA. RNA from immunized mice spleens was used for cDNA synthesis and VH amplification. A VH-displaying phage library was created by cloning VH amplicons into pComb3-hy phagemid. Phages binding to sol-PBP5 were selected via biopanning, and their VH sequences were identified. The 3D structure of VH43 and its interaction with sol-PBP5 were modeled, and VH43 was produced recombinantly. Binding tests were performed with BOCILLIN-FL. Synergistic effects of VH43 with cephalosporin were tested using bacterial growth analysis and modified Kirby-Bauer/spot tests.

Results: Cloning of sol-PBP5 into the pET-30a(+) vector was confirmed by sequence analysis. Expression and purification were validated via SDS-PAGE, with BOCILLIN-FL treatment confirming the protein's native conformation. Immunization was confirmed by ELISA (p-value = 9.02e-05). cDNA from total RNA of immunized mice spleens showed 500-600 bp bands on agarose gel. VH amplicons were cloned into the phagemid, and a phage library was produced via M13K07 infection. Affinity phages for sol-PBP5 were selected by biopanning, and VH sequences were determined after the third biopanning cycle. VH43's amino acid sequence and CDRs were identified, with a molecular weight of 10.3 kDa, 12 alpha helices, and 61 beta chains. Seven hydrogen bonds were found between VH43 and sol-PBP5. Recombinant VH43, confirmed via SDS-PAGE to be 20.3 kDa including His-tags, showed reduced brightness in BOCILLIN FL binding experiments. Combining VH43 with cephalosporin significantly inhibited bacterial growth, with increased zone diameter in modified Kirby-Bauer tests compared to cephalosporin alone.

Conclusion: The increase in antibiotic-resistant *E. faecium* strains requires the development of new molecules. VH43 discovered in this thesis study is the first antibody/antibody fragment developed against *E. faecium* and whose sequence was determined. Combining recombinant VH43 with cephalosporin showed synergetic effect and significantly inhibited bacterial growth. This result underscores the potential of VH43 to enhance the effectiveness of cephalosporins that are normally ineffective, offering a promising approach for combating bacterial infections.

Keywords: D,D-transpeptidase, *Enterococcus faecium*, PBP5, Recombinant antibiotic

ÖZET

ENTEROCOCCUS FAECİUM'A KARŞI REKOMBİNANT ANTİBİYOTİK GELİŞTİRİLMESİ

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Amaç: *E. faecium*'un sahip olduğu beta laktamlara düşük afinite gösteren PBP5 proteinine karşı recombinant antibiyotik geliştirilmesi amaçlandı.

Gereç ve Yöntem: PBP5 proteininin çözünür formunu kodlayan gen pET-30a(+) ekspresyon vektörüne klonlandı, rekombinant olarak üretildi ve IMAC ile saflaştırıldı. Cd-1 fareleri saf sol-PBP5 proteini ile immunize edildi ve ELISA ile doğrulandı. İmmünize farelerden RNA izolasyonu, cDNA sentezi ve VH amplifikasyonu yapıldı. VH amplikonları pComb3-hy fajmidine klonlanarak VH gösteren faj kütüphanesi oluşturuldu. Sol-PBP5'e bağlanan fajlar biopanning yöntemiyle seçildi ve VH sekansları belirlendi. VH43'ün 3 boyutlu yapısı ve sol-PBP5 ile interaksiyonu modellendi. VH43 klonlandı ve rekombinant olarak üretildi. BOCILLIN-FL kullanılarak karşılaştırmalı bağlanma testi yapıldı. VH43'ün antibakteriyel etkisi tek başına ve sefalosporin ile kombine edilerek test edildi. İstatistiksel analizde bağımsız örneklemler için t-testi, tek yönlü varyans analizi ve Tukey's HSD testleri kullanıldı.

Bulgular: sol-PBP5'in pET-30a(+) vektörüne klonlandığı koloni PCR ve sekans analizi ile doğrulandı. sol-PBP5 proteininin ekspresyonu ve saflaştırıldığı SDS-PAGE analizinde yaklaşık 75 kDa boyutunda bant görülmesiyle doğrulandı. Proteinin BOCILLIN-FL ile muamelesi sonucunda SDS-PAGE analizinde parlama görüldü. Saf sol-PBP5 ve PBS ile enjeksiyon sonrasında, sol-PBP5 ile kontrol grubu arasında anlamlı antikor yanıtı görülmesi ile doğrulandı (p-değeri = 9.02e-05). İmmünize farelerden izole edilen izole edilen total RNA'dan izole edilen cDNA agaroz jelde yaklaşık 500-600 boyunda bant verdi. cDNA'den çoğaltılan VH amplikonları fajmid vektörüne klonlandı ve koloni PCR ile doğrulandı. Faj kütüphanesinden sol-PBP5'e afinite gösterenler biopanning ile seçildi ve biopanning öncesi ve sonrası arasında faj sayısında beklenen düşüş gözlendi. 3. Biopanning döngüsü sonrasında afinite gösteren fajın kodladığı VH sekanslandı. VH43'e ait amino asit sekansı ve bulundurduğu CDR sekansları belirlendi. Keşfedilen VH43'ün moleküler ağırlığı 10.3 kDa ve proteinin yapısında 12 alfa

heliks ve 61 beta zincir bulunduğu ortaya kondu. VH43 ve sol-PBP5 arasında 7 hidrojen bağı oluştuğu ve bağların lokasyonları belirlendi. VH43 rekombinant protein olarak ekspresyonunda üretildi ve N ve C terminalindeki Histag ile birlikte toplamda 20.3 kDa olduğu SDS-PAGE analizi ile gösterildi. VH43 ve VH kodlayan fajların sol-PBP5'e bağlandığı BOCILLIN FL ile yapılan karşılaştırılmalı bağlanma deneyinde parlaklığın azalması ile kanıtlandı. VH43 tek başına antibakteriyel etki göstermemiştir, ancak ceftazidime/cefazoline ile kombinlendiğinde, ceftazidime/cefazoline tek başına uygulandığında göre önemli bir inhibitör etkiye sahip olmuştur. Bu, VH43 ve ceftazidime'nin sinerjik etkisini doğrulamaktadır.

Sonuç: Antibiyotik dirençli *E. faecium* suşlarının artışı, yeni moleküllerin geliştirilmesini gerektirmektedir. Bu tez çalışmasında keşfedilen VH43 *E. faecium*'a karşı geliştirilen ve sekansı tespit edilen ilk antikor/antikor fragmentidir. VH43'ün sefazolin/seftazidim ile kombine halde uygulandığında *E. faecium*'un büyümesini önlemesi onun recombinant antibiyotik potansiyelini ortaya koymuştur.

Anahtar kelimeler: D,D-transpeptidaz, *Enterococcus faecium*, PBP5, Rekombinant antibiyotik

1. INTRODUCTION

Dissemination of antibiotic resistance among major bacterial pathogens is recognized as one of the 10 threats for global health by WHO in 2019. Developing new, innovative drugs for the treatment of infectious diseases is very important to overcome this emerging threat for public health. *Enterococcus faecium* is an opportunistic pathogen as well as a member of the intestinal flora. It typically exhibits mild pathogenicity; however, it has the potential to lead to serious illnesses like bloodstream infections, endocarditis, and peritonitis in specific situations. *Enterococcus faecium* was identified as one of the bacterial priority pathogens by WHO in 2024 which is particularly important for public health due to its ability to transmit resistance elements. Recently, the prevalence of *Enterococcus faecium* resistant to vancomycin, which is frequently used in treatment, has been increasing and such treatment options are limited (Casalini 2024).

Protein-protein interaction is important for a variety of biological processes. To stop a biological process targeting the main enzymes and inactivating them by specifically binding molecules is commonly used for treatment. For example, β-lactam antibiotics bind specifically to D,D-transpeptidase enzymes (also called penicillin-binding proteins [PBPs]) and inhibit bacterial cell wall synthesis to exert their bactericidal activity. This study aims to develop a new protein that binds and prevents the activity of the D,D-transpeptidase PBP5 of *Enterococcus faecium* (PBP5) which is specific to *E. faecium* and has a low affinity for beta-lactams (especially cephalosporins).

The thesis aimed to develop a recombinant antibiotic originating from the VH sequence of antibodies obtained from PBP5-immunized mice. This study hypothesizes that the obtained and recombinantly produced VH protein named recombinant antibiotic will have an antibacterial effect against *E. faecium* alone or in combination with an antibiotic from the cephalosporin group.

2. GENERAL INFORMATIONS

2.1. Antibiotic resistance concerns

Antimicrobial resistance has become one of the major health care problems in the world. WHO declares antimicrobial resistance as one of the 10 most important health care problems worldwide. More than 750 000 people die every year due to antimicrobial resistance in the world and this number is increasing each year (Dadgostar 2019). Nowadays, it is not very pretentious to speak about post-antibiotic era but necessary and alternative approaches should be developed for treatment of infectious diseases. One of the rising fight systems against infectious disease problem is to use biological products. *E. faecium* was indicated as bacterial priority pathogen by WHO and it was mentioned that the importance of developing new strategies to prevent and control antimicrobial resistance (Casalini 2024).

2.2. *Enterococcus faecium*

2.2.1. General information

Enterococcus faecium is a gram-positive bacterium belonging to the Enterococcus genus. It was firstly discovered in fecal flora and is a member of the human gut flora. However, it can reveal as opportunistic pathogen and cause bacteremia, endocarditis, urinary tract infection, intra-abdominal infection and skin infection (Nelson 2014). *E. faecium* was associated with hospital acquired (nosocomial) outbreak and can cause opportunistic infection in hospitalized patients (Zhou 2020). Its intrinsic and acquired antibiotic resistance significantly supports the ability of *Enterococcus faecium* to survive and spread in hospital environments. The presence of virulence genes that facilitate biofilm formation and colonization is another property that makes *E. faecium* resistant to hospital conditions.

2.2.2. Antimicrobial resistance of *E. faecium*

E. faecium have intrinsic resistance to beta lactams especially cephalosporins and penicillinase-resistant penicillins, low concentrations of aminoglycosides, clindamycin, fluoroquinolones. Especially, beta lactams are widely used in clinical treatment and inability the use of them restricts the treatment options. Acquired resistance is common in *Enterococcus faecium* and is manifested by b-lactams in high concentrations (via penicillin-binding proteins or b-lactamase), aminoglycosides in high concentrations, and glycopeptides (vancomycin and teicoplanin) (Eliopoulos 2001). The resistance developed by *E. faecium* against antibiotics such as vancomycin, teicoplanin and linezolid greatly limits treatment options. These resistance mechanisms allow bacteria to develop resistance to different classes of antibiotics. The antbiotic resistance mechanism can reveal with different ways. For example, vancomycin resistance prevents vancomycin binding by making changes in the cell wall through the VanA and VanB gene clusters, while ampicillin resistance is achieved by mutation of the PBP5 protein. Linezolid resistance occurs as a result of mutations on 23S rRNA (Zhou 2020, Moon 2023).

In Europe and the USA, particularly heavy use of antibiotics and inadequate infection control measures have contributed to the spread of antibiotic resistant *E. faecium*. Increasing antimicrobial resistance creates major challenges in the treatment of *E. faecium* infections (Xuan 2021). It was found that approximately 40% of device-related infections in intensive care units were caused by *E. faecium*, and 80% of them were resistant to vancomycin and 90.4% to ampicillin (Nelson 2014). According to data collected globally by the One Health Trust, over 75% of *E. faecium* strains are resistant to aminopenicillins. In the United States, Argentina, and Australia, the prevalence of vancomycin-resistant *E. faecium* exceeds 50%. High-level aminoglycoside resistance is around 50% in many countries. Although global resistance to linezolid, an alternative treatment for vancomycin and aminopenicillin-resistant *E. faecium* strains, is below 25%, this rate exceeds 50% in Argentina and Australia (Moon 2023). Also, it should be considered that some countries have not conducted studies on this issue (Figure 1).

This situation necessitates the development of new treatment strategies and antibiotics. Understanding the resistance mechanisms of *E. faecium* will contribute to the development of more effective treatment methods. It is of great importance to develop new recombinant antibiotics against antibiotic resistant *E. faecium* strains, especially as one of the leading causes of hospital-acquired infections and epidemics (Cattoir 2014, Moon 2023).

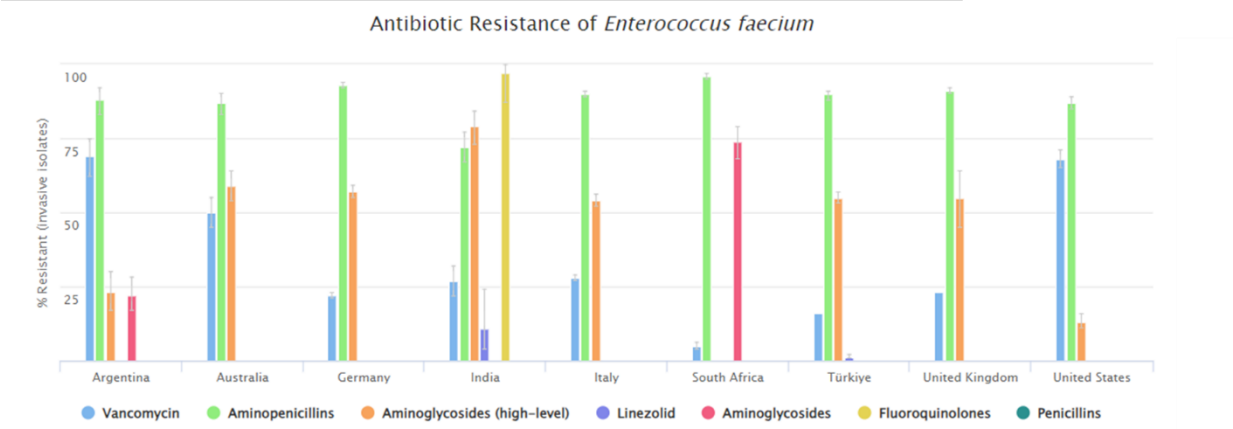


Figure 1. The antibiotic resistance profile of *E. faecium* isolates in Türkiye, United States, United Kingdom, Argentina, Australia, Germany, Italy, India and South Africa.

2.2.3. The cell wall structure

The cell wall of *E. faecium*, which belongs to the gram-positive bacterial group, contains a thick peptidoglycan layer. This peptidoglycan layer provides protection against external stress by ensuring the structural integrity and shape preservation of the bacteria. The cell wall of *E. faecium* contains teichoic and lipoteichoic acids. These acids cause the cell wall to carry a negative charge and increase the durability of the cell wall by interacting with metal ions. Additionally, teichoic acids increase the resistance of bacteria to environmental stresses by providing flexibility to the cell wall. Cell wall components carry antigenic properties that the immune system recognizes and responds to, contributing to *E. faecium* playing an important role in the infection process. These features are critical factors that enable *E. faecium* to survive and proliferate in the host organism (Gilmore 2013).

2.2.4. Penicillin Binding Proteins

Penicillin-binding proteins (PBPs) are membrane-associated proteins involved in the biosynthesis of peptidoglycan (PG) and play a critical role in the antibiotic resistance of this bacterium. PBPs are enzymes involved in peptidoglycan synthesis and maintain the structure and integrity of the bacterial cell wall. The peptidoglycan layer consists of chains in which sugars called N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) are arranged

alternatively, and these chains are cross-networked by PBPs to create a stable structure. PBPs are classified by their enzymatic activity class A, bifunctional PBPs with both glycosyltransferase and transpeptidase activities class B, transpeptidases class C, carboxypeptidases and endopeptidases (Hendrickx 2013).

E. faecium has 6 penicillin binding proteins (PBPs), which are class A (bifunctional enzymes with glycosyltransferase and d,d-transpeptidase activity) and class B (monofunctional d,d-transpeptidases) (Rice 2009). PBPs of *E. faecium*, especially PBP5, are closely associated with antibiotic resistance. PBP5, unlike other PBPs, is a low-affinity penicillin-binding protein, and this feature increases the resistance of *E. faecium* to beta-lactam antibiotics. B-lactam antibiotics exert their bactericidal activity by specifically binding to D,D-transpeptidase enzymes (also called penicillin-binding proteins [PBPs]). Beta-lactam antibiotics inhibit PBPs, stopping peptidoglycan synthesis and thus killing the bacteria. However, low-affinity PBPs reduce the effect of these antibiotics, allowing the bacteria to survive (Mainardi 2002).

Besides PBP5, other PBPs are found in the genome of *E. faecium*, and these PBPs are involved in the development of resistance to various antibiotics. Genomic analyzes revealed the genetic diversity and adaptability of PBPs of *E. faecium*. These adaptations increase the bacteria's ability to survive and spread in the hospital environment (Galloway-Peña 2012).

2.2.5. Genetic determinants of pbp5

Penicillin binding protein 5 (PBP5), the main determinant of beta-lactam resistance in *Enterococcus faecium*, is found at different expression levels in different clades. It is also known that sequence variances occurring in the pbp5 gene at different expression levels, mutations in the psr (PBP synthesis repressor) gene located upstream of PBP5, and changes in the genetic environment cause decreased sensitivity to beta lactams (Montealegre 2017).

2.3. Current strategies to fight bacterial infections

2.3.1. Bacteriophages

Bacteriophages, also named phages, are viruses specific to bacteria. They can infect bacteria and replicate within them. Specificity of phages is used for the treatment of human and animal infected with bacteria. Some properties of bacteriophages make them attractive for therapy. In recent years, the research about *E. faecium* phages has increased significantly. There are several bacteriophages against *Enterococcus faecium* have been characterized. It was shown that the phages can effectively inhibit the growth of *E. faecium* and prevent biofilm formation. *E. faecium* phages can disrupt these biofilms and control the infection. In clinical trials, the safety and effectiveness of phage therapy are evaluated. Challenges such as stabilizing phages, determining the appropriate dosage, and preventing them from being recognized and inactivated by the immune system are being addressed by researchers (Canfield 2021). Furthermore, there are several clinical experiments by using phages for the treatment of *E. faecium* (Paul 2021).

2.3.2. Antimicrobial peptides

Antimicrobial peptides (AMPs), also named as host defense peptides (HDPs), are synthesized by bacteria, fungus, plants, mammalians etc. Length of AMPs is mostly between 20-50 amino acids with exceptions, until 100 amino acids. They have cationic and amphipathic properties. They can have anti-bacterial, anti-viral, anti-fungal, anti-parasitic and anti-tumor activities (Roque-Borda 2024). As with many other pathogens, the effectiveness of antimicrobial peptides against *E. faecium* is being tested (Vasilchenko 2015). There are also antimicrobial peptides that are designed and effective based on natural antimicrobial peptides (Mishra 2022).

2.3.3. Antibodies

Nowadays, antibodies are used to treatment of many diseases like cancer and autoimmune disease therapy, transplant rejection and infections. The usage of antibodies to fight infections is a very old therapy, before even antibiotics. In 1890, Emil von Behring with his colleagues firstly used passive immunization against diphtheria and tetanus. The discovery of serum therapy against diphtheria was brought the 1901 Nobel Prize in Physiology or Medicine to Emil von Behring. They immunized guinea pigs with infectious agents that are induced diphtheria and tetanus. To treat infected animals, the serum of immunized animals was applied to infected animals. The serum therapy technique has been used 20th century for many infectious diseases such as pneumococcal pneumonia, Ebola (Ebola Virus Disease), meningococcal meningitis and anthrax. It would appear that serum therapy will never go out of existence. Faced with a novel infectious agent, the well-known serum therapy is used as a lifesaver. For example, serum therapy was used against SARS-CoV-2 effectively in COVID-19 pandemic (Joyner et al, 2021). Even so, different strategies are tried to develop to fight infections, because of disadvantages of the serum therapy. They are mild adverse effects, immunological reactions such as serum sickness and anaphylaxis, risk of transfusion-associated infections. Also, some challenges are found in serum therapy. The challenges can be a lack of antibodies in donor serum and necessary large infusion volumes (Nagoba et al, 2020). The serum of infected animals contains antibodies that are rocket of the immune system.

The use of antibody therapy in *E. faecium* infection began with the use of human serum isolated from healthy people against *E. faecium* infection (Hufnagel 2005). Recently, there has been the use of monoclonal antibody therapy against *E. faecium*. There are studies showing that opsonic mouse monoclonal antibodies have been developed against multidrug-resistant enterococci and that these antibodies can be used as a potential therapeutic agent in the treatment of infections (Kalfopoulou 2019). Opsonic antibodies offer a new treatment strategy against antibiotic resistance by enabling the immune system to recognize and destroy pathogens. Future studies may take an important step in fighting with hospital-acquired infections by further investigating the use and effectiveness of these antibodies in clinical applications.

2.3.4. CRISPR-Cas Technology

Genome editing is one of the techniques to combat antimicrobial-resistant pathogens. Although the method is new and not many applications both in research and clinic, it promises favourable outcomes. The nucleases are important accessories in genome editing. The one of the most famous and used RGN is Cas9 enzyme that is produced by Streptococcus pyogenes. Cas9 endonuclease is part of CRISPR/Cas9 method. The method is used to edit genome of several organisms like various bacteria, yeast, plant and animals. The nucleases can be imported into host cell in different forms and different ways. The form of nucleases and delivery of nucleases is various. Transferred Cas9 can be found DNA form on plasmid, Cas9 mRNA form or Cas9 protein. They can be transferred into host cells with viral or non-viral vectors. In viral delivery, a viral vector that contains nuclease DNA sequence, is constructed and gene editing occurs in vivo and in vitro conditions. However, this method has some limitations. The viral vector can integrate itself into host genome or cause mutations. In non-viral delivery, the physical methods, are micro injection and electroporation, and the chemical methods, are modification of cargo and encapsulation of cargo, are used (Gong et al, 2020). There are approaches such as reducing the virulence of pathogens or silencing antibiotic resistance genes using various CRISPR-Cas systems (Tao 2022). The genome of *E. faecium* is edited with CRISPR-Cas technology (de Maat 2019).

2.3.5. Nanoparticles

Nanoparticles can be used to increase the effectiveness of antibiotics and other antimicrobial agents. Nanoparticles enable antibiotics to reach target bacteria more effectively and increase the bioavailability of drugs. There are many studies that showing antibacterial and antibiofilm activities of nanoparticles (Halkai 2018). Additionally, it has been shown that the anti-biofilm activity of linezolid against *E. faecium* increases by coating antibiotics with silver and gold nanoparticles (Sabzi 2022).

2.3.6. Other approaches

Vaccination, Probiotics and Prebiotics, photodynamic therapies and RNA-interferance technology are other strategies to overcome infection diseases (Parmanik 2022).

2.4. Antibody Phage Display Technology

2.4.1. Antibody based therapies

Although serum containing antibodies has been used in the treatment of infectious diseases for a long time, their use is not preferred today due to their significant side effects. Instead, the use of safer monoclonal antibodies is both common and safe. The antibodies were targeted toxin, polysaccharides and surface proteins (Motley 2019). However, monoclonal antibody production is very expensive and still causes serious side effects. For this reason, with the developing recombinant DNA/protein technology, it is possible to use the effective parts of antibodies in the treatment of infectious diseases. Beside treatment, the antibodies also used for diagnosis of infection diseases (Pantaleo 2022).

2.4.2. Antibody structure

Antibodies are proteins that have a critical role in the immune system's defence against pathogens. Antibodies are responsible for recognising specific antigens and neutralising them or signalling them to immune system cells. The structural components of antibodies are of great importance in their functioning. The intact antibody consists of 3 functional parts: 2 Fab (Fragment antigen-binding) regions and 1 fragment crystallizable (Fc) region (Figure 2).

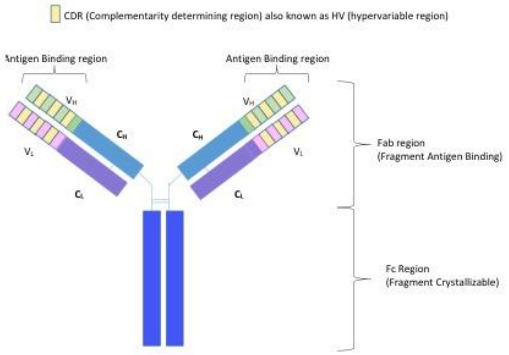


Figure 2. The structure of antibody.

