VILNIUS UNIVERSITY

LIFE SCIENCES CENTRE

GABIJA LAUCIŪTĖ

Molecular Biotechnology study program

Master Thesis

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST ANTIBIOTIC RESISTANCE PROTEINS OXA-48, OXA-134, SHV-42, SME-3, AND ADC-144

Supervisor dr. Martynas Simanavičius

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Abbreviations

Ab – antibody

ADC - *Acinetobacter*-derived cephalosporinases

Ag – antigen

APS – ammonium persulfate

AST – antimicrobial susceptibility testing

BSA – bovine serum albumin

dH_2O – deionized water

DMEM - Dulbecco's Modified Eagle Medium

DMSO - dimethyl sulfoxide

DTT - Dithiothreitol

ELISA - enzyme-linked immunosorbent assay

FBS - Fetal bovine serum

GES - Guiana extendedspectrum β-lactamase

GS – gentamicin sulphate

HAT –hypoxanthine, aminopterin, thymidine

HRP - horseradish peroxidase

HT –hypoxanthine, thymidine

Ig – immunoglobulin

IMI/NMC-A - imipenemase/non-metallocarbapenemase-A

IPTG - isopropyl β- d-1-thiogalactopyranoside

K_D – dissociation constant

KPC - *K. pneumoniae* carbapenemase

LB – Luria-Bertani

LF – lateral flow

MAb – monoclonal antibody

MBP – maltose binding protein

MIC – minimal inhibitory concentration

MRSA - Methicillin-resistant *Staphylococcus aureus*

NAAT – nucleic acid amplification test

OD – optical density

OXA – oxacillinase

PBP – penicillin-binding protein

PBS - phosphate-buffered saline

PBST – phosphate-buffered saline with 0.1 % Tween-20

PCR – polymerase chain reaction

PEG-400 - polyethylene glycol 400

PMSF - phenylmethylsulphonyl fluoride

POC – point-of-care

PVDF - polyvinylidene difluoride

SDS - sodium dodecyl sulfate

SDS-PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SFC - *Serratia fonticola* carbapenemase

SHV - sulfhydryl variable lactamase

TMB - 3,3',5,5' tetramethylbenzidine

WB – Western Blot

WHO – World Health Organization

INTRODUCTION

In the beginning of the 20th century, infectious diseases were one of the most common causes of deaths worldwide (Achievements in Public Health, 2022). Everything changed with the discovery of penicillin by Alexander Fleming in 1928, marking the era of antimicrobial revolution (Fleming, 1929). The following few decades were considered the golden age with the discoveries of many new antibiotics. However, since 1970s, "discovery" of new drugs was reduced to the modifications of already existing antibiotics (Aminov, 2010).

Since the discovery of penicillin, antibiotics have been one of the most successful forms of treatment in the history of medicine. According to Center for Disease Control (CDC) statistics, leading causes of deaths in the US changed from infectious diseases to non-communicable diseases such as cancer and cardiovascular diseases. Antibiotics also helped to raise life expectancy to 78.8 years. Communicable diseases nowadays are usually the problem of immunocompromised patients (elderly, cancer and transplant patients, people with prolonged hospitalization (Yoshikawa, 2002).

Most of the discovered antibiotics are still used in the clinical environment, however, their effectiveness has been decreased by the rise of antimicrobial resistance. The rapid discovery of a number of classes of antibiotics during a short period of time led to the excessive use of these drugs promoting antibiotic resistance (Katz, 2016). This was and still is a significant threat to the achievements of the golden era.

Although the emergence of resistant microorganisms would occur even without selective pressure (sublethal doses) from the healthcare environment, overuse and inappropriate use of antibiotics has a significant effect on the evolution of resistance (Burnham et al., 2017). Smarter use of the drugs is considered one of the goals for addressing the rising antimicrobial resistance. To achieve that, rapid point-of-care diagnostics that can detect antibiotic-resistant pathogens are critical to ensure that the patient receives effective treatment before getting unnecessary broad-spectrum antimicrobial drug (Shanmugakani et al., 2020).

Immunodetection is one of the methods that is used to identify bacterial pathogens. Based on antigen detection by an antibody, it is a specific and sensitive tool that can be applied in antimicrobial resistance testing (Vasala et al., 2020). The number of tests to this day is limited, thus, generation of new monoclonal antibodies against antimicrobial resistance factors would increase the chance of developing more diagnostic tools.

The aim of the thesis is to generate and characterize monoclonal antibodies against antibiotic resistance proteins OXA-48, OXA-134, SME-3, SHV-42, and ADC-144.

Objectives of the thesis:

1. To express and purify OXA-48 and OXA-134 β-lactamases from *E. coli* expression strains.
2. To develop hybridoma cell lines, that secrete monoclonal antibodies against OXA-48, OXA-134, SME-3, SHV-42, ADC-144 β-lactamases.
3. To characterize the generated monoclonal antibodies using immunochemical methods.

1. LITERATURE ANALYSIS

1.1 Antibiotic resistance

Antibiotic resistance is a major health threat in the 21st century. Historically, infectious diseases have been a major cause of mortality. Specifically, during the 20th century, out of the top 10 leading causes of death in the United States, infectious diseases accounted for one-third of all deaths. However, developments in public health systems and medicine during the last 100 years helped to reduce the load linked with infectious diseases. The discovery of penicillin, a key medical breakthrough, together with improved sanitation, played crucial roles in reducing infectious disease mortality (Achievements in Public Health, 2022). Alexander Fleming's discovery of penicillin lead to the "golden era of antibiotics", during which many new antibiotics were developed and introduced, causing many to believe that infectious diseases could be conquered (Conly and Johnston, 2005). Unfortunately, infectious diseases are on the rise. Infectious pathogens can evolve and over time many have developed resistance to currently prescribed antibiotics causing significant impacts at the global level. For example, Methicillin-resistant *Staphylococcus aureus* is responsible for the deaths of 50 000 people in the United States and Europe every year (O'Neill J., 2014).

Antibiotic resistance emerges due to a variety of reasons around the world. In a study, conducted by A. Chokshi and her colleagues, it was concluded that in developing countries, the main contributors to antibiotic resistance were: "(1) lack of surveillance of resistance development, (2) poor quality control of available antibiotics, (3) clinical misuse, and (4) ease of availability", while developed countries were contributing to rising antibiotic resistance by: (1) poor hospital-level regulation and (2) overuse of antibiotics in food-producing animals. The authors also stressed that a factor that affects both developing and developed countries is the lack of research on new antibiotics (Chokshi et al., 2019). Antibiotic resistance has substantial burdens on the healthcare system and economy and could reach a global annual cost of 10 million deaths and US$100 trillion by 2050 (O'Neill, 2014).

1.1.1 Clinical misuse of antibiotics

Clinical misuse together with excessive use of antibiotics promotes resistance. Often there's lack of proper diagnostic methods in infection treatments which results in unnecessary antibiotic prescriptions. For example, in 2003, a Chinese study showed that of 1025 infection cases, only 39 had infection source identified by microbiological methods, although all 1025 of patients were treated with one or more antibiotics (Hu et al., 2003). Another study conducted in China found that of 57 009 cases, only 8,7% received appropriate antibiotic prescriptions. (Chang et al., 2019). One more study from

Indonesia highlighted that 94% of diarrhea cases in children were treated with antibiotics, even though most doctors suspected viral infections (Obong et al., 2000).

World Health Organization (WHO) has done significant work to create international standards that would improve clinical diagnosis of tuberculosis. However, due to the cost of molecular tests for tuberculosis, developing countries perform the tests on specific (e.g., HIV-associated or multidrug-resistant) cases rather than all tuberculosis infection cases (StopTB Partnership Survey, 2015). Developing countries rarely perform microbiological bacterial culture and drug sensitivity tests and other tests used to confirm infections are uncommon, so many doctors rely on clinical symptoms instead of recommended laboratory tests. Consequently, there is an increased use of broad-spectrum antibiotics and that results in selection of resistant intestinal microorganisms which could be the cause of future pathogenic infections (Chokshi et al., 2019). Tuberculosis is only one of many common infections that require antibiotic treatment. Salmonella, various sexually transmitted infections, urinary tract infections and pneumonia and other infection treatments promote antibiotic resistance, too (Frieri et al., 2017).

1.1.2 Lack of research on new antibiotics

Despite the evidence that antibiotic resistance is an increasingly important issue and already a global threat, research on new antibiotic pharmaceuticals to combat infectious diseases is slowing down. The period between the 1940s and 1980s lead to the discoveries of many new antibiotics, however, no new antibiotic class has been discovered since then (Aminov, 2010). Between 1980 and 1984 there were 19 new drug application approvals by Food and Drug Administration, where only 6 antibiotic approvals were given between 2010 and 2014 (Ventola, 2015). In 2017 World Health Organization published a report stating the issue that antibiotics which are now being developed, are not sufficient to meet rising antibiotic resistance. During the same year WHO published the global priority pathogens list, which includes 12 groups of pathogens in three priority categories (critical, high, medium), highlighting the threat of gram-negative bacteria that are resistant to multiple antibiotics to ensure R&D response to urgent public health needs (WHO, 2017).

1.2 Antibiotic resistance mechanisms

Treatment of infectious diseases requires understanding of pathogenesis and antibiotic resistance. There has been substantial progress in understanding how antibiotics work and the major mechanisms by which bacteria can resist the inhibitory or killing effects of antibiotics (Fig.1.1). Some mechanisms are intrinsic, where the cell can use genes it already possesses to survive antibiotic exposure, and some are acquired, where gain of new genetic material provides new factors that contribute to the survival (Ruppé et al., 2015).

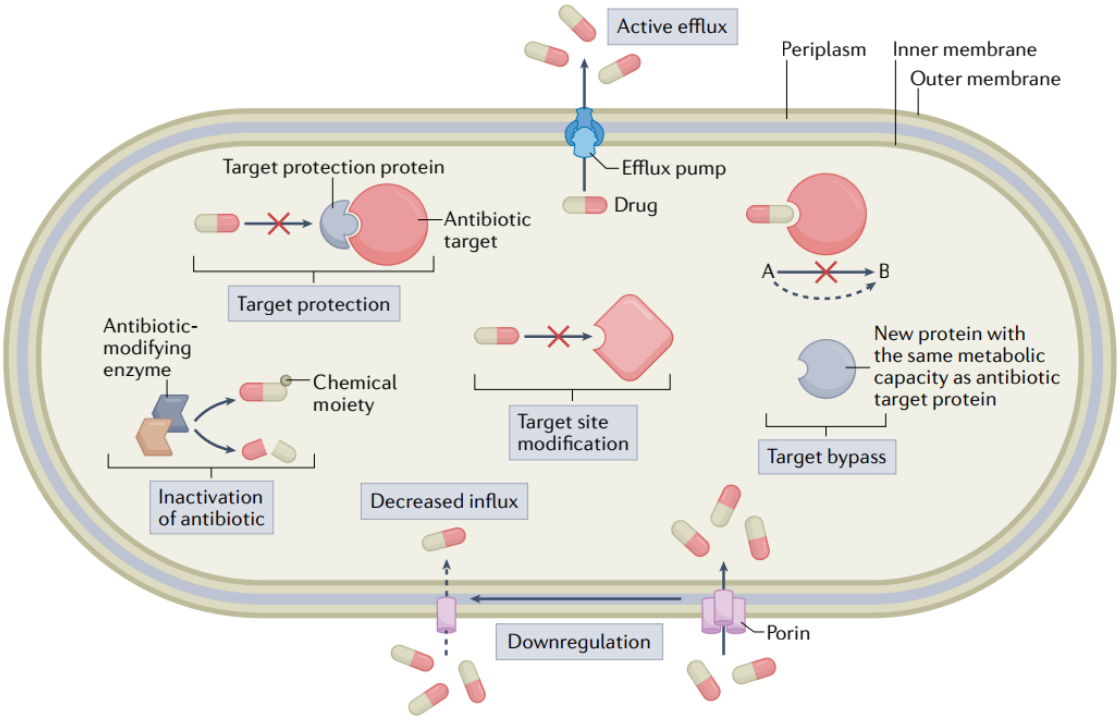

Fig. 1.1. Overview of the molecular mechanisms of antibiotic resistance: target protection, active efflux, target site modification, target bypass, decreased influx, downregulation and inactivation of antibiotic (Darby et al., 2022).

Many antimicrobials must cross bacterial cell membrane in order to reach their target and have an effect. Gram-negative bacteria have the advantage of double-membrane structure that makes the cellular envelope rather impermeable, providing resistance to many antibiotics that work against Gram-positive pathogens. Antimicrobial resistance increases with additional alterations to envelope structure, such as changes to phospholipid and fatty acid content in the cytoplasmic membrane or porin loss, both factors affecting the ability of the drug to enter the cell (Fernández and Hancock, 2012). It was recently shown that permeability of the outer membrane of Enterobacterales dynamically changes during bacterial growth, which also affects how much of the drug will get into the cell (Whittle et al., 2021). Compared to Gram-negative, Gram-positive bacteria are naturally more permeable to many drugs, however, cytoplasmic membrane composition changes have been shown to reduce antibiotic entrance into the cell, too (Mishra et al., 2012). In addition, some Gram-positive bacteria possess an outer lipid layer and a polysaccharide capsule coat, which prevents hydrophilic molecules from penetrating the membrane (Batt et al., 2020).

Even if antibiotics get into the cell, bacteria have ways to export them outside, specifically, by utilizing efflux pumps. These transmembrane proteins transport various toxic compounds using energy. Efflux pumps are especially significant in Gram-negative bacteria as they contribute to antimicrobial

resistance. These proteins contribute to the multidrug resistant phenotype in clinically important pathogens such as *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa* and *Neiserria gonorrhoeae.* Together with the double membrane, efflux pumps provide from low to high levels of resistance and act as a crucial mechanism that enables other antimicrobial resistance mechanisms (Nazarov, 2022).

Another mechanism by which antibiotics cause inhibition of growth or death is inhibiting essential cellular functions (Baquero and Levin, 2021). Antimicrobial agents are selective and bind their cellular targets with high specificity and affinity, so if the structure of the primary target is altered by mutation or chemical moieties, it is likely that antibiotic binding will be hindered leading to resistance. For example, mutations in genes coding for penicillin-binding proteins (PBPs) decrease susceptibility to β-lactam antibiotics (Periasamy et al., 2020). Target alterations can be a result of random point mutations, which expand under antibiotic pressure, or by recombination between alleles, generating high frequencies of mutant genes and conferring resistance rapidly (Huber et al., 2021). Alternatively, mutant alleles of target genes can be acquired through transformation – alternative alleles are gained from related species and mosaic genes are generated by recombination, such as PBP2x, PBP2b & PBP1a mutations in clinical isolates of *Streptococcus pneumoniae* (Hakenbeck et al., 2012).

1.2.1 Inactivation or modification of antibiotics and their spread in clinically relevant bacteria

A widespread mechanism of resistance in many pathogenic bacteria is the modification or inactivation of the antimicrobial drug itself (Forsberg et al., 2015). Such mechanism is often implemented through enzymatic functions and does not require altering core components of the cell. Antibiotics can be modified through inactivation by degradation or modification of the antibiotic by the transfer of a chemical group. The former mechanism damages antimicrobial molecule structure making it less effective, which reduces treatment success of the patient (Schaenzer and Wright, 2020). The latter makes antibiotic ineffective by the transfer of a chemical group, examples of which are enzymes modifying aminoglycosides, macrolides, rifamycins, streptogramins, lincosamides and phenicols. Aminoglycosides are modified on the hydroxyl or amino group of the molecule and enzymes responsible for that are acetyltransferases, phosphotransferases and nucleotidyltransferases (Ramirez and Tolmasky, 2010). Many aminoglycoside-modifying enzymes are encoded on mobile genetic elements, as well as in chromosomes, and they are found in both Gram-positive and Gram-negative species (Timilehin et al., 1970).

Antibiotic-inactivating enzymes are also encoded on chromosomes and mobile genetic elements and one group of such enzymes are β-lactam antibiotics-hydrolyzing β-lactamases. Specifically,

β-lactamases degrade the drug by hydrolyzing the amide bond of the β-lactam ring as shown in Fig. 1.2 (Tooke et al., 2019). The enzymes have been studied since 1940s and new β-lactamases are constantly being characterized, the list of characterized Beta Lactamase DataBase now containing over 7700 different proteins (Naas et al., 2017).

