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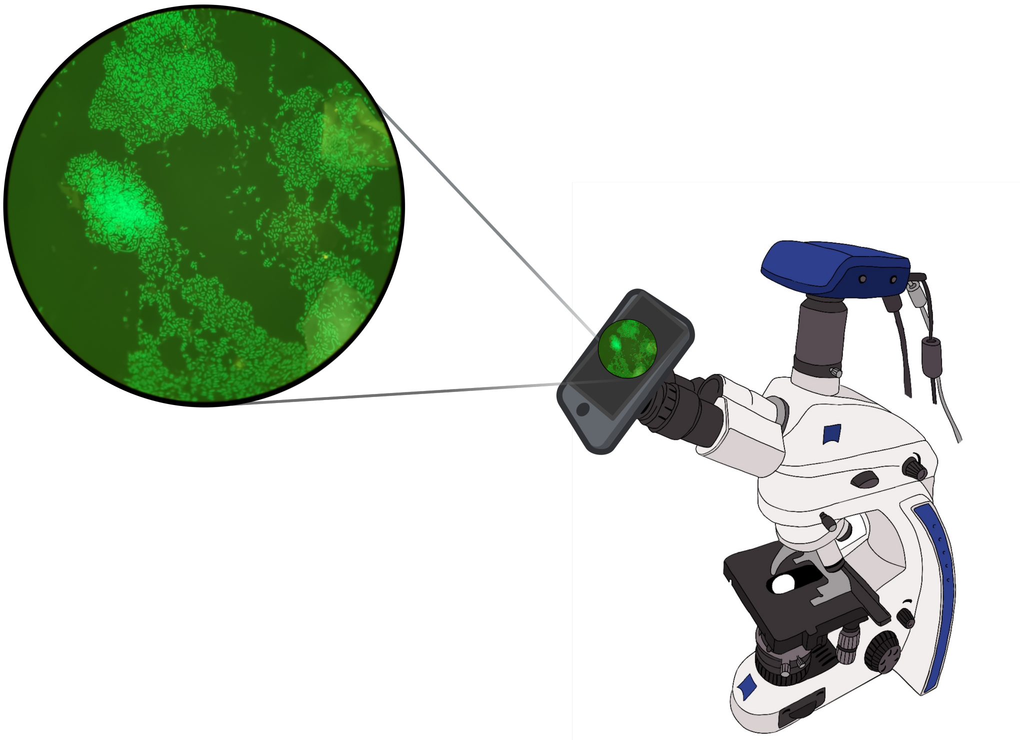

Detection of ESBL-Producing Bacteria in the Neonatal Unit at Muhimbili National Hospital Using PNA-FISH and Smartphone-Based Fluorescence Microscopy

Master's thesis in Biotechnology

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Cover: The setup of the fluorescence microscope with the attached smartphone to detect ESBL-producing bacteria after the PNA-FISH procedure. Created in Biorender.com.

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Abstract

Sub-Saharan Africa has the highest neonatal mortality rate, accounting for 43% of the global neonatal deaths. This is due to bacterial infections caused by bacteria such as *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP). The global threat of antimicrobial resistance complicates the treatment of bacterial infections, particularly those caused by Extended-spectrum beta-lactamase (ESBL)-producing bacteria. ESBL-producing bacteria destroy most commonly prescribed antibiotics for the treatment of bacterial infections. This project aimed to develop and implement a fluorescence *in situ* hybridization (FISH)-based assay to identify ESBL-producing bacteria in neonates (0-28 days) at Muhimbili National Hospital (MNH), Tanzania. The two target bacteria was EC and KP which were detected with a fluorescently labeled peptide nucleic acid (PNA) probe. Starting with a preparatory study at Chalmers University of Technology (CTH), initial tests were performed with the PNA probe for detection of EC and KP. Additionally, tests were conducted to evaluate whether the PNA-FISH assay could be performed faster, at lower temperatures, and with fewer chemicals. In the second phase at MNH, the PNA-FISH assay was established to detect for ESBL-producing EC in all admitted and discharged neonates over four days. Results analysis was done using a simple fluorescence microscope commonly found in labs in low and middle-income countries.

The preparatory study at CTH concluded that the EC probe showed good results, with high fluorescence signal, for detection of EC, while the KP probe showed a low fluorescence signal for detection of KP. This could be due to low degree of hybridization. For EC, the hybridization temperature should not exceed 50°C, with optimal results even at 22°C for a laboratory strain of EC. Reducing the hybridization time from 1 hour to 15 minutes for the EC PNA-probe did not compromise with the quality of results from what could be concluded visually. Reducing the number of chemicals used in the PNA-FISH assay did not impact the results either. However, similar tests must be performed on fecal samples before definite conclusions can be drawn. The field studies at MNH showed that the PNA-FISH results of all patient samples matched the expected outcomes from the project's verification step. The project's findings did not only demonstrate the potential for using PNA-FISH as a diagnostic tool, but also stressed the importance of developing simple tools to detect antibiotic resistant bacteria in low-income countries, where it is most needed.