2.4.2.1. Fab (Fragment antigen-binding) region

The Fabs are linked to Fc through the hinge region. The hinge provides flexibility to Fabs. The Fab (Fragment antigen-binding) region of antibodies is where antigens specifically bind. The Fab region consists of variable and constant heavy chains and light chains. The heavy and light chains are connected by disulfide bonds, creating the stable structure of the antibody. Variable frgment of Fab contains variable heavy and variable light chains. These regions are highly variable, allowing antibodies to recognize millions of different antigens. The variable regions of the Fab domain contain complementary determining regions (CDRs), which consist of three short amino acid sequences. These CDRs are the parts that interact directly with the antigen and provide binding specificity (Chiu 2019).

2.4.2.2. Heavy chain

Heavy chains are two polypeptide chains weighing approximately 50 kDa each. Each heavy chain contains a variable region and a constant region. Variable regions of heavy chains (VH) form the antigen binding site (paratope) and play a critical role in the specific recognition of antigens. The constant regions (CH) of the heavy chains form the Fc region of the antibody. These regions are involved in interactions that determine the biological activity of the antibody. The constant regions of the heavy chains determine the class of antibodies (IgG, IgM, IgA, IgE, IgD), and each class supports different immune functions (Chiu 2019).

2.4.2.3. Light chain

Light chains are two polypeptide chains weighing approximately 25 kDa each. Light chains also contain a variable region (VL) and a constant region (CL). The variable regions, together with the variable regions of the heavy chains, form the antigen binding site. There are two types of light chains: kappa (κ) and lambda (λ). Each antibody molecule contains either two kappa or two lambda light chains, but an antibody molecule does not contain both types (Chiu 2019).

2.4.2.4. Fragment crystallizable region

The fragment crystallizable (Fc) region consists of constant regions of heavy chains and enables antibodies to interact with immune system cells and molecules. The Fc region enables the biological effects of antibodies, such as recognition by phagocytic cells and activation of the complement system. These functions of the Fc region help antibodies modulate immune responses and effectively eliminate pathogens (Chiu 2019).

2.4.3. Antibody libraries

An antibody library is a collection containing a wide variety of antibodies. These libraries contain many different antibody sequences produced in vitro or obtained from natural sources. Antibody libraries play an important role, especially in biotechnology and biomedical research. There 4 types of antibody libraries. These are immune, naïve, semi-synthetic and synthetic libraries. An immune antibody library contains antibody genes obtained from individuals immunized with a specific antigen. These libraries enable the selection of high affinity and specific antibodies and reflect the immune response. Naïve Antibody Library contains antibody genes obtained from individuals not immunized with a specific antigen. It offers a wide variety of antibodies and allows antibody discovery without a specific immune response to any antigen. Semi-Synthetic Antibody Library is created by modifications of natural antibody genes. By adding specific mutations to natural antibody sequences, they are expanded,

and their diversity increased, resulting in more targeted antibodies against specific antigens. Synthetic Antibody Library consists of antibody genes designed and synthesized entirely in the laboratory environment. It provides a wide variety of antibodies that are not naturally occurring but biologically functional and contains completely artificial sequences.

2.4.4. Innovational antibody fragments

Different types of antibodies are used for viral, bacterial and fungal infections (Wu et al, 2017). The antibodies can be found at divergent purities like monoclonal, polyclonal or mixed (blend of distinct monoclonal antibodies). Recently, antibodies with different formats have been produced recombinantly (Figure 3). The formats affect the biological functions and therapeutic potential of antibodies. Full-length antibodies (IgG) are the most common antibody format and consist of two heavy chains and two light chains. This format has advantages such as strong antigen binding ability and long half-life. Fab (antigen-binding fragment) is derived from the full-length antibody and contains only the antigen-binding domains (VH and VL). Fab fragments can penetrate tissues better due to their smaller size. F(ab')2 fragments are formed by the fusion of two Fab domains and offer the advantage of having two antigen-binding domains. Single-chain variable fragment (scFv) is formed by connecting the VH and VL regions to a single polypeptide chain and can easily reach tissues with its small size. Nanobodies (VHH) consist of a single variable heavy chain region and are distinguished by their very small size and high stability. Domain antibodies (dAb) consist of a single variable region (either VH or VL) and are notable for their small size and high stability. Bispecific antibodies can target two different antigens and thus have the capacity to target different pathogens or cellular pathways simultaneously. These diverse antibody formats offer a wide range of applications in research, diagnostics and therapeutics (Pirkalkhoran 2023).

In addition to them, single domain antibodies that are nanobodies and domain antibodies (dAbs), facilitate to realize innovative ideas. The antibodies contain only antigen specific part of intact antibody, and they are very small molecules. Nanobodies are originated from camels and dAbs are originated from Immunoglobulin G (IgG) (Bates and Power, 2019).

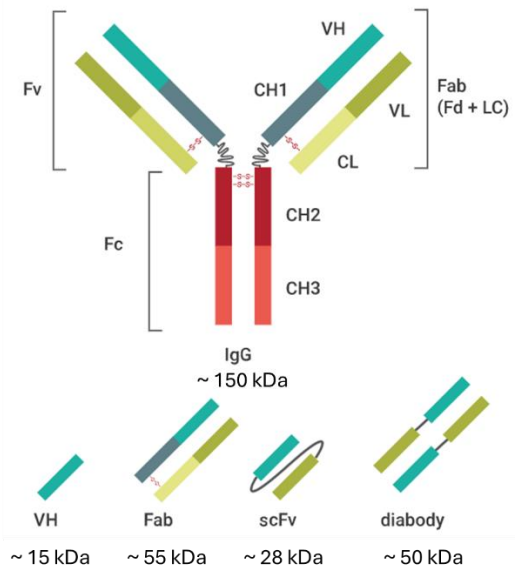


Figure 3. The structures of IgG antibody, VH, Fab, scFv and diabody, and the molecular weight of them were given as 150, 15, 55, 28 and 50 kDa, respectively. (https://www.bioatla.com/appendix/antibody-structure/).

2.3.4.1. Domain antibodies

Domain antibodies (DAbs) are small, stable protein fragments derived from conventional antibodies that contain antigen-binding domain. DAbs are derived from the variable region of antibodies and have high binding specificity and affinity. Thanks to their small size, high stability and ease of biotechnological production, DAbs have a wide range of uses in research, diagnostic and therapeutic fields.

Domain antibodies are derived from the heavy chain variable region (VH) or light chain variable region (VL) of the antibodies. These small protein fragments weigh approximately 12-15 kDa and consist of a single polypeptide chain. The molecular weight of domain antibodies is significantly lower than full-length antibodies (150 kDa). VH and VL gain their specificity with the Complementarity-Determining Regions (CDR) found in their structures (Figure 4). CDRs are short amino acid sequences that provide the antigen binding specificity of DAbs. VH has three CDRs, and these CDRs interact directly with the antigen (Mirsky 2015).

Both VH and VL contain 3 CDRs in their structures. However, the variable heavy chain (VH) contains V (Variable), D (Diversity) and J (Joining) segments, while VL contains only V and J domains. The Diversity segment, unique to VH, allows the heavy chain to gain greater diversity and high-affinity binding capabilities. This allows antibodies to bind with high

specificity to a variety of antigens. VH is more advantageous than VL because it has higher diversity and binding affinity and is more resistant to environmental factors such as temperature, pH and proteases (Wang 2011, Mirsky 2015).

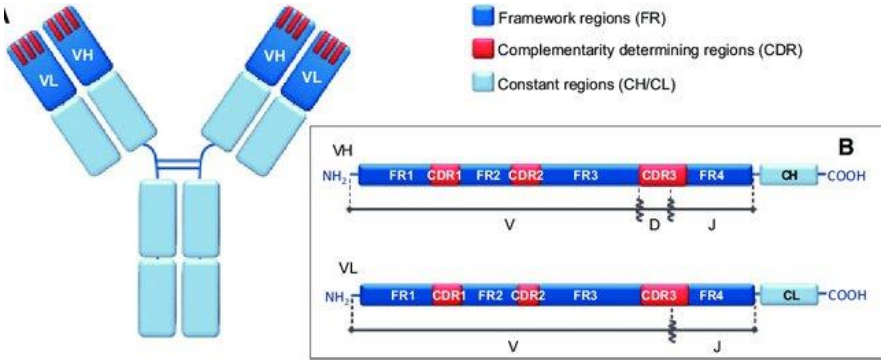


Figure 4. Antibody structure and locations of CDRs in variable chains (Mirsky 2015)

DAbs are produced in bacterial, yeast or mammalian cell systems using genetic engineering techniques. These production processes are highly efficient and cost-effective, allowing large-scale production. Additionally, the affinity, specificity, and stability of DAbs can be optimized by genetic engineering. DAbs are advantageous because of their small size, high specificity, high stability and easy production. The immunogenicity and short half-life of DAbs are disadvantages for them.

2.4.5. Phage Display library for VH

Phage display technology is a powerful and innovative bioengineering method used in the selection of VH (Variable Heavy) antibodies. This technology enables the display of variable regions of antibodies on the surface of bacteriophages (phages). The phage display method enables rapid and efficient selection of VH antibodies with high affinity to specific antigens from a large antibody library.

2.4.5.1. M13 phage

M13 phage and phage display technology are based on the creation of a bacteriophage coat protein-linked polypeptide library. Although T4, T7 and λ phages are also used, M13 and fd filamentous phages are most preferred because they do not lyse bacteria but continue their life cycle by releasing virus particles. M13 bacteriophage has circular single-stranded DNA (ssDNA) and a flexible, rod-shaped structure approximately 1 μm long and 6 nm in diameter. The pVIII protein of the M13 phage constitutes 87% of the total virion mass with 2,700 copies. Even though the pVIII protein is suitable for displaying short peptide sequences, it is limited for large proteins. The pIII protein is ideal for the displaying of the larger proteins (Huang 2012). The life cycle of filamentous phages was shown at Figure 5.

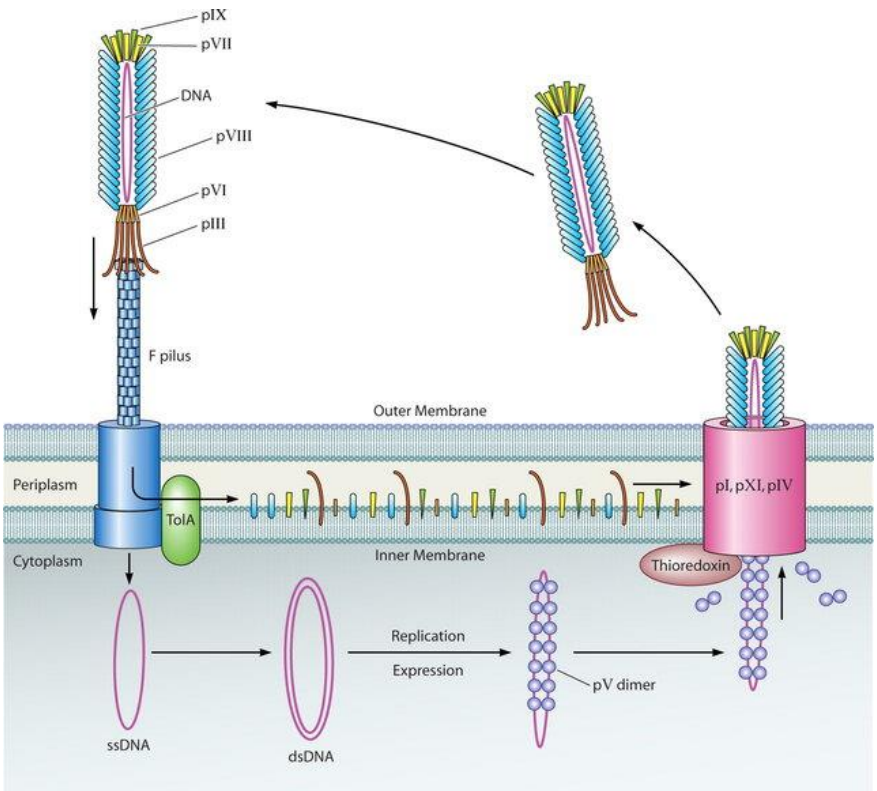


Figure 5. Life cycle of filamentous M13 phage (Huang 2012).

Phage display technology uses phage coat proteins to display various proteins. This technology is favourable for antibody discovery, study of protein-protein interactions, and development of new therapeutic molecules. The flexibility and stability of the M13 phage have made it a suitable and preferred tool for phage display.

2.4.5.2. VH displaying phage library construction

To discover high-affinity antibodies against various antigens, VH displaying phage library is essential. For library construction, RNA isolation was processed from sera, spleen or B cells. cDNA synthesis from the isolated RNA is performed and VH sequences are amplified. VH protein is cloned into the phagemid vector by fusion with the coat protein. Transformation of the vectors into proper host results in formation of phage library (Huang 2012).

2.4.5.3. Biopanning

Biopanning method is used to select VH with high affinity that can bind to the targeted antigen from the VH displaying phage library. In this method, the phage library is applied to the antigen-coated surface and elution is performed following washing steps. The eluted phages are cycled again, and this process is performed at least 3 times. Thus, the VH sequences showing the highest affinity are obtained (Figure 6).

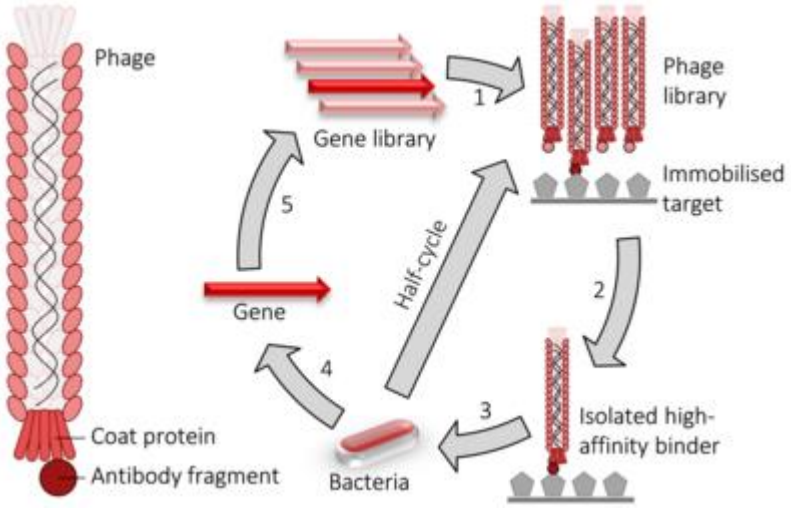


Figure 6. Biopanning with VH displaying phage library

3. MATERIALS AND METHOD

3.1. Materials

3.1.1. Devices

The devices which are available in Aydın Adnan Menderes University Recombinant DNA and Recombinant Protein Center (REDPROM) were used. The devices used in this study are LONGGENE A300 thermal cycler for PCR, MSC-100 Refrigerated Block Heater Shaker and Memmert INB200 incubator for incubation, Hirayama HV-50L for sterilization, Hettich MIKRO 200 Microlitre Centrifuge for centrifugation, LM10 microfluidizer Processor for bacteria harvest, spectrophotometer for ELISA and measurement of bacterial absorbance, NGC 10 Medium-Pressure Chromatography System for protein purification.

3.1.2. Chemicals

RNAlater solution (AM7020, Invitrogen) for storage of RNA, IPTG Isopropyl-beta-D-thiogalactoside (Thermo scientific, US), BOCILLIN FL (Thermo scientific, US), Tween-20 (Merck, Germany), imidazole (Merck, Germany), sodium chloride (Merck, Germany), TEMED (Merck, Germany), APS (Merck, Germany), SDS (Merck, Germany), Tris (Merck, Germany), Sodium dihydrogen phosphate (Merck, Germany) and glycine (Thermo scientific, US) were used.

3.1.3. Bacteria and phage strains

Escherichia coli BL21 (DE3) for recombinant protein production and *Escherichia coli* XL1-Blue for phage display were used. *Enterococcus faecium* HM1070 as DNA source for cloning of sol-PBP5 and for antibacterial activity of developed VH. The 50 clinical vancomycin

resistant *Enterococcus faecium* isolates which are found in the culture collection of REDPROM. M13K07 phage was used for phage display.

3.1.4. Media and Solutions

3.1.4.1. Tryptic Soy Agar

To prepare the medium, 11.1 g of tryptone soy agar (Merck, Germany) was dissolved in 300 mL of distilled water. The medium, which was sterilized in an autoclave at 121 °C for 15 minutes, was poured into sterile petri dishes. It was used in the activation of bacteria and in cloning studies.

3.1.4.2. 2X YT medium

To prepare the medium, 16 g of tryptone, 10 g of yeast extract and 5 g of sodium chloride were dissolved in 1 L of distilled water. The medium was sterilized in an autoclave at 121 °C for 15 minutes. It was used in the culture of bacteria, cloning and protein expression studies. 2X YT with 1% glucose (w/v) was used in some specific parts of the study.

3.1.4.3. Soft agar

To prepare soft agar from 2X YT, supplemented with agar (0.5% agar, Biokar Diagnostics) and calcium chloride (2.5 mM, Merck Laboratory, Germany). The soft agar media used for phage experiments.

3.1.4.4. Phosphate buffered saline

One phosphate buffered saline (PBS) tablet (Merck Laboratory, Germany) was dissolved in 200 ml distilled water. It was sterilized in an autoclave at 121 °C for 15 minutes and used in microbiological and molecular experiments.

3.1.4.5. RNAlater solution

RNAlater solution (AM7020, Invitrogen) was used for storage of RNA.

3.1.4.6. Bicarbonate buffer

0.0125 M Sodium bicarbonate and 0.0875 M Sodium carbonate (anhydrous) were dissolved in 1 L distilled water and used in molecular experiments.

3.1.4.7. DNA4PCR

DNA4PCR was used DNA extraction for PCR

3.1.5. Plasmids

pET-30a(+) plasmid as expression vector and pComb3 as phagemid were used.

3.1.6. Enzymes

Restriction enzymes (Thermo scientific, US), T4 DNA ligase (Thermo scientific, US), Taq DNA Polymerase (Thermo scientific, US), smART Reverse Transcriptase (EURx, Poland) were used for molecular experiments.

3.1.7. Protein experiments

3.1.7.1. Protein purification columns

Ni-charged EconoFit Nuvia IMAC Columns and EconoFit Bio-Gel P-6 Desalting Columns were used for protein purification.

3.1.7.2. Buffers

Elution buffer (500 mM NaCI, 50 mM KH_2PO_4/NaH_2PO_4, 500 mM imidazole, pH 8.0) and wash buffer (500 mM NaCI, 50 mM KH2PO4/NaH2PO4, pH 8.0) were used for protein purification with IMAC.

3.1.8. Animals

Female CD-1 mice were used for immunization.

3.1.9. Antibiotics

Kanamycine (Sigma-Aldrich, US), Ampicilline (Sigma-Aldrich, US), Tetracycline (Sigma-Aldrich, US), Ceftazidime (Menarini, Türkiye), Ceftriaxone (Menarini, Türkiye) and Cefotaxime (Menarini, Türkiye) antibiotics were used for microbiological experiments.

3.1.10. Markers

Lambda-PstI DNA was used as DNA marker and it was obtained by digesting 0.1 mg Lambda DNA with PstI enzyme.

Color Prestained Protein Standard (NEB, US) marker/ladder was used for protein experiments.

3.1.11. Primers

The designed and used primers in this study were given at Table 1.

Table 1. The used primers for the study. The flanked Restriction enzyme sequences were underlined.

Primer	Primer sequence
sol-pbp5efF-BamHI	GACGGATCCCAAGAAAGCCAAGCAGTAG
sol-pbp5efR-EcoRI	GACGAATTCTTATTGATAATTTTGGTTGAGG
T7F	TAATACGACTCACTATAGGG
T7R	GCTAGTTATTGCTCAGCGG
mIGHG RT	AGCTGGGAAGGTGTGCACAC
template-switch oligo.	AAGCAGTGGTATCAACGCAGAGTACATGrGrGr
ISPCR	AAGCAGTGGTATCAACGCAGAG
mIGHGPCR	GGGATCCAGAGTTCCAGGTC
Fpuc19	CACACAGGAAACAGCTATGACC
M13F	GTAAAACGACGGCCAGT
VH43F-BamHI	TCGGGATCCCAGATCCAGTTGGTGCAGT
VH43R-HindIII	CTGAAGCTTTTATGAGGAGACGGTGACTGA

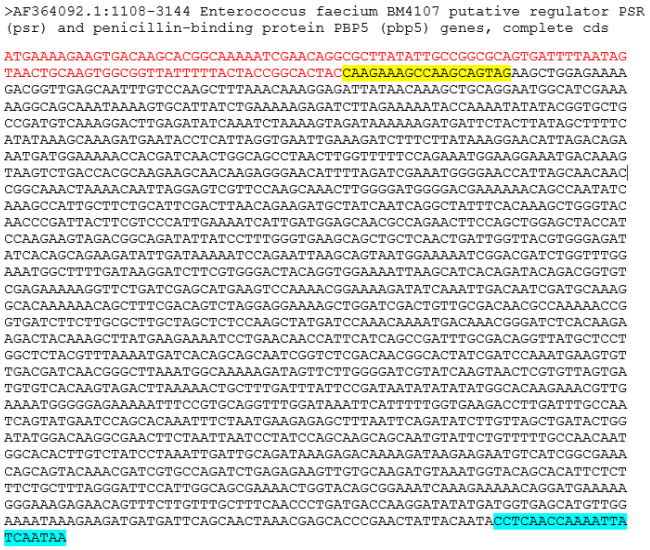
3.2. Method

3.2.1. The cloning of sol-pbp5

3.2.1.1. The primer design for soluble sol-PBP5cloning

E. faecium sol-PBP5 DNA sequence was extracted from the record (AF364092.1) in Genbank. To obtain soluble form of sol-PBP5protein (sol-PBP5), the transmembrane domain of the protein which is not contribute to transpeptidase activity of the protein was discarded

from the sequence. It was reported that the domain with its hydrophobic characteristics complicates the purification step by decreasing the solubility of the recombinant protein (Hu 2007). Besides, in a study in which sol-PBP5 was cloned by excluding its transmembrane domain, it was observed that it preserved its natural form (Moon 2018). The forward sol-PBP5 and reverse sol-PBP5primers were designed from the obtained sequence (Figure 7). To clone the sol-pbp5 into pET-30 a (+) expression vector in same orientation with promoter and 6X histag, the restriction enzyme sequences were flanked at the 5' of the primers (Table 1). BamHI and EcoRI enzyme sequences were used for forward and reverse primers, respectively. It was checked the enzymes are non-cutter for the gene. Consequently, if the gene is amplified with the designed primers and cloned into the pET-30 a (+) vector, the recombinant protein with 6X histag at N terminal should be produced.