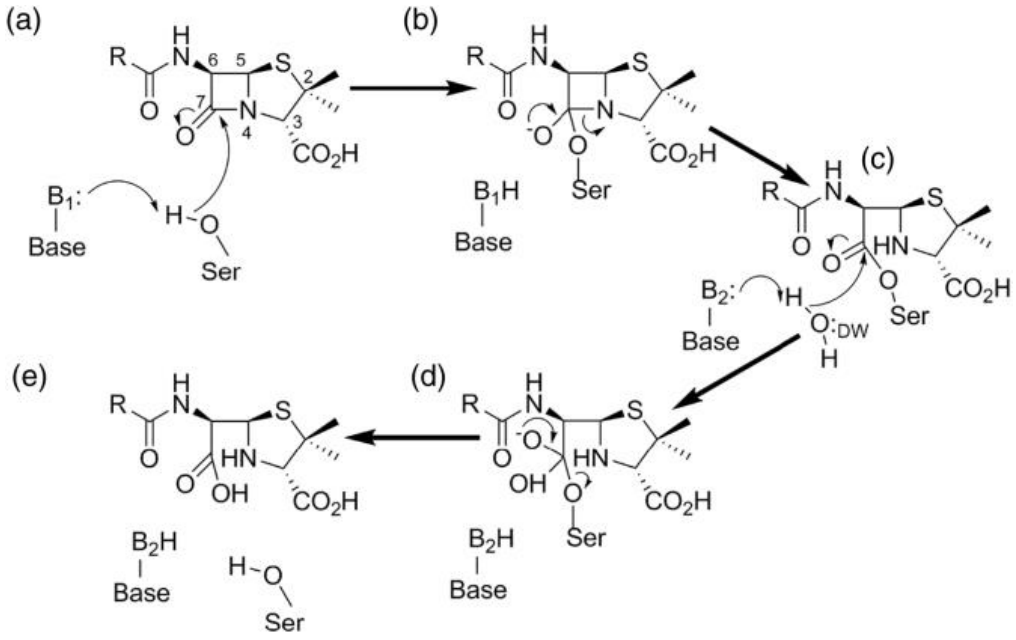

Fig. 1.2. Overview of serine β-lactamases, hydrolyzing generic penicillin substrate. (a) General base B1 activates Ser for nucleophilic attack on the amide carbonyl carbon (C7) generating covalent acylenzyme (c) via tetrahedral oxyanionic acylation transition state (b). General base B2 activates incoming deacylating water molecule (DW) for nucleophilic attack on the acylenzyme carbonyl liberating penicilloate product (e) via tetrahedral deacylation transition state (d) (Tooke et al., 2019).

1940s was the time when the first chromosomal β-lactamase was identified in *E. coli* K-12. The gene encoding the enzyme was then designated *ampC* (Burman et al., 1973). AmpC proteins are cephalosporinases – they hydrolyze most penicillins, class-A β-lactamase inhibitors, narrow-spectrum cephalosporins. These β-lactamases present stronger hydrolytic activity towards cephalosporins rather than penicillins (Jacoby, 2009). Regarding extended-spectrum cephalosporinases, they show increased hydrolytic activity towards broad-spectrum β-lactams, examples of which would be cefotaxime, ceftazidime, and cefepime (Nordmann and Mammeri, 2007). The spread of cephalosporinases has been concerning in such nosocomial pathogens as *A. baumannii*, as it has lead to significant morbidity and mortality in β-lactam resistant cases (Karlowsky et al., 2003; Rodríguez-Martínez et al., 2010). Resistance to β-lactam antibiotics in *A. baumannii* – ceftazidime and cefotaxime- is linked to overexpression of bla_{AmpC} gene (Bratu et al., 2008; Hujer et al., 2005). Overproduction of the protein is

achieved by strong promoter in the insertion sequence IS*Aba1* (Bratu et al., 2008). ISs can mediate the transfer of antibiotic resistance genes between different sites within the bacterial genome, as well as between different bacteria (Galiot et al., 2023). This can lead to the spread of antibiotic resistance within *A. baumannii* populations, and between *A. baumannii* and other bacterial species. To date, there has been 256 different cephalosporinases submitted to Comprehensive Antibiotic Resistance Database designated as *Acinetobacter*-derived cephalosporinases (ADCs) (Alcock et al., 2023.). This suggests a high level of genetic diversity and the potential for the emergence of new resistance mechanisms related to inactivation of antibiotics.

Inactivation of β-lactams is not limited to one class of β-lactamases. Functionally, there are four (A–D): classes A, C and D are serine β-lactamases and members of class B are zinc-dependent metallo-β-lactamases (Tooke et al., 2019). Extended-spectrum β-lactamases confer resistance to extended-spectrum cephalosporins and monobactams (Nepal et al., 2017). Carbapenem resistance can be mediated by carbapenemases (class A, B and D β-lactamases) or by production of an extended-spectrum β-lactamase in combination with porin loss. As carbapenems are one of the most powerful antibiotics, resistance to this type is particularly concerning (Lima et al., 2020). Specifically, carbapenems have a 5-membered ring that is fused to the beta-lactam ring which enhances their stability against most metallo-β-lactamases as well as extended spectrum β-lactamases (Knapp and English, 2001). Despite their powerful nature, carbapenem resistance is on the rise as one study, analyzing multidrug resistant bacteria from intensive care units in hospitals from the United States and Pakistan, found that β-lactam antibiotic resistance genes were the most abundant of all and that different pathogen species frequently shared resistance genes. Alarmingly, 40.3% of the genes encoded for putative carbapanemases (D'Souza et al., 2019).

OXA β-lactamases – class D β-lactamases – are structurally diverse group of enzymes, which were first shown to possess enhanced activity towards semisynthetic penicillins, such as oxacillin (thus being named oxacillinases, or OXA), and reduced activity towards penicillin (Hedges et al., 1974). The group includes five subgroups of carbapenem-hydrolyzing enzymes: OXA-23-like, OXA-24/40-like, OXA-51-like, and OXA-58-like β-lactamases, found only in *A. baumannii*, and OXA-48-like β-lactamases, which migrated to other *Enterobacteriaceae* (Hirvonen et al., 2021). Some OXA enzymes, e.g., OXA-2 and OXA-10, although classified as narrow-spectrum β-lactamases, have demonstrated carbapenem hydrolysis, indicating that most oxacillinases could be considered carbapanemases (Antunes et al., 2014). The emergence of OXA enzymes that can confer resistance to carbapenems, especially in

A. baumannii, has transformed these β-lactamases from a minor hindrance into a major problem that threatens clinical efficacy of carbapenem antibiotics.

One of the most widespread OXA-type-β-lactamases in *Klebsiella pneumoniae* and other *Enterobacteriaceae* is OXA-48 β-lactamase. It was obtained from multidrug resistant *K. pneumoniae* isolate in Turkey and shown to have carbapenemase activity (Poirel et al., 2004). OXA-48 β-lactamase and its variants now represent one of the most concerning developments in carbapenem resistance in the last decade. Kinetic measurements of OXA-48 report that the enzyme has a low level of hydrolytic activity against carbapenems with much greater activity against imipenem than meropenem and only modestly increases minimal inhibitory concentrations (MICs) of the carbapenems after being cloned into sensitive *E. coli* strains (Poirel et al., 2004). However, when two of OXA-48 variants, OXA-181 and OXA-232, were cloned into sensitive *E. coli* strains that lacked porins OmpF and OmpC, MIC values increased significantly (Potron et al., 2013). This indicates that the presence of OXA-48-like enzymes together with other resistance mechanisms can confer high levels of carbapenem resistance.

Even though there are many characterized OXA-β-lactamases that are major problems in clinical environment, it is important to avoid overlooking potential threats to antibiotic treatments, too. Studies have been conducted to identify OXA-type enzymes that belong to *Acinetobacter* species, one of which found OXA-134a in an *Acinetobacter lwoffii* isolate. Further screening of *A. lwoffii* isolates has revealed OXA-186 to OXA-191 enzymes, all universal to the species. *A. lwoffii* isolates are not resistant to β-lactam antibiotics, which correlates with the fact that $bla_{OXA\text{-}134a\text{-}like}$ (OXA-134a-like β-lactamase-encoding) genes are not highly expressed. However, when the genes encoding OXA-134a and OXA-187 were cloned into susceptible *E. coli* strain, they reduced susceptibility to carbapenems, most cephalosporins and conferred resistance to penicillins. Since OXA-134-like enzymes have been found only in *A. lwoffii*, they do not present clinical problems yet. However, they represent a reservoir of enzymes, which have potential to evolve and confer higher levels of resistance to β-lactams that could be transferred to other pathogen species (Figueiredo et al., 2010).

Similarly to class-D, class-A β-lactam-inactivating proteins threaten the clinical environment and have potential to expand resistance profile, too. One of such class examples are SHV β-lactamases, identified in *E. coli* in the 1970s (Pitton, 1972). SHV enzymes such as SHV-1 have evolved from being able to degrade limited spectrum penicillins – early cephalosporins – to posessing extended spectrum β-lactamase qualities by efficiently degrading cefotaxime (e.g., SHV-2 or SHV-42) (Bradford, 2001; Mulvey et al., 2004). Finally, at the end of the 20th century class A β-lactamases mutated to become carbapenem hydrolyzing enzymes (Walther-Rasmussen and Høiby, 2007). There have been six types of

class A carbapanemases reported: SME (*Serratia marcescens* enzyme), SHV (sulfhydryl variable lactamase), GES (Guiana extendedspectrum β-lactamase), KPC (*K. pneumoniae* carbapenemase), SFC (*Serratia fonticola* carbapenemase), and IMI/NMC-A (imipenemase/non-metallocarbapenemase-A) (Sawa et al., 2020). The genes encoding KPCs are the most abundant transmissible sequences found in Enterobacteriaceae (Patel and Bonomo, 2013). These enzymes are capable of hydolyzing all β-lactam antibiotics, as strains bearing bla_{KPC} are often resistant to amynoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole. This way the spread of KPCs remains the most succesful cause of multi-drug resistance pandemics in Gram-negative bacteria (Nordmann et al., 2009; Patel and Bonomo, 2013). SFC-1 and SME β-lactamases have been reported to be similar to KPCs, having broad hydrolysis spectrum that includes penicillins, aztreonam, cephalosporins, and carbapenems (e.g., SME-3), but only found in *S. marcescens*, opportunistic pathogen causing digestive, respiratory, urinary tract, and surgical wound infections (Khanna, 2013; Queenan et al., 2006).

1.3 Immunodiagnostic tests – a way to mitigate antibiotic resistance pandemic

Lack of affordable and rapid diagnostics challenges the understanding of the true global burden of antimicrobial resistance. In regions where most prescribing is based on clinical symptoms, there is an urgent need for point-of-care (POC) rapid diagnostics to tackle the misuse of antibiotics. It is especially important in the case of children who present undifferentiated fever, where bacterial, viral, and malarial infections predominate in low-resource settings. Most commonly used diagnostic test to distinguish bacterial from viral infection is C-reactive protein, which might be elevated in non-bacterial infections such as malaria or severe dengue fever (Wilairatana et al., 2021). Current commercially available diagnostics are not applicable in low-resource environments, as they are not affordable and at the same time accurate, rapid, and easy to use. What is more, distinguishing between bacterial and viral infections is only a part of the diagnosis − the next step is identifying pathogen's resistance profile. Nucleic acid-based tests allow medical staff to identify resistance genes quickly, however, some methods, such as next generation sequencing, still require positive bacterial culture prior to the test. It is also important to note that resistance mutations interpretation has it's challenges, one of which is the difference between mutation and phenotypic drug resistance (Miotto et al., 2017).

While many novel methods claim to perform antimicrobial susceptibility testing (AST) in a few hours, it is rarely the case, because such statements do not consider growing or isolation of the cultures as it sometimes takes up to two days (Fig. 1.3). For example, methods based on nucleic acid hybridization (NAAT), immunodiagnostics or nucleic acid hybridization allow the use of non-purified samples. However, only after a short cultivation with a pre-determined antibiotic load and NAAT, antibiotic

resistance is determined, and rough MIC value is known. It is unfortunate that fast, precise, easy-to-use and inexpensive AST systems are still inaccessible in hospitals (Belkum et al., 2019).

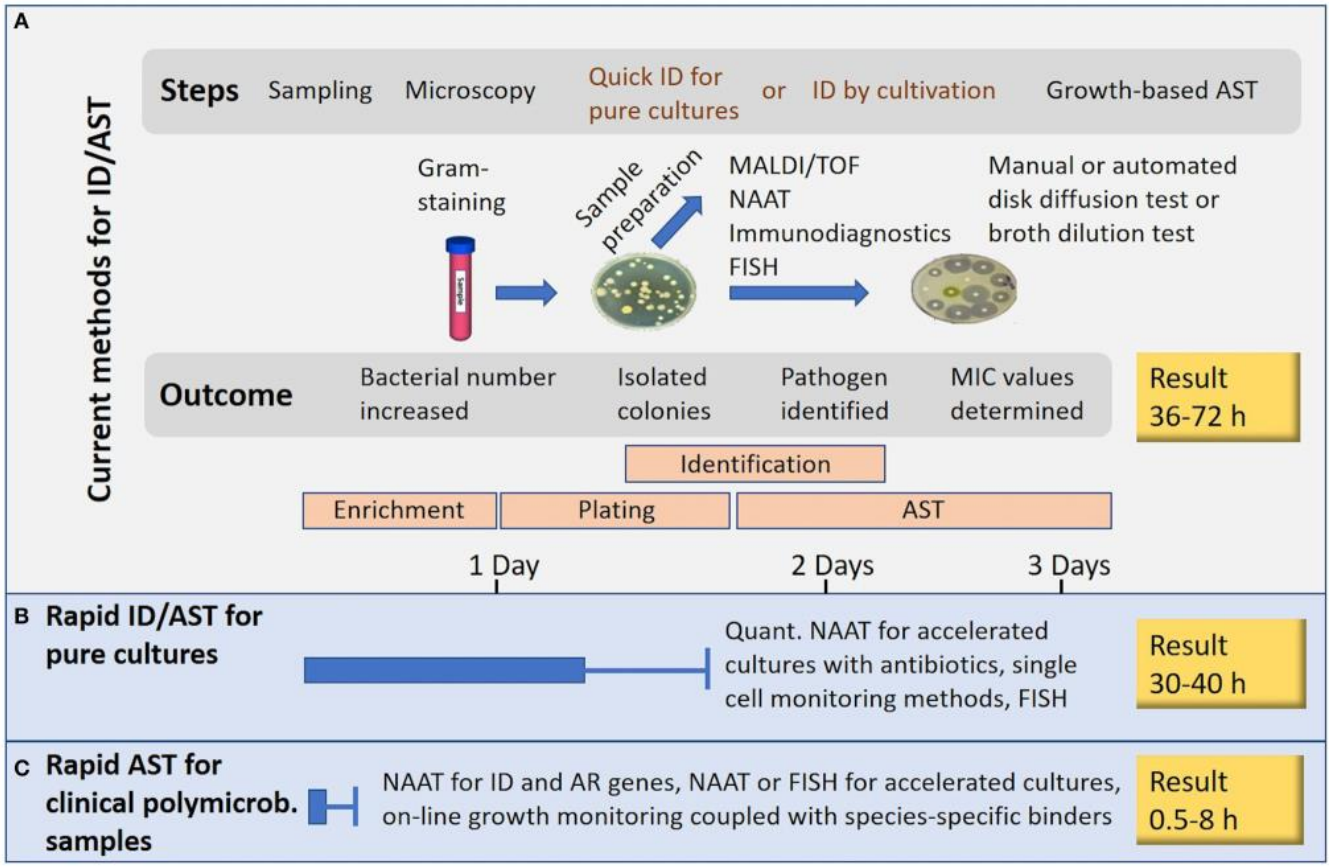

Fig. 1.3. Time consumption comparison of different diagnostic tests. (A) Current technologies. (B) Rapid AST applicable to pure cultures. (C) Rapid AST for clinical polymicrobial samples. Abbreviations: ID – identification, AR – antibiotic resistance, FISH – Fluorescence *in situ* Hybridization. The presented times are rough estimates and generalizations. (Vasala et al., 2020).

Immunodetection is one of the most specific and sensitive method for bacterial identification (Verma et al., 2013). It is based on the reaction between antigen (Ag, protein of interest) and a selective antibody (Ab), while the efficacy mainly depends on the efficiency of Ab-Ag complex formation (Di Nardo et al., 2021). In such tests antibodies are immobilized onto strips, nanoparticles or biosensor surfaces resulting in efficient and specific target binding. The detection antibody can be labeled fluorescently or using redox enzymes to produce a quantitative signal. Utilizing this method, pathogen identification and growth monitoring could potentially be achieved in a single step, since it does not always require bacteria lysis. Immunodetection can be applied as a simple lateral flow (LF) test, as well as integrated in microfluidics, biosensor technology and even DNA/RNA-based analysis (Vasala et al., 2020). Immunodetection in the form of LF is advantageous for its simplicity, cost-effectiveness, rapidity and no requirement for equipment or laboratory training (Di Nardo et al., 2021).