Keywords: Fluorescence *in situ* hybridization (FISH), Diagnostic FISH, Peptide nucleic acid (PNA) probes, Extended-spectrum beta-lactamases (ESBLs), Antimicrobial resistance (AMR), Low and middle-income countries (LMICs), Neonatal mortality, *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), Fluorescence microscopy

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Moa Andersson & Maya Awada, Gothenburg, June 2024

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1
Introduction

In 2020, 2.4 million infants died within the first month of life. Most of the newborn deaths are reported in low and middle-income countries (LMIC) and Sub-Saharan Africa is the region worldwide that has the highest neonatal mortality rate, covering 43% of the global neonatal deaths [1]. Neonatal deaths are primarily caused by bacterial infections, including pneumonia, sepsis, and meningitis [2]. The bacterial infections are mainly caused by Coagulase-negative *Staphylococcus* (CoNS), Group B *Streptococcus* (GBS), *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP) [3]. Neonates are extra prone to infections due to their lack of established protective gut microbiota at birth [4]. Premature neonates also have immature skin and lack proper protective barriers in the gastrointestinal tract [5].

The situation is further complicated by the spread of antimicrobial resistance (AMR). For example, Enterobacterales, such as KP and EC, may produce enzymes called extended-spectrum Beta-lactamases (ESBLs), which will destroy most beta-lactam antibiotics and make them ineffective [6]. Therefore, infections by ESBL-producing bacteria are complex to treat. Antimicrobial stewardship is crucial for preventing the spread of AMR [7]. LMICs suffer from limited resources regarding antimicrobial stewardship, efficient infection controls, and diagnostic laboratories etc. These resource limitations put LMICs in a vulnerable spot. Therefore, it is crucial that tools and assays are developed to be suitable for use in LMICs.

The choice of treatment depends on the specific bacteria causing the infection and hence developing tools to identify these bacteria is of paramount interest. Since colonization often precede infection, knowledge about colonization can improve treatment outcome if the neonate develops an infection.

This project is a part of a larger collaboration between Chalmers University of Technology, Karolinska Institute, Azevedo lab at University of Porto and Muhimbili National Hospital (MNH) in Dar es Salaam, Tanzania. The collaboration aims to develop and implement a fluorescence *in situ* hybridization (FISH)-based assay to identify pathogenic bacteria that affects neonates in low-resource settings. The probe of the PNA-FISH assay can be modified to detect different bacteria. Imaging will be done using the Zeiss® Primostar iLED microscope, a microscope available in many LMICs due to a tuberculosis diagnosis initiative by Zeiss®. The results of this study could support future studies with the ultimate goal of reducing the mortality rate for neonates.

1.1 Aim

The main aim of the project is to use a fluorescence *in situ* hybridization (FISH) based assay to identify pathogenic bacteria affecting neonates (0-28 days) in low-resource settings, at Muhimbili National Hospital (MNH) in Tanzania. The two target bacteria are ESBL-producing KP and EC. The initial aim of the project is to establish the FISH assay and test the hybridization efficiency of the probes that will be used to detect the target bacteria. At MNH, rectal swabs will be collected from all admitted and discharged neonates during one month. The fecal samples will be cultured in the presence of antibiotics and screened using the PNA-FISH assay combined with a simple fluorescence microscope (Zeiss® Primostar iLED) [8]. ESBL-selective chromogenic agar plates will be used to confirm the results of PNA-FISH on the patient samples.

As a part of the project, a 4.5-month-long field study will be performed to explore the possibilities of implementing the assay as routine at MNH, Dar es Salaam, Tanzania. The field study will take place at the Bacteriology unit of MNH. This part of the project will be preceded by six months of preparatory studies at Chalmers University of Technology (CTH). The project will be done in the Westerlund research group which has an established collaboration with MNH.

1.1.1 Specific aims

The specific aims for the preparatory study at CTH are:

- Set up the PNA-FISH assay at CTH by testing both the EC and KP probes to evaluate their hybridization efficiency.
- Simplify the solutions of the PNA-FISH assay by using fewer chemicals to suit low-resource settings.
- Optimize the time and temperature of the PNA-FISH assay with the EC and KP probe.