>AF364092.1:1108-3144 Enterococcus faecium BM4107 putative regulator PSR (psr) and penicillin-binding protein PBP5 (pbp5) genes, complete cds

ATGAAAAGAAGTGACAAGCACGGCAAAAATCGAACAGGCGCTTATATTGCCGGCGCAGTGATTTTAATAG
TAACTGCAAGTGGCGGTTATTTTTACTACCGGCACTACCAAGAAAGCCAAGCAGTAGAAGCTGGAGAAAA
GACGGTTGAGCAATTTGTCCAAGCTTTAAACAAAGGAGATTATAACAAAGCTGCAGGAATGGCATCGAAA
AAGGCAGCAAATAAAAGTGCATTATCTGAAAAAGAGATCTTAGAAAAATACCAAAATATATACGGTGCTG
CCGATGTCAAAGGACTTGAGATATCAAATCTAAAAGTAGATAAAAAAGATGATTCTACTTATAGCTTTTC
ATATAAAGCAAAGATGAATACCTCATTAGGTGAATTGAAAGATCTTTCTTATAAAGGAACATTAGACAGA
AATGATGGAAAAACCACGATCAACTGGCAGCCTAACTTGGTTTTTCCAGAAATGGAAGGAAATGACAAAG
TAAGTCTGACCACGCAAGAAGCAACAAGAGGGAACATTTTAGATCGAAATGGGGAACCATTAGCAACAAC
CGGCAAACTAAAACAATTAGGAGTCGTTCCAAGCAAACTTGGGGATGGGGACGAAAAAACAGCCAATATC
AAAGCCATTGCTTCTGCATTCGACTTAACAGAAGATGCTATCAATCAGGCTATTTCACAAAGCTGGGTAC
AACCCGATTACTTCGTCCCATTGAAAATCATTGATGGAGCAACGCCAGAACTTCCAGCTGGAGCTACCAT
CCAAGAAGTAGACGGCAGATATTATCCTTTGGGTGAAGCAGCTGCTCAACTGATTGGTTACGTGGGAGAT
ATCACAGCAGAAGATATTGATAAAAATCCAGAATTAAGCAGTAATGGAAAAATCGGACGATCTGGTTTGG
AAATGGCTTTTGATAAGGATCTTCGTGGGACTACAGGTGGAAAATTAAGCATCACAGATACAGACGGTGT
CGAGAAAAAAGGTTCTGATCGAGCATGAAGTCCAAAACGGAAAAGATATCAAATTGACAATCGATGCAAAG
GCACAAAAAACAGCTTTCGACAGTCTAGGAGGAAAAGCTGGATCGACTGTTGCGACAACGCCAAAAACCG
GTGATCTTCTTGCGCTTGCTAGCTCTCCAAGCTATGATCCAAACAAAATGACAAACGGGATCTCACAAGA
AGACTACAAAGCTTATGAAGAAAATCCTGAACAACCATTCATCAGCCGATTTGCGACAGGTTATGCTCCT
GGCTCTACGTTTAAAATGATCACAGCAGCAATCGGTCTCGACAACGGCACTATCGATCCAAATGAAGTGT
TGACGATCAACGGGCTTAAATGGCAAAAAGATAGTTCTTGGGGATCGTATCAAGTAACTCGTGTTAGTGA
TGTGTCACAAGTAGACTTAAAAACTGCTTTGATTTATTCCGATAATATATATATGGCACAAGAAACGTTG
AAAATGGGGGAGAAAAATTTCCGTGCAGGTTTGGATAAATTCATTTTTGGTGAAGACCTTGATTTGCCAA
TCAGTATGAATCCAGCACAAATTTCTAATGAAGAGAGCTTTAATTCAGATATCTTGTTAGCTGATACTGG
ATATGGACAAGGCGAACTTCTAATTAATCCTATCCAGCAAGCAGCAATGTATTCTGTTTTTGCCAACAAT
GGCACACTTGTCTATCCTAAATTGATTGCAGATAAAGAGACAAAAGATAAGAAGAATGTCATCGGCGAAA
CAGCAGTACAAACGATCGTGCCAGATCTGAGAGAAGTTGTGCAAGATGTAAATGGTACAGCACATTCTCT
TTCTGCTTTAGGGATTCCATTGGCAGCGAAAACTGGTACAGCGGAAATCAAAGAAAAACAGGATGAAAAA
GGGAAAGAGAACAGTTTCTTGTTTGCTTTCAACCCTGATGACCAAGGATATATGATGGTGAGCATGTTGG
AAAATAAAGAAGATGATGATTCAGCAACTAAACGAGCACCCGAACTATTACAATACCTCAACCAAAATTA
TCAATAA

Figure 7. *E. faecium* sol-PBP5 gene sequence, the designed primers, and their locations on the sequence. sol-pbp5F primer, sol-pbp5R primer and the transmembrane region of sol-PBP5were colored with yellow, blue and red, respectively.

3.2.1.2. The cultivation and DNA extraction of *E. faecium* HM1070 strain

E. faecium HM1070 from REDPROM culture collection was grown on TSA and a colony was picked up and homogenized in 1 ml PBS. The sample was centrifuged at 10000 x g for 5 minutes. The supernatant was discarded, and the pellet was dissolved in 100 µl

DNA4PCR solution. After the sample was incubated at 56℃ for 20 minutes, it was vortexed and incubated at 100℃ for 8 minutes. After the sample was cooled at room temperature, it was centrifuged for at 10000 x g for 3 minutes and the supernatant was used as a DNA source.

3.2.1.3. The amplification of sol-PBP5 gene

The PCR conditions were as follows: 6 minutes at 95°C, followed by 35 cycles of {94°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds}, and a final extension at 72°C for 8 minutes. The enzyme used was Taq DNA Polymerase according to the manufacturer's recommendations.

3.2.1.4. The restriction and ligation of sol-PBP5PCR products and pET-30 a (+) vector

The pET-30 a (+) vector, present in our laboratory, was isolated from the *E. coli* BL21 strain according to the protocol of the Presto™ Mini Plasmid Kit (PDH100) and the PCR product was purified according to the protocol of the Geneaid PCR Cleanup Kit (DFC100/DFC300). The purified sol-pbp5ef amplicon and pET-30 a (+) vector were restricted with BamHI and EcoRI enzymes by incubating at 37°C for 30 minutes, as shown in Table 2. 5 µl of the obtained products were run on a 1% agarose gel to confirm the restriction. The remaining 15 µl product from each reaction was purified using phenol-chloroform-isoamyl alcohol (25:24:1). The ligation reaction was set up using the purified restriction products and T4 DNA Ligase enzyme and incubated overnight at 22°C (Table 3). The next day, the ligated product was purified using phenol-chloroform-isoamyl alcohol (25:24:1) (Sambrook et al., 1989).

Table 2. Restriction Reaction Components and Amounts

Component	Amount
sol-PBP5amplicon / pET-30 a (+) vector	~5 µg in 10 µl
10X enzyme buffer	2 µl
Restriction enzyme 1	1 µl
Restriction enzyme 2	1 µl
Molecular water	6 µl
Total	20 µl

Table 3. Ligation Reaction Components and Amounts

Component	Amount
Digested sol-PBP5amplicon + pET-30 a(+)	17 µl
10X T4 DNA Ligase buffer	2 µl
T4 DNA Ligase (5 Weiss U/µl)	1 µl
Total	20 µl

3.2.1.5. Transformation of *E. coli* BL21 (DE3) with ligands

The chemical $CaCl_2$ method was used to transform purified ligands into the *Escherichia coli* BL21 strain (Sambrook, 1989). For the preparation of competent cells, a single colony from *E. coli* BL21 (DE3) culture was picked up, inoculated into 10 ml of 2X YT liquid medium and incubated overnight 37°C. Next day, 1 ml of the *E. coli* BL21 culture from the liquid medium was added to 50 ml of liquid medium and incubated at 37°C for approximately 3 hours until the log phase was reached. The *E. coli* BL21 (DE3) culture at the log phase was placed on ice for 25 minutes and then centrifuged at 5000 rpm for 10 minutes at +4°C. The supernatant was discarded, and resuspension was done with 10 ml of water. After washing again with water, the resulting pellet was resuspended in 10 ml of 10% glycerol water and centrifuged at 5000 rpm for 10 minutes at +4°C. The supernatant was discarded, and resuspension was done with 30 ml of 80 mM MgCl2-20 mM CaCl2 solution. Then, it was centrifuged at 5000 rpm for 10 minutes

at +4°C. The pellet was resuspended in 1 ml of 0.1 M CaCl2 solution. To 100 µl of competent cells, 10 µl of ligand was added and mixed. The competent cell-ligand mixture was kept on ice for 20 minutes. Subsequently, it was incubated at 42°C for 1 minute and then placed on ice for 2 minutes. 1000 µl of liquid medium was added and incubated at 37°C with shaking at 220 rpm for 1 hour. After incubation, 200 µl of the sample was taken and plated on solid medium containing 50 µg/ml kanamycin.

3.2.1.6. The confirmation of cloning of sol-PBP5 gene

The next day, colony PCR was performed under previously used PCR conditions with primers specific to colonies growing on selective medium. The amplicons obtained from colony PCR were sequenced using T7 forward primer with Sanger sequencing (Medsantek, Turkey). Also, plasmids were extracted from colonies with positive PCR results, and after cutting with the enzymes used in cloning, the restriction products were run on the gel.

3.2.2. The expression and purification of sol-pbp5

3.2.2.1. The recombinant sol-PBP5 expression and induction in *E. coli* BL21 (DE3)

To observe the recombinant sol-PBP5 production in *E. coli* BL21 (DE3) cells, the colony which is carrying pET-30a (+) Ω sol-PBP5 plasmid was inoculated into 10 ml 2X YT liquid medium with 50 µg/ml kanamycin at final concentration and incubated overnight at 37°C. Next day, 1 ml culture was inoculated into 50 ml 2X YT liquid medium with 50 µg/ml kanamycin at final concentration and when it reached OD600:0.8, 1 ml culture was taken and centrifuged. The supernatant was discarded, and the pellet was stored at 4°C until it is used. The remaining culture was induced with 5 g/L lactose and incubated overnight at room temperature. Next day, 1 ml culture was taken from the culture and centrifuged. The supernatant was discarded, and the pellet was stored at 4°C until it is used.

3.2.2.2. The confirmation of recombinant sol-PBP5 expression in *E. coli* BL21 (DE3) with SDS-PAGE analysis

To observe recombinant sol-PBP5 protein before and induction and 24 hours post-induction, the cell lysates were examined with SDS-PAGE analysis. SDS-PAGE, reaction components were mixed as given in table 4 and added to the gel cassette. First, the 12.5% separating gel was prepared as in Table 5 and poured and covered with 96% ethanol. After the gel was frozen, the ethanol was removed, and the stacking gel as in Table 6 was poured, and the comb was installed to create loading wells. The pellet samples obtained before induction and 24 hours post-induction were dissolved in 100 µl PBS and they were mixed with 100 µl 2X Laemmli loading buffer for denaturation. They were incubated at 100°C for 10 minutes and were put on ice immediately. The samples were loaded into the wells and the proteins were run in SDS-PAGE under denaturing conditions in 1X running buffer. The protein concentration of each sample was measured and equalized to approximately 1 mg/ml. After the samples were loaded into the wells, they were run at 40 mA, 150 V for 90 minutes.

Table 4. SDS-PAGE reagents

Seperation Buffer;	**1.5M Tris base + HCl pH 8.8**	
Stacking Buffer;	0.5M Tris base + HCl pH 6.8	
2X Laemmli loading buffer;	Bromophenol Blue	0.01% (w/v)
	2-mercaptoethanol	10% (v/v)
	Glycerol	25% (v/v)
	SDS	2% (w/v)
	Tris-HCl	0.125M
1X Running Buffer (pH 8.3)	Tris Base	25mM
	Glycine	200mM
	SDS	0.1% (w/v)
10% SDS (10 mL)	1 gr	To 10 mL ddH_2O

10% Ammonium Persulphate (10 mL)	1 gr	To 10 mL ddH_2O
TEMED		
Bradford Reagent (100 mL)	Coomassie Brilliant Blue G-250	10 mg
	95% Ethanol	5 mL
	85% Phosphoric Acid	10 mL

Table 5. The preparation of separation gel

Acrylamide %	**20%**	**17.5%**	**15%**	**12.5%**	**10%**	**7.5%**	**5%**
40% acrylamide	2000 µl	1750 µl	1500 µl	1250 µl	1000 µl	750 µl	500 µl
Seperation Buffer	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl
10% SDS	40 µl	40 µl	40 µl	40 µl	40 µl	40 µl	40 µl
10% APS	40 µl	40 µl	50 µl	60 µl	60 µl	60 µl	60 µl
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
ddH_2O	915 µl	1165 µl	1405 µl	1645 µl	1895 µl	2145 µl	2395 µl

Table 6. The preparation of stacking gel

Reagents	**Amount**
40% acrylamide	225 µl
Stacking Buffer	500 µl
10% SDS	20 µl
10% APS	30 µl
TEMED	5 µl
ddH_2O	1220 µl

3.2.2.3. The purification of sol-PBP5 with IMAC

E. coli BL21 culture with cloned sol-PBP5gene was inoculated into 20 ml liquid medium overnight. The 20 ml liquid culture was transferred to 1 liter of 2X YT medium containing 50 μg/ml kanamycin and it was incubated at room temperature until it reached OD600:0.8. Then, it was induced with 5 g/L lactose and incubated at room temperature for 24 hours. After incubation, cells were sedimented and the pellet was dissolved in about 50 ml of IMAC wash buffer (500 mM NaCI, 50 mM KH_2PO_4/NaH_2PO_4, pH 8.0). The cell suspension was lysed with microfluidizer 5 times at 15000 psi in ice. The lysate was centrifuged at 5000 g for 20 minutes and the supernatant was filtered using a 0.45 μm filter. The lysate containing the sol-PBP5 protein was used in the purification step.

Immobilized Metal Ion Affinity Chromatography was used to purify sol-PBP5 (Spriestersbach 2015). Purification was performed through the affinity of the 6X His-tag tail located at the N terminus of sol-PBP5 to Ni^{+2} ions. First, the IMAC column was washed with distilled water 5 times the column volume. To equilibrate the column, at least 5 times the column volume of wash buffer was passed through at a flow rate of 0.5 ml/min. The cell lysate containing the sol-PBP5protein was then loaded onto the column. After loading the lysate, the column was washed with wash buffer equivalent to 10 times the column volume. The purified protein was obtained by passing 5 times the column volume of elution buffer (500 mM NaCI, 50 mM KH_2PO_4/NaH_2PO_4, 500 mM imidazole, pH 8.0) through the column. During purification process, fractions from the cell lysate, flow-through, wash and elution were collected. The collected fractions were visualized on 10% SDS-PAGE after staining with Coomassie blue, as explained in section 3.2.2.1.

3.2.2.4. The removal of salts from elution fraction with desalting column

In order to remove imidazole and other salts from elution fraction and obtain the protein in PBS, the desalting column was used. First, the desalting column was washed and equilibrated with PBS 5 times the column volume. Then, the sample was loaded, and the fractions were collected as the UV value started to increase. The collected fractions were visualized on 10% SDS-PAGE after staining with Coomassie blue, as explained in section 3.2.2.1. The concentration of the protein was measured with Nanodrop spectrophotometer and PBS was

used as a blank. Also, the measurement was confirmed with Bradford assay, with bovine serum albumin (BSA) used as the standard at different concentrations (Kruger, 2009). A standard curve was generated using Excel, and the protein concentration was calculated based on this curve.

3.2.3. Confirmation of Recombinant sol-PBP5 Protein in Native Conformation

To confirm that the cloned and purified sol-PBP5 protein is in its native conformation, the fluorescent antibiotic BOCILLIN FL was used (Hujer et al., 2005). This penicillin-based antibiotic binds to the sol-PBP5 protein. If the produced recombinant sol-PBP5 is in its native form, it is expected to bind the antibiotic and emit fluorescence under UV light after SDS-PAGE.

Purified recombinant sol-PBP5 (0.5 µg) and BSA protein (0.5 µg) used as control were incubated with 200 µM BOCILLIN FL for 4 h and overnight at 37°C. They were denatured as mentioned at 3.2.2.1. section, and SDS-PAGE was performed. After washing the gel with ultrapure distilled water, imaging was performed under UV. Additionally, the same samples were loaded by SDS-PAGE and then stained with Coomassie blue to confirm their presence and marker size dimensions.

3.2.4. Immunization of mice with recombinant sol-PBP5

Female CD-1 mice aged 5-6 weeks were used for immunization. The mice were obtained from the Production and Research Center of the Faculty of Veterinary Medicine at Aydın Adnan Menderes University (Aydın, Turkey). For immunization with sol-PBP5ef, 8 mice were used, and 1 mouse was used as a control. Mice in the control group were administered the same volume of PBS and underwent the same procedures. Recombinant sol-PBP5, purified using the IMAC method, was desalted into PBS using a desalting column. 25 µg sol-PBP5 protein was emulsified in 100 µl Freund's adjuvant. On day 0, mice were immunized subcutaneously with 100 µl of a solution containing 25 µg of purified sol-PBP5protein emulsified in Complete Freund's Adjuvant. Subsequently, the mice received two booster injections of the same antigen emulsified in Incomplete Freund's Adjuvant on days 15 and 30 (Majelan et al, 2019). One week after the final dose, the mice were sacrificed by cervical

dislocation. Blood samples (approximately 100 µl) from the immunized mice were collected into EDTA tubes and stored at -80°C until further use. Spleens were aseptically harvested from the mice and transferred to RPMI 1640 medium containing 10% FBS, 100 I.U./mL penicillin, and 100 µg/mL streptomycin. Each spleen was divided into approximately 25 mg pieces, distributed into tubes, and 100 µl of RNAlater solution was added to each tube. Samples were stored at -80°C until further use.

3.2.5. Confirmation of the Immune Response Against Recombinant sol-PBP5 using ELISA

To confirm the immunization of mice against recombinant sol-PBP5, ELISA was performed using blood samples from the immunized mice. A 96-well ELISA plate was coated with 100 µl of sol-PBP5 protein solution (5/10 µg/ml in 100 mM bicarbonate/carbonate buffer) and incubated at 4°C overnight. The wells were washed with PBS, blocked with 5% skim milk in PBS, and incubated at 37°C for 2 hours. Blood samples at various dilutions (1:20, 1:40, 1:80, 1:160) were added, incubated at 37°C for 1 hour, and washed. A secondary antibody (HRP-conjugated Anti-Mouse IgG, 1:1000) was added and incubated at 37°C for 2 hours. After washing, 50 µl of TMB substrate was added and incubated at room temperature for 30 minutes, followed by stop solution, and absorbance was measured at 450 nm. To compare the absorbance values between the immunized and control groups, an independent two-sample t-test was performed using Excel.

3.2.6. The construction of VH library and the cloning of variable heavy (VH) chain library into pComb3-hy phagemid

3.2.6.1. Total RNA isolation from the spleens

Total RNA isolation of spleens of immunized mice confirmed to have an immune response against sol-PBP5was performed using NucleoZOL, one phase RNA purification solution, in accordance with the manufacturer's instructions. 500 µl of NucleoZOL solution was added to 25 mg of spleen tissue and homogenized. 200 µl of RNase-free water was added and

mixed for 15 seconds. After a 5-minute incubation at room temperature, the mixture was centrifuged at 12,000 x g for 15 minutes at room temperature. 500 µl of the supernatant was transferred to a new tube, 500 µl of isopropanol was added, and the mixture was incubated at room temperature for 10 minutes. Afterwards, it was centrifuged at 12000xg for 10 minutes. The supernatant was removed and 500 µl 75% ethanol was added and centrifuged at 8000xg for 3 minutes at room temperature. After the supernatant was removed, the pellet was left to dry. The dried pellet was dissolved with 50 µl RNAse-free water. Of 5 µl isolated RNA was run on agarose gel and stored at -80°C until used in the next steps.

3.2.6.2. cDNA synthesis of VH region from total RNA and amplification of VH via PCR

Reverse transcription and polymerase chain reaction (PCR) experiments were performed based on the work by Meyer et al. (2019). In this study, cDNA was synthesized from total RNA isolated from immunized mice, which expressed antigen-specific antibodies. Specific primers and a unique reverse transcriptase enzyme were used for cDNA synthesis. The specific primer was complementary to the constant sequence, while the reverse transcriptase was designed to terminate synthesis with a CCC sequence. The complementary strand of cDNA was synthesized using the CCC sequence, employing a GGG primer with a universal primer at the 5’ end to serve as a template for subsequent PCR. This method is known as SMART (switching mechanism at 5’ end of RNA transcript) technology.

In the SMART (Switching Mechanism at the 5' end of RNA Transcript) method, Moloney murine leukemia virus (MMLV) reverse transcriptase adds a CCC extension at the end of cDNA synthesis. The next step involves a template-switch oligo with a GGG extension at its 3’ end, which binds to the CCC extension of the cDNA. MMLV reverse transcriptase then synthesizes the complementary DNA of the oligo. As a result, the cDNA will have a designed universal sequence at the 3’ end, a known constant region sequence at the 5’ end, a variable leader sequence, and the entire variable region. For cDNA synthesis, a heavy chain mIGHG RT primer specific to conserved constant regions of mouse IgG antibodies and a template-switch oligo primer were used. The reverse transcription reaction was initiated with the template-switch oligo and heavy chain primer pair, following the manufacturer's recommendations with SMART Reverse Transcriptase (EURX, Poland). To clone the variable heavy (VH) region of the antibody produced against sol-PBP5protein, a PCR reaction was set up with ISPCR and

mIGHGPCR primers under the recommended conditions by using Taq polymerase. The ISPCR primer sequence is identical to the template-switch oligo used in cDNA synthesis.

3.2.6.3. The construction of pComb3-hy phagemid

The pComb3 phagemid is used for cloning variable heavy and light chains in antibodies. However, this vector is known to have low stability, likely due to the presence of two pelB sequences at different sites. The pelB sequence downstream of the M13 phage pIII sequence was removed using restriction enzymes. The pComb3 plasmid was digested with NheI and XbaI according to the manufacturer's instructions. These enzymes are compatible, creating cohesive ends (CTAG) suitable for ligation. The resulting ligated product was transformed into *E. coli* DH10B strain (Sambrook 1989), and plasmid isolation was performed from colonies grown on selective media. The targeted DNA sequence to be removed is 275 bp and contains one EcoRI site. Thus, the pComb3 vector has two EcoRI sites, whereas the modified pComb3-H vector will have one EcoRI site. Isolated plasmids and the original pComb3 plasmid were digested with EcoRI and analyzed by agarose gel electrophoresis.

3.2.6.4. The generation of TA vector from pComb3-hy phagemid

One hundred micrograms of the pComb3 vector was linearized by digesting with the SmaI restriction enzyme and purified using the phenol:chloroform:isoamyl alcohol (25:24:1) method as previously described. The linearized vector was then treated with terminal deoxynucleotidyl transferase (TdT). For this, 104 µl of linearized DNA, 30 µl of 5X TdT buffer, 3 µl of ddTTP (1 mM), and 13 µl of TdT (20 units/µl) were mixed. The mixture was incubated at 37°C for 2 hours and subsequently purified. The purified product was aliquoted into 10 parts.

3.2.6.5. The cloning of VH amplicons into pComb3-hy and construction of VH library

The VH amplicons and TA vector of pComb3-hy phagemid were ligated and transferred into *E. coli* XL1-Blue competent cells. The transformants were inoculated into agar plate containing 1% glucose and ampicillin (100 µg/ml)-tetracycline (10 µg/ml), and it was incubated

overnight at 37°C. Next day, colony PCR with Fpuc19-IPTG and M13F primers was performed to determine the ratio of colonies containing the insert to the total number of colonies. All colonies on the petri dish surface were collected and transferred to LB medium containing 15% glycerol, ampicillin (100 µg/ml), and tetracycline (10 µg/ml). The cultures were then stored at -80°C for future use.

3.3. The phage display of VH library obtained from sol-PBP5 immunized mice

3.3.1. The infection of *E. coli* XL1-Blue cells with M13K07 helper phage

3.3.1.1. Infection of XL1-Blue cells with M13K07 helper phage

XL1-Blue cells were cultured in 2 ml of 2X YT medium containing 10 µg/ml tetracycline and incubated with shaking until an OD600 of 0.8 was reached. Subsequently, 10^9 pfu M13K07 phage was added to the culture and incubated with shaking at 37°C for 1 hour. Following this incubation, kanamycin was added to a final concentration of 70 µg/ml, and the culture was incubated at 37°C for an additional hour. Then, the culture was incubated overnight at 30°C to allow for phage production.

3.3.1.2. Phage precipitation and phage titer calculation

The cultures were centrifuged at 2500 x g for 20 minutes to pellet the cells, and the supernatant containing the phages was filtered through a 0.22 µm filter. The filtrate was divided into tubes and centrifuged at 17000 x g for 3 hours. The supernatant was discarded, and the phage pellet was resuspended in 100 µl of TBS containing 2% BSA. 10 µl of precipitated phage and 100 µl of XL1-Blue culture at log phase were mixed and incubated at 37°C for one hour. Then, it was diluted ten-fold until 10^{-7}. The dilutions were spotted on kanamycin (50 µg/ml) and tetracycline (10 µg/ml) containing plate. It was evaluated that the growth of colonies on the selective medium indicated successful infection of the cells by the M13K07 phage. The titer (pfu/ml) of phage suspension was calculated as an estimate. The used formula is dilution factor x bacteria count x 100. Also, to confirm the infectivity of M13K07 helper phage, spot test was

performed. Briefly, 100 µl of the log phase bacterial culture was added to the soft agar, mixed, and then poured onto the bottom agar. After allowing the plates to sit at room temperature for half an hour, 10 µl of the phage suspension was spotted onto marked locations. The plates were left at room temperature for another half hour to allow the drops to dry, then incubated overnight at 37°C. The next day, plaque formation was examined.