While LF tests are widely used to diagnose many types of diseases in the clinical field, the lack of highly sensitive and specific antibodies limits the number of tests developed to detect antimicrobial resistance (Di Nardo et al., 2021). One of the examples is the detection of aminoglycoside 6'-N-acetyltransferase AAC(6')-Iae, which confers aminoglycoside resistance in *P. aeruginosa* samples: amikacin, dibekacin, kanamycin, netilmicin, isepamicin, sisomicin, and tobramycin (Kitao et al., 2010; Sekiguchi et al., 2005). Abbott Inc. has launched an immunochromatography test to detect methicillin resistance in *S. aureus* (MRSA) isolates. The assay is able to detect resistance proteins from cell lysates of 1 colony on agar plate (10^8 colony forming units) and the test is based on a PBP2a-specific chicken IgY antibody (Yamada et al., 2013). Coris Bioconcept from Belgium has launched tests for the detection of carbapenemases OXA-48-like, KPC, and NDM type from Enterobacterial isolates (Glupczynski et al., 2017). These LF tests work well with isolated clinical pathogens. However, they are limited in the direct analysis of clinical samples, once again highlighting the lack of truly rapid diagnostics.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents

Table 2.1. Reagents that were used to prepare this work.

Reagent (Source)	Purpose
DreamTaq Green PCR Master Mix (2×) (Thermo Scientific, Lithuania)	Molecular cloning, bacteria cultivation, and induction of gene expression
Water, nuclease-free (Thermo Scientific, Lithuania)	
FastDigest XhoI Restriction Endonuclease (Thermo Scientific, Lithuania)	
FastDigest BamHI Restriction Endonuclease (Thermo Scientific, Lithuania)	
DNA Gel Loading Dye (6×) (Thermo Scientific, Lithuania)	
10× FastDigest Buffer (Thermo Scientific, Lithuania)	
FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, Lithuania)	
Rapid DNA Ligatio Kit (Thermo Scientific, Lithuania)	
GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania)	
GeneJET Plasmid Miniprep Kit (Thermo Scientific, Lithuania)	
GeneRuler 1kb Plus DNA Ladder (Thermo Scientific, Lithuania)	
GeneRuler 50bp DNA Ladder (Thermo Scientific, Lithuania)	
50× TAE Electrophoresis Buffer (Thermo Scientific, Lithuania)	
Top Vision Agarose (Thermo Scientific, Lithuania)	
LB Broth (Roth, Germany)	
LB Agar (Roth, Germany)	
Kanamycin (Sigma, USA)	
Ampicillin (Roth, Germany)	
IPTG (Thermo Scientific, Lithuania)	
Sodium chloride (NaCl) (Roth, Germany)	Buffers
Tris (Tris) (Sigma, USA)	
32 % hydrogen chloride solution (HCl) (Sigma-Aldrich, USA)	
Potassium chloride (KCl) (Roth, Germany)	
Glycine (Sigma, USA)	
Sodium hydroxide (NaOH) (Roth, Germany)	
Potassium dihydrogen phosphate (KH2PO4) (Roth, Germany)	
Disodium phosphate dodecahydrate (Na2HPO4 · 12H2O) (Sigma-Aldrich, USA)	
Tween-20 (Roth, Germany)	
β-mercaptoethanol (Fluka Chemie, Switzerland)	Protein sample preparation
Guanidine Hydrochloride (Roth, Germany)	
Imidazole (Sigma, USA)	
PMSF Protease Inhibitor (Thermo Scientific, Belgium)	
Glycerol (Roth, Germany)	
Antifoaming agent (Sigma, USA)	
Sodium dodecyl sulphate (SDS) (Roth, Germany)	SDS-PAGE
Acrylamide (Roth, Germany)	
N,N′-(1,2-dihydroxyethylene)bisacrylamide (Sigma, USA)	
Ammonium persulfate-APS (Sigma-Aldrich, USA)	

Table 2.1. Reagents that were used to prepare this work (continued).

N,N,N',N'-tetramethylethylenediamine (TEMED) (Roth, Germany)	SDS-PAGE
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific, USA)	
Pierce™ Lane Marker Reducing Sample Buffer (5×) (Thermo Fisher Scientific, USA)	
PageBlue Protein Staining Solution (Thermo Scientific, Lithuania)	
95–98 % sulfuric acid, (H_2SO_4) (Sigma, USA)	ELISA, Western blot
NeA-Blue Aqueous TMB substrate (Clinical Science Products, USA)	
Goat polyclonal antibody, against mouse immunoglobulin G (IgG), conjugated with horseradish peroxidase (Biorad, USA)	
Milk powder (Roth, Germany)	
4-chloro-1-naphthol (Sigma, USA)	
30 % hydrogen peroxide solution (H_2O_2) (Sigma, USA)	
Methanol (Roth, Germany)	
ROTI block (Roth, Germany)	
Ammonium sulphate ($(NH_4)_2SO_4$) (Roth, Germany)	Hybridoma technology
Freund‘s Complete Adjuvant (Sigma, USA)	
Freund‘s Incomplete Adjuvant (Sigma, USA)	
Dulbecco's Modified Eagle Medium (DMEM) - high glucose (Sigma-Aldrich, USA)	
HyClone Dulbecco's Modified Eagle Medium (DMEM) - high glucose (Cytiva, USA)	
HAT Media Supplement (50×) (Sigma, USA)	
HT Media Supplement (50×) (Sigma, USA)	
96 % ethanol (Vilniaus degtinė, Lithuania)	
Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)	
Fetal bovine serum (FBS) (Biochrom, Germany)	
BD Mouse Immunoglobulin Isotyping ELISA Kit (BD Biosciences, USA)	
Gentamycin sulphate (GS) (Roth, Germany)	
Horseradish peroxidase (HRP) (Merck KGaA, USA)	Antibody labeling
Sodium acetate ($NaCH_3COO$) (Roth, Germany)	
Sodium borohydride ($NaBH_4$) (Sigma-Aldrich, USA)	
Sodium carbonate (Na_2CO_3) (Sigma-Aldrich, USA)	
Sodium bicarbonate ($NaHCO_3$) (Roth, Germany)	
Sodium periodate ($NaIO_4$) (Sigma-Aldrich, USA)	

2.1.2. Solutions, buffers, and cell growth media

Table 2.2. Buffers, solutions, and cell growth media prepared in this work. Deionized water was used to prepare the solutions, if not stated otherwise.

Solutions, buffers, and cell growth media	Composition	Purpose
Bacterial freezing medium	2× LB, 40 % glycerol	Bacteria cultivation, preservation, and chemo transformation
Luria-Bertani **(**LB) medium	25 g of LB broth in 1 l of dH_2O.	
LB agar	35 g of LB agar in 1 l of dH_2O.	
NaCl solution	100 mM NaCl	
$CaCl_2$ solution	100 mM $CaCl_2$	
DNA electrophoresis buffer	1× electrophoresis buffer (diluted from 50×**)**	
Ethidium bromide solution	1 µg/mL EtBr	
Biomass washing solution	20 mM Tris-HCl, 0.14 M NaCl, pH 7.4	Protein purification under denaturing conditions
2% SDS solution	2 % SDS (w/v) in PBS	
Resuspension buffer	20 mM Tris-HCl, pH 8.0	
Isolation buffer	2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2 % Tween-20, pH 8.0	
Binding buffer	6 M Gua-HCl, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0	
SDS-PAGE Running buffer	0.025 mol/l Tris, 0.2 mol/l Gly, 0.1 % SDS, pH 8.3-8.6	SDS-PAGE
Acrylamide-bisacrylamide solution	30 % acrylamide, 0,8 % N,N′-(1,2-dihydroxyethylene)bisacrylamide	
0.5 mol/L Tris-HCl, pH 6.8	0.5 mol/l Tris-HCl, pH 6.8	
1.5 M Tris–HCl, pH 8.8	1.5 M Tris–HCl, pH 8.8	
10 % APS	10 % (w/v) APS	
10 % SDS	10 % (w/v) SDS	
Phosphate-buffered saline (PBS)	0.0027 mol/l KCl, 0.0015 mol/l KH_2PO_4, 0.0081 mol/l $Na_2HPO_4 \cdot 12H_2O$, 0.137 mol/l NaCl	ELISA, Western blot
PBST	PBS with 0.1 % Tween-20 (w/v)	
Coating buffer	0.05 mol/l $NaHCO_3$, pH 9.5	
Stop solution	3.6 % (w/v) H_2SO_4	
ELISA Blocking solution	2 % BSA (w/v) in PBST	
WB Blocking solution	2% milk powder (w/v) in PBS	
Washing solution	PBST : dH_20 (1:1)	
Protein transfer buffer	0.025 mol/l Tris, 0.15 mol/l Gly, 10 % (w/v) methanol	
WB substrate solution	10 mL of PBS, 2 ml of chloronaphthol solution, 30 µl of 30 % H_2O_2	
4-chloro-1-naphthol solution	One 4-chloro-1-naphthol tablet diluted in 10 ml of methanol	
Sp2/0 cells growth medium	DMEM with 9 % FBS (w/v), 100 µg/ml GS	Hybridoma technology
Serum-free medium	DMEM with 300 µg/ml GS	

Table 2.2. Buffers, solutions, and cell growth media prepared in this work. Deionized water was used to prepare the solutions, if not stated otherwise (continued).

Hybridoma cells growth medium	DMEM with 15 % FBS (w/v), 100 µg/ml GS	Hybridoma technology
HAT growth medium	DMEM with 15 % FBS (w/v), 100 µg/mL GS, 10^{-4} mol/l hypoxanthine, 4×10^{-7} mol/l aminopterin, 1.6×10^{-5} mol/l thymidine	
HT growth medium	DMEM with 15 % FBS (w/v), 100 µg/mL GS, 10^{-4} mol/l hypoxanthine, 1.6×10^{-5} mol/l thymidine	
Cells cryopreservation medium	90 % FBS (w/v), 10 % DMSO (w/v)	
Binding/wash buffer	1.5 M Glycine, 3 M NaCl, pH 8.9	Antibody purification
Elution buffer	0.1 M Glycine, pH=3	
0.01 mol/l sodium carbonate	0.01 mol/l sodium carbonate, pH 9.5	Antibody labeling
Sodium periodate solution	0.2 mol/l $NaIO_4$	
0.2 mol/l sodium carbonate	0.2 mol/l sodium carbonate, pH 9.5	
1 mmol/l sodium acetate	1 mmol/l sodium acetate, pH 4.5	
Sodium borohydride solution	$NaBH_4$ 4 mg/mL	

Recombinant SME-3-MBP (697 amino acids, 76.5 kDa), SHV-42-MBP (689 amino acids, 75.4 kDa), and ADC-144-MBP (786 amino acids, 83.3 kDa) proteins were constructed and produced in *E. coli* Tuner (DE3) cells by PhD student Laima Čepulytė from the Department of Eukaryote Gene Engineering. Protein purification was carried out by PhD student Vytautas Rudokas from the Department of Immunology.

2.1.3. Bacteria strains, cell lines, and laboratory animals

Table 2.3. Bacteria strains, eukaryotic cell lines, and laboratory animals used in this work.

Name	**Description**
E. coli DH10B	*F– mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ (ara-leu)7697 galU galK λ– rpsL(Str^R) nupG*. Used for molecular cloning.
E. coli BL21(DE3)	F$^–$ *omp*T *gal dcm lon hsdS$_B$*($r_B^- m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB$^+$*]$_{K\text{-}12}$(λ^S). Used for recombinant protein synthesis.
E. coli Tuner(DE3)	F$^–$*omp*T *hsd*S$_B$ (r_B^- m_B^-) gal *dcm lac*Y1(DE3). Used for recombinant protein synthesis.
E. coli BL21 Star(DE3)	F$^-$*omp*T *hsd*S$_B$ (r_B^-, m_B^-) *galdcmrne*131. Used for recombinant protein synthesis.
E. coli Rosetta(DE3)	F$^–$ *ompT gal dcm lon*? *hsdS$_B$*($r_B^- m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)pLysSRARE[*T7p20 ileX argU thrU tyrU glyT thrT argW metT leuW proL ori*$_{p15A}$](Cm^R)
Murine peritoneal macrophages	Isolated from BALB/C mice in this work. Used as feeder cells for hybridoma cultivation.

Table 2.3. Bacteria strains, eukaryotic cell lines, and laboratory animals used in this work (continued).

Mice Sp2/0-AG14	Sp2/0-AG14 is a hybrid between BALB/c spleen cells and the myeloma cell line P3X63AG8. Cells were described as not synthesizing or secreting immunoglobulin chains, being resistant to azaguanine at 20 μg/ml, and not growing in HAT medium. Cell line is used as a fusion partner for the production of hybridomas secreting specific monoclonal antibodies.
BALB/c mice	8 weeks old female mice were used in the experiments. Permit for using mice for immunizations in this research was issued by the Lithuanian Ethical Committee for Animal Testing under the State Food and Veterinary Service no. G2–117.

2.1.4. Vectors

Table 2.4. Vectors used or constructed in this work.

Name	Description	Source
pUC57_OXA-48	pUC57 plasmid with synthetic *bla*$_{OXA-48}$ gene. Harbors ampicillin resistance gene. Used for molecular cloning of *bla*$_{OXA\_48}$.	General Biosystems, USA
pUC57_OXA-134	pUC57 plasmid with synthetic *bla*$_{OXA-134}$ gene. Harbors ampicillin resistance gene. Used for molecular cloning of *bla*$_{OXA\_134}$.	General Biosystems, USA
pET28a(+)	Vector for expression of N-terminally 6xHis-tagged proteins with a thrombin site. Harbors kanamycin resistance gene and *lac* operator. Used for cloning of *bla*$_{OXA-48}$ and *bla*$_{OXA-134}$ genes.	Addgene, USA
pET28-MBP-TEV	Vector for expression of N-terminally MBP-tagged proteins with a thrombin site. Harbors kanamycin resistance gene and *lac* promoter. Used for cloning of *bla*$_{OXA-48}$ and *bla*$_{OXA-134}$ genes.	Addgene, USA
pET28a(+)-OXA-48	IPTG-inducible vector, harboring *bla*$_{OXA-48}$. Used for synthesis of OXA-48.	This work
pET28a(+)-OXA-134	IPTG-inducible vector, harboring *bla*$_{OXA-134}$. Used for synthesis of OXA-134.	
pET28-MBP-TEV-OXA-48	IPTG-inducible vector, harboring *bla*$_{OXA-48}$. Used for synthesis of OXA-48-MBP.	
pET28-MBP-TEV-OXA-134	IPTG-inducible vector, harboring *bla*$_{OXA-134}$. Used for synthesis of OXA-134-MBP.	

2.1.5. Oligonucleotide primers

Table 2.5. Oligonucleotide primers used in this work. Source – Metabion, Germany.

Name	Sequence 5'→3'	Description
T7prom-frw	TAATACGACTCACTATAGGG	Used for screening and sequencing of successful constructs after DNA cloning.
T7term-rev	GCTAGTTATTGCTCAGCGG	
MBP-F	GAAGCCCTGAAAGACGCG	

2.1.6. Materials and equipment

- 0.5 × 25 mm and 0.6 × 25 mm needles (BD Biosciences, USA)
- 0.5 mL, 1.5 mL, 2 mL tubes (Eppendorf, Germany), 15 mL and 50 mL centrifuge tubes (TPP, Switzerland)
- 1 mL cryogenic storage vials (Thermo Scientific, USA)
- 1 mL, 2.5 mL medical syringe (Lab Unlimited, United Kingdom)
- 12-channel pipette (30-300 µL) (Eppendorf, Germany), 8-channel pipette (30-300 µL) (FinnPipette, Thermo Scientific, USA; CAP, Denmark)
- 25cm2, 75cm2 cell culture flasks (Thermo Scientific, USA)
- 5 mL, 10 mL, 25 mL, 50 mL sterile polystyrene disposable serological pipets (Thermo Scientific, United Kingdom)
- 6, 12, 24, 96-well tissue culture test plates (TPP, Switzerland)
- 96-well ELISA plates (Nerbe plus, Germany), 96-well ELISA plates MaxiSorp (Nunc, Denmark), 96-well ELISA plates PolySorp (Thermo Fisher Scientific, Denmark)
- Amicon® Ultra 0.5mL centrifugal filters for protein concentration (Merck Millipore, Ireland)
- Biometra Standard Power Pack P25 (Biometra, Germany)
- Bürker hemocytometer for cell counting (Sigma, USA)
- Bottle-top filter system (Roth, Germany)
- Centrifuge “Eppendorf 5424R” (Eppendorf, Germany)
- Centrifuge “SL-16” (Thermo Scientific, USA)
- Centrifuge Universal 32 (Hettich Zentrifugen, Germany)
- Chromatography column (Bio-rad, USA)
- CO_2 incubator (Galaxy R, United Kingdom)
- Desalting Column Sephadex G-25 in PD-10 (GE Healthcare Bio-Sciences Corp., USA)
- Dialysis membrane Membra-Cel™ (Carl Roth, Germany)
- D-Tube™ Dialysers mini-maxi (Merck, USA)
- Electro-blotting device “Fastblot B33” (Biometra, Germany)
- Electrophoresis power supply (Consort, Belgium)
- ELISA reagent reservoir (Titertek, United Kingdom)

- HiTrap Protein A HP antibody purification column (GE Healthcare Bio-Sciences Corp., USA)
- Homogenisator “Sonopuls HD 3100” (Bandelin, Germany)
- Horizontal gel system “multiSUB” (Cleaver Scientific, UK)
- Laminar flow hood (ESCO, Singapore; Thermo Scientific, USA)
- Magnetic stirrer “Variomag MONO” (Thermo Scientific, USA)
- Membrane filters, Ø: 47 mm, 0.22 µm and 0.45 µm (Roth, Germany)
- Microscope „Nicon ECLIPSE TS100“ (Nikon, USA)
- MiniBIS-Pro imaging system (SERVA electrophoresis, Germany)
- Mini-centrifuge/vortex Micro–Spin “Biosan FV-2400” (Biosan, Latvia)
- pH-meter (Metter Toledo, USA)
- Pipette tips: (0-10 µL), (2-200 µL), (5-300 µL), (100-1000 µL), (0.5-5 mL), (1-10 mL) (Nerbe plus, Germany; Thermo Scientific, USA)
- Pipettes: (0.2-10 µL), (2-20 µL), (10-100 µL), (20-200 µL), (100-1000 µL), (0.5-5 mL), (1-10 mL) (Eppendorf, Germany; Thermo Scientific, USA; Gilson, USA; Rainin, JAV)
- Plate shaker (Titertek, Flow Laboratories, United Kingdom; IKA, Germany)
- Poly(vinylidene fluoride) (PVDF) membrane (Roth, Germany)
- Power Pack P25 “Biometra Standard” (Biometra, Germany)
- Protein purification system “ÄKTA start” (GE Healthcare Life Science, USA)
- Round fraction collector “Frac30” (GE Healthcare Life Science, USA)
- Scales “Kern PCB 1000-12” (KERN & SOHN, Germany)
- Scanner (HP Inc., USA)
- Shaker “Innova 40R Inc/Ref” (Eppendorf, Hamburg)
- Spectrophotometer “Multiscan GO” (Thermo Scientific, USA)
- Spectrophotometer “NanoDrop“ (Thermo Scientific, USA)
- Spectrophotometer “BioPhotometer plus” (Eppendorf, Germany)
- Syringe Filters (Roth, Germany)
- Thermal Cycler “SimpliAmp” (Applied Biosystems, Singapore)
- Thermoshaker (Eppendorf, Germany)
- Tube shaker (IKA, Germany)

- Whatman Grade GB005 Gel Blotting Papers, Cellulose, 1.5 mm, 580 × 580 mm, (GE Healthcare Life Science, USA)

2.1.7. Software

- Microsoft 365
- OriginPro 8 (OriginLab)
- SoftMax Pro 4.0 (Molecular Devices)
- GraphPad Prism 9.5.1 (GraphPad Software)
- NanoDrop 2000 1.6.198 (NanoDrop Software)
- Gel Capture MiniBis v 1.0.1.0 (gel documentation system)

2.2. Methods

2.2.1. Vector isolation

Plasmid DNA for molecular cloning as well as constructed vectors are isolated from overnight cultures using GeneJET Plasmid Miniprep Kit. DNA is purified according to manufacturer's instructions. The concentration and purity of isolated plasmid DNA is measured with NanoDrop spectrophotometer.

2.2.2. Restriction reaction and dephosphorylation

To prepare isolated plasmid DNA for subsequent molecular cloning steps, restriction reactions are performed. Synthetic genes bla_{OXA-48} and $bla_{OXA-134}$ are excised from respective vectors pUC57_OXA-48 and pUC57_OXA-134 using Fast Digest XhoI and Fast Digest BamHI restriction endonucleases. pET28a(+) and pET28-MBP-TEV vectors are cut using the same restriction enzymes in order to achieve DNA overhangs, which are compatible for ligation with the mentioned synthetic genes. To avoid vector re-circularization during ligation step, the 5' ends of pET28a(+) and pET28-MBP-TEV are removed using FastAP (alkaline phosphatase). All reactions are performed according to enzyme manufacturer's instructions. Double digestion and dephosphorylation is achieved in Fast Digest buffer.

2.2.3. DNA electrophoresis in agarose gel

Products of restriction reactions and colony PCR are analyzed by electrophoresis through 1% agarose gel, prepared by melting agarose (1% w/v) in DNA electrophoresis buffer. Samples are dyed by mixing restriction products with 6× DNA Gel Loading Dye and GeneRuler DNA ladders are used as size standards. Electrophoresis is performed at a voltage gradient of 7 V/cm until the bromophenol dye migrates approximately two-thirds of the gel length. After the run ends, the gel is stained in ethidium bromide solution for 10 minutes and DNA fragments are visualized under UV light.

2.2.4. DNA purification from agarose gel

pET28a(+) and pET28-MBP-TEV restriction reaction products are purified from the agarose gel by firstly excising the longer bands- backbones. In the case of pUC57_OXA-48 and pUC57_OXA-134 restriction reaction products, the bands corresponding to the length of the synthetic genes are excised from the gel. After obtaining the bands with specific sequences, DNA is purified using GeneGET Gel Extraction Kit following manufacturer's instructions. The concentration and purity of DNA is measured with NanoDrop spectrophotometer.

2.2.5. Ligation reaction

T4 DNA Ligase is used to covalently link cut backbone and insert DNA. Experiments are performed according to the manufacturer's instructions. Ligation reaction is performed at three different vector:insert ratios – 1:1, 1:3, 1:5. Reaction mixtures are incubated at 16 ℃ for 16 hours. Products of the reaction are stored at 4 ℃ or -20 ℃ until use.

2.2.6. Preparation of chemically competent *E. coli* DH10B and *E. coli* DE3 cells and chemical transformation of bacteria

Overnight bacteria culture is grown in 5 ml of LB medium at 37 ℃ for 16 − 18 hours. 50 µl of overnight culture is transferred into a new sterile LB medium tube and bacteria are grown until culture is at exponential phase (2 − 2.5 hours). All steps are continued at 4 ℃ from here on. Cells are centrifuged for 5 min at 3000 rpm and washed with 5 ml of cold NaCl solution. Then cells are centrifuged again and after removing supernatant, they are suspended in 3 ml of cold $CaCl_2$ solution, vortexed and for 30 min kept on ice. Following the incubation, cells are centrifuged one more time, and after decanting supernatant, they are suspended in the remaining drop of $CaCl_2$ solution.

Chemo transformation if achieved by mixing 50 µl of previously prepared cell solution together with 1 µl of plasmid DNA (concentration – 0.1 µg/µl). In the case of transforming cells with DNA after ligation reaction, the volume of ligation mixture must not exceed 1/10 of the volume of cell suspension. After incubating cells and DNA mixture on ice for 30 min, the tube is placed into 42 ℃ thermoblock for 2 minutes and immediately cooled on ice. Bacteria solution is then diluted with 1 ml of LB medium and incubated at 37 ℃ for 1 hour. Following the incubation, cells are centrifuged at 3000 rpm at room temperature for 5 min, supernatant is decanted, and bacteria are suspended in the remaining drop. 20 µl and 50 µl of cell culture are plated on LB agar, containing respective antibiotic. For cells, transformed with vectors harboring ampicillin resistance gene, final concentration of the antibiotic in LB agar was 100 µg/ml, while in the case of vectors conferring resistance to kanamycin, final concentration of the antibiotic was 30 µg/ml. Plated cells are incubated in a 37 ℃ incubator overnight. Next day a few

colonies from each plate are streaked on new LB agar plates with respective antibiotic and incubated overnight at 37 ℃.

2.2.7. Colony PCR

Once the colonies after chemo transformation are observed and streaked, they are screened for the presence of an insert. In this work, backbone-specific primers and DreamTaq Green PCR Master Mix are used to perform colony PCR. Reaction mixtures and conditions are selected according to the manufacturer's instructions. A small amount of the colony is picked using a sterile pipette tip and swirled in the reaction mixture. PCR products are analyzed in 1% agarose gel. If the gene is inserted, the length of the PCR product is the length of the gene plus the length of the short sequence from the vector. If the gene is not inserted, PCR product results only from the sequence in the vector, thus is short.

2.2.8. Bacteria cryopreservation

For long-term storage of strains, containing verified successful constructs, bacteria are prepared to be frozen. First, 5 ml of overnight culture is grown for 16 hours with appropriate antibiotic. 2 × 150 µl of the culture inoculated in 2 × 5 ml of fresh LB medium, again containing appropriate antibiotic. Bacteria is incubated at 37 ℃ for ~2.5 hours. Following the incubation, cells are centrifuged at 3000 rpm for 5 min at room temperature. After discarding the supernatant, bacteria are suspended in 250 µl of LB medium. Then cell suspension is transferred to the second tube with centrifuged cells and mixed thoroughly. The suspension is transferred to collection tubes and 300 µl of bacterial freezing medium is applied. The tubes with strains are stored in -70 ℃ freezer.

2.2.9. Recombinant gene expression using IPTG

For evaluation of recombinant protein solubility, 0.5 ml of overnight culture is inoculated into 30 ml of LB medium, containing 30 µg/ ml kanamycin. Cells are cultivated at 37 ℃ until cell growth is at mid-log phase OD_{600}≈0.6 − 0.8. Transcription of the recombinant gene is then induced with various concentrations of IPTG ranging from 0.2 to 1 mM. Synthesis is carried out at temperatures from 16 to 37 ℃ with time being dependent on the temperature selected and ranging from 16 to 2.5 hours. After induction is over, cells are collected by centrifugation at 3000 rpm at 4 ℃ for 5 min. Bacteria are then suspended in 3 ml of biomass washing solution containing 1 mM PMSF and 2 µl of antifoaming agent and vortexed well.

Recombinant protein synthesis for purification under denaturing conditions is carried out as follows: 5 ml of overnight culture is inoculated into 300 ml of LB medium, containing 30 µg/ml kanamycin. Cells are cultivated until OD_{600}≈0.6-0.8. Recombinant gene transcription is induced with

1 mM of IPTG and bacteria are incubated at 37 ℃ for 2.5 hours. Cells are centrifuged at 3000 rpm for 15 min at 4 ℃ and stored on ice until further use.

2.2.10. Sonication of bacteria cells

Recombinant protein producing cells are lysed by exposing them to high frequencies of sound using a sonicator. First, bacteria cell suspension, prepared as described in the previous section (2.2.9), is cooled on ice. Sonication is carried out at 60 % amplitude for 4 min (5 s on, 5 s off). This step is repeated three times. After sonication, suspension is centrifuged at 14000 rcf at 4 ℃ for 15 min. Supernatant is analyzed in SDS-PAGE for the presence of soluble recombinant protein.

When purifying recombinant protein under denaturing conditions, biomass, collected in the previous section (2.2.9), is suspended in 12 ml of resuspension buffer. Then cells are disrupted using the same settings: 60 % amplitude for 4 min (5 s on, 5 s off) three times.

2.2.11. Protein purification under denaturing conditions

After recombinant proteins are synthesized, biomass is collected as described in section 2.2.9, and cells are disrupted as outlined in section 2.2.10, the solution is centrifuged at 10000 rcf at 4 ℃ for 15 min. Purification procedure is adapted from "Affinity Chromatography" handbook by GE Healthcare (GE Healthcare, 2016). Supernatant is removed and pellet is then suspended in 9 ml of isolation buffer. Sample is sonicated as stated previously and centrifuged again. Resuspension, sonication and centrifugation are repeated one more time and then inclusion body pellet is suspended in 15 ml of binding buffer. Sample is stirred for 60 min at ambient temperature. Following the incubation, sample is centrifuged at 14000 rcf at 4 ℃ for 15 min to separate insoluble proteins from the solution. Any remaining particles are removed by passing the sample through 0.45 µm filter. The sample with soluble recombinant protein fraction are transferred into a dialysis bag and dialyzed in PBS overnight. Next day, sample from the dialysis bag is centrifuged at 14000 rcf at 4 ℃ for 15 min to remove any formed aggregates. Protein concentration is measured using NanoDrop. Finally, purified proteins are formulated by adding DTT (final concentration 2 mM) and glycerol (final concentration 50 %) and stored at -20 ℃.

2.2.12. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis

To investigate protein synthesis and evaluate their solubility, samples were analyzed sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method, proposed by Laemmli in 1970. Polyacrylamide gel consists of separating 12 % and stacking 4 % parts. Composition of gel solutions is shown in Table 2.6:

Table 2.6. Composition of polyacrylamide gel parts.

Reagent	Separating 12% gel	Stacking 4% gel
Acrylamide-bisacrylamide solution	2 ml	335 µl
1.5 M Tris-HCl, pH 8.8	1.25 ml	625 µl
10% SDS solution	50 µl	25 µl
dH_2O	1.6 ml	1.5 ml
10% APS solution	25 µl	12.5 µl
TEMED	2.5 µl	2.5 µl

First, 12% gel solution is poured in the assembled cassette. To prevent inhibition of gel polymerization by oxygen, the surface of the gel is covered with isopropanol. After 30 minutes, isopropanol is removed and stacking 4 % gel solution is poured on the top of the separating gel. The gel is left to fully polymerize with the comb inserted into the stacking gel part. Samples are prepared in a way so that 0.1-2 µg of protein are loaded into the well. Protein solution is mixed with the loading dye in appropriate volumes and the sample is heated at 100 ℃ for 10 min before loading it into the gel. When analyzing cell lysate, sample is additionally heated with 2% SDS solution at 100 ℃ for 10 min prior to mixing it with the loading dye. Electrophoresis is carried out in SDS-PAGE running buffer at ~10 V/cm of gel until the dye is at the bottom. Lastly, gel is washed in dH_2O and stained with PageBlue protein staining solution according to manufacturer's instructions. After the background has faded enough, gel is visualized using a scanner.

2.2.13. Western Blot

Western Blot is performed to determine whether generated monoclonal antibodies recognize linear epitopes. First, proteins are separated in polyacrylamide gel, as described in section 2.2.12. After that, they are transferred onto the PVDF membrane by semi-dry electrophoretic transfer as follows: PVDF membrane is submerged in methanol for 1 min., two pieces of Whatman paper for WB are soaked in protein transfer buffer and a "sandwich" on a cathode plate is assembled, consisting of one piece of Whatman paper, PVDF membrane, protein gel, and second piece of Whatman paper. Then an anode plate is placed on top and protein transfer is performed for 45 min at 1 mA/1 cm^3. Following the transfer, membrane is incubated in WB blocking solution at 4 ℃ overnight. Next day, blocking solution is removed and the membrane is washed with PBST 3 times. After that membrane is incubated at ambient temperature for 1 hour in the primary antibody solution, consisting of 2 µg/ml monoclonal antibody in 2 % milk powder solution in PBST. The primary antibody solution is then removed, followed by washing of the membrane in PBST 3 times. The membrane is incubated in secondary antibody solution in 2 % milk powder in PBST and then thoroughly washed 5 times with PBST. Finally, the membrane is

incubated in 4-chloro-1-naphthol solution until bands are visible, but not more than 40 min. Reaction is stopped by washing the membrane in dH_2O.

2.2.14. Enzyme-linked immunosorbent assay

96-well plate is coated with 50 µl of 1-10 µg/ml antigen solution. The plate is incubated in in either 4 ℃ overnight or at 37 ℃ for 1 hour. Following the coating step, antigen solution is removed and the uncoated surface is blocked with 300 µl per well of ELISA blocking solution for an hour at room temperature while shaking. After the blocking solution is removed, primary antibody solution is applied. If needed, titration method is used to dilute antibody in column of a row. Primary antibody solution is considered either of specific purified antibody concentration, prepared in PBST, or hybridoma growth medium. The plate with such solution is incubated at ambient temperature for 1 hour while shaking. Then the wells are washed 4 times with washing solution. Secondary antibodies, linked with horse radish peroxidase (HRP) detect primary antibodies and after enzymatic reaction, signal is detected. For this reason, 50 µl of secondary antibody solution is applied to each well and the plate is incubated at room temperature for another hour while shaking. To avoid background signal, conjugate solution is washed thoroughly – 6 times with washing solution. If background signal observed, the wells are additionally washed 2 times with dH_2O in the follow-up experiments. For the colorimetric reaction, 50 µl of TMB substrate solution is applied and depending on when the signal is visible, the plate is incubated at room temperature for up to 15 min. TMB produces a deep blue color during the enzymatic degradation of hydrogen peroxide by HRP. The reaction is stopped by applying 25 µl of STOP solution, which gives a yellow color that absorbs at 450 nm. Using Multiscan GO spectrophotometer optical density is measured at 450 and 620 nm. Final OD value is calculated as the difference between the corresponding OD values.

2.2.15. Collection of mouse polyclonal antibodies

To collect polyclonal antibodies from BALB/c line mouse, it is first euthanized by rapid cervical dislocation and disinfected with 70 % ethanol. After opening the mouse's chest, heart tissue is homogenized, and blood is collected by diluting it with 2 ml of PBS. Blood is centrifuged at 310 × g for 20 min. As polyclonal antibodies remain in the supernatant, the latter is collected and mixed with equal volume of saturated ammonium sulphate solution to precipitate proteins. Sample is incubated at 4 ℃ for at least 24 hours and centrifuged at 14000 × g for 10 min. After that, supernatant is removed and precipitated antibodies are dissolved in PBS. Polyclonal antibodies are mixed with saturated ammonium sulphate solution (ratio 1:1) again and stored at 4 ℃ for further experiments.