3.3.2. The infection of VH library in *E. coli* XL1-Blue cells and Production of VH Phage Library

The XL1-Blue cells carrying the VH library obtained from mice immunized with sol-SOL-PBP5were cultured in liquid medium containing 1% glucose, tetracycline (10 µg/ml), and ampicillin (100 µg/ml) until an OD600 of 0.8 was reached. The cells were then infected with 10^9 pfu M13K07 helper phage and incubated at 37°C for one hour. Subsequently, the culture was transferred to 20 ml of 2X YT medium containing 0.1 mM IPTG, tetracycline (10 µg/ml), ampicillin (100 µg/ml), and kanamycin (50 µg/ml), and incubated overnight at 30°C with shaking. The next day, the culture was centrifuged, and the supernatant was filtered through a 0.22 µm filter. The produced phages were precipitated as described in section 3.3.1.2. The phage pellet was resuspended in 100 µl of TBS containing 2% BSA. the phage library was subjected to the biopanning procedure against sol-PBP5.

3.3.3. Biopanning for the Selection of M13K07 Phages Displaying sol-PBP5-Specific VH Fragments

For biopanning, recombinant sol-SOL-PBP5was prepared at a final concentration of 5 µg/ml in 100 mM sodium bicarbonate buffer. The same procedure was followed for a 6X His-tag protein to prevent non-specific binding. 100 µl of the prepared protein solution was added to each well of a 96-well plate. The plate was incubated overnight at 4°C. The next day, the liquid was removed from the wells, and the wells were washed once with TBS. Then, 300 µl of TBS containing 2% BSA was added to each well for blocking and incubated at 37°C for one hour. After blocking, the wells were washed once with TBS. Previously prepared phage suspensions (VH phage library) were added to the wells coated with 6X His-tag and incubated

at 37°C for one hour. The suspension containing phages that did not bind to the His-tag was then transferred to the wells coated with sol-PBP5and incubated at 37°C for one hour. After incubation, the liquid was removed from the wells, and the wells were washed five times with 3-minute intervals using TBS. For elution, 100 µl of glycine buffer (pH:2.2) was added to each well and incubated at room temperature for 5 minutes. The eluted phages were neutralized by adding 6 µl of Tris-HCl buffer (pH:9). Following neutralization, 100 µl of an overnight culture of XL1-Blue culture was added to the wells and incubated at 37°C for one hour. After incubation, the entire content of each well was plated on TSA containing ampicillin and tetracycline and incubated overnight. The next day, the growing colonies were collected in 1 ml 2X YT with 15% glycerol. Of 90% culture was stored in -80°C. The remaining culture was performed as in 3.3.3 section. Briefly, when the culture reached late log phase, it was infected with M13K07 phage and incubated overnight. Next day, the produced phages were used for biopanning. These steps were repeated at 3 times for subsequent rounds of selection to enrich for phages with high affinity to sol-PBP5. After last biopanning process, randomly four colonies were chosen for further experiments, and they were produced as mentioned in 3.3.1.1-2.

3.3.4. The examination of VH displaying phage affinity to sol-PBP5

3.3.4.1. The sequencing and bioinformatic analysis of VH sequence

The 4 colonies randomly picked from ampicillin containing plate obtained from the last biopanning process and they were amplified as mentioned in 3.2.5.5 section. The VH amplicons were sequenced with Sanger sequencing. The sequence results were obtained as a fasta file. From the DNA fasta file, amino acid sequence of the VHs which are potential recombinant antibiotics were obtained. The complementarity-determining region (CDR) of VHs were predicted by using AbRSA tool. The sequence was compared against known germ line sequences using IgBLAST with IMGT.

3.3.4.2. The prediction of 3D structure of VH and sol-PBP5 and their protein-protein interactions

The 3D structures of VH and sol-PBP5 were predicted using Alphafold and the interaction between the proteins was explored using AlphaFold (Jumper 2021). Firstly, to create high-accuracy 3D models of the proteins, the amino acid sequences of the proteins were submitted to Alphafold. These models were used to investigate protein-protein interactions. The types and locations of bonds including hydrogen bonds, ionic bonds, and hydrophobic interactions were detected at the interaction surfaces. This comprehensive approach provided detailed insights into the binding motifs and interaction regions of the proteins.

3.4. The cloning of VH showing affinity to sol-PBP5

The primers were designed to cloning of the VH based on sequence result. The VH was amplified with VH43F1-BamHI and VH43R-HindIII primers. The amplicon and pET-30 a (+) vector were restricted with HindIII and BamHI enzymes. They were ligated with T4 DNA ligase and the ligand was transferred to *E. coli* BL21 (DE3) as mentioned in 3.2.1.4-5 sections. After the confirmation of the cloning, the expression and purification of VH was performed as mentioned in 3.2.2 section and the concentration of the recombinant VH was measured with Nanodrop spectrophotometer.

3.5. Comparative binding studies of VH displaying phages and recombinant antibiotic

To test the inhibition capacity of the four phages, M13K07 phage and recombinant VH43 protein, BOCILLIN FL was used. First, 5 µl of sol-PBP5 (200 µg/ml) and 10 µl of TBS were added into 9 separate tubes. Then, 5 µl of four phages (10^5 pfu/ml)/ PBS/ ampicillin (1 mg/ml)/ ceftazidime (1 mg/ml)/ recombinant VH43 (1 µg) or 5 µl of TBS were added into each tube, separately. The samples were incubated at 37°C for one hour. Then, 1 µl of 100 mM BOCILLIN FL was added and mixed. They were incubated at 37°C for one hour. After the incubation, SDS-PAGE analysis was performed as described in section 3.2.2.1. Following electrophoresis, the gel was washed with ultrapure distilled water, and imaging was performed

under UV light. Additionally, the same samples were analyzed by SDS-PAGE and stained with Coomassie blue to confirm their presence and determine marker size dimensions.

3.6. Determination of antibacterial activity of the recombinant VH43 with high binding affinity to sol-PBP5

3.6.1. Determination of minimum inhibitory concentration of recombinant VH43 for HM1070

To determine the minimum inhibitory concentrations (MIC) of VH43 which is recombinant antibiotic for *E. faecium* HM1070, broth microdilution method was used. Two groups were created for each antibiotic. In the first group, 2X YT medium and PBS were mixed at a 1:1 ratio. In the second group, 2X YT medium and PBS containing VH (20 µg/ml) were mixed at a 1:1 ratio. The overnight *E. faecium* HM1070 culture was diluted to 10^7 cfu/ml with water, and 10 µl of the diluted culture was added into the wells. The wells for growth control and negative control were prepared. The plate was incubated at 37°C overnight.

3.6.2. Determination of minimum inhibitory concentration of cephalosporin for *E. faecium* HM1070 and clinical *E. faecium* isolates

To determine the minimum inhibitory concentrations (MIC) of antibiotics for *E. faecium* HM1070, broth microdilution method was used. One third-generation cephalosporins (ceftazidime) and one first-generation cephalosporin (cefazolin) were used. Stock antibiotic solutions were prepared at concentrations of 100 mg/ml and diluted to 1 mg/ml. For the MIC test, 2X YT medium diluted 1:1 with PBS was used. The antibiotics were added to the wells at final concentrations ranging from 1024 to 0.003 µg/ml. The overnight *E. faecium* HM1070 culture was diluted to 10^7 cfu/ml with water and 10 µl of the diluted culture was added into the wells. Each plate included a growth control well containing only bacteria without antibiotic and a medium control well containing only the medium without bacteria and antibiotic. The same steps were applied to determine MIC of ceftazidime for randomly picked 10 *E. faecium* isolates.

3.6.3. Synergistic Effects of Combined Use of Recombinant VH43 with antibiotic on *Enterococcus faecium* HM1070

3.6.3.1. Bacterial growth curve analysis

The antibacterial activity of purified recombinant antibiotic and its combination with cephalosporin against *E. faecium* HM1070 was determined using MIC and bacterial growth curve analysis by measuring optical density. Two groups were created for each antibiotic. In the first group, 2X YT medium and PBS were mixed at a 1:1 ratio. In the second group, 2X YT medium and PBS containing VH (20 µg/ml) were mixed at a 1:1 ratio. The samples were distributed into wells, and antibiotics were added to the wells at final concentrations ranging from 1024 to 0.003 µg/ml. The overnight *E. faecium* HM1070 culture was diluted to 10^7 cfu/ml with water, and 10 µl of the diluted culture was added into the wells. For each group, a growth control and wells containing only medium or medium with VH were prepared. The plate was incubated at 37°C overnight. For bacterial growth analysis, the plate was incubated overnight by shaking and the absorbance was measured at one-hour intervals. The significance of the difference between the two groups, one administered cephalosporin alone and the other administered recombinant VH43 combined with cephalosporin, was determined statistically by a t-test.

3.6.3.2. Kirby-Bauer Disk Diffusion Test/Spot test

The antimicrobial activity of recombinant VH43 was assessed using the Kirby-Bauer test and the spot test, with modifications to the standard protocols (Hudzicki, 2009). For this assay, two different strategies were carried out.

First, the plates ceftazidime at 2 µg/ml, 4 µg/ml and 8 µg/ml concentrations were prepared. The concentrations were 1/2XMIC, 1XMIC and 2XMIC, respectively. The overnight culture of *E. faecium* was diluted with physiological saline to achieve a 0.5 McFarland turbidity. The bacteria were streaked onto the antibiotic-containing agar using a sterile cotton swab. The agar plates were dried for 15 minutes. The plates were divided into four sections. The left side of agar was used to apply recombinant VH43 and right side of agar was used to apply PBS. Sterile filter paper discs were placed onto upper left and right sides of agar with forceps, and

the discs were wetted with 30 µl of recombinant VH43 (0.6 µg) and PBS, respectively. The recombinant VH43 and PBS were spotted onto lower left and right sides of agar. The plates were incubated at room temperature until the drops were absorbed, and then incubated at 37°C overnight. Then, the plates were incubated at 37°C overnight. Next day, the plates were observed.

Differently from first method, ceftazidime was not added into the agar and TSA was used for experiment. Similarly, the overnight culture of *E. faecium* was diluted with physiological saline to achieve a 0.5 McFarland turbidity. The bacteria were streaked onto the antibiotic-free agar using a sterile cotton swab. The agar plates were dried for 15 minutes. 6 sterile filter paper discs were placed onto agar at equal distance. The 6 prepared solutions were used to wet discs. They are 8 µg ceftazidime, 8 µg ceftazidime + recombinant VH43 (0.6 µg), 4 µg ceftazidime, 4 µg ceftazidime + recombinant VH43 (0.6 µg), recombinant VH43 (0.6 µg), and PBS. The plate was incubated at room temperature for 30 minutes and then incubated at 37°C overnight. Then, the plates were incubated at 37°C overnight. Next day, the plates were observed.

3.6.4. Determination of antibacterial activity of the VH for clinical *Enterococcus faecium* isolates

3.6.4.1. Antibacterial effect of recombinant VH43 for clinical E. faecium isolates

The 10 clinical E. faecium isolates from REDPROM culture collection were cultured. To determine the minimum inhibitory concentrations (MIC) of recombinant antibiotic VH43 for clinical *E. faecium* isolates, broth microdilution method was used. Two groups were created for each antibiotic. In the first group, 2X YT medium and PBS were mixed at a 1:1 ratio. In the second group, 2X YT medium and PBS containing VH (20 µg/ml) were mixed at a 1:1 ratio. The overnight culture was diluted to 10^7 cfu/ml with water, and 10 µl of the diluted culture was added into the wells. The wells for growth control and negative control were prepared. The plate was incubated at 37°C overnight.

3.6.4.2. Effect of Combined Use of VH Cephalosporin on the selected clinical E. faecium isolates

To observe antibacterial effect of recombinant antibiotic on the selected 4 clinical *E. faecium* isolates, the bacterial growth curve analysis by measuring optical density was performed. Firstly, MIC values were determined as in section 3.6.2. The steps in 3.6.3. section was applied. The significance of the difference between the two groups, one administered cephalosporin alone and the other administered recombinant VH43 combined with cephalosporin, was determined statistically by a t-test.

3.7. Cell cytotoxicity assay

The cytotoxic effect of VH was tested with WST-1 assay on an immortalized human keratinocytes cell line HaCaT and cervical carcinoma HeLa cell line (Sola 2020, Dastan 2023). Both group of cells were prepared as suspensions in 10^5 cell/ml density. Suspensions were inoculated into sterile 96-well polystyrene flat bottom microtiter plates in 100 µL volumes. After incubating for 24 hours at 37°C, adherences to the bottom of plate were observed. Wells were gently washed with 100 µl of sterile phosphate buffered saline (PBS). 50 µl of the treatments which were 0.20 µg/ml recombinant VH, 0.10 µg/ml, 0.05 µg/ml and PBS as a control, and 50 µl antibiotic free and 10% FBS containing DMEM was added to the wells. After 24 hours incubation at 37°C, 10 µl of Cell Proliferation Colorimetric Reagent, WST-1 (Biovision, USA) was added to each well. Also, mixture of 50 µl medium and 50 µl PBS was used as a blank. The plate was incubated for 4 hours, and the cell cytotoxicity was measured at 4. hour in 450 nanometer (nm) wavelength. The absorbance results were evaluated.

4. RESULTS

4.1. The cloning of sol-PBP5 gene

4.1.1. The amplification of sol-PBP5 gene

The sol-PBP5 gene was amplified by using the designed specific primers with PCR. The extracted DNA from *E. faecium* HM1070 was used as a DNA source. The PCR products were run on agarose gel (Figure 8). The amplicons were approximately 1900 bp long, as expected. After the amplicons were purified with the PCR clean-up kit, they were stored at -20°C for use in later stages.

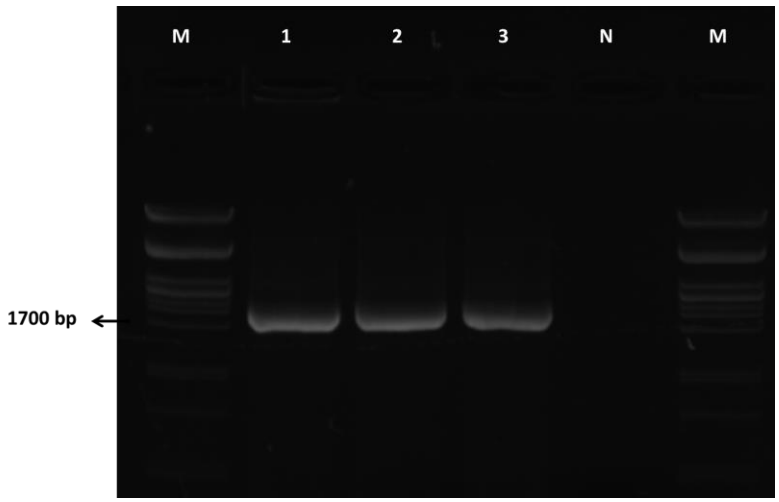


Figure 8. The sol-PBP5 amplicons. Lane 1,2,3: PCR amplicons obtained from *E. faecium* HM1070 with PCR. The band was near 1930 bp as expected. N: Negative control. M: Lambda-PstI marker.

4.1.2. The restriction and ligation of sol-PBP5 PCR products and pET-30 a (+) vector

The purified sol-pbp5 amplicon and pET-30 a (+) vector were cut with BamHI and EcoRI enzymes, 5 µl of the restriction product was loaded on agarose gel and visualized (Figure 9). The amplicon size was 1930 bp as expected and the pET-30 a (+) vector was linearized at

about 5400 bp. After the remaining products were purified, ligation was established with T4 DNA ligase enzyme and incubated overnight at room temperature.

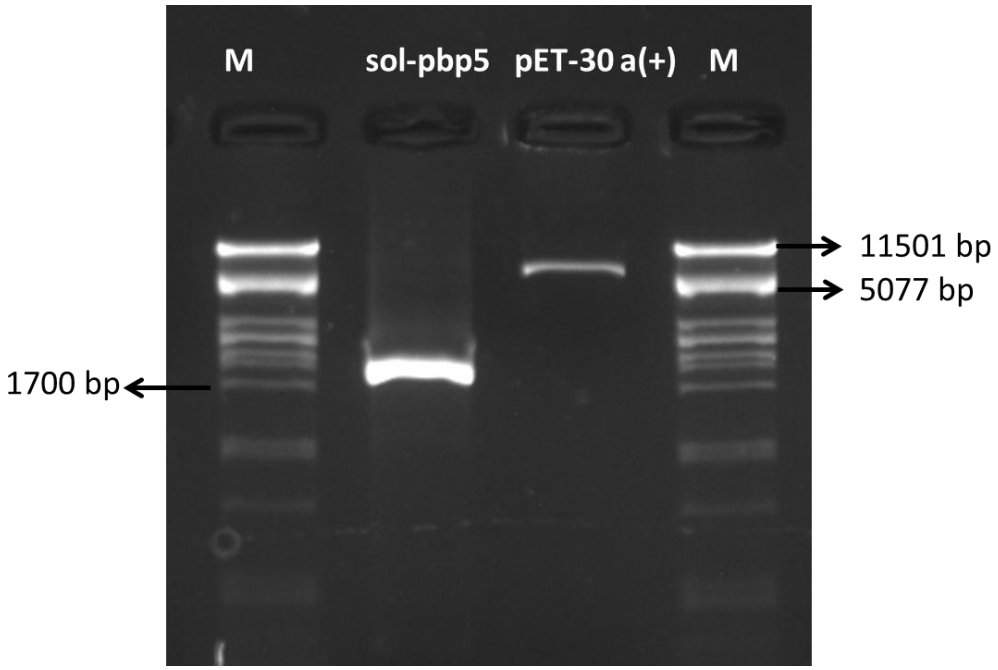


Figure 9. The restriction of sol-pbp5 amplicon and pET-30 a (+) vector with EcoRI and BamHI enzymes. The restricted sol-pbp5 amplicon was 1900 bp and the pET-30 a (+) vector was approximately 5400 bp. M: Lambda-PstI marker.

4.1.3. The transformation of pET-30 a (+) Ω sol-PBP5 construction into *E. coli* BL21 (DE3) competent cells

The obtained pET-30 a (+) Ω sol-PBP5 ligands were transferred into *E. coli* BL21 (DE3) competent cells via chemical transformation and the transformed cells were selected on kanamycin containing TSA plate.

4.1.4. The confirmation of cloning of sol-pbp5 gene

4.1.4.1. Colony PCR

Colony PCR was performed on colonies growing on selective medium using sol-pbp5ef gene-specific primers. The obtained PCR products were run on agarose gel (Figure 10). A positive result was obtained in the majority of colonies subjected to PCR with sol-pbp5-specific primers. The amplified fragment was observed to be approximately 1900 bp in length, the same as the sol-pbp5ef gene.

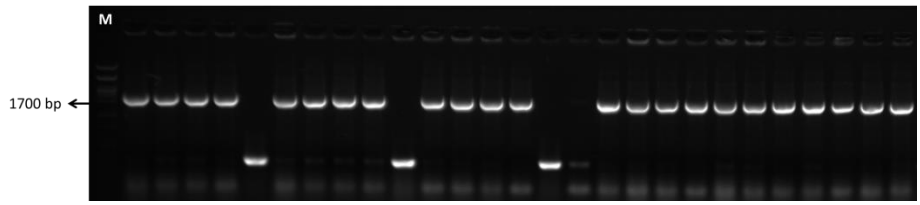


Figure 10. Colony PCR results of colonies grown on selective medium. Colony PCR confirmed that the majority of the colonies contained the sol-pbp5ef gene. The expected band of approximately 1900 bp was obtained. M: Lambda-PstI marker.

4.1.4.2. The plasmid isolation and restriction

Plasmid isolation was performed on a randomly selected colony that tested positive in the colony PCR. The isolated plasmid was digested with the EcoRI and BamHI enzymes used in cloning. The undigested plasmid and the restriction product were run on an agarose gel (Figure 11). Due to the circular plasmid topology, the expected band was observed above approximately 8000 bp. The plasmid digested with restriction enzymes revealed bands corresponding to both the pET-30a(+) vector (5400 bp) and the sol-PBP5ef gene (1900 bp). This confirmed that the sol-pbp5ef gene was cloned into this vector using the BamHI and EcoRI restriction enzymes.

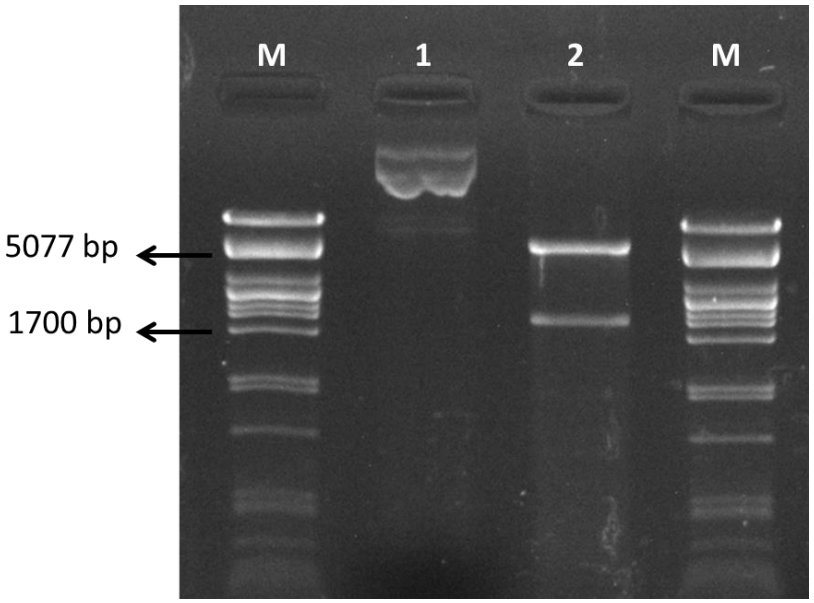


Figure 11. Circular and restriction enzyme-digested forms of the pET-30a(+)-sol-pbp5ef plasmid. 1: Circular form of the pET-30a(+)-sol-pbp5ef plasmid, 2: Product obtained after digestion of the pET-30a(+)-sol-pbp5ef plasmid with BamHI and EcoRI enzymes, M: Lambda PstI

4.1.4.3. The sequencing of amplicons obtained from colony PCR

The cloning of sol-PBP5was confirmed by sequencing of PCR amplicons obtained from colony PCR with T7 forward primer. The sequence result was analysed via BLASTx. It showed 100% similarity with "TPA: penicillin-binding transpeptidase domain-containing protein [*Enterococcus faecium*]" (Figure 12).

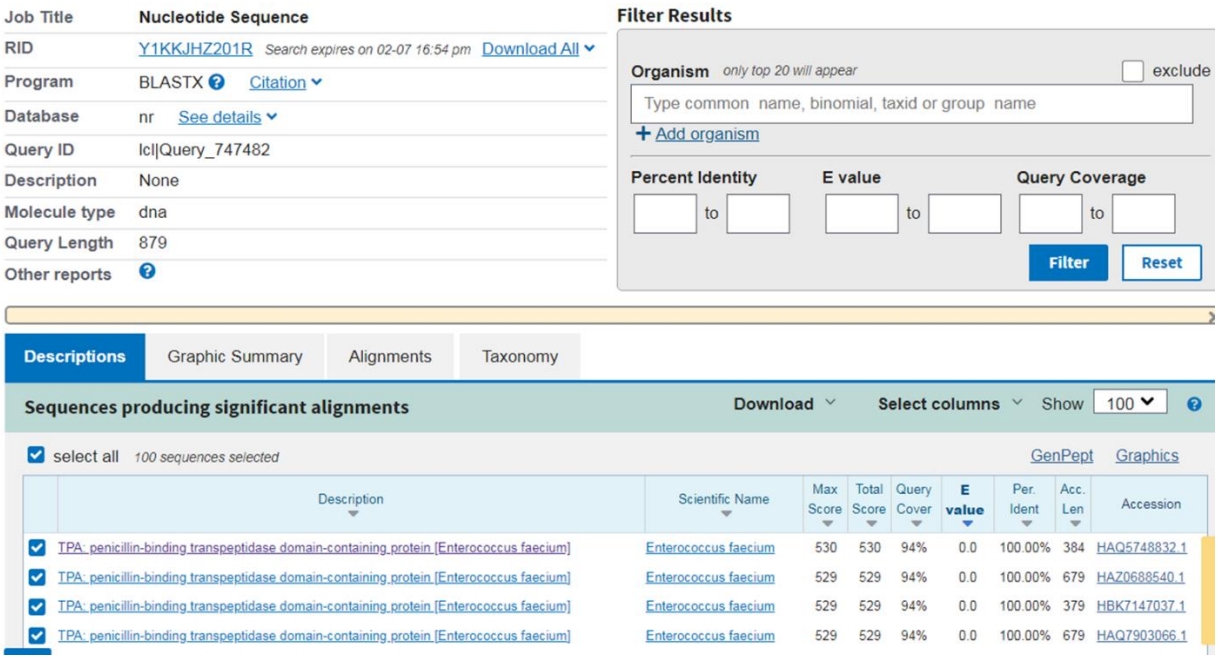


Figure 12. The BLASTX analysis of the cloned sol-PBP5 sequence

In addition, it was confirmed that the DNA fragment encoding sol-sol-pbp5ef in the obtained sequence and the 6xHistag tail intended to be used in purification were located in the same ORF, and thus the recombinant protein produced would be produced with a 6xHistag tail (Figure 13).