2.2.16. Cultivation of Sp2/0 cells

Sp2/0 mice myeloma cells are used for hybridoma technology and grown in suspension culture at 37 ℃ with 5 % CO_2. The cells are thawed by suspending them in serum-free medium. Then, they are centrifuged at 200 × g for 5 min, resuspended in 9 % FBS DMEM, and cultured in a culture flask. For better growth cells are subcultured every 1 −2 days, obtaining better cell viability. Depending on the confluency of the cell culture, cells are subcultured at split ratio 1:2 − 1:5.

2.2.17. Isolation of murine peritoneal macrophages

Primary murine peritoneal macrophage feeder layer is used in hybridoma technology for secretion of interleukins and phagocytosis of cellular debris, which promotes cell growth (Otero-González et al, 2013). First, BALB/c line mouse is euthanized by rapid cervical dislocation and disinfected with 70 % ethanol. Intact peritoneal wall is exposed by making a small incision along the midline using sterile scissors and retracting the abdominal skin. After that, the peritoneal wall is washed with 3 ml of serum-free medium. A small incision is then made in the abdominal area of the peritoneal wall and macrophages are collected by suspending them in 10 ml of serum-free medium. Suspension is centrifuged at 200 × g for 5 min. After removing supernatant, cells are suspended in 3 ml of serum-free medium and counted using Bürker hemocytometer as shown in Fig 2.1. The total number of macrophages is calculated using (1) formula.

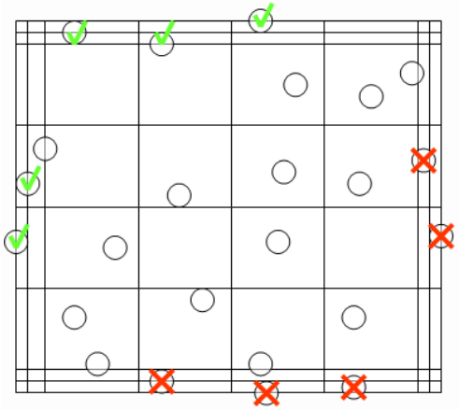

Fig. 2.1. Counting system using Bürker hemocytometer. Green check mark symbol indicates a cell, that is included, while red cross symbol indicates a cell, that is excluded when counting.

$$Total\ number\ of\ macrophages = A \times B \times C \times 10^4 \quad (1)$$

A – number of cells in the hemocytometer square; B – sample dilution factor; C– volume of serum-free medium, used to suspend the cells.

Isolated macrophages are grown at 37 °C with 5 % CO_2 at appropriate density. Before applying other cells to the culture, the macrophages are allowed to adhere to the plastic surface for at least 1 hour.

2.2.18. Hybridoma technology

Hybridoma technology is one of the most common methods used to produce monoclonal antibodies (MAbs). In this method, after immunizing a mouse with specific antigen, antibody-producing B lymphocytes are isolated from the spleen and fused with immortal myeloma cells to form hybrid cells, called hybridoma cells (Mitra and Tomar, 2021). Hybrid cells have the characteristics of both cells – they produce MAbs limitlessly. The process of hybridoma technology is represented in Fig. 2.2.

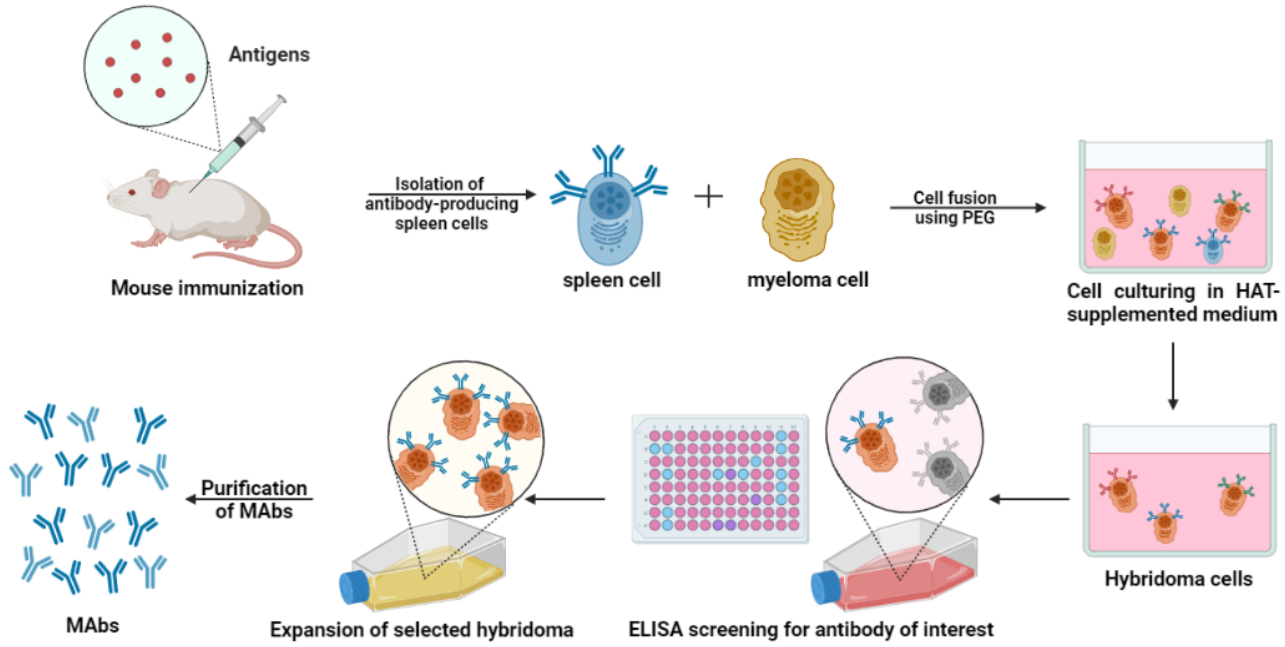

Fig. 2.2. General representation of the hybridoma method. Created using Biorender.com.

<u>BALB/c mice immunization</u>

8-week female mice are immunized three or four times every 28 days. For the first immunization, 200 µl of solution, consisting of 50 µg of antigen and Freund's complete adjuvant (volume ratio 1:1) is injected into a mouse. Complete Freund's adjuvant is composed of a light mineral oil, a surfactant agent mannide monooleate, and heat-killed and dried mycobacterial cells, which promote activation and proliferation of CD+ lymphocytes (Stils, 2005). Second immunization solution contains 50 µg of antigen mixed with incomplete Freund's adjuvant, which is free from mycobacterial cells. This step induces CD4+ lymphocyte activation and proliferation, resulting in production of high-titer, high-affinity, and high-avidity antibodies (Billiau and Matthys, 2001; Stils, 2005). Subsequent immunization solutions

contain the previous amount of antigen in PBS. To check antibody titer, blood is collected by tail snip before every round of immunization. The blood is diluted 25 × with PBS and stored at -20 ℃.

A week before hybridization, ELISA is performed to test mice blood samples and determine mice antibody titers. Antibody titer refers to the level of specific antibodies produced by the immune system of a mouse in response to a particular antigen. It is expressed as the highest dilution of antibody sample at which an immune response is still detectable. The mouse with the highest antibody titer is used to proceed with the following steps of hybridoma technology and receives a booster shot – another immunization with 50 µg of antigen in PBS.

Sp2/0 and spleen cell preparation

Myeloma cells are cultured as described in section 2.2.16. Prior to hybridization, the cells are subcultured several times, grown to logarithmic phase. Myeloma cells are suspended and centrifuged at 300 × g for 5 min. Growth medium is saved for hybridization medium preparation, while cells are suspended in serum-free medium and counted using Bürker hemocytometer (section 2.2.17).

Spleen cells are isolated after the mouse is euthanized and peritoneal macrophages are isolated as described in section 2.2.17. First, spleen carefully dissected and placed in a sterile plate with serum-free medium. Then, it is crushed thoroughly to remove all the cells from the spleen capsule. Cells are collected and centrifuged at 300 × g for 5 min, suspended in serum-free medium and counted using Bürker hemocytometer (section 2.2.17). Polyclonal antibodies are collected (section 2.2.15) to be used as control in ELISA experiments later.

Cell fusion

Cell fusion is carried out at spleen to myeloma ratio from 1:4 to 1:8 (preferably 1:5 or 1:6). All spleen cells are used for the procedure, while the number of myeloma cells used is calculated accordingly. After mixing the cells, the suspension is centrifuged at 300 × g for 5 min and every drop of supernatant is removed, leaving the cells as dry as possible. The cells are then spread out at the bottom of the tube, and 1 ml of PEG-400 is slowly added over a period of 1 minute. PEG fuses the plasma membranes of adjacent myeloma and spleen cells, forming a single cell with two or more nuclei (Mitra and Tomar, 2021). Then, the tube is slowly rotated for 2 min, followed by 10 ml of serum-free medium over the next 2 min. 40 more ml are added and the suspension is centrifuged at 300 × g for 5 min. After removing supernatant, the cells are suspended in hybridization medium composed DMEM supplemented with 15 % FBS, 1 × HAT, Sp2/0 growth medium and macrophages. The suspension is finally dispensed into sterile

96-well plates, with a cell density of 2.5×10^5 cells/well (200 µl). Hybridoma cells are left undisturbed in an incubator at 37 °C with 5 % CO_2 for a week.

Changing of hybridoma medium

After a week, hybridoma growth medium is changed by removing 80 µl from each well and applying 90 µl of fresh DMEM, supplied with 1 × HT. After 4 days, the medium is changed once again with the same 1 × HT DMEM.

Screening of hybridoma cells

2 days after the second medium change every well is tested by ELISA to determine the presence of antibodies of interest. 65 µl of hybridoma cell growth medium from each well are transferred into the corresponding well in ELISA reaction plate and 80 µl fresh DMEM, supplemented with 15 % FBS, are applied to the cells. Polyclonal antibodies collected from the same mouse are used as positive control in ELISA. After the reaction described in section 2.2.14 is completed, positive wells are examined under the microscope for the presence of distinct and viable clones (groups of identical cells).

The next day, the second screening is performed by testing positive wells with single clones. This time, 120 µl of growth medium is divided into two plates: one containing target antigen, the other containing a different antigen, which should not be detected by specific antibodies. The same positive control is used as previously. Clones that produce the best-reacting antibodies, are selected for cloning.

Cloning of hybridoma cells

To create stable cell lines for MAb production, hybridoma cells are cloned by serial dilution in 96-well plates. Cloning is performed in 96 well plates prepared the day before (ideally) as described in section 2.2.17. The cells are incubated at 37 °C with 5 % CO_2 for 6 − 10 days depending on the size of the clones and examined under the microscope. ELISA is performed to test the wells with 1 to 2 clones and after positive re-clones are examined, they are re-cloned again. The method is repeated until stable line is obtained.

Hybridoma cell cultivation

Once a stable MAb-producing cell line is achieved, the cells are inoculated in 24-well plate, containing murine macrophage culture, prepared as described in section 2.2.17. Then, hybridoma cells are transferred to larger tissue culture vials to facilitate the collection of hybridoma growth medium. Finally, after enough medium is obtained, it is collected for MAb purification and stored at 4 °C. The

cells of several clones are collected and frozen for long-term preservation as described in the following section (2.2.19).

2.2.19. Cryopreservation of hybridoma cell lines

First, the cells are examined under the microscope to ensure that they are in logarithmic growth phase. Then, they are collected by centrifugation at 300 × g for 5 min. After removing growth medium, cells are suspended in cell cryopreservation medium at the density of 1×10^6 to 1×10^7 cells/ml and transferred to cryopreservation vials. The cells are left in – 70 ℃ overnight and moved to a liquid nitrogen dewar.

2.2.20. Antibody isotyping

To choose the strategy for MAb purification, MAb class (e.g., IgG vs. IgM) and subclass (e.g., IgG1 vs. IgG2) are determined. Antibody isotyping is performed using "BD Mouse Immunoglobulin Isotyping ELISA Kit". This kit also identifies kappa or lambda light chain. The method was carried out according to manufacturer's instructions.

2.2.21. MAb purification from hybridoma growth medium

MAbs are purified from the cell growth medium using affinity chromatography. Purification conditions are selected according to the isotype determined as described in the section above (2.2.20). In this work, MAbs are purified according to protocol for IgG1 type purification. First, medium is centrifuged at 1 000 × g for 10 min to remove any remaining cell debris. Sample is prepared by diluting it two-fold using binding buffer and filtering through 0.2 µm filter. Then, ÄKTA start protein purification system as well as HiTrap Protein A HP antibody purification column are prepared by running loading and elution buffers through the system. Sample is loaded onto the column and unbound proteins are washed using binding buffer. The bound MAbs are eluted by step gradient using elution buffer. After collecting peak fractions and measuring antibody concentration, the MAbs are dialyzed in PBS, and if needed, concentrated using Amicon Ultra 0.5 ml filters. Finally, purified MAbs are sterile filtered using 0.2 µm filter and stored at 4 ℃.

2.2.22. Horseradish peroxidase (HRP) labeling of the MAbs

HRP-labeled MAbs allow direct detection of the antigen in Western Blot and ELISA experiments. Purified MAbs are labeled using HRP : antibody ratio of 1:1. HRP is dissolved in dH_2O and a final concentration of 0.2 M of $NaIO_4$ solution is added. HRP is activated by spinning the mixture in the dark at ambient temperature for 20 min. Then HRP solution is desalted with 1 mM sodium acetate solution using "Sephadex G- 25" desalting column. Activated HRP is mixed with 0.2 M sodium carbonate, where

the volume of the latter is 1/10 total solution volume. The mixture is incubated at room temperature for 1 hour. Then, final concentration of 0.4 mg/ml of $NaBH_4$ solution is applied and the mixture is incubated at room temperature for another hour. After the incubation, MAb-HRP conjugate buffer is exchanged with PBS using Amicon Ultra 0.5 ml filters. Finally, labeled MAb is formulated with BSA (2 %) and glycerol (50 %).

2.2.23. Sandwich ELISA

Sandwich ELISA is used for direct detection of the antigen using HRP-labeled antibodies. First, 5 µg/ml MAb capture antibody solution is prepared in PBS or coating buffer and aliquoted into the wells of a 96-well plate (100 µl/well). The plate is incubated at 4 ℃ overnight. Next day, antibody solution is removed and 300 µl of blocking solution, either 2 % BSA in PBST or "ROTI Block", is applied to each well. After incubating the plate on a shaker at room temperature for 1 hour, blocking solution is removed and 100 µl of antigen solution, prepared in either 3 % milk powder in PBST or 2 % BSA in PBST, is applied to each well. When making a standard curve, specific concentration is used and titration method is applied to dilute the antigen through row or column. The reaction plate is incubated on a shaker at room temperature for another hour, after that, the plate is washed 4 times with washing solution. To detect captured antigen, 100 µl of appropriately diluted HRP-labeled antibody is applied. 3 % milk powder in PBST or 2 % BSA in PBST is used for the dilution of the antibody. Following another incubation, the wells are washed 6 times and after the plate is dried, 100 µl of TMB substrate is applied to each well. The plate is incubated on a shaker at room temperature for 10 min and the reaction if stopped by applying 50 µl of STOP solution to each well. Absorbance is measured at 450 and 620 nm using "Multiscan GO" spectrophotometer. The final OD value is the difference between the corresponding OD values at the mentioned wavelengths.

2.2.24. Statistical analysis

MS Excel and GraphPad Prism 9.5.1 were used for statistical analysis. For comparison of two samples, a paired T-test was used. If the p-value is less than 0.05, the difference between the samples is considered significant.

3. RESULTS AND DISCUSSION

Antibiotic resistance has been a problem of deep concern in clinics of both outpatients and inpatients for the last two decades. Increased use and misuse of antibiotics in medicine, when the patient is not diagnosed properly before prescribing treatment primarily contributes to the phenomenon (Alekshun and Levy, 2007; Chang et al., 2019; Hu et al., 2003). The emergence of antibiotic resistance genes is particularly alarming in multidrug resistant pathogens such as *K. pneumoniae*, *A. baumannii*, methicillin-resistant *S. aureus*, extensively drug-resistant *M. tuberculosis* and other *Enterobacteriaceae* (Alekshun and Levy, 2007).

To prescribe appropriate antibiotics, rapid detection tools must be available. This is especially important for low-resource settings, where there is no possibility of performing complex laboratory tests as they require certain equipment as well as trained staff. Immunodetection is a simple, cost-effective, and rapid method that can be applied to detect antibiotic resistance proteins. (Di Nardo et al., 2021). To this day, there have been only a few antibody-based POC tests developed for the detection of antibiotic resistance in *P. aeruginosa,* MRSA, and Enterobacterial isolates (Glupczynski et al., 2017; Kitao et al., 2010; Yamada et al., 2013) and they are significantly limited by their inability to directly analyze clinical samples.

As β-lactams are the most commonly used antibiotics to treat bacterial infections, they continue to be the cause of resistance emergence in Gram-negative bacteria. The continuous exposure of clinically significant pathogens to these antibiotics has induced the production and spread of β-lactamases in these bacteria, further expanding their activity towards newer versions of β-lactams (Shaikh et al., 2015). The focus of this work is to develop monoclonal antibodies against β-lactamases OXA-48, OXA-134, SHV-42, SME-3, ADC-144 to contribute to the development of improved diagnostic strategies for the detection of β-lactamase-mediated antibiotic resistance.

3.1. Production of recombinant OXA-48, OXA-134, OXA-48-MBP, and OXA-134-MBP proteins

To generate OXA-48-specific and OXA-134-specific monoclonal antibodies, β-lactamases were used as antigens in hybridoma technology. First, genes bla_{OXA-48} and $bla_{OXA-134}$, encoding the proteins were cloned and expressed in *E. coli* expression strains. β-lactamases are located in the periplasm, where they inactivate β-lactams before they reach penicillin binding proteins – their targets on the cytoplasmic membrane (Ciofu et al., 2000). Overexpression of the gene often results in inclusion body formation. For this reason, MBP-fused versions of the protein were constructed, as MBP is shown to increase solubility and stability of the recombinant proteins.

3.1.1. Molecular cloning of *bla*$_{OXA-48}$ and *bla*$_{OXA-134}$ genes

To produce recombinant OXA-48 and OXA-134 proteins, *bla*$_{OXA-48}$ and *bla*$_{OXA-134}$ were cloned into expression vectors pET28a(+) and pET28-MBP-TEV. pET28a(+) construct allows purification of the protein of interest using affinity chromatography, as the protein in pET28a(+) is fused with 6 × His-tag. pET28-MBP-TEV vector is an expression vector in which recombinant gene is fused with MBP encoding sequence, separated by TEV protease recognition sequence for the cleavage of fusion partner after expression and purification is done.

Once the presence of the insert was confirmed, vectors were sent for sequencing to verify successful cloning (no mutations/errors, correct reading frame and orientation of the gene). Finally, the constructs were transformed into *E. coli* expression strains: BL21 (DE3), BL21 Tuner (DE3), BL21 Star (DE3), Rosetta (DE3).

3.1.2. Assessment of recombinant OXA-48, OXA-134, OXA-48-MBP, and OXA-134 solubility

As mentioned previously, expression and purification of β-lactamases is a challenging process. To assess the solubility of the recombinant enzymes, multiple expression conditions were tested. These include: IPTG concentrations (0.2 mM, 0.5 mM, 0.8 mM, 1 mM), induction time (2.5 h, 3 h, 5 h, 16 h) and temperature (37 ℃, 30 ℃, 25 ℃, 20 ℃, 16 ℃), as well as *E. coli* BL21 Tuner (DE3), BL21 Star (DE3), and Rosetta (DE3) strains, used to increase solubility of recombinant protein.

Following the induction, bands corresponding to the target recombinant protein were observed in *E. coli* cell lysates, indicating successful expression. The gels shown in Fig. 3.1 (A and B) illustrate the pattern where the recombinant β-lactamases OXA-48 and OXA-134 consistently appeared as insoluble aggregates under all tested conditions. Fusion with MBP tag increased the yield of soluble protein, however approximately 50 % remained in an aggregated of insoluble form. The biggest yield of soluble protein was observed when inducing protein synthesis in *E. coli* Tuner (DE3) at 30 ℃ for 3 hours (Fig. 3.1. C and D). These conditions were used to obtain bacterial biomass for purification of OXA-48-MBP and OXA-134-MBP. Purification of the proteins using affinity chromatography was carried out by PhD student Vytautas Rudokas.

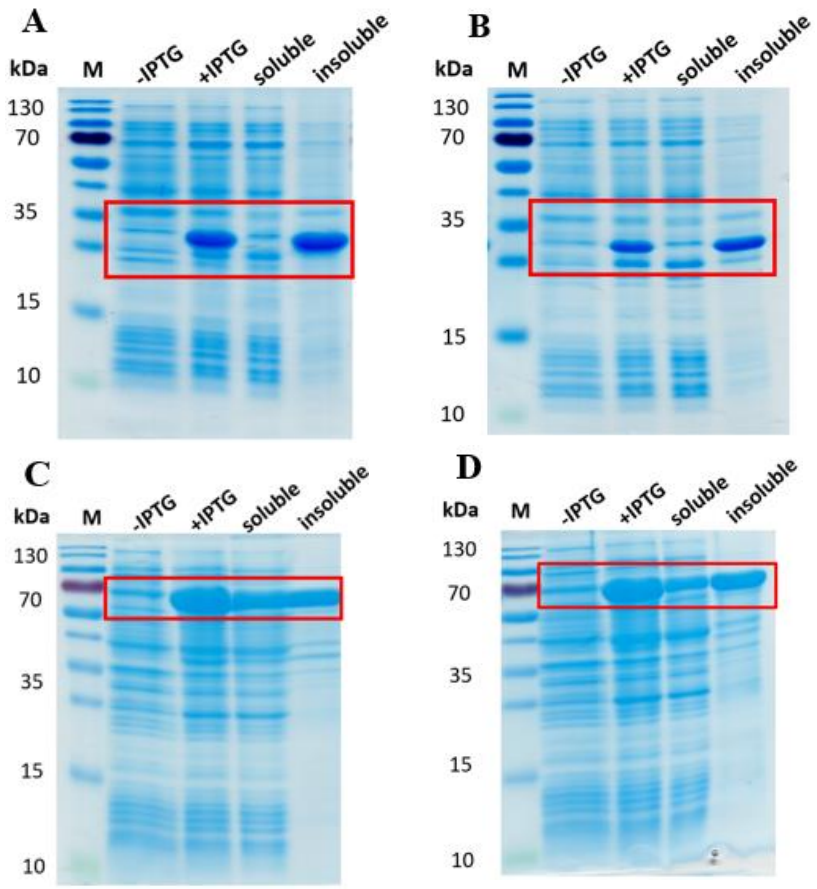

Fig. 3.1. Solubility of recombinant OXA-48, OXA-134, OXA-48-MBP, and OXA-134-MBP proteins in *E. coli* lysates. Analyses were carried out in 12 % SDS-PAGE gel. A – OXA-48; B – OXA-134 ; C – OXA-48-MBP ; D – OXA-134-MBP. Syntheses of all the proteins shown in the representative gels were induced with 1 mM IPTG in *E. coli* Tuner (DE3) cells at 30 ℃ for 3 hours.

3.1.3. Purification of OXA-48 and OXA-134 under denaturing conditions

Antigens for the immunizations were selected considering the observations and experience of colleagues from the department. They reported limited success in generating monoclonal antibodies when fusing the antigen of interest with the MBP tag in hybridoma technology. More MBP-specific MAbs rather than antigen-specific MAbs were obtained in the process, which suggested that MBP tag might hinder the availability of antigen epitopes. To potentially increase the number of generated antibodies, both variants of the proteins were used separately for immunizations.

OXA-48 and OXA-134 were obtained by purifying the proteins from inclusion bodies under denaturing conditions (described in section 2.2.11). The conditions in which the OXA protein constituted the majority of the insoluble protein fraction were selected for purification (Fig. 3.1 A and B). During the purification process, both soluble and insoluble protein fractions were obtained (Fig. 3.2 A and B). The protein gels reveal the presence of OXA-48 and OXA-134 bands in the soluble fractions, indicating the successful isolation of part of the proteins in their soluble form. Denaturing conditions appeared to

be a solution for the purification of difficult to synthesize recombinant β-lactamases. However, it is significant that despite purification efforts, some portion of the target proteins remained in the insoluble fraction, highlighting the challenge in obtaining completely soluble β-lactamases.

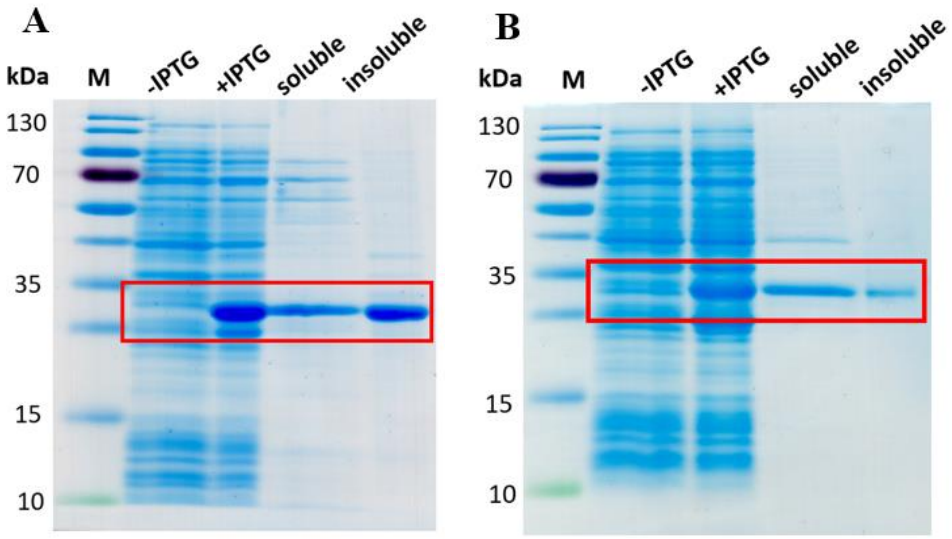

Fig. 3.2. Solubility of OXA-48 and OXA-134 proteins, purified under denaturing conditions. Analyses were carried out in 12 % SDS-PAGE gel. A – OXA-48; B – OXA-134. Syntheses of both proteins were induced with 1 mM IPTG in *E. coli* Tuner (DE3) cells at 30 ℃ for 3 hours.

3.2. Generation of monoclonal antibodies against OXA-48 and OXA-134 β-lactamases

Monoclonal antibodies are immunological tools, widely used in diagnostics for the detection of antigens as well as antibodies against bacterial or viral diseases (Siddiqui, 2010). Immunodetection serves as a simple, cost-effective, and rapid point-of-care method. Unfortunately, a limited number of tests are available for the detection of antibiotic resistance. To increase the available number of monoclonal antibodies that could detect significant antimicrobial resistance proteins, mice immunization with β-lactamases and subsequent monoclonal antibody producing cell lines development by hybridoma technology was performed.

For the generation of OXA-48 and OXA-134-specific MAbs, two groups of tree mice were immunized four times with the corresponding antigens. OXA-48-MBP and OXA-134-MBP-specific MAb generation was carried out by immunizing one mouse three times per antigen. Mice blood samples were collected before the first and after each immunization to evaluate immune response to the antigens.

3.2.1. Evaluation of immunogenicity of OXA-48, OXA-134 and their fusion proteins in mice

Before proceeding with the hybridization step in hybridoma technology, blood samples after each immunization were collected and tested in indirect ELISA to assess general immunogenicity of the

antigens and, if needed, select the highest antibody titer responder mouse from the group for the generation of monoclonal antibodies by hybridoma technology.

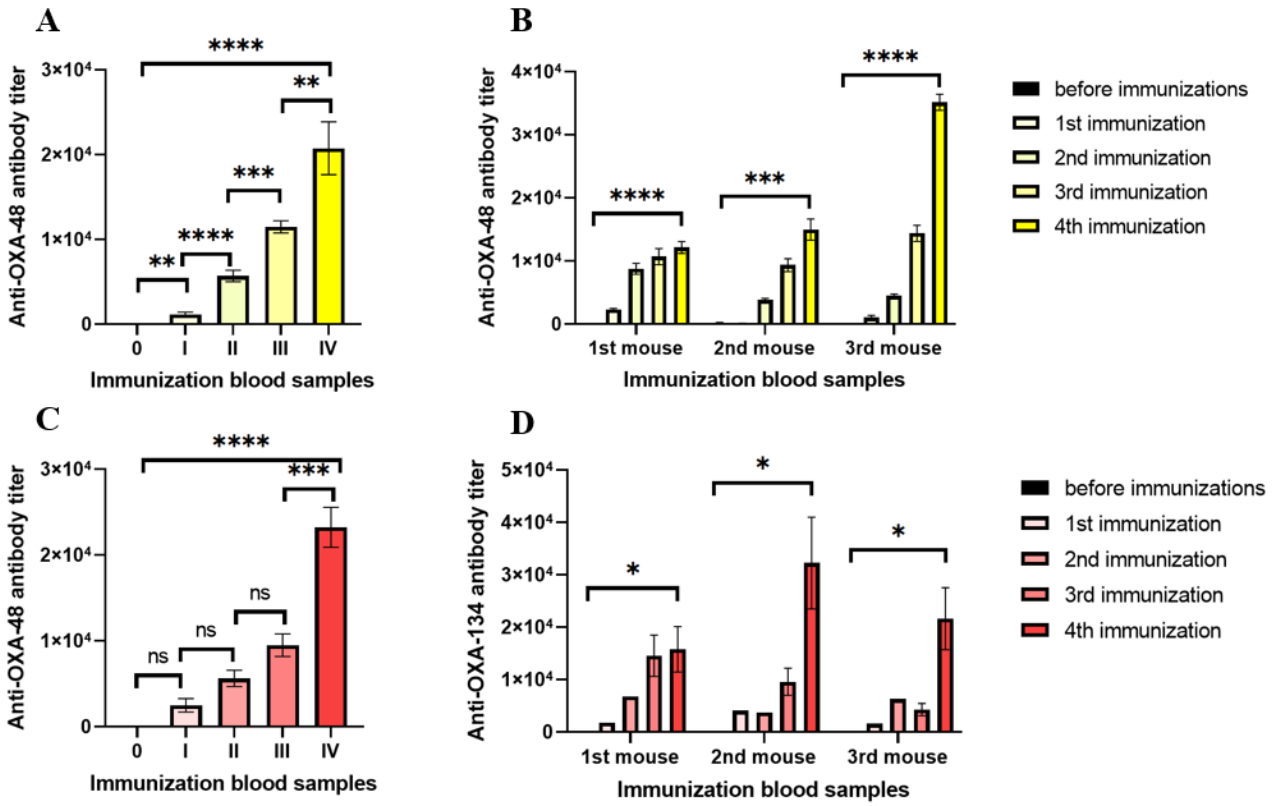

Fig. 3.3. Evaluation of OXA-48 and OXA-134 immunogenicity in mice. A, C – the means of antibody titers determined after each immunization with OXA-48 and OXA-134 respectively; B, D – mean titer values of each mouse after immunizations with OXA-48 and OXA-134 respectively. Every two values were compared by applying T-test (ns – $p>0.05$, *- $p<0.05$, ** - $p<0.01$, ***-$p<0.001$, **** - $p<0.0001$, N=3). Error bars represent standard error of mean.

To assess the significance of changes in antibody titers, a paired T-test analysis comparing the antibody titers after each immunization was conducted. The results of the analysis revealed a statistically significant increase in antibody titers after each immunization with OXA-48 ($p<0.05$) (Fig. 3.3 A). Analysis of blood samples from OXA-134 revealed that although individual paired T-tests comparing antibody titers after each immunization to the prior one did not show significant differences ($p>0.05$) (except after third immunization), the overall increase in antibody titers was significant. Specifically, a significant difference was observed when comparing the antibody titers in blood prior to any immunizations with the antibody titers after the fourth immunization ($p<0.05$). These results indicate that

the immunizations successfully elicited an immune response, and the antigens are immunogenic in BALB/c mice.

Immune response was elicited in mice immunized with OXA-48-MBP and OXA-134-MBP as statistically significant overall increase in anti-OXA-48-MBP and anti-OXA-134-MBP antibody titers was observed (Fig. 3.4 A and B). These findings suggest that both OXA fusion proteins are immunogenic and suitable to use in hybridoma technology as antigens. OXA-48-MBP and OXA-134-MBP mice were sacrificed to collect spleen cells and use them in the fusion step.

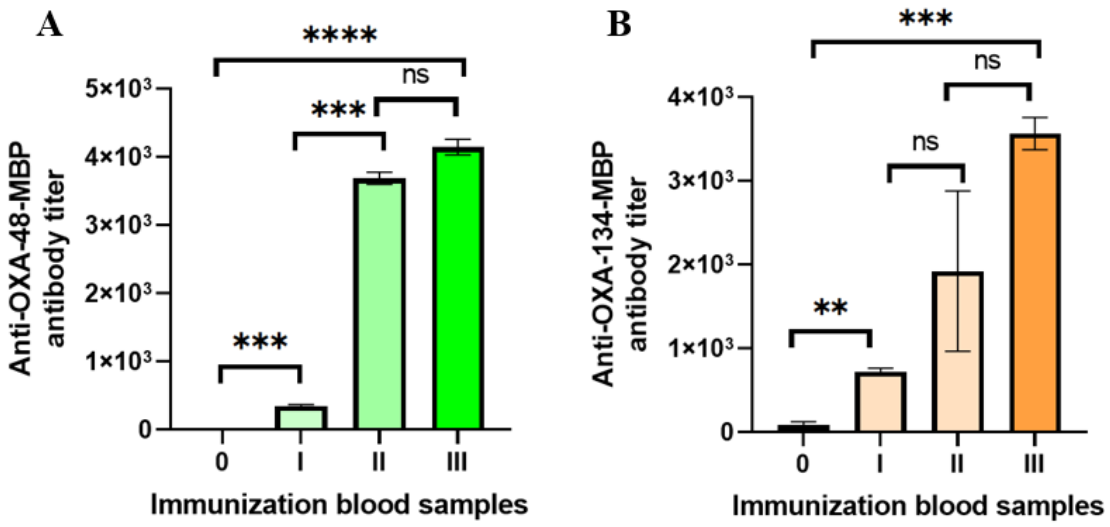

Fig. 3.4. Evaluation of OXA-48-MBP and OXA-134-MBP immunogenicity in mice. Graphs show the means of antibody titers determined after each immunization with respective antigens. Every two values were compared by applying T-test (ns – $p>0.05$, *- $p<0.05$, ** - $p<0.01$, ***-$p<0.001$, **** - $p<0.0001$). Error bars represent standard error of mean.