Figure 13. The display of sequence result of amplicons from colony PCR. It appears that the sol-pbp5 gene is located in the same ORF as the 6xHistag tail found in the pET-30 a vector and therefore will be produced in fusion with the sol-PBP5 protein.

4.2. The expression and purification of sol-pbp5

4.2.1. The expression of recombinant sol-PBP5 protein in *E. coli* BL21 (DE3)

The molecular weight of the sol-PBP5was calculated from the sequence result and it was found as 75.2 kDa. The colony carrying sol-PBP5encoding plasmid was cultured and induced with lactose when it reached late-log phase. The samples were taken before induction and after 24 hours induction. The samples were examined with SDS-PAGE analysis. In the two lines, the amount of loaded proteins were same. There is an increased intensity (or thickening) observed above the 72 kDa band on the SDS-PAGE gel (Figure 14).

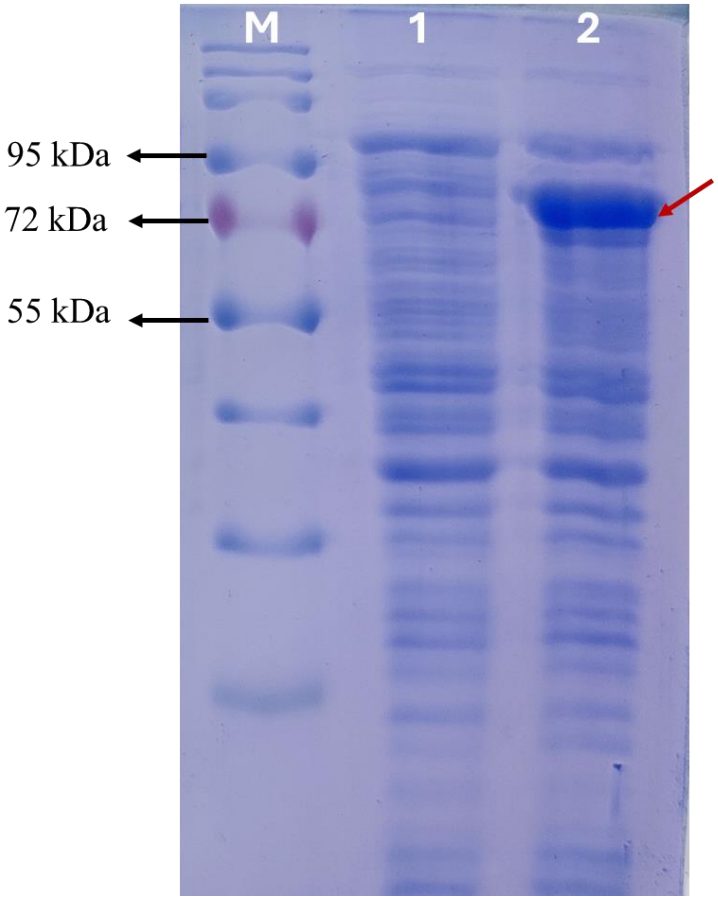


Figure 14. The expression of sol-PBP5 in *E. coli* BL21 (DE3). 1: before induction, 2: 24 hours post-induction, M: Marker. When line 1 and line 2 compared, the increased intensity was seen at line 2 above 72 kDa. Protein ladder: 10638393, Fischer bioreagents.

4.2.2. The purification of sol-PBP5protein

Recombinant sol-PBP5 protein with 6XHistag was purified with IMAC. The collected fractions were run on SDS-PAGE (Figure 15). The fractions were cell lysate, flow-through, wash and elution, respectively. All fractions were collected during elution step. It was seen an intense band above 72 kDa at cell lysate. The intense band was weakened at flow-through fraction and a small amount of the band with many non-specific bands was seen at wash fraction. At the elution step, 6 fractions were collected, and the purest fraction was the last fraction which had highest imidazole concentration. The imidazole and the other salts were removed by using the desalting column from purest sol-PBP5fraction. The obtained protein fraction after desalting process was run on SDS-PAGE. The concentration of sol-PBP5was 2.5 mg/ml and it was used for further experiments.

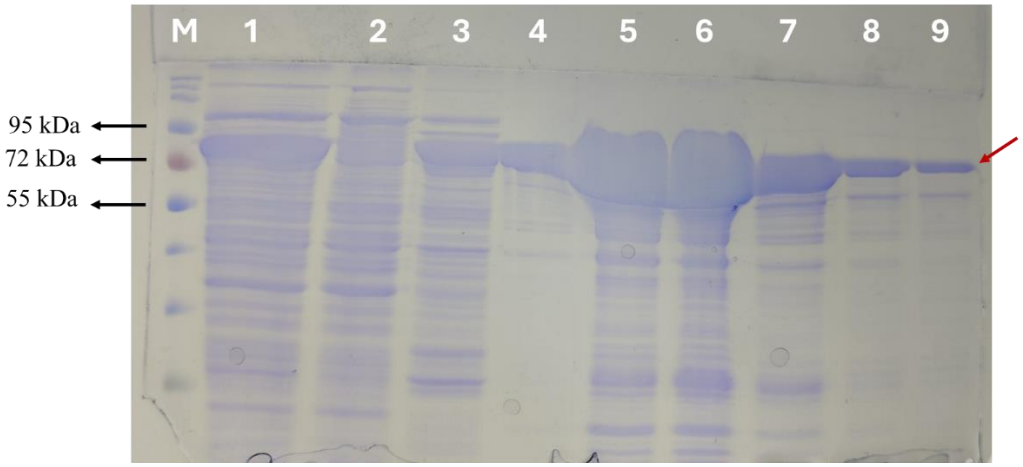


Figure 15. SDS-PAGE analysis of cell lysate and fractions during sol-PBP5 protein purification with IMAC. M: Marker, 1: cell lysate of sol-PBP5producing *E. coli* BL21 (DE3), 2: flow-through, 3: wash, 4-9: elution fractions. An intense band above 72 kDa was observed in the cell lysate (Line 1). This band was weakened in the flow-through fraction (Line 2) and showed a small amount with many non-specific bands in the wash fraction (Line 3). During elution, 6 fractions (Line 4-9) were collected, with the purest fraction being the last one, containing the highest imidazole concentration. Protein Ladder: 10638393, Fischer Bioreagents.

4.2.3. Confirmation of Recombinant sol-PBP5ef Protein in Native Conformation

To verify the native conformation of the cloned and purified sol-PBP5 protein, the purified sol-PBP5 was treated with fluorescent antibiotic BOCILLIN FL.

This penicillin-based antibiotic, BOCILLIN FL, has a binding affinity for the sol-PBP5 protein. After treatment and SDS-PAGE analysis, while light was visible in the wells containing the sol-PBP5protein, but not in the control (Figure 16). Thus, it was proven that the purified recombinant sol-PBP5 proteins were in their native form.

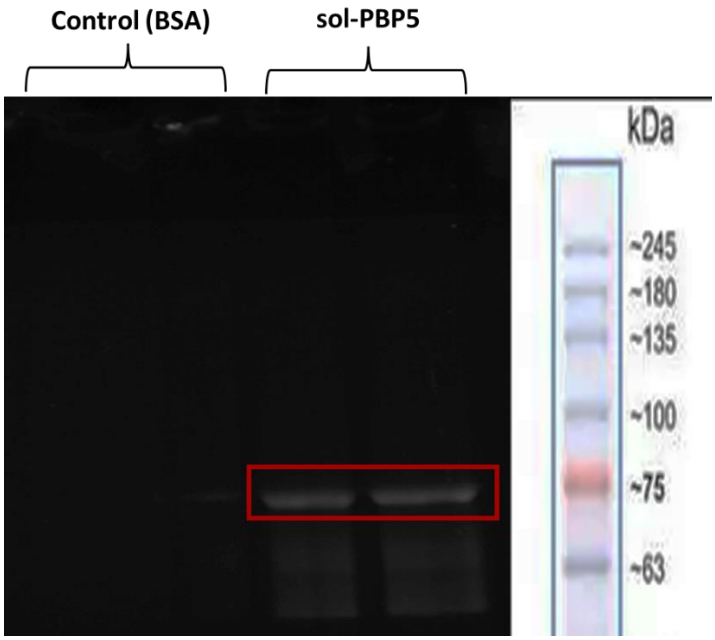


Figure 16. SDS-PAGE analysis of recombinant sol-PBP5 protein and BSA (control) treated with Bocillin FL. Fluorescence was observed in the well containing sol-PBP5 protein, but not in the control. Protein Ladder: G02101, Lambda biotech.

4.3. Confirmation of immunization of mice with recombinant sol-PBP5

One week after immunization with sol-PBP5/PBS, blood and spleens were collected from the mice. Serum was obtained from the blood samples by centrifugation and used at different concentrations in ELISA. No significant differences were observed between the two tested sol-PBP5concentrations (5/10 µg/ml) used for surface coating. Absorbance values for the immunized group were: 5597.00, 6800.00, and 6800.00. For the control group, absorbance

values were found: 0.287, 0.152, and 0.112. An independent two-sample t-test was performed to determine whether there was a statistically significant difference in absorbance between the two groups. P-value (two-tailed) was 9.02e-05. The low p-value ($p < 0.05$) indicates a statistically significant difference between the absorbance values of the immunized group and the control group, supporting the conclusion that the immunization with sol-PBP5 was successful.

4.4. The construction of VH library and the cloning of variable heavy (VH) chain library into pComb3-H phagemid

4.4.1. Total RNA extraction and cDNA synthesis

Total RNA was isolated from the spleens of mice confirmed to be immunized with sol-PBP5. The concentration of the isolated total RNA was measured using a Nanodrop spectrophotometer, resulting in a concentration of 780 ng/µl. cDNA synthesis was performed from the total RNA using the SMART method with VH-specific primers and a template switch oligo. The resulting cDNA product was analyzed by agarose gel electrophoresis (Figure 17), and a band of approximately 500-600 bp was observed as expected.

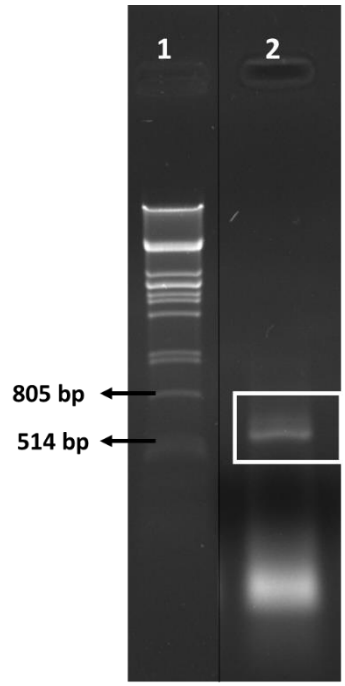


Figure 17. cDNA synthesis from total RNA isolated from spleens of immunized mice. The cDNA synthesized using the SMART method was observed to be approximately 500-600 bp in size.

4.4.2. The amplification of VH

The synthesized cDNA was used as a DNA template to amplify VH fragments. The resulting PCR amplicons were analyzed by agarose gel electrophoresis. The amplicons were observed to be approximately 600 bp in size (Figure 18).

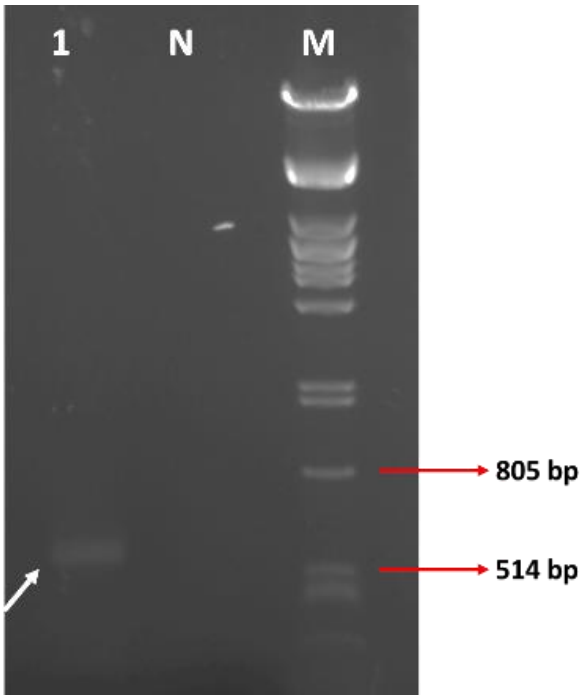


Figure 18. Amplification of VH. A band of approximately 600 bp was obtained from PCR using cDNA synthesized from total RNA isolated from the spleens of mice immunized with sol-PBP5.

4.4.3. The construction of pComb3-hy phagemid

Following digestion of the pComb3 vector with XbaI and NheI enzymes and subsequent ligation, the plasmid was transformed into *E. coli* DH10B cells. Plasmids isolated from colonies grown on selective media were analyzed to determine whether the desired vector was constructed. For this purpose, both the original pComb3 vector and the plasmids presumed to be pComb3-hy were digested with the EcoRI enzyme. The restriction products were analyzed by agarose gel electrophoresis. As expected, digestion of the pComb3 vector resulted in two bands of 3166 bp and 864 bp, while digestion of pComb3-hy produced a single band of 3758 bp (Figure 19). This confirmed the successful construction of the desired pComb3-hy vector. The maps of the pComb3 and pComb3-hy plasmids were given at Figure 20.

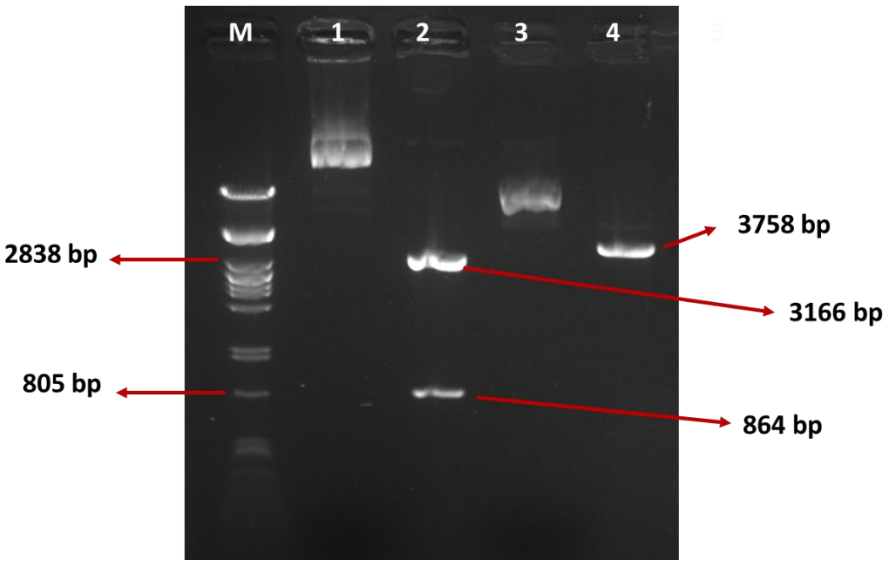


Figure 19. Circular and restriction enzyme-digested forms of pComb3 and pComb3-hy vectors. The pComb3 vector is 4030 bp in length and, in its circular form, produced bands of various sizes. Digestion of pComb3 with EcoRI resulted in two bands of 3166 bp and 864 bp. The pComb3-hy vector is 3758 bp in length and also produced bands of various sizes in its circular form. Digestion of pComb3-hy with EcoRI produced a single band of 3758 bp. This confirmed the successful removal of the target DNA fragment containing the EcoRI site. Lane 1: pComb3 vector, Lane 2: pComb3 vector (EcoRI digested), Lane 3: pComb3-hy vector, Lane 4: pComb3-hy vector (EcoRI digested), Lane M: Lambda-PstI marker.

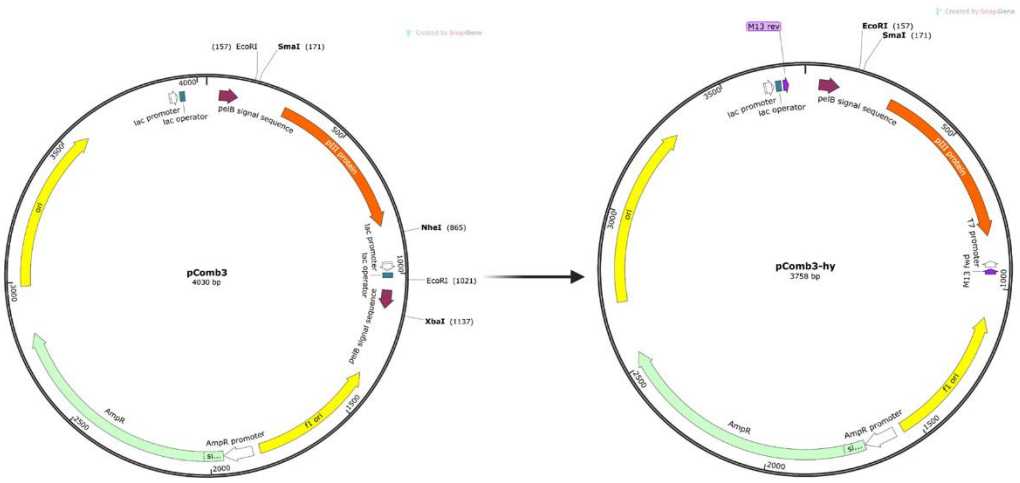


Figure 20. The maps of the pComb3 and pComb3-hy plasmids. The extraction of pelB sequence at the end of M13 GeneIII was shown in the figure. The figures were created using SnapGene Software.

4.4.4. The generation of TA vector from pComb3-hy phagemid and the ligation of VH amplicons and TA vector

The isolated pComb3-hy vector was linearized by digestion with the SmaI enzyme, and its linear form was confirmed by agarose gel electrophoresis. Subsequently, the linearized vector was treated with TdT enzyme and used for ligation with VH amplicons.

4.4.5. Characterization of the VH Library Constructed by Cloning VH Amplicons into the pComb3-hy Vector

Following the transformation of *E. coli* XL1-Blue cells with pComb3-hyΩVH ligands, the cells were plated on selective solid media containing ampicillin and incubated overnight (Figure 21). To assess the insert ratio and the diversity of inserts based on their sizes, colony PCR was performed on the transformants using Fpuc19-IPTG and M13F primers. The PCR products were analyzed by agarose gel electrophoresis (Figure 22). As expected, the PCR with the pComb3-hy vector yielded a band of approximately 1029 bp. The PCR with the transformants resulted in bands ranging from 1500 to 1800 bp. Colony PCR confirmed the presence of inserts in all transformants.

Figure 21. Colonies grown on selective media following the transformation of *E. coli* XL1-Blue competent cells with pComb3-hyΩVH ligands.

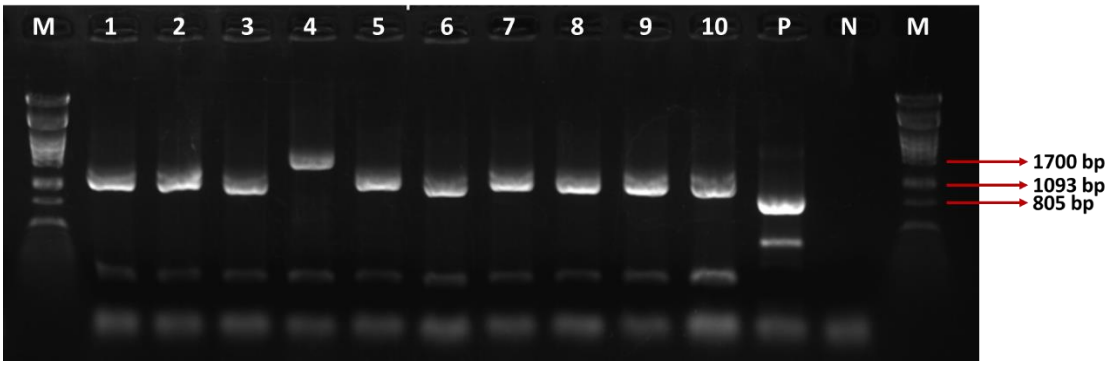


Figure 22. Colony PCR analysis of the pComb3-hyΩVH library transformants. The PCR products were obtained using Fpuc19-IPTG and M13F primers and analyzed by agarose gel electrophoresis. The pComb3-hy vector produced a band of approximately 1029 bp, while transformant bands ranged from 1500 to 1800 bp, indicating successful insert incorporation.

4.5. The phage display of VH library obtained from sol-PBP5 immunized mice

4.5.1. Production of M13K07 helper phage and VH Phage Library

The infectivity of M13K07 phage was confirmed by spot test and selection of bacteria able to grow on kanamycin containing plate. The spot test showed that M13K07 was able to infect XL1-Blue at log phase (Figure 23). Also, the colonies were growing on kanamycin containing plate were stocked and used for further experiments.

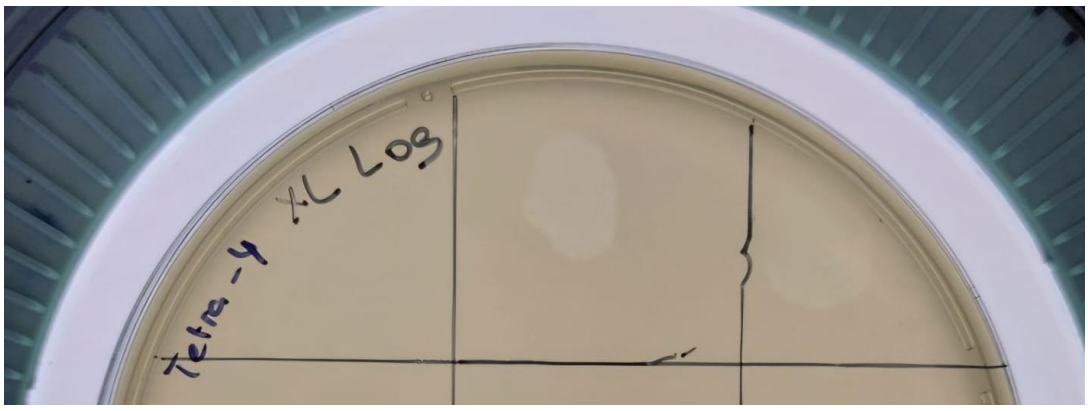


Figure 23. The spot test of M13K07 with *E. coli* XL1-Blue

In order to create VH phage library, the pComb3-hyΩVH carrying XL1-Blue cells were infected by M13K07 helper phages. Then, they were selected on ampicillin, tetracycline and kanamycin containing plates.

4.5.2. Biopanning for the Selection of M13K07 Phages Displaying sol-PBP5 Specific VH Fragments

To select VH fragments with affinity to sol-PBP5, the biopanning method was employed. Initially, the VH phage library was incubated with 6X His-tag in the wells of 96-well plate, and the phages that did not bind to the 6X His-tag were subsequently applied to sol-PBP5. Following several washes, the phages bound to sol-PBP5 were eluted and incubated with XL1-Blue cells. The incubated culture was then plated on agar containing tetracycline and ampicillin. In each biopanning round, the inoculated plates were examined, and it was found that the colony number decreased from 1. round to 3. round (Figure 24). It indicated that selection occurs through biopanning and low affinity phages are discarded at each stage, resulting in the selection of the highest affinity phages in the final round. Thus, during the biopanning process, the diversity of phages decreased, but the specificity of the obtained phages towards sol-PBP5 increased. Growth of bacteria on the agar plates containing ampicillin indicated successful infection by the M13K07 phage carrying the pComb3-hyΩVH plasmid. The grown colonies on ampicillin and tetracycline containing plate were collected and cultured.