It is worth emphasizing that to ensure that the observed antibody reactivity was not solely attributed to the MBP portion of the fusion protein, additional testing using tag-free OXA proteins was performed. The positive results obtained from the analysis using tag-free proteins confirmed that the immune response detected was specifically directed against the OXA-48 and OXA-134 components as well (data not shown).

3.2.2. Development of OXA-48, OXA-134-specific monoclonal antibodies

Selection of the mouse used for hybridization was based on the evaluation of antibody titer increase in each mouse separately. Statistical analysis revealed that a significant increase in OXA-48 or OXA-134-specific antibodies was detected in all immunized mice (Fig. 3.3 B and D). In the OXA-48 group, the antibody titer of the third mouse was the highest, thus, this animal was sacrificed for the experiments. OXA-134-specific antibody titer was the highest in the corresponding group's decond

mouse, so this mouse was sacrificed as well. First hybridizations yielded one cell line secreting OXA-48-specific MAbs, and two OXA-134-specific MAb secreting cell lines. For the purpose of generating more OXA-specific MAbs, mice with second highest antibody titers were sacrificed for the experiments (the second from OXA-48 group and the third mouse from OXA-134 group). Following the hybridizations, one more OXA-134 MAb secreting cell line was obtained, while OXA-48 hybridization was unsuccessful.

Regarding the MBP-fused protein versions, two cell lines secreting anti-OXA-48-MBP MAbs were produced, while no OXA-134-MBP-specific MAbs were obtained. In total, three hybridomas secreting OXA-134 MAbs, one hybridoma secreting OXA-48 MAbs, and two hybridomas secreting OXA-48-MBP MAbs were generated.

3.3. Generation of monoclonal antibodies against SME-3, SHV-42, and ADC-144 β-lactamases

Since there was no notable difference in the number of MAbs specific to tag-free OXA protein, compared to MBP-fused versions, MAbs against SME-3, SHV-42, and ADC-144 were generated using already purified MBP-fused versions of those proteins.

Immunizations with SME-3-MBP, SHV-42-MBP, and ADC-144-MBP were carried out in groups of two mice. SME-3-MBP mice were immunized three times, while SHV-42-MBP and ADC-144-MBP mice received four immunizations. The decision to administer a fourth immunization was based on the observed increase of antigen-specific antibody titers in OXA-48 and OXA-134 mice (Fig. 3.3). Mice blood samples were collected before the first and after each immunization to evaluate immune response to the antigens.

3.3.1. Evaluation of immunogenicity of SME-3-MBP, SHV-42-MBP, and ADC-144-MBP proteins in mice

Blood samples were analyzed by performing indirect ELISA to assess immunogenicity of the fusion proteins used for immunizations. Statistical analysis using T-test comparing antibody titers after each immunization was conducted. As shown in Fig. 3.5. A, significant increase in anti-SME-3-MBP antibody titers was observed after each immunization ($p<0.01$). Regarding the SHV-42-MBP group, fourth immunization had no significant effect on the number of anti-SHV-42-MBP antibody titers, however, immunization with this fusion protein elicited overall significant immune response (Fig. 3.5. C). Furthermore, immunizations with ADC-144-MBP fusion protein also elicited significant immune response in mice, the fourth one being significant in increasing anto-ADC-144-MBP antibody titers (Fig. 3.5. E).

3.3.2. Development of SME-3, SHV-42, and ADC-144 - specific monoclonal antibodies

Selection of the more suitable mouse for hybridization was based on individual mouse blood sample evaluation. Analysis of SME-3-MBP immunized mice revealed that antibody titers were slightly higher in the first mouse (Fig. 3.5. B), just like the first mouse presented higher anti-SHV-42-MBP antibody titer in the SHV-42-MBP group. What is more, higher anti-ADC-144-MBP antibody titer was detected in the second mouse from the corresponding group. All the mentioned mice were sacrificed to generate respective β-lactamase-specific MAbs.

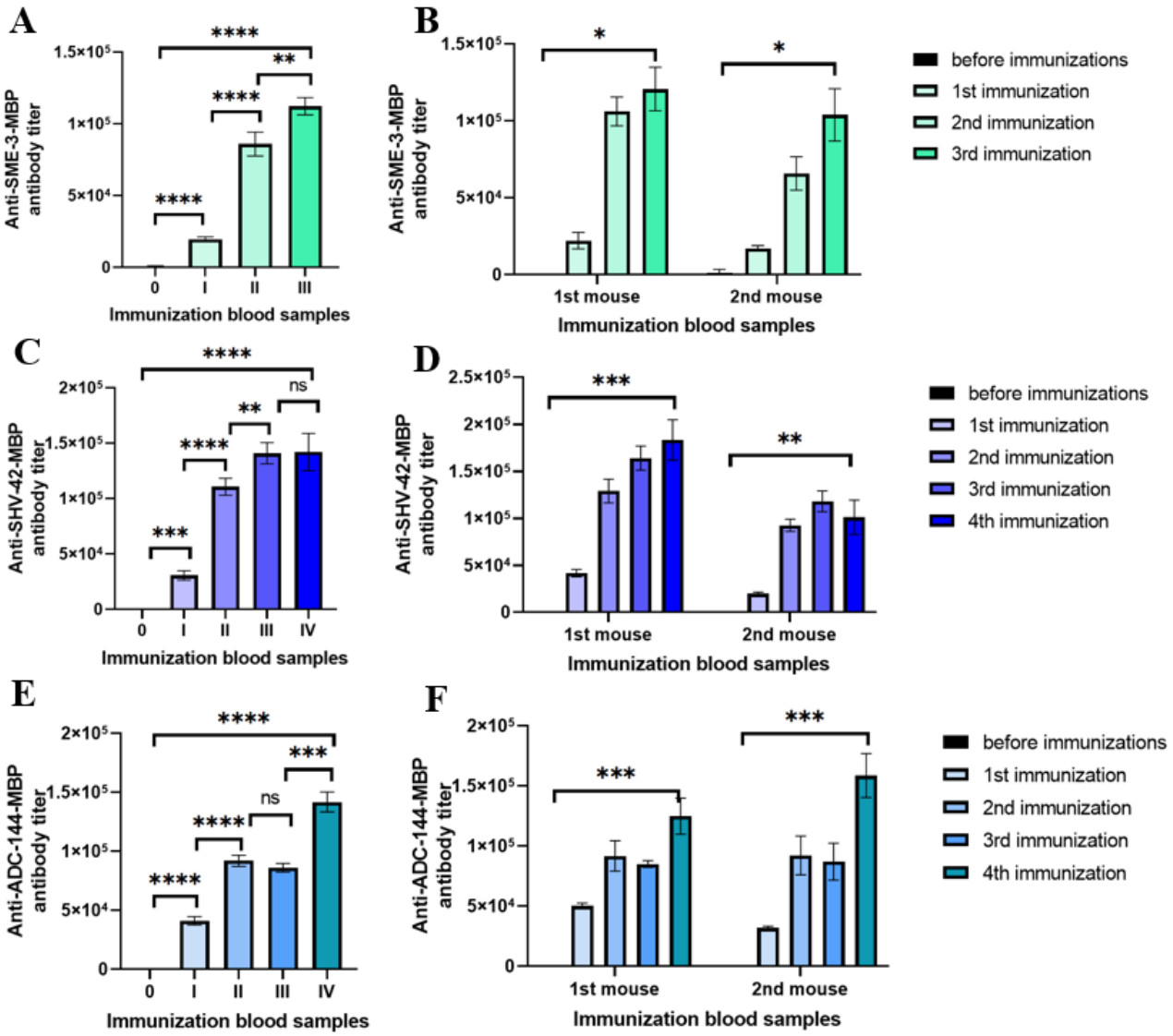

Fig. 3.5. Evaluation of SME-3-MBP, SHV-42-MBP, and ADC-144-MBP immunogenicity in mice. A, C, D – the means of antibody titers determined after each immunization with SME-3-MBP, SHV-42-MBP, ADC-144-MBP respectively; B, D, F – mean titer values of each mouse after immunizations with OXA-48 and OXA-134 respectively. Every two values were compared by applying T-test (ns – $p>0.05$, *- $p<0.05$, ** - $p<0.01$, ***-$p<0.001$, **** - $p<0.0001$, N=2). Error bars represent standard error of mean.

Following the first hybridizations, one anti-SHV-42-MBP secreting cell line, three anti SME-3-MBP secreting cell lines, and no anti-ADC-144-MBP secreting cell lines were produced. To obtain more SHV-42-MBP-specific MAbs, a second mouse from the corresponding group was sacrificed. Also, previously unsuccessful hybridization of the ADC-144-MBP group was repeated sacrificing the other mouse from the group. Finally, a total of two anti-SHV-42-MBP secreting cell lines, two ADC-144-MBP secreting cell lines and three SME-3-MBP secreting cell lines were generated.

3.4. Characterization of monoclonal antibodies against target β-lactamases

All generated MAbs were characterized by immunochemical methods: indirect ELISA (used for determination of apparent equilibrium constant and assessment of specificity), sandwich ELISA (isotyping), and Western Blot (used to determine specificity of the MAbs and whether they recognize linear or conformational epitopes).

3.4.1. Isotype determination of monoclonal antibodies

In this study, the first step in characterizing MAb involved identifying their antibody class, subclass, and type of light chain. To achieve this, BD Mouse Immunoglobulin Isotyping ELISA Kit was employed. The newly generated MAbs were captured by two out of eight rat monoclonal antibodies, which are designed to recognize a specific mouse immunoglobulin isotype (one antibody determines the class/subclass and another determines the light chain type). Subsequently, the captured antibodies were detected using HRP-conjugated rat anti-mouse Ig antibody. Following the colorimetric reaction, a signal appeared in the wells corresponding to the respective isotype and light chain type. The findings of isotype determination are presented in Table 3.1.

Table 3.1. β-lactamase-specific monoclonal antibody isotyping.

	OXA-48	OXA-48-MBP		OXA-134			SHV-42-MBP		ADC-144-MBP		SME-3-MBP		
	11H2	24B4	24C9	3F1	12F3	13C3	12G4	15G4	3F7	19B9	12G7	19C12	24B10
IgG1	+	+	+	+	+	+	+	+	+	+	+	+	+
IgG2a													
IgG2b													
IgG3													
IgA													
IgM													
κ	+	+	+	+	+	+	+	+	+	+	+	+	+
λ													

All thirteen monoclonal antibodies against target β-lactamases were shown to be of IgG1 subclass and possessed λ light chains. Consequently, affinity chromatography using protein A was chosen as the

strategy for MAb purification. All generated monoclonal antibodies were purified for further characterization, with four antibodies (11H2, 3F1, 12F3, 13C3) purified in this study, while the remaining (24B4, 24C9, 12G4, 15G4, 3F7, 19B9, 12G7, 19C12, 24B10) antibodies were purified by Ignas Ušinskas.

3.4.2. Monoclonal antibody apparent equilibrium dissociation constant determination

Antibody affinity is the strength with which an antibody binds to the epitope on an antigen. Apparent equilibrium dissociation constant (K_D) of an antibody is a quantitative measurement of antibody affinity. It is a ratio of k_{off}/k_{on} between the antibody and its antigen epitope. As K_D and affinity are inversely related, the lower the K_D value, the higher the affinity of the antibody. The value of apparent equilibrium dissociation constant is the molar concentration of an antibody required to occupy half the available antigen molecules an a solution (Abbas et al., 2016). K_D determination is an important aspect of characterizing the generated monoclonal antibodies since the values are helpful in choosing MAbs for the development of diagnostic tests.

Table 3.2. Apparent equilibrium dissociation constants of target β-lactamase-specific MAbs, as determined by indirect ELISA.

Antigen	MAb	K_D value (mol/l)	± SEM
OXA-48	11H2	$3{,}69 \times 10^{-10}$	$9{,}04 \times 10^{-12}$
OXA-48-MBP	24B4	$7{,}42 \times 10^{-10}$	$2{,}64 \times 10^{-11}$
	24C9	$4{,}21 \times 10^{-8}$	$4{,}02 \times 10^{-9}$
OXA-134	3F1	$1{,}15 \times 10^{-10}$	$1{,}14 \times 10^{-11}$
	12F3	$3{,}21 \times 10^{-11}$	$1{,}08 \times 10^{-12}$
	13C3	$4{,}83\times 10^{-11}$	$2{,}07 \times 10^{-12}$
ADC-144-MBP	3F7	$3{,}65 \times 10^{-10}$	$9{,}52 \times 10^{-11}$
	19B9	$1{,}76 \times 10^{-10}$	$3{,}93 \times 10^{-11}$
SHV-42-MBP	12G4	$9{,}04 \times 10^{-11}$	$2{,}80 \times 10^{-11}$
	15G4	$9{,}79 \times 10^{-8}$	$8{,}37 \times 10^{-8}$
SME-3-MBP	12G7	$7{,}84 \times 10^{-11}$	$2{,}78 \times 10^{-11}$
	24B10	$5{,}37 \times 10^{-10}$	$9{,}29 \times 10^{-11}$
	19C12	$1{,}02 \times 10^{-9}$	$2{,}02 \times 10^{-10}$

In this work, K_D was determined by indirect ELISA. It is calculated as the value, corresponding to the MAb concentration when the absorbance is at half the maximum OD measured. The experiment was carried out by titration of the MAb starting from 3.3×10^{-8} mol/l to 5.6×10^{-13} mol/l. The results are presented in Table 3.2.

Apparent dissociation constant values of the generated monoclonal antibodies fell in the range from 1×10^{-11} mol/l to 1×10^{-9} mol/l. High-affinity antibodies are generally considered to be in the range of 10^{-9} mol/l, with very high-affinity antibodies being in the 10^{-12} mol/l range. The findings of K_D values indicate that eleven monoclonal antibodies generated in this study exhibit high affinity towards their corresponding antigen epitopes, while two MAbs deviate from the range associated with high affinity. Stronger interaction between antigen and antibody result in a more sensitive diagnostic test that can detect lower concentrations of the target protein with greater accuracy. As a result, the majority of the MAbs generated in this study hold the potential for application in the development of highly sensitive diagnostic tools.

3.4.3. Reactivity of the monoclonal antibodies in Western Blot

Further characterization of β-lactamase-specific monoclonal antibodies was achieved by Western Blot analysis. The method was employed to ascertain the types of epitopes recognized by the antibody, specifically whether they are linear or conformational. *E. coli* Tuner (DE3) was included in the analysis as the strain was used for the expression of the antigens.