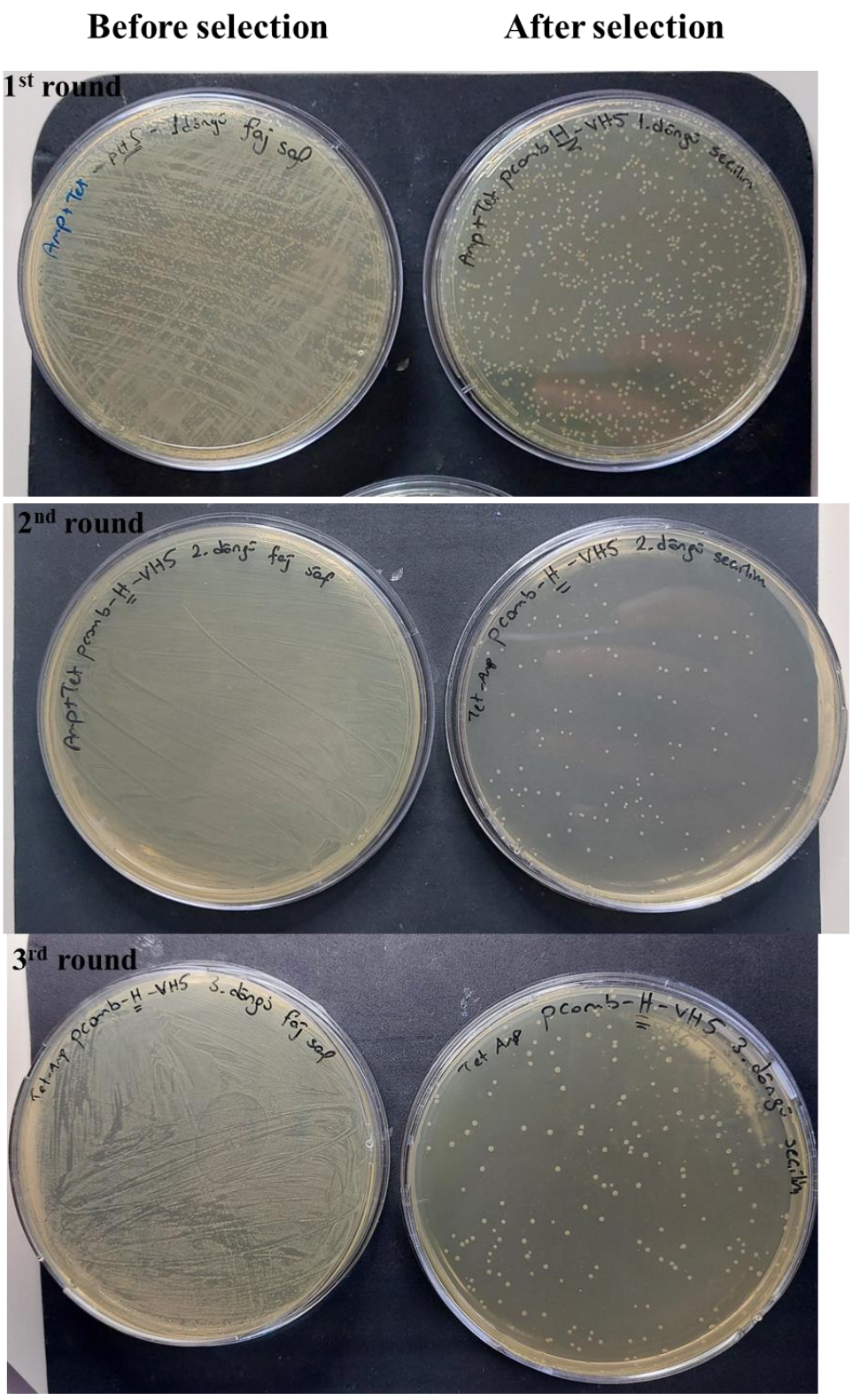


Figure 24. Selection of sol-PBP5 specific VH displaying phage via biopanning. The plates were obtained before and after selection of phages via biopanning. *E. coli* XL1-Blue was infected separately with the precipitated phages and eluted phages from biopanning was plated on selective medium. The colony counts were decreased after biopanning process for each round.

4.5.3. Production of phages showing affinity to sol-PBP5

The randomly selected 4 colonies were isolated and used for phage production. They were named VH43, VH45, VH60 and VH63. The isolated phages displaying recombinant antibiotics in their structures. After production of the phages, to calculate titer they were grown on selective medium and bacterial colonies were counted (Figure 25). The estimated titers of phages were calculated as 10^9, 10^9, 10^8, 10^9 and 10^9 pfu/ml for M13K07, VH43, VH45, VH60 and VH63 phages. The phage titers were equalized for further experiments.

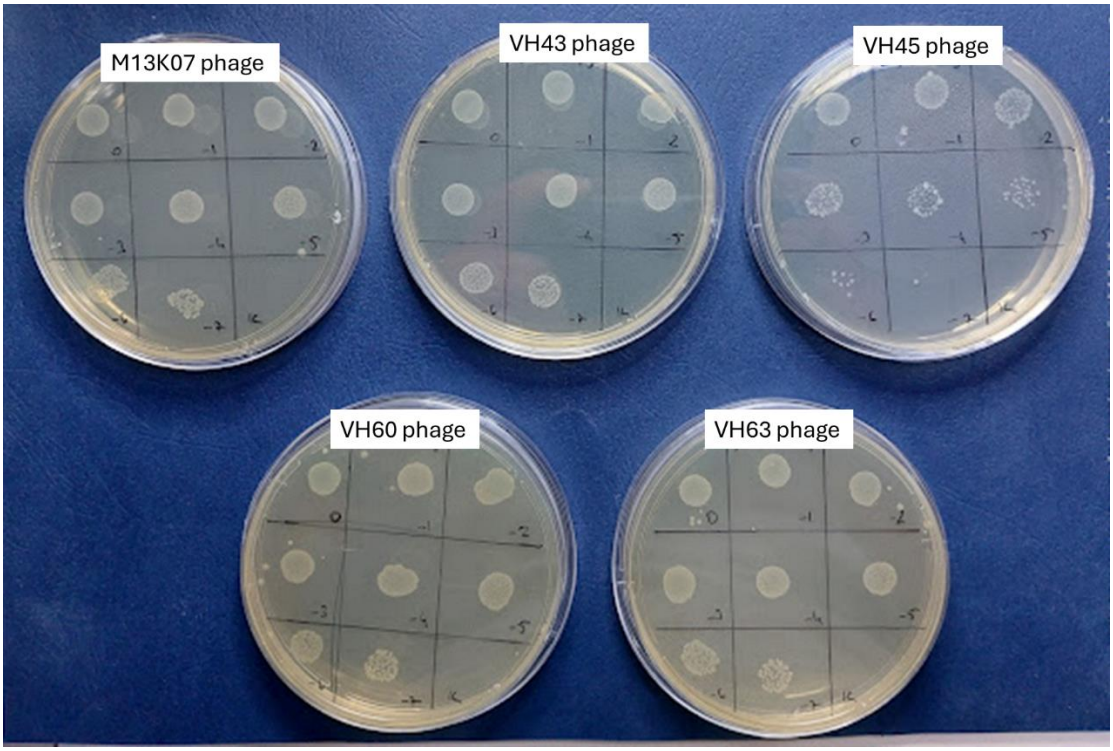


Figure 25. Production of VH43-45-60-63 and M13K07 Phages

4.6. The examination of VH displaying phage affinity to sol-PBP5

4.6.1. The sequencing of the examined four VHs

VHs that are potential recombinant antibiotics were amplified and sequenced. The DNA sequence of the four VHs were examined and the potential ORFs were determined. The predicted VH43, VH45, VH60 and VH63 sizes were 118 aa, 144 aa, 144 aa and 123 aa, respectively. The CDRs and VH domain were predicted with AbRSA tool. It was found that only VH43 has CDRs and indicated its type as VH. No antibody variable domain Sequence was Detected for VH45-60-63. The amino acid sequences of CDR1, CD2 and CDR3 were given at Figure 26. The whole amino acid sequence of VH43 was found as heavy chain region. The locations of CDRs in VH were shown at Figure 27. The VH43 sequence was analyzed with IgBLAST and the results showed the germline V, D, and J gene matches. The V, D, and J segments of VH43 show high sequence similarity with IGHV3-2301, IGHD3-1001 and IGHJ4*02 alleles. The result confirmed the chain type is VH and the VH sequence is productive with V-J in-frame. These results noted that a specific combination of gene segments contributing to the structure of VH43, suggesting a diverse immune response against the target antigen (Table 7).

Summary of CDRs

Name	Type	CDR1	CDR2	CDR3
VH43	VH	GYTFTDYS	INTETGEP	TRAYGYYEMDY

Figure 26. The sequences of CDRs in VH43

```
>VH43

  1 QIQLVQSGPELKKPGETVKISCKSSGYTFTDYSIHWVKQAPGKGLKWMGWINTETGEPAY   60
 61 ADDFKGRFAFSLETSTNTAYLQINNLKNEDTTTYFCTRAYGYYEMDYWGQGTSVTVSS
```

Figure 27. The locations of CDRs in VH43

Table 7. IgGBLAST analysis of VH43. The results showed the V-(D)-J rearrangement of VH43.

Top V gene match	Top D gene match	Top J gene match	Chain type	stop codon	V-J frame	Productive
IGHV9-2-1*01	IGHD6-5*01	IGHJ4*01	VH	No	In-frame	Yes

4.7. The prediction of 3D structure of VH43 and sol-PBP5 and their protein-protein interactions

The structure of VH43, sol-PBP5, and their interactions with each other were predicted with Alphafold. The number of alpha helix and beta strands in VH43 were 12 and 61, respectively. The identified CDRs in VH43 were labelled with red in Figure 28A. The number of alpha helix and beta strand in sol-PBP5 were 231 and 126, respectively. The active site of sol-PBP5 (transpeptidase) was colored with yellow in Figure 28B. The protein-protein interaction of VH43 and sol-PBP5 was modelled. The 7 H bonds between CDRs of VH43 and sol-PBP5 were labelled with green (Figure 28,D). The 4 of 7 hydrogen bonds were between CDR3 of VH43 and sol-PBP5. The bonds were sol-PBP5 THR 632 ↔ VH43 TYR 103, sol-PBP5 LYS 638 ↔ VH43 MET 105, sol-PBP5 ASN 669 ↔ VH43 TYR 100, sol-PBP5 ASP 672 ↔ VH43 GLY 101. None of the 7 hydrogen bonds were at the active site of sol-PBP5 which provides transpeptidase activity.

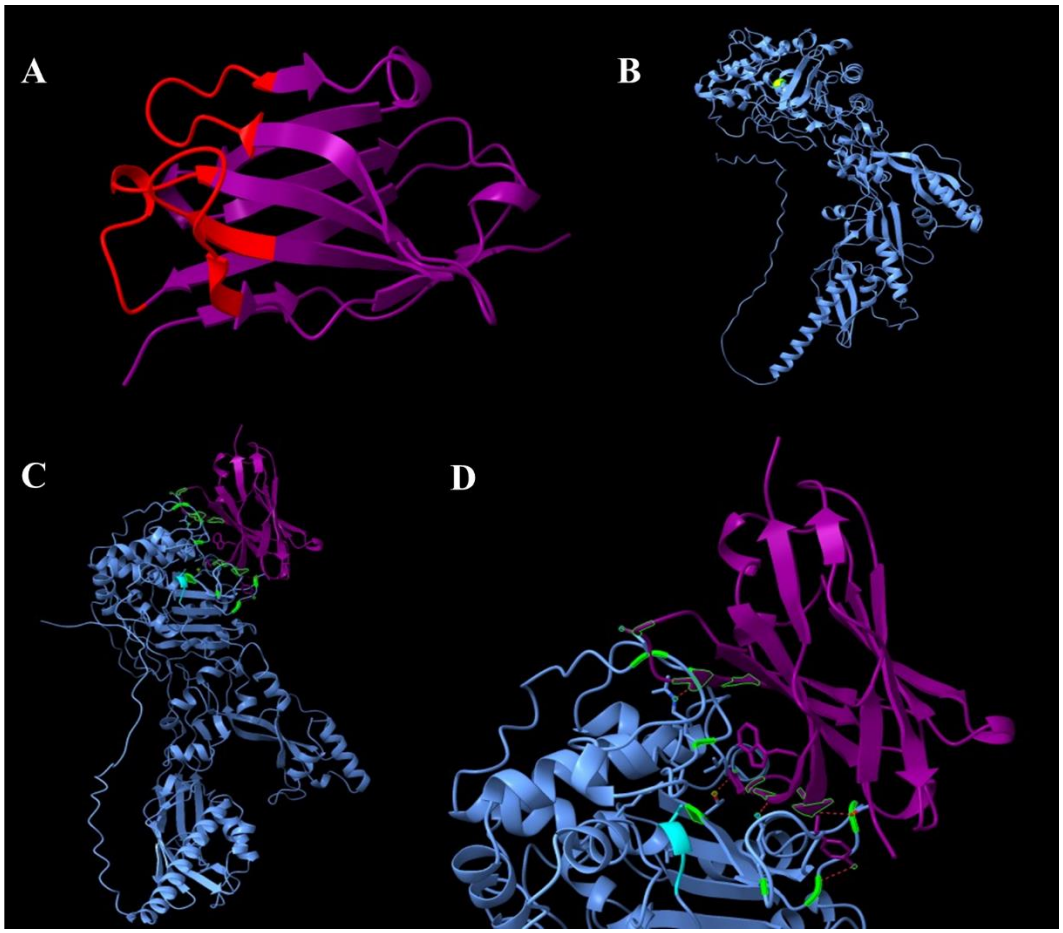


Figure 28. The 3D structures of VH43 and sol-PBP5 proteins and their interactions with each other. A. The 3D structure of VH43 protein was colored with purple and the CDRs were labelled with red. B. The 3D structure of sol-PBP5 protein was colored with blue and the active site was labelled with mint. C. The protein-protein interaction of VH43 and sol-PBP5. The H bonds between them colored with green. D is a zoomed-in view of C, showing detailed structural features.

4.8. The expression and purification of recombinant VH43

4.8.1. The expression of recombinant VH43 protein in *E. coli* BL21 (DE3)

While the molecular weight of VH43 which is recombinant antibiotic is 13.3 kDa, the molecular weight of the VH43 which is recombinant antibiotic with 6X His-tag at N and C terminals was calculated from the sequence result and it was found as 20.3 kDa. The colony carrying VH43 encoding plasmid was cultured and induced with lactose when it reached late-log phase. The samples were taken before induction and after 24 hours induction. The samples were examined with SDS-PAGE analysis. In the two lines, the amount of loaded proteins were

same. There is an increased intensity (or thickening) observed above the 20.3 kDa band on the SDS-PAGE gel (Figure 29).

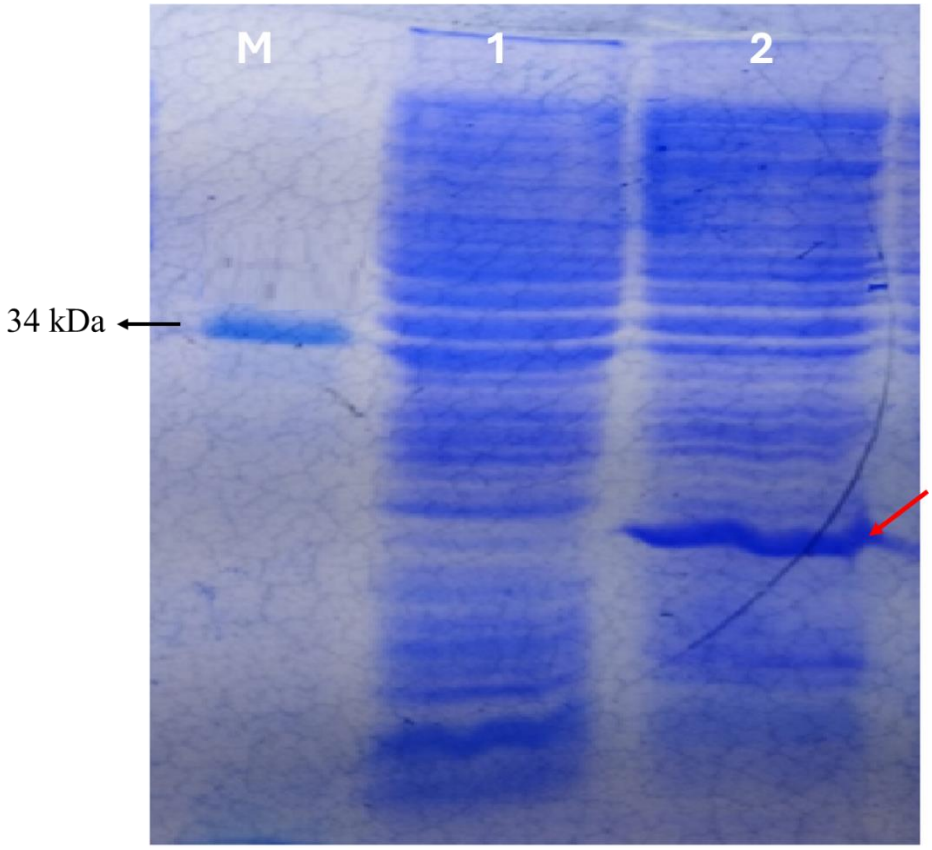


Figure 29. The expression of VH43 in *E. coli* BL21 (DE3). 1: before induction, 2: 24 hours post-induction, M: Marker. When line 1 and line 2 compared, the increased intensity was seen at line 2 above 20.3 kDa. M: 34 kDa recombinant protein.

4.8.2. The purification of recombinant VH43 protein

Recombinant VH43 which is recombinant antibiotic protein with 6XHistag was purified with IMAC. The collected fractions were run on SDS-PAGE (Figure 30). The fractions were cell lysate, flow-through, wash and elution, respectively. All fractions were collected during elution step. It was seen an intense band above 20.3 kDa at cell lysate. The intense band was weakened at flow-through fraction and a small amount of the band with many non-specific bands was seen at wash fraction. At the elution step, 6 fractions were collected, and the purest fraction was the last fraction which had highest imidazole concentration. The imidazole and the other salts were removed by using the desalting column from purest VH43 fraction. The

obtained protein fraction after desalting process was run on SDS-PAGE. The concentration of sol-PBP5 was 0.2 mg/ml and it was used for further experiments.

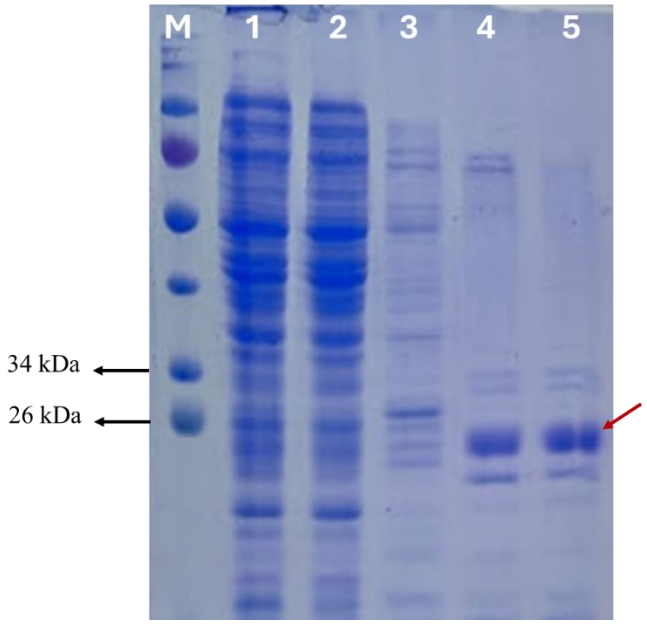


Figure 30. The SDS-PAGE analysis of purification of recombinant VH43. M: Marker, 1: cell lysate of VH43 producing *E. coli* BL21 (DE3), 2: flow-through, 3: wash, 4-5: elution fractions. A slightly intense band near 20 kDa was observed in the cell lysate (Line 1). This band was weakened in the flow-through fraction (Line 2) and showed a small amount with many non-specific bands in the wash fraction (Line 3). During elution, 2 fractions (Line 4-5) were collected for further experiments. Protein Ladder: P7719, NEB.

4.9. Comparative binding studies of VH displaying phages and recombinant VH43

A competitive binding assay using BOCILLIN FL was conducted to measure the binding capacity of VH displaying phages, recombinant VH43 treatment, M13K07 phage, and antibiotics to sol-PBP5. A decrease in fluorescence after treatment with BOCILLIN FL would indicate successful binding of the protein or antibiotic to sol-PBP5. To perform this assay, sol-PBP5 was first exposed to VH displaying phages, recombinant VH43 treatment, M13K07 phage, and antibiotics. Following this exposure, the samples were treated with BOCILLIN FL and analyzed by SDS-PAGE. No decrease in fluorescence was observed when treated with PBS, ceftazidime, or M13K07 phage. In contrast, VH43-45-60-63 displaying phages nearly completely reduced fluorescence levels. Additionally, the recombinant VH43 protein reduced

fluorescence by approximately 70% (Figure 31). After SDS-PAGE was observed under UV, it was stained with Coomassie blue (Figure 32).

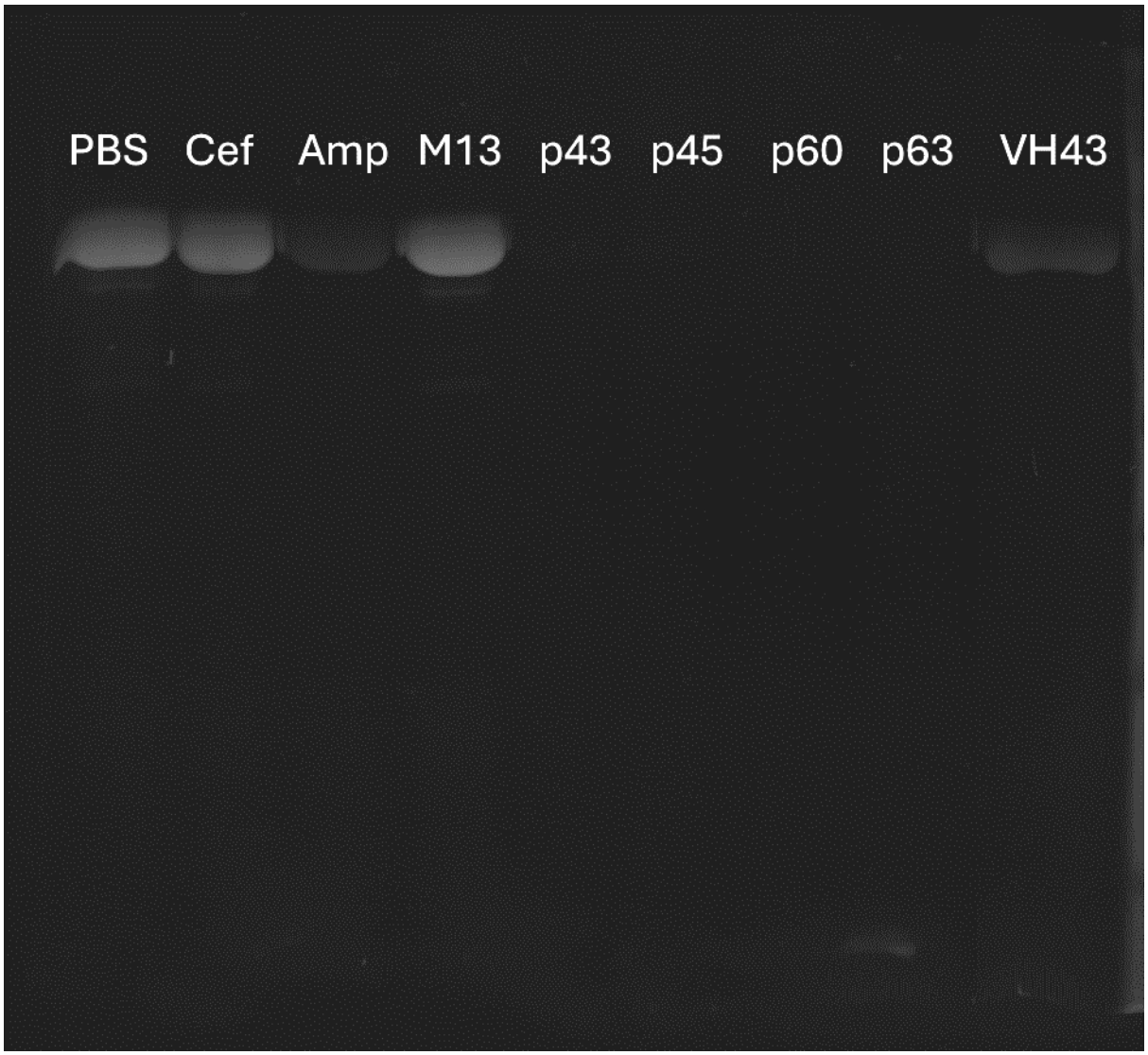


Figure 31. SDS-PAGE analysis showing the binding capacity of various treatments to sol-PBP5. Treatments included PBS, ceftazidime, M13K07 phage, VH43-45-60-63 displaying phages, and recombinant VH43 protein, followed by BOCILLIN FL incubation. The fluorescence intensity remained unchanged with PBS, ceftazidime, or M13K07 phage, indicating no or very low binding. However, the fluorescence was nearly completely diminished by VH43-45-60-63 displaying phages. Recombinant VH43 protein reduced the fluorescence by approximately 70%, demonstrating significant binding.

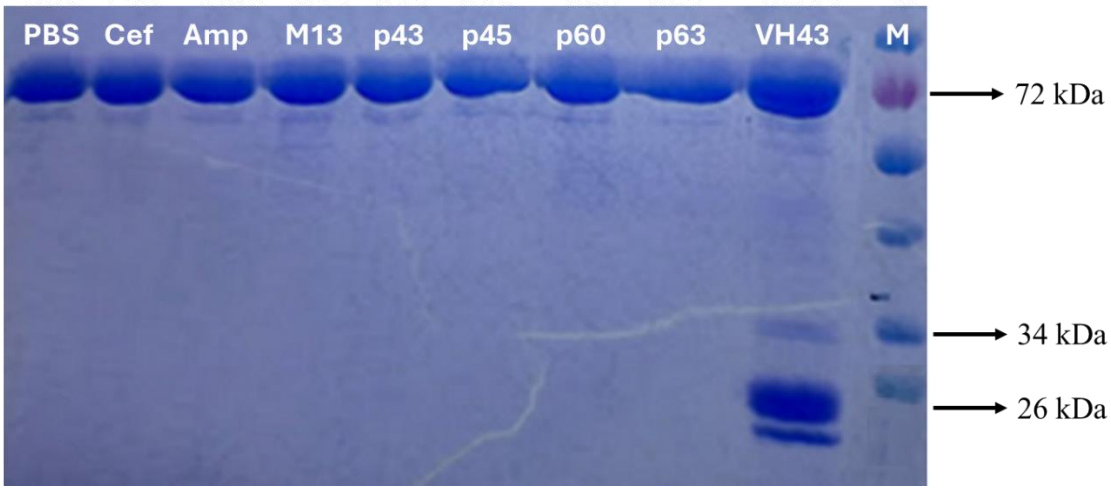


Figure 32. The Coomassie staining of SDS-PAGE from comparative binding assay. It was shown that the amount of PBP5 protein were same for all wells and in VH43 line, the band representing VH43 was seen. Protein Ladder: P7719, NEB.

4.10. Determination of antibacterial activity of the recombinant VH43 with high binding affinity to sol-PBP5

4.10.1. Minimum inhibitory concentration of cephalosporin and VH43 for *E. faecium* HM1070 and clinical E. faecium isolates

The minimum concentrations of ceftazidime and cefazolin for *E. faecium* HM1070 were determined. The MIC values were 4 µg/ml and 0.25 µg/ml for ceftazidime, and cefazolin, respectively. The values were used for bacterial growth curve analysis. There is no antibacterial effect at any concentration of VH43 on *E. faecium* HM1070. The minimum concentrations of ceftazidime for the randomly picked 10 clinical clinical isolates were found as >1024 µg/ml.

4.10.2. Synergistic Effects of Combined Use of Recombinant VH43 with antibiotic on *Enterococcus faecium* HM1070

4.10.2.1. Bacterial Growth Curve Analysis of Ceftazidime/Cefazoline Alone and in Combination with VH43

The growth curves of *E. faecium* HM1070 were analyzed under two conditions: treatment with cefazolin at different concentration ranging from 1024 to 0.003 µg/ml, alone and treatment with a combination of cefazolin and VH43. The bacterial growth was monitored over time using OD600 measurements, the most effective result was obtained with a cefazolin concentration of 0.25 µg/ml. The growth curves for both conditions were plotted (Figure 33). Our results demonstrate that the combination of cefazolin and VH43 significantly inhibits the growth of *E. faecium* HM1070 compared to the treatment with cefazolin alone. Statistical analysis confirmed that this difference is statistically significant (t-value = 2.168, p-value = 0.03585).

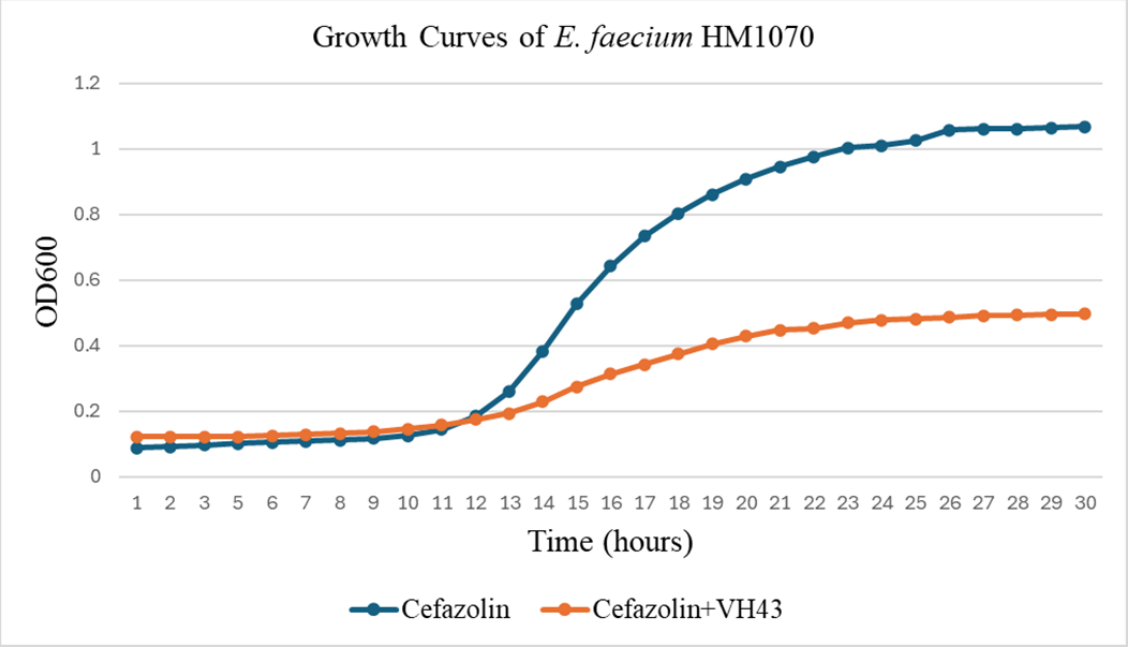


Figure 33. Growth Curve Analysis of *E. faecium* HM1070 treated with cefazolin alone and in combination with VH43. The concentration of cefazolin was 0.25 µg/ml for both treatments.

The same procedures conducted with cefazolin were also performed with ceftazidime, a third-generation cephalosporin, and a growth curve analysis was carried out. the most effective result was obtained with a ceftazidime concentration of 4 µg/ml (Figure 34). The results demonstrate that the combination of ceftazidim and VH43 significantly inhibits the growth of *E. faecium* HM1070 compared to treatment with ceftazidim alone. Statistical analysis confirmed the significance of this difference, with a t-value of 3.93732 and a p-value of 0.00066, indicating a statistically significant enhancement of antibacterial efficacy.

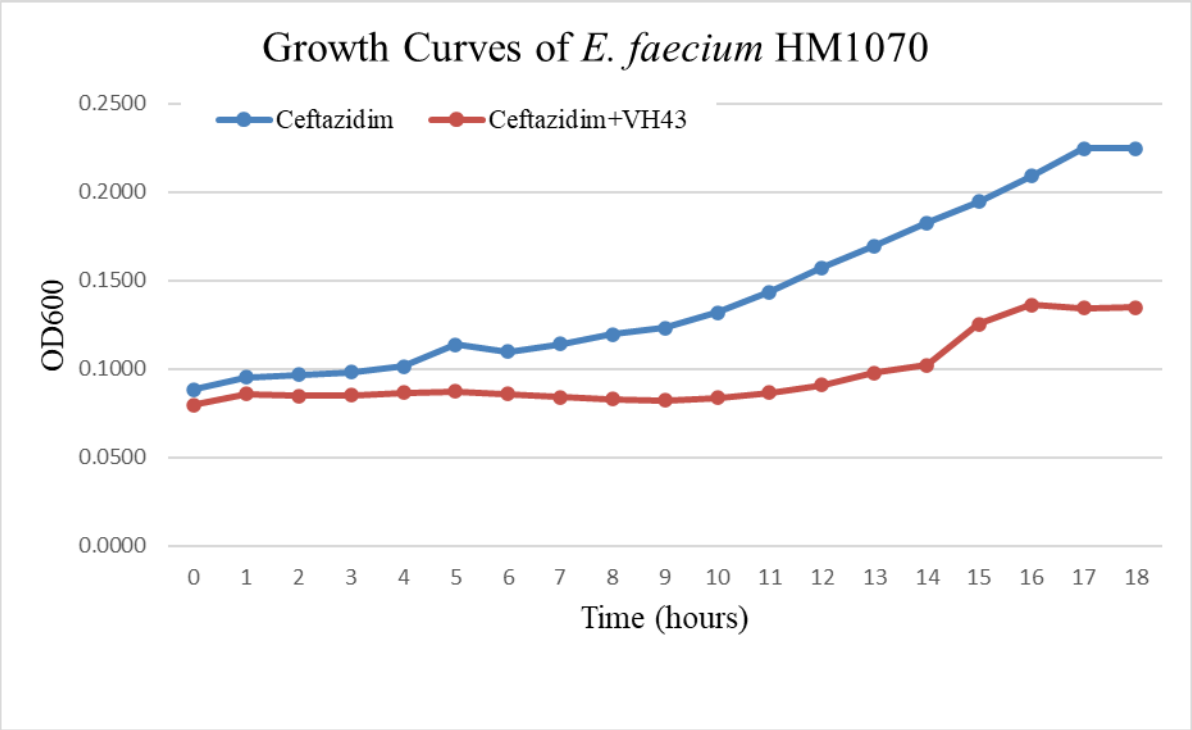


Figure 34. Growth Curve Analysis of *E. faecium* HM1070 Treated with Ceftazidime Alone and in Combination with VH43

4.10.2.2. Kirby-Bauer Disc Diffusion Test/Spot test of Ceftazidime/Cefazoline Alone and in Combination with VH43

Kirby-Bauer test and spot test were performed to observe the antibacterial activity of recombinant VH43. The Kirby-Bauer test was essentially performed in two different ways. In one method, petri dishes containing ceftazidime at concentrations of 2xMIC (8 µg/ml), MIC (4 µg/ml), and 1/2xMIC (2 µg/ml) were prepared. Bacterial growth was observed only on the agar plate containing 2 µg/ml of ceftazidime. While zone formation was observed around the disk

impregnated with recombinant VH43, no zone was observed around the disk impregnated with PBS. Additionally, in the spot test, no bacterial growth was observed in the area where recombinant VH43 was spotted, whereas bacterial growth was observed in the area where PBS was spotted (Figure 35).

In the other method, no antibiotics were used in the agar plate, but they were impregnated onto the antibiotic discs. As shown in Figure 36, the largest zone of inhibition was observed around disk 1. Disk 1 was impregnated with 8 µg of ceftazidime and recombinant VH43, while disk 2 was impregnated with only 8 µg of ceftazidime. The application of only 8 µg of ceftazidime resulted in a weak inhibition zone, while the combination of ceftazidime and VH43 induced a large double zone around the disc (Figure 36). While the inner zone was clear and 2 mm in diameter, the outer zone showed faint growth and was 44 mm in diameter. The application of only 4 µg of ceftazidime (disc 3) and the combination of 4 µg of ceftazidime with VH43 (disc 4) resulted in weak inhibition zones. PBS (disc 5) and VH43 (disc 6) alone had no inhibitory effect on the growth of *E. faecium* HM1070. The results indicate that the combination of recombinant VH43 with ceftazidime at concentrations higher than the MIC has a significant inhibitory effect compared to ceftazidime alone. This confirms the synergistic effect of VH43 and ceftazidime.

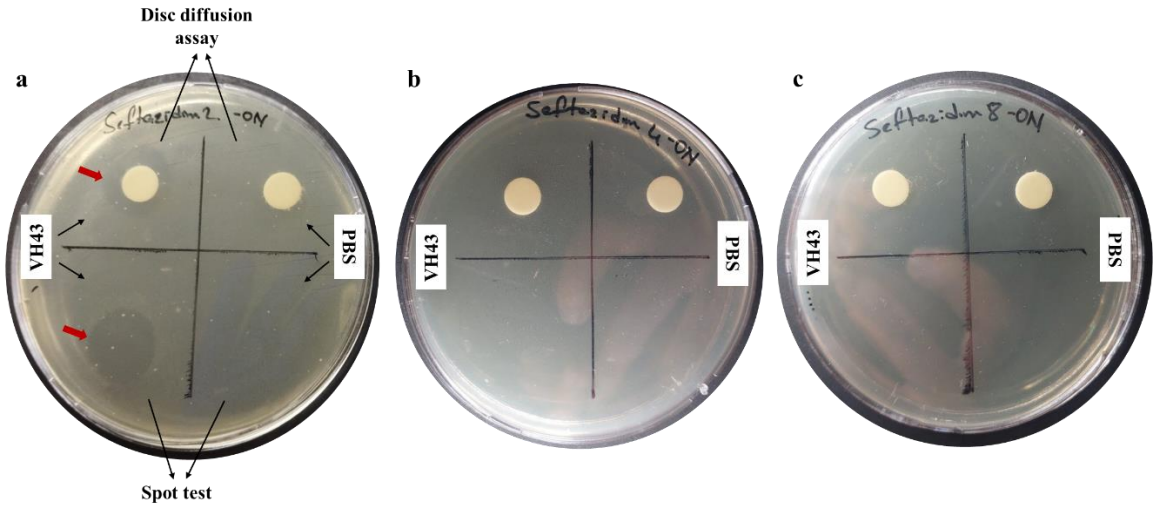


Figure 35. Kirby-Bauer Disc Diffusion Test/Spot test with combination of recombinant VH43 and ceftazidime. a. The agar plate containing 2 µg/ml ceftazidime, b. The agar plate containing 4 µg/ml ceftazidime, c. The agar plate containing 8 µg/ml ceftazidime. Only bacterial growth was seen at a. At the upper sides of agar, inhibition zone was observed around the disc impregnated recombinant VH43, not PBS. At the lower sides of the agar, a clear area was observed where recombinant VH43 was dropped, not PBS.

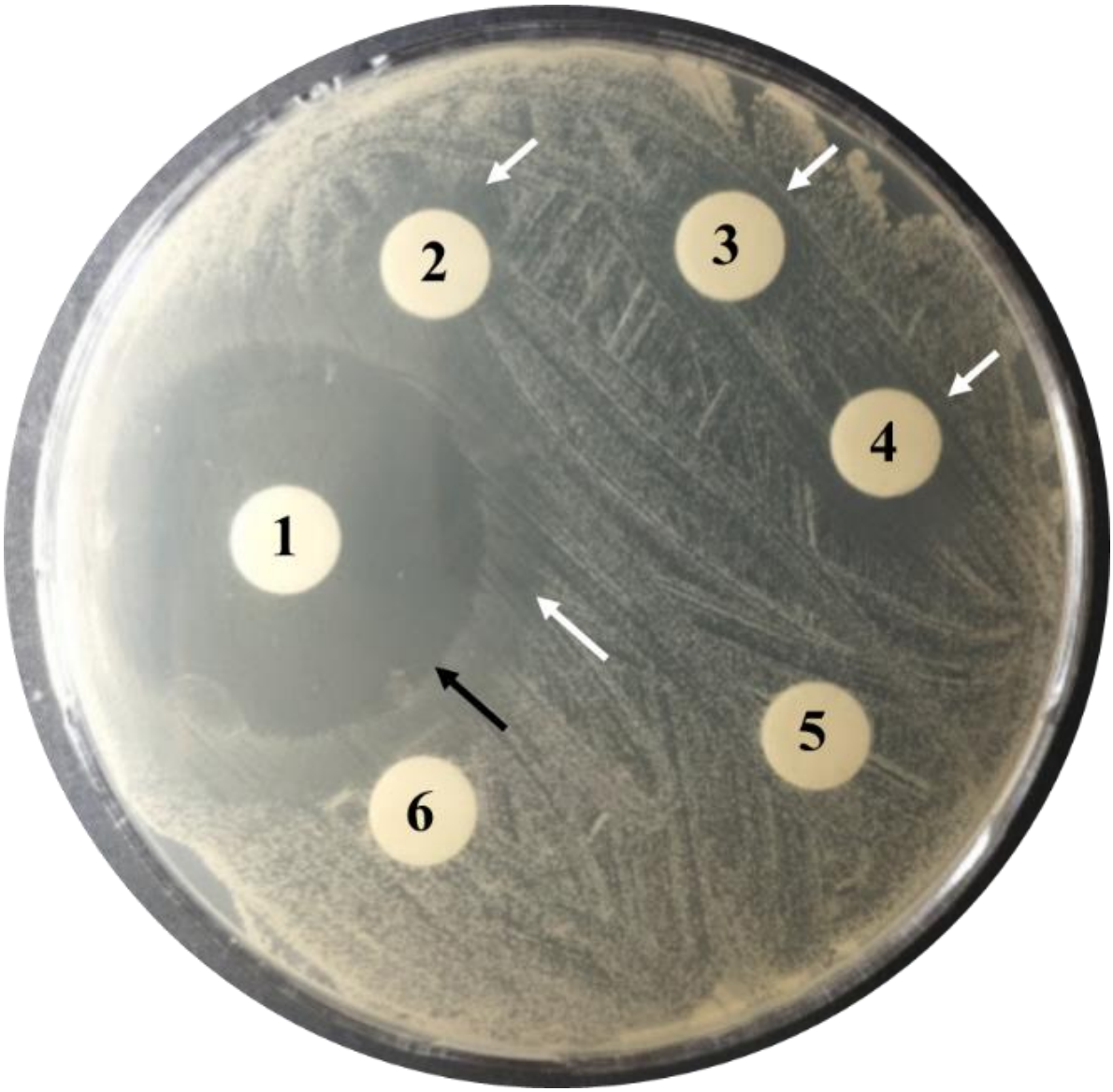


Figure 36. Kirby-Bauer Disc Diffusion Test by antibiotic/VH43 impregnated discs. Disc 1 impregnated with 8 µg of ceftazidime and recombinant VH43, disc 2 impregnated with only 8 µg of ceftazidime, disc 3 impregnated with 4 µg of ceftazidime and recombinant VH43, disc 4 impregnated with 4 µg of ceftazidime, disc 5 impregnated with PBS, disc 6 impregnated with recombinant VH43. While the larger inhibition zone was seen at disc 1, the weaker zones were seen at disc 2,3 and 4.

4.11. Determination of antibacterial activity of the VH for clinical *Enterococcus faecium* isolates

The VH43 did not show antibacterial activity alone and growth curves of the randomly selected 10 clinical *E. faecium* isolates were analyzed under two conditions: treatment with cefazolin alone and treatment with a combination of cefazolin and VH43. The bacterial growth was monitored over time using OD600 measurements and the significance of absorbance values was evaluated statistically with t-test, and no statistically significant difference was detected in any of the pairwise comparisons.

4.12. Cell cytotoxicity assay

To test the cytotoxic effect of VH43, the cell cytotoxicity assay was performed on an immortalized human keratinocytes cell line HaCaT and cervical carcinoma HeLa cell lines with WST-1 agent. The absorbance values were normalized according to PBS treatment group and the normalized value was 100% (Figure 37). The cell viability rates of HaCaT cells were 127.8%, 117.6% and 107.26% for 0.20 µg/ml VH43, 0.10 µg/ml VH43 and 0.05 µg/ml VH43 treatments, respectively. The cell viability rates of HeLa cells were 102%, 100.8% and 103.4% for 0.20 µg/ml VH43, 0.10 µg/ml VH43 and 0.05 µg/ml VH43 treatments, respectively. The cell viability rates equal or above 100% indicate that VH43 at these concentrations did not harm the HaCaT cells and may have even promoted cell growth.

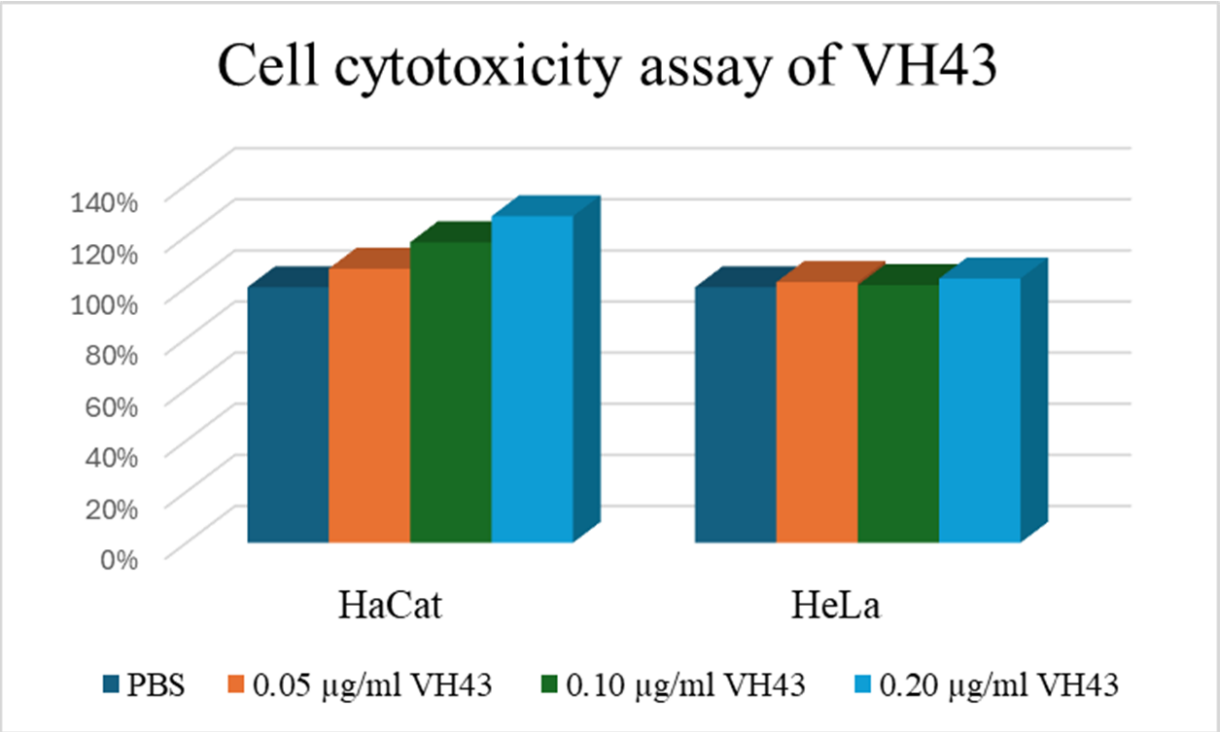


Figure 37. The cell cytotoxicity assay for VH43. The cytotoxic effect of VH43 was tested on HaCat and HeLa cell lines using the WST-1 reagent, and it was shown that VH43 did not exhibit any cytotoxic effect.

5. DISCUSSION

Although *E. faecium* is naturally found in the gastrointestinal tract, it is a bacterium that can cause serious infections in hospital environments. Serious infections such as urinary tract infection, sepsis, and endocarditis are examples of these. Increased antibiotic resistance in *E. faecium* causes traditional antibiotics used to treat the infection to become ineffective. Vancomycin-resistant *Enterococcus faecium* (VRE) strains, specifically, have acquired resistance to widely utilized vancomycin and other beta-lactam antibiotics. Consequently, the range of effective antibiotics available for treating infections is considerably diminished. Infections caused by resistant *E. faecium* are linked to elevated rates of morbidity and mortality. Especially in patients with weakened immune systems, these infections can be fatal. Moreover, *E. faecium* infections that are resistant to treatment frequently necessitate extended periods of therapy and more costly antibiotic protocols. These resistant infections can lead to prolonged hospitalization and escalate the expenses associated with treatment, including additional laboratory examinations. Consequently, this places a significant financial strain on both patients and healthcare systems (Cattoir 2014, Moon 2023).