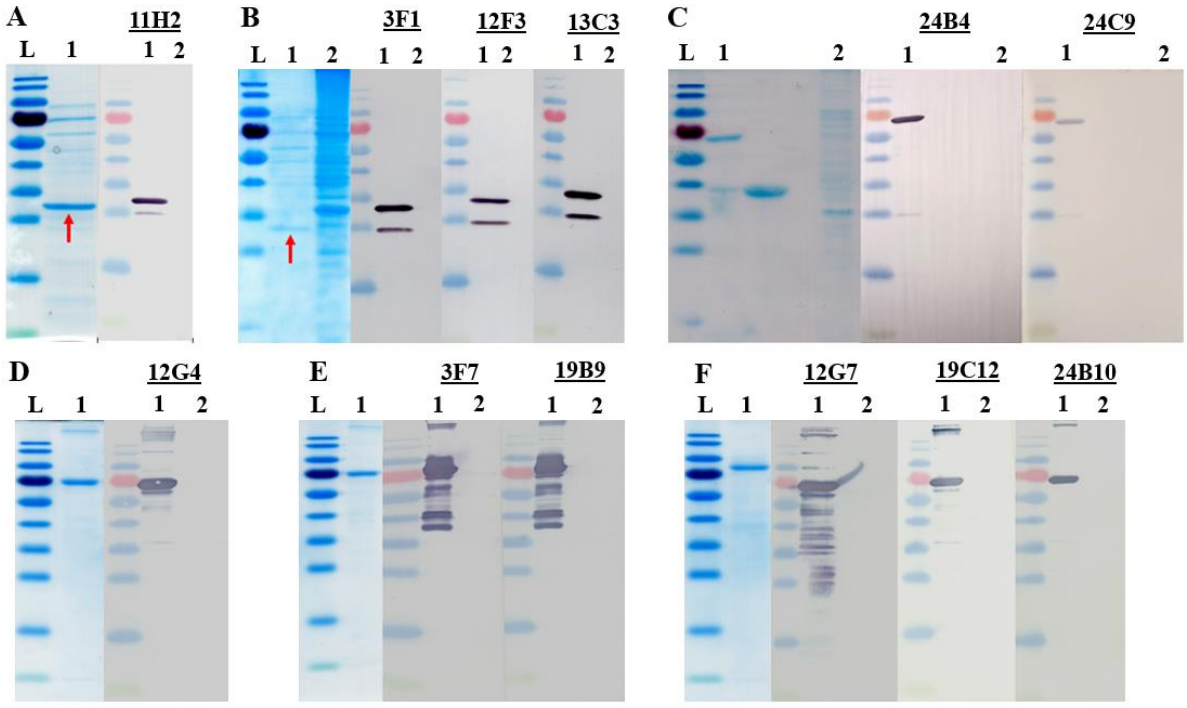

Fig. 3.6. Western Blot analysis of β-lactamase-specific monoclonal antibodies. Proteins were analyzed in 12% SDS-PAGE gel. A – OXA-48-specific MAb 11H2; B – OXA-134-specific MAbs 3F1, 12F3, 13C3; C – OXA-48-MBP-specific MAbs 24B4, 24C9; D – SHV-42-MBP-specific MAb 12G4; E – ADC-144-MBP-specific MAbs 3F7, 19B9; F- SME-3-MBP-specific MAbs 12G7, 19C12, 24B10. In the electrophoresis gel: L – PageRuler Prestained Protein Ladder; 1 – 0.2 µg of respective antigen; 2 – *E. coli* Tuner (DE3) lysate.

MAb analysis by Western Blot revealed that twelve out of thirteen MAbs react with their respective antigens (except SHV-42-MBP-specific 15G4, data not shown) and recognize their linear epitopes. What is more, none of the antibodies detected any proteins in expression strain lysate, indicating that generation of monoclonal antibodies was not affected by the impurities in the antigen mix.

3.4.4. Cross-reactivity of generated monoclonal antibodies

Assessing cross-reactivity of monoclonal antibodies developed for diagnostic purposes is critical to ensure specificity and overall performance of the diagnostic assay. Maintaining high specificity is important to avoid false-positive or false-negative results. However, some cross-reactivity might be desirable in diagnostics of antibiotic resistance, as that expands detection range: such MAbs could recognize not only the specific target but also its variants. This can be particularly beneficial in high-throughput screening scenarios, where multiple samples need to be analyzed rapidly. Therefore, MAbs against target β-lactamases were evaluated by conducting indirect ELISA. The wells were coated with β-lactamases from this work, as well as other β-lactamases synthesized by colleagues in the laboratory. This allowed for the evaluation of the antibodies' potential interaction with closely related enzymes. Cross-reactivity assessment results are presented in Table 3.3.

These results indicate that while some monoclonal antibodies are specific only towards their target antigen (anti-ADC-144-MBP 3F7, anti-SHV-42-MBP 15G4, anti-SME-3-MBP 19C12), others exhibit cross-reactivity with similar β-lactamases (anti-ADC-144-MBP 19B9 reaction with OXA-134-MBP, anti-SHV-42-MBP 12G4 reaction with ADC-144-MBP, anti-SME-3-MBP MAb reactivity towards ADC-144-MBP and IMI-1-MBP proteins). Similarities in amino acid sequences indicate a higher likelihood of cross-reactivity. For example, tag-free IMI-1 and SME-3 contain 70.69% sequence identity (as determined using NCBI BLASTp tool), which might be the reason behind IMI-1-MBP detection by anti-SME-3 24B10 monoclonal antibody. However, regarding the application of 24B10 in diagnostic tool development, it is important to note that the MAb is not necessarily ineffective. In fact, due to shared ability of both enzymes to confer resistance to carbapenems (Queenan et al., 2006; Rasmussen et al., 1996), the 19B9 monoclonal antibody might still have valuable implications in the diagnostics of carbapenem resistance.

Table 3.3. Cross-reactivity of monoclonal antibodies, as determined by indirect ELISA. "+" indicates a positive signal in ELISA reaction plate.

MAb \ Antigen		ADC-144-MBP	SHV-42-MBP	SME-3-MBP	OXA-48-MBP	OXA-48	OXA-134	OXA-134-MBP	IMP-1-MBP	IMI-1-MBP	OXA-51Δ27	DHA-1-MBP
Anti-ADC-144-MBP	3F7	+	-	-	-	-	-	-	-	-	-	-
	19B9	+	-	-	-	-	-	+	-	-	-	-
Anti-SHV-42-MBP	12G4	+	+	-	-	-	-	-	-	-	-	-
	15G4	-	+	-	-	-	-	-	-	-	-	-
Anti-SME-3-MBP	19C12	-	+	-	-	-	-	-	-	-	-	-
	24B10	+	-	+	-	-	-	-	-	+	-	-
	12G7	+	-	+	-	-	-	-	-	-	-	-
Anti-OXA-48-MBP	24C9	-	-	-	+	+	-	-	-	-	-	-
	24B4	-	-	-	+	+	-	-	-	-	-	-
Anti-OXA-48	11H2	-	-	-	+	+	-	-	-	-	-	-
Anti-OXA-134	3F1	-	-	-	-	-	+	+	-	-	-	-
	12F3	-	-	-	-	-	+	+	-	-	-	-
	13C3	-	-	-	-	-	+	+	-	-	-	-

Similarly, it was observed that anti-OXA-48 MAbs demonstrated reactivity towards OXA-48-MBP protein, the same way that anti-OXA-48-MBP MAbs reacted with OXA-48. What is more, the same pattern was observed in OXA-134-related reactions. Here, cross-reactivity is even more likely caused by the shared epitopes rather than unspecific reactions with another antigen. These findings suggest that monoclonal antibodies developed against OXA-48 and OXA-48-MBP can be used interchangeably in assays targeting either antigen.

3.5. Development of sandwich ELISA for detection of β-lactamases

Sandwich ELISA serves as an effective method for antigen detection, making it an appropriate platform to assess the suitability of the MAbs for diagnostic purposes. In this work, sandwich ELISA was carried out to assess the potential of β-lactamase-specific monoclonal antibodies for application in diagnostic tools development. The purpose of this experiment was to identify and optimize MAb pairs that would detect their targets, leading to a highly sensitive detection method.

First, purified MAbs and their HRP-labeled versions were used to carry out antibody pairing – determination of capture and detection antibodies. The capture antibody was immobilized on the microplate well, while the other (HRP-labeled) antibody was used to detect the captured antigen (Fig. 3.7 A). Achieving antibody pairing involved selecting the antibody pairs that exhibited the strongest signal while excluding the pairs that either showed no signal or the signal was detected only at the highest concentrations of the target antigen. The pairs selected for further optimization of the method were: anti-OXA-134 12F3 + 13C3* (* indicates HRP-labeling), anti-SME-3-MBP 19C12 + 12G7*, and anti-ADC-144-MBP 19C9 + 19C9*. It is important to mention that in sandwich ELISA for OXA-134 detection, OXA-134-MBP protein was used in further experiments. This decision was made based on the observation that anti-OXA-134 MAbs can detect OXA-134-MBP (Table 3.3) and the selected MAbs exhibit high affinity towards OXA-134-MBP (anti-OXA134 12F3 $K_D=3.24 \times 10^{-11}$ mol/l, anti-OXA-134 13C3 $K_D=4.92 \times 10^{-11}$ mol/l).

For the improvement of method's sensitivity, various concentrations of capture antibody as well as dilution factors of detection antibody were tested. To decrease background signal and prevent nonspecific binding, several blocking solutions, and solutions for the dilution of antigen and labeled antibody were examined. What is more, TMB substrate and sulfuric acid reaction volume was increased to achieve higher absorbance in the positive wells. Optimized sandwich ELISA conditions are represented in Table 3.4.

Table 3.4. Optimized sandwich ELISA conditions for the detection of β-lactamases.

	OXA-134-MBP 12F3+13C3*	**SME-3-MBP 19C12+12G7***	**ADC-133-MBP 19C9+19C9***
Immobilization buffer	PBS	PBS	Coating buffer
Capture antibody concentration	10 µg/ml	10 µg/ml	10 µg/ml
Immobilization time and temperature	4 ℃ overnight	37 ℃ 1 hour	37 ℃ 1 hour
Blocking solution	2 % BSA in PBST	2 % BSA in PBST	3 % milk powder in PBST
Solution for antigen and detection antibody dilution	PBST	3 % milk powder in PBST	ROTI block
Detection antibody dilution factor	3000X	2000X	300X

Following the above indicated conditions, three standard curves were obtained (Fig. 3.7. B, C, D), each representing a specific antibody pair in the assay. These standard curves demonstrate the relationship between β-lactamase and signal intensity, providing a framework for the detection of antimicrobial resistance. Anti-OXA-134 monoclonal antibodies detected their analyte as low as 4.57×10^{-11} mol/l (6.86×10^{-3} µg/ml), while anti-SME-3-MBP and anti-ADC-144-MBP MAbs exhibited positive signal at 9.15×10^{-11} mol/l (1.37×10^{-2} µg/ml) of their respective antigens. The threshold value for these assays was established based on the background signal observed in the negative control samples. In this case, a positive signal was defined as an OD value exceeding 0.1. Interpreting these results and drawing definite conclusions about the sensitivity of the assay poses challenges as antimicrobial resistance proteins are usually detected in cell lysates (Glupczynski et al., 2017; Kitao et al., 2010; Oueslati et al., 2021; Yamada et al., 2013). Cell components add another variable, which should be assessed to truly understand the performance of this sandwich ELISA.

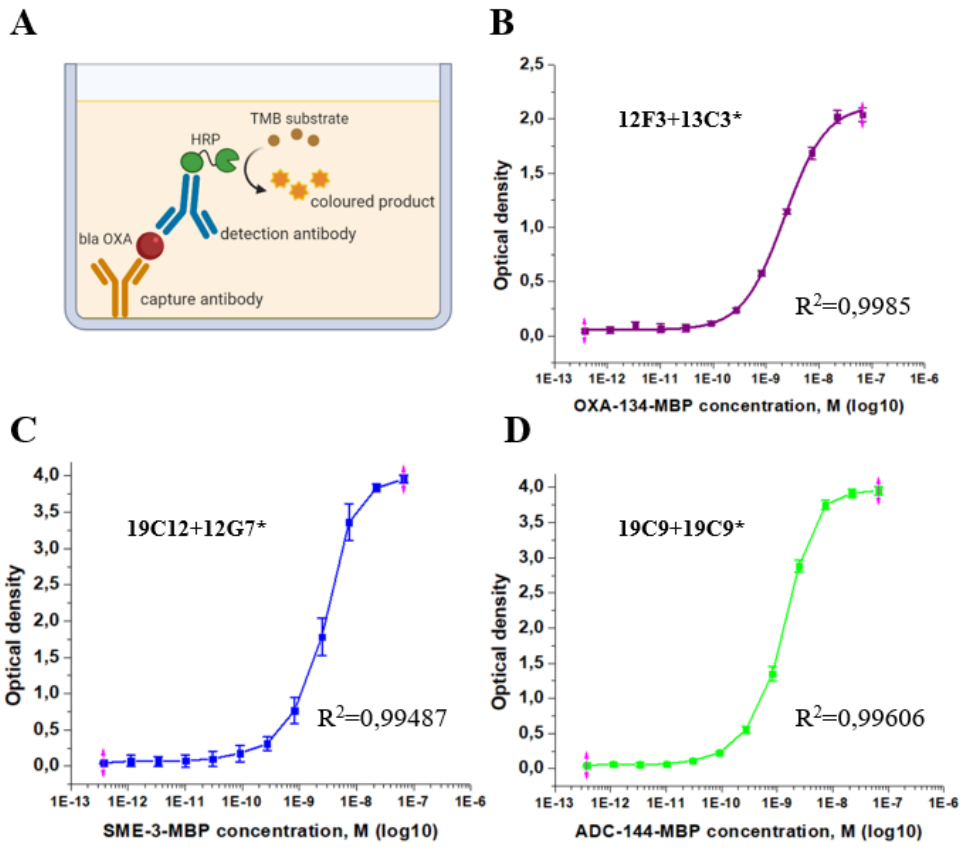

Fig. 3.7. Sandwich ELISA method used to detect recombinant antibiotic resistance protein. A – schematic representation of sandwich ELISA; B, C, D – standard curves generated for the detection of OXA-134-MBP, SME-3-MBP, ADC-144-MBP respectively. Error bars represent standard error of mean.

Due to unavailability of clinical isolates containing target β-lactamases a pilot experiment was conducted using *E. coli* expression strain with induced antigen synthesis. This approach allowed for rough assessment of the method's performance. The assay conducted using the cell lysates demonstrated promising results: a signal was detected when analyzing lysates of the cells in which target antigen expression was induced. Furthermore, control lysate in which synthesis of the antigen was not induced did not yield a positive signal, implying that the assay does not produce false-positive results because of the presence of the cellular proteins. Also, the successful detection of the target antigen in the induced lysates suggests that there is no strong hindrance from the other proteins in the reaction mix – the assay is capable of identifying the target antigen in complex protein mix (data of the pilot experiment is not shown). While clinical isolates would have been ideal to evaluate generated monoclonal antibody potential for the application in immunodiagnostic tools development, the use of expression strain served as a proof-of-concept experiment. Further characterization of the MAbs is needed to ensure their successful implementation in the diagnostics and monitoring of antibiotic resistance.

CONCLUSIONS

1. Recombinant OXA-48 and OXA-134 β-lactamases were successfully expressed and purified under denaturing conditions from *E. coli* Tuner (DE3) expression strain. Denaturing conditions appeared to be a solution for the purification of difficult to synthesize recombinant β-lactamases.
2. OXA-48, OXA-134, OXA-48-MBP, OXA-134-MBP, SME-3-MBP, ADC-144-MBP, and SHV-42-MBP were immunogenic in mice inducing high antibody titers above 1063. It revealed that these recombinant proteins are suitable immunogens for monoclonal antibody generation by hybridoma technology.
3. Thirteen hybridoma cell lines were successfully obtained secreting: one anti-OXA-48, three anti-OXA-134, two anti-OXA-48-MBP, two anti-SHV-42-MBP, two anti-ADC-144-MBP, SME-3-MBP monoclonal antibodies of IgG1 isotype, exhibiting high-affinity towards antigens. Generated antibody characterization demonstrated wide-applicability nature of these biotechnological tools.
4. Sandwich ELISAs for the detection of recombinant OXA-134, SME-3 and ADC-144 β-lactamases were developed and demonstrated in a pilot study imitating clinical samples; however, further optimization using clinical isolates is needed for the analysis of clinical samples.

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Master thesis

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST ANTIBIOTIC RESISTANCE PROTEINS OXA-48, OXA-134, SHV-42, SME-3, AND ADC-144

SUMMARY

Antibiotic resistance is one of the biggest problems healthcare systems face in the 21st century. Although the development of antibiotics during the last 100 years has significantly decreased the load of infectious diseases, they are on the rise again as poor regulation of antibiotic use is causing the development of antimicrobial resistance.

The lack of affordable and rapid diagnostics challenges the correct prescription of antibiotics. Immunodetection is a specific, sensitive, and cost-effective approach to detect antibiotic resistance proteins. However, the development of such diagnostic tests is limited by the lack of monoclonal antibodies against antibiotic resistance proteins. The aim of this work was to generate mouse monoclonal antibodies against OXA-48, OXA-134, SME-3, SHV-42, and ADC-144 proteins which confer resistance to various types of β-lactam antibiotics.

Monoclonal antibodies (MAbs) were produced by hybridoma technology. Initially, OXA-48 and OXA-134 β-lactamases were purified, all β-lactamases were used as immunogens and thirteen MAb-secreting cell lines were generated. MAbs were then characterized by immunochemical methods. It was shown that eleven out of thirteen MAbs exhibit high affinity towards their respective antigens, and twelve of all the antibodies recognize linear epitopes. Furthermore, sandwich ELISA assays were developed detecting three different recombinant β-lactamases. Additional testing is needed to assess the MAbs true diagnostic potential.

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ACKNOWLEDGEMENTS

This research was funded by EUREKA project “Novel affinity binders for immunodetection of antimicrobial resistance” (Nr.01.2.2-MITA-K-702-05-0003).

I want to express my gratitude to my supervisor dr. Martynas Simanavičius for sharing his experience and knowledge, offering solutions during challenging moments, and patience.

I am grateful to prof. dr. (HP) Aurelija Žvirblienė for an opportunity to conduct my thesis within her department and sharing her knowledge.

I want to thank my laboratory colleagues for their kindness, helpfulness, and creating a pleasant working environment.

Lastly, I am grateful to my loved ones for being my pillars of support.