In this study, it was aimed to develop a recombinant antibiotic that binds to the PBP5 protein responsible for D,D transpeptidation of *Enterococcus faecium*. For this purpose, PBP5 protein was cloned to be obtained in soluble form, produced recombinantly and obtained in high purity. Female CD-1 mice were immunized with sol-PBP5, and serum and spleen were collected from the mice 2 weeks after the last injection. In order to confirm the immunization, ELISA was performed with the plate coated with sol-PBP5. It was proven that there was a statistically significant difference between the control group injected with PBS and the group immunized with sol-PBP5, and the immunization was successful. This result supports studies showing that immunization with recombinant proteins obtained from pathogenic bacteria can elicit strong antibody responses (Nascimento 2012). PBP5 protein is seen as a potential vaccine in vaccines intended to be developed against *Enterococcus faecium*. However, so far its immunogenicity has only been tested in rabbits (Wagner 2023). The intense production of specific antibodies against sol-PBP5 in mice confirmed that it could be considered as a potential vaccine.

In order to obtain VH sequences from spleens obtained from immunized mice, RNA isolation, VH specific cDNA synthesis and VH amplification were performed. In VH

amplification, the SMART method has been used so far, unlike classical methods, to amplify the variable regions of antibodies (Meyer 2019). Determination of the variable region sequence by PCR-based approaches is difficult due to difficulties in designing universal primers that amplify all possible variable region sequences. This problem arises as a result of the inherent low sequence identity in the variable regions themselves as well as in the 5' leader sequence of the antibody light and heavy chains directly upstream of the variable regions (Dübel 1994). There are studies aiming to amplify the 5' region using degenerate primers (Koren 2008). These degenerate primers sometimes result in amplification success rates of only 80–90% due to nonspecific binding or lack of binding; this means that 10–20% of antibody variable regions cannot be amplified by these methods. It is estimated that the SMART method used in this study is suitable for creating a maximum diversity VH library and has a positive contribution to obtaining VH specific to the targeted antigen.

To obtain stable phagemid vector, pComb3-hy vector was constructed by using pComb3 vector. Even though pComb3 vector has some advantages such as high-copy number, the instability of pComb3 causing deletions in the cloned fragments is an important problem (Barbas 1991). The one of the two PelB sequences was removed from the phagemid. The constructed new pComb3-hy vector was used to clone VH sequences and form VH displaying phage library. The phages are binding to sol-PBP5 were selected and obtained via biopanning. Various VH libraries derived from different species including mice, humans, camels, and rabbits have been utilized to identify VH binding to a range of antigens. In the study, VHs generation of a phage-display library of single-domain antibody was performed against *Chlamydomonas reinhardtii* antigens. Unlike this study, Variable Heavy-chain only (VHH) antibody which is camelid antibody was produced in the study and the specific binding of the produced VHs to *C. reinhardtii* was proved (Jiang 2013). In a study which aimed to discover the VHs prevent the binding of SARS-COV-2 to ACE2 receptor and neutralize of SARS-COV-2, used synthetic human VH-phage library (Bracken 2021). The single domain antibodies also used to discover binders against tumor cells, and it was predicted that the obtained VHs could be used as immunotherapy agents. The VH single domains were produced via rabbit VH phage display method (Feng 2020).

The use of the VH phage display method to develop anti-infective strategies against infectious agents has gained great popularity in recent years and many studies have been conducted in this field. Phage display method has been used in the production of VH and other

antibody fragments against viral agents such as HIV-1, SARS-CoV, SARS-CoV-2 and Respiratory Syncytial Virus (RSV) for diagnosis and treatment purposes (Mustafa 2024).

A wide spectrum of recombinant antibodies or antibody fragments, from gram-negative to gram-positive, have been developed for use in diagnosis or treatment. For instance, phage libraries displaying antibodies from humans, mice, and llamas have been created against Bacillus anthracis, from humans and llamas against Clostridium difficile, and from humans against Salmonella Typhimurium (Roth 2021). So far, a VH-displaying phage library has been constructed against *Enterococcus faecium*. As far as I know, this is the first study to obtain *E. faecium* specific antibody/antibody fragments.

The phages carrying VHs which are binding to sol-PBP5 were selected through biopanning and the VHs were sequenced. The sequence analysis of VH43 was performed via IgBLAST. These results noted that a specific combination of gene segments contributing to the structure of VH43, suggesting a diverse immune response against the target antigen. The 3D structure of VH43 showed it has been 12 alpha helix and 61 beta strands. The abundance of beta strands in VH structure is consistent with the overall architecture and stability requirements of immunoglobulin domains. This fold provides a stable framework that allows for significant variability in the antigen-binding site, enabling antibodies to recognize a vast array of antigens (Chiu 2019). The modelling of protein-protein interaction between PBP5 and VH43 showed that none of the 7 hydrogen bonds were at the active site of sol-PBP5 which provides transpeptidase activity. Thus, the inhibition of transpeptidation can be limited although the VH43 bind to PBP5.

Very comprehensive results were obtained that support the experimental findings from the laboratory by modeling the protein-protein interaction between proteins VH43 and sol-PBP5. This indicates that utilizing the computational tool for designing artificial VH and testing its interaction with the target antigen could serve as a viable alternative. (Hummer 2022). From a different point of view, the sequencing of all VHs in the phage library can enable the characterization of all possible VHs which bound to sol-PBP5 in this case. In this way, VHs with a sequence of natural origin from the immune system of an organism but artificially synthesized can be obtained. Thus, selection can be carried out in a wet lab without the need for surface display techniques.

The comparative binding assay of VH displaying phages and VH43 indicates that they reduced binding of BOCILLIN FL. Even though both VH43 and VH displaying phages binds

to PBP5, while VH displaying phages completely prevent the binding of BOCILLIN FL, VH43 partially blocks it. It can be explained by the fact that VH displaying phages are larger in volume compared to VH43 and that they better prevent the binding of BOCILLIN by creating steric hindrance (De Michele 2016).

VH43 showed an antibacterial effect on *E. faecium* HM1070 when combined with ceftazidime/cefazoline. However, VH43 did not show an antibacterial effect on *E. faecium* HM1070 or clinical *E. faecium* strains when applied alone. This is because there are bi-functional PBPs that can perform transpeptidation in *E. faecium*, other than PBP5, which carries out transpeptidation. These proteins are class A PBPs. As a result, even though VH43 bound to PBP5 and inhibited transpeptidation, the bacteria survived because other PBPs continued the synthesis of peptidoglycan (Rice 2009). PBPs except PBP5 of *E. faecium* have affinity to cephalosporin. For this reason, the combined use of cephalosporin and VH43 recombinant antibiotic was predicted to have an antibacterial effect, and combined use and use of cephalosporin alone were tested for *E. faecium* strain. First of all, MIC values of ceftazidime and cefazolin for *E. faecium* were determined. Then, the combined use of VH43 and antibiotic at MIC value was exposed. Our study investigated the effects of combining a recombinant protein with an antibiotic on bacterial growth inhibition. The results from both the bacterial growth analysis and Kirby-Bauer disk diffusion test strongly indicate a synergistic effect between the recombinant protein and the antibiotic. In the bacterial growth analysis, the antibiotic alone exhibited only a weak inhibitory effect, as reflected by the relatively high absorbance values. However, when combined with the recombinant protein, there was a significant reduction in absorbance, indicating a marked inhibition of bacterial growth. This suggests that the recombinant protein enhances the antibacterial efficacy of the antibiotic. Furthermore, the Kirby-Bauer disk diffusion test supported these findings. The disk impregnated with the antibiotic alone produced a small inhibition zone, demonstrating limited antibacterial activity. In contrast, the disk impregnated with both the antibiotic and the recombinant protein produced a significantly larger inhibition zone. This substantial increase in the inhibition zone diameter indicates a strong synergistic interaction between the two agents, resulting in enhanced antibacterial activity. The synergistic effect observed can be attributed to the complementary mechanisms of action of the antibiotic and the recombinant protein. While the antibiotic targets PBPs except PBP5, the recombinant protein specifically targets PBP5, it is leading to an increased overall antibacterial effect. These findings align with previous studies that have shown similar synergistic interactions between antimicrobial peptides and

conventional antibiotics (Mhlongo 2023). This synergistic combination has significant implications for the treatment of bacterial infections, particularly those involving antibiotic-resistant strains. By enhancing the efficacy of existing antibiotics, such combinations could help overcome resistance and reduce the required dosage of antibiotics, thereby minimizing potential side effects. In conclusion, the combination of the recombinant protein with the antibiotic demonstrates a potent synergistic effect, significantly enhancing bacterial growth inhibition compared to the antibiotic alone. Further research into the mechanisms underlying this synergy and its potential applications in clinical settings is warranted. Since VH43 is not effective alone, it must be used together with a cephalosporin if used in treatment. Similarly, the Efungumab antibody combined with amphotericin B has been used successfully in the treatment of invasive candidiasis (Karwa 2009).

Although polyclonal antibodies against *E. faecium* are commercially available, there are limited studies on the development of antibodies or antibody fragments against it. Rakita et al. obtained polyclonal antibodies against *E. faecium* by immunizing rabbits. They collected the sera of the rabbits and purified the IgGs in the sera. They generated $F(ab')_2$ and Fab fragments from the purified IgGs. They showed that the generated *E. faecium* specific IgGs, $F(ab')_2$ and Fab fragments support opsonization and PMN-mediated killing against *Enterococcus faecium*. However, none of the antibody/antibody fragments were monoclonal. Thus, amino acid sequences of them are unknown. Also, it was indicated that the generated antibodies potentially directed against bacterial carbohydrate (Rakita 2000). In a more recent study, opsonic B-cell clones from healthy human B cells were selected and 2 VH and 1 VL were amplified with PCR. Then, cloned into a mammalian expression vector which enables production of fully human IgG1 monoclonal antibody and the potential use of the antibodies the treatment of *E. faecium* infection. They were indicated the target of antibodies are unknown, but the target was predicted as polysaccharide. The VH and VL sequences were not released in publicly available databases (Rossmann 2015). Different from this thesis, both two studies did not construct VH library, directly not use domain antibody such as VH and the sequence of the antibody was not reported. VH43 is the first antibody/antibody fragment that shows binding affinity to *E. faecium* and for which sequence has been reported. Moreover, *E. faecium* has been shown to bind to the PBP5 protein.

Phage display technology is a powerful tool used to develop peptides and antibodies with high specificity for bacterial pathogens. These molecules can be employed in diagnostic assays to accurately identify bacterial infections, thanks to their ability to bind selectively to

unique bacterial proteins. This approach enhances the precision and speed of diagnostics, making it an invaluable method for detecting bacterial infectious diseases (Zhao 2023). The VH43 protein developed in this study has the potential to be used in the diagnosis of bacterial infectious diseases, specifically targeting *Enterococcus faecium*. This potential arises from its unique specificity for the PBP5 protein, which is found exclusively in *E. faecium*. The ability of VH43 to selectively bind to PBP5 suggests its promising application in diagnostic procedures, enhancing the detection and identification of *E. faecium* infections.

There are numerous antimicrobial peptides being researched for their use against various bacteria. While MRSA is the most targeted bacterium, there are also extensive studies against *E. faecium*. These antimicrobial proteins have various origins. These antimicrobial peptides can be part of the innate immune system produced by different organisms. Various Proline-rich antimicrobial peptides (PrAMPs) produced by cetacean species have been reported to exhibit antimicrobial activity against *E. faecium*. Among these, Del1 demonstrates antibacterial effects by causing membrane permeabilization. However, due to this property, it has cytotoxic effects on mammalian cells, particularly causing hemolytic activity. On the other hand, Bal1 and Lip1 inhibit protein synthesis and have no reported hemolytic effect (Sola 2020). Antimicrobial peptides (AMPs) are generally not species-specific, as illustrated by the examples that are not specific to *E. faecium*. AMPs are typically cationic peptides, which allows them to interact with bacterial membranes. This interaction is a primary reason for their cytotoxic effects (Inui Kishi 2018). To eliminate the undesired side effects and weaknesses of antimicrobial peptides, synthetic novel AMPs are produced based on natural antimicrobial peptide sequences (Ringstad 2010). Although it has been observed that chemical modifications, such as lipidation or aromatic acid addition to AMPs, can increase antimicrobial activity in novel AMPs, these modifications can also lead to increased cytotoxic effects (Kamysz 2020). Bellavita et al. reviewed the literature and reported that peptide cyclization, N-methylation, PEGylation, glycosylation, and lipidation are used to extend the half-lives of AMPs and make them resistant to serum proteases (Bellavita 2023). Due to their general membrane-targeting nature, AMPs often exhibit cytotoxicity; however, VH43, which does not show cytotoxic effects, is comparatively safer. Plectasin, which is a defensin, showed antimicrobial effect against *E. faecium* and was produced recombinantly (Chen 2015). Additionally, some strategies were developed to deliver antimicrobial peptides. It was reported that the probiotic organism Lactococcus lactis was used to deliver enterocin to *E. faecium* (Geldart). In conclusion, the antimicrobial proteins being developed against *E. faecium* are generally AMPs, and due to their

high likelihood of being cytotoxic, various strategies are employed to mitigate this. VH43, developed in this study, is preferable due to its antibacterial activity and lack of cytotoxicity. However, its ineffectiveness against clinical strains with high cephalosporin levels indicates that further research and new strategies are needed.

6. CONCLUSION and SUGGESTIONS

In this study, which aimed to develop a recombinant antibiotic in antibody fragment structure against the PBP5 protein of *E. faecium*, the sol-PBP5 protein was produced recombinantly and a VH displaying phage library was created against PBP5. VH, contained in one of the phages obtained by selection from this library, was sequenced and the amino acid sequence of VH43, the 3D structure of the protein and its interaction with the PBP5 protein were determined by computational analysis. As a result of the analysis, it was determined that VH43 had the desired structure and its interaction with PBP5 was determined by modeling. It was demonstrated that VH43 has the potential to be used as a recombinant antibiotic by inhibiting the growth of *E. faecium* when used with cefazolin/ceftazidime.

Suggestions:

- New VH with higher binding affinity or that can bind to the active site of PBP5 can be obtained by random mutations in the gene encoding VH43,
- In bacterial growth analysis, application of VH43 at higher concentrations and/or more frequent doses may be tried.
- To achieve recombinant antibiotic which is effective against clinical *E. faecium* isolates with high cephalosporin resistance, the genetic determinants of PBP5 can be investigated by sequencing and used to improve new strategies.

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APPENDIX

APPENDIX 1

ADU Local Ethics Committee for Animal Experiments (HADYEK)

T.C.
AYDIN ADNAN MENDERES ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(AYDIN ADÜ-HADYEK)

Aydın 22/06/2023

Oturum : Hayvan Deneyleri Yerel Etik Kurulu 2023 Yılı VI. Oturum

Sayı : 64583101/2023/82

Proje Başlığı : *Enterococcus faecium*'a karşı rekombinant antibiyotik geliştirilmesi

Proje Yürütücüsü : Bülent BOZDOĞAN

Proje Ekibi : Hanife SALİH DOĞAN

	Bu çalışmanın hiçbir bölümünde
	İnsan embriyosu ve fötüsü kullanılması
	İnsan embriyosu ve fötüsü dokularının kullanılması
	Diğer insan doku ve hücrelerinin kullanılması
Hayvan Çalışması	İnsanlarda araştırma
	İnsan olmayan primatların kullanılması
	Transgenik hayvanların kullanılması
	Hayvanlarda genetik modifikasyon öngörülmemiştir.

Bu çalışmanın yapılmasında etik açıdan bir sakınca bulunmamaktadır.

Prof. Dr. Musa SARIERLER, Başkan
Prof. Dr. M. Dinçer BİLGİN, Başkan Yardımcısı
Prof. Dr. Nurhan DOST, Üye
Prof. Dr. Işıl SÖNMEZ, Üye
Prof. Dr. Serkan BAKIRCI, Üye
Dr. Öğr. Üyesi Solmaz KARAARSLAN, Üye(İzinli)
Dr. Öğr. Üyesi A. Önder ÜSTÜNDAĞ, Üye
Dr. Öğr. Üyesi Aysun KOÇ GÜLTEKİN, Üye
Dr.Öğr.Üyesi Aşide Gülce ORYAŞIN, Sor. Vet. Hek. Üye
Hidayet ADMAN, Serbest Vet. Hek. Üye
Öğr. Gör. Dr. Mehlem ÖZTÜRK AYDIN, Sor. Vet. Hek. Üye
Şenay TEKİNBAŞ HAYTAP, Üye

Bu rapor, sadece Adnan Menderes Üniversitesi'nde yapılacak çalışmalar için geçerlidir.

T.C.

AYDIN ADNAN MENDERES ÜNİVERSİTESİ

SAĞLIK BİLİMLERİ ENSTİTÜSÜ

BİLİMSEL ETİK BEYANI

"Development of Recombinant Antibiotics Against *Enterococcus faecium*" başlıklı Doktora tezimdeki bütün bilgileri etik davranış ve akademik kurallar çerçevesinde elde ettiğimi, tez yazım kurallarına uygun olarak hazırlanan bu çalışmada, bana ait olmayan her türlü ifade ve bilginin kaynağına eksiz atıf yaptığımı bildiririm. İfade ettiklerimin aksi ortaya çıktığında ise her türlü yasal sonucu kabul ettiğimi beyan ederim.

Hanife SALİH DOĞAN

/ /

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EDUCATION

2018: Graduate in Molecular Biology and Genetics, Izmir Institute of Technology, Turkey

2020: MSc in Molecular Biotechnology Department, Aydın Adnan Menderes University, Turkey

2024: PhD candidate in Molecular Biotechnology Department, Aydın Adnan Menderes University, Turkey

FELLOWSHIPS AND AWARDS

- 218S464, The phenotypic and genotypic characterization of efficient bacteriophages that are isolated in our laboratory against Pseudomonas Aeruginosa strains isolated from diabetic foot infections, TÜBİTAK 1001-Research, Executive, Scholar
- YOK 100/2000 fellowship program, Council of Higher Education (CoHE), 2020-ongoing
- 2211/A National PhD Scholarship Program, The Scientific and Technological Research Council of Turkey, 2021-ongoing
- Congress Attendance Grant-10th FEMS Congress of European Microbiologists, Federation of European Microbiological Societies, 2023

PUBLICATIONS

- Salih, H., Oryaşın, E., & Bozdoğan, B. (2020). pADU94, a Cloning and Expression Vector with Chloramphenicol Resistance Marker, Turk Mikrobiyol Cemiy Derg, 50(1), 027-034.

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- Karaynir, A., Salih, H., Bozdoğan, B., Güçlü, Ö., & Keskin, D. (2022). Isolation and Characterization of Brochothrix phage ADU4. Virus Research, 198902.

- Ali, S., Karaynir, A., Salih, H., Öncü, S., & Bozdoğan, B. (2023). Characterization, genome analysis and antibiofilm efficacy of lytic Proteus phages RP6 and RP7 isolated from university hospital sewage. Virus research, 326, 199049.

- Karaynir, A., Bozdoğan, B., & Salih Doğan, H. (2023). Environmental DNA transformation resulted in an active phage in *Escherichia coli*. Plos one, 18(10), e0292933.

- Salih Doğan, H., Oryaşın, E., & Bozdoğan, B. (2024). K fragment as a polymerase chain reaction-based vector for antibiotic resistance gene hunting. GPD null, 0(0), 1674. https://doi.org/10.36922/gpd.1674

- Salih Doğan, H., Karaynir, A., Yilmaz, Ü. İ., Başgöz, B. B., Hoşbul, T., & Bozdoğan, B. (2024). Two novel phages, Klebsiella phage GADU21 and Escherichia phage GADU22, from the urine samples of patients with urinary tract infection. Virus Genes, 1-14.

- Uskudar Guclu, A., Unlu, S., Salih Doğan, H., Yalcin, S., & Basustaoglu, A. (2024). Biological and Genomic Characteristics of Three Novel Bacteriophages and a Phage-Plasmid of Klebsiella pneumoniae. Canadian Journal of Microbiology, (ja).

PROJECTS

- TPF-19052, pRTA, a new vector and TA cloning: A method for characterization of novel antibiotic resistance genes, BAP (Aydın Adnan Menderes University)-MSc thesis project, 2020 (Researcher)

- 119Z640, Resistance Gene Hunting with Marker-free Plasmid Amplicon, TÜBİTAK 1002, 2019-2020 (Reseacher)

- 218S464, The phenotypic and genotypic characterization of efficient bacteriophages that are isolated in our laboratory against Pseudomonas Aeruginosa strains isolated from diabetic foot infections, TÜBİTAK 1001, 2019-2022 (Scholar, Researcher)

- TPF-20027, Development of DNA and RNA Vaccines for COVID-19, BAP (Aydın Adnan Menderes University)-Research project, 2020-2023 (Researcher)

- REDPROM-22001, Development of rapid diagnostic test to use in respiratory tract infections, BAP (Aydın Adnan Menderes University)-Research project, 2022-ongoing (Researcher)

- TPF-22024, Development of recombinant antibiotics against *Enterococcus faecium*, BAP (Aydın Adnan Menderes University)-PhD thesis project, 2022-ongoing (Researcher)

- KA22/25, Isolation and characterization of bacteriophage effective against Klebsiella pneumoniae isolates showing clinical ESBL and carbapenemase activity from water sources, BAP (Başkent University), Research project, 2022-2024 (Researcher)

CONFERENCE PAPER

- Salih, H., Oryaşın, E., Bozdoğan, B. (2023). K fragment, a PCR-based vector for antibiotic resistance gene hunting. 10th FEMS Congress of European Microbiologists.

- Dastan, N., Salih, H., Kurt, İ., Sakarya S. (2023). Effects of hypochlorous acid on bronchial epithelium, in vitro study. XXII. Turkish Clinical Microbiology and Infectious Diseases Congress

- Salih Doğan, H., Orbayoğlu, G. A., Doğan, Y., Karaynir, A., Bozdoğan, B. Phage-encoded novel DP21 enzyme, a polysaccharide depolymerase, prevents and destruct biofilms of various species. ECCMID 2024 (34th European Congress of Clinical Microbiology and Infectious Diseases).

- Karaynir, A., Ali, S., Salih Doğan, H., Nachimuthu, R., Bozdoğan, B. Degradation of preformed Gram-positive and Gram-negative bacteria biofilms using disintegrated and intact Phages. ECCMID 2024 (34th European Congress of Clinical Microbiology and Infectious Diseases).

- Ozkan, R. G., Salih Doğan, H., Karaynir, A., Zafer, E., Bozdoğan, B., Basbulbul, G. Vaginal microbiome and phageome associated with bacterial vaginosis (BV). ECCMID 2024 (34th European Congress of Clinical Microbiology and Infectious Diseases).

- Uskudar Guclu, A., Unlu, S., Salih, H., Yalcin, S., Basustaoglu, A. Whole genome sequences revealed a novel phage-plasmid infecting Klebsiella pneumoniae. ECCMID 2024 (34th European Congress of Clinical Microbiology and Infectious Diseases).

- Uskudar Guclu, A., Unlu, S., Salih, H., Yalcin, S., Basustaoglu, A. A novel Klebsiella pneumoniae bacteriophage kpn17 with depolymerase activity: it's efficacy on biofilm formation. ECCMID 2024 (34th European Congress of Clinical Microbiology and Infectious Diseases).

CERTIFICATES

- NGC Chromatography Systems and Chromlab Software Therotical and Hands-on Application trainings. 26-29 July 2021. Aydın Adnan Menderes University

- Advanced Bioinformatics Training developed for the GPS and JUNO projects. 29th Jun-2nd Feb 2024. Hacettepe University-Wellcome Sanger Institute.

- Experimental Animal Use Certificate Training Course, 4-13 April 2023. Eskişehir Osmangazi University Animal Experiments Local Ethics Committee.

- Occupational Health and Safety Basic Training Program, 2018. Aydın Adnan Menderes University.

WORKSHOP INSTRUCTOR

- 4th Gene Cloning Course, Aydın Adnan Menderes University, 13-17 May 2019, Educator

- 1st Bacteriophage Course, Aydın Adnan Menderes University, 20-23 March 2023, Educator

MEMBERSHIPS

- ESCMID (The European Society of Clinical Microbiology and Infectious Diseases)-Young Scientist Member of ESCMID
- Turkish Society for Microbiology
- Turkish Society for Microbiology, Young Scientists Commission (TMC-GençBil)
- Turkish Society for Microbiology, Bacteriophage Study Group