The specific aims of the field studies at the Bacteriology Department of MNH in Tanzania are:

- To establish the PNA-FISH assay at the Bacteriology Department of MNH.
- To prepare the laboratory at MNH to receive samples from the neonatal ward screening by ensuring proper setup of transport media tubes.
- To identify an antibiotic-supplemented media suitable for cultivating fecal samples for ESBL-producing EC and KP.
- To detect KP and EC in fecal samples from neonates using the PNA-FISH assay.

- To compose simple instruction manuals of the PNA-FISH assay for the Bacteriology department staff to adhere to, guaranteeing the continuation of the assay.
- To instruct and train two persons from the staff at the Bacteriology department in performing the PNA-FISH, ensuring the further continuation of the assay.

1.2 Limitations

Many bacteria can cause infections but the project will cover the two bacteria; EC and KP. The reason for this is their relevance for healthcare-associated infections, particularly in low-resource settings where the field studies are planned to be conducted. For the field studies, the screening will be performed on a limited number of patient samples at MNH.

Antibiotic resistance is a problem, especially in LMICs. The PNA-FISH assay detects bacteria but does not give information on whether the bacteria are resistant to antibiotics or not. To include the antibiotic resistance in the project it will be controlled by cultivation of EC and KP in the presence of antibiotics and not by the PNA-FISH assay itself.

Inoculation of the patient samples on ESBL-selective chromogenic agar plates will be used as the verification to compare to the results obtained from PNA-FISH. These selective plates are used as verification since they are available at the lab at MNH.

2

Theory

This chapter provides an overview of the fundamental concepts and techniques relevant to the study. It covers the structure of DNA and the cells of bacteria, as well as, antibiotics and antibiotic resistance. It also describes healthcare-associated infections and the current situation at the neonatal unit of MNH. It also introduces the technique PNA-FISH, along with fluorescence microscopy principles.

2.1 DNA

Deoxyribonucleic acid (DNA) is the molecule that carries the genetic information of an organism's function and development [9]. DNA is two long strands held together with hydrogen bonds to form a double helix. The strands consist of four different nucleotide subunits. Each nucleotide comprises a five-carbon sugar (deoxyribose) attached to a phosphate group and a nitrogen-containing base. The four different bases are adenine (A), cytosine (C), guanine (G) and thymine (T) and they form covalent bonds with the deoxyribose and phosphate. This forms the backbone, a chain with alternating sugar-phosphate-sugar-phosphate in a single-stranded DNA. It is the order of the bases that acts like a template for the genetic information of the DNA molecule [9]. Two complementary single-stranded DNA molecules bind to each other by hydrogen bonding between the bases and form a double-stranded DNA molecule. Three hydrogen bonds pair G with C, while two hydrogen bonds pair T with A (see Figure 2.1).

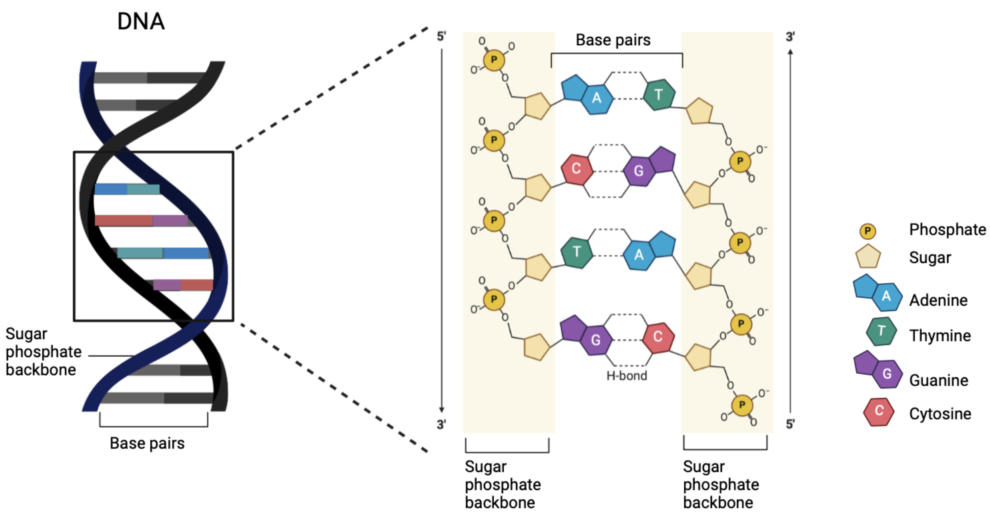

Figure 2.1: Structure of the double helix DNA molecule. A zoomed-in illustration of the hydrogen bonds and the base pairs. Created in Biorender.com

The sugar-phosphate backbone is positioned outside the double helix and the nucleotide base pairs on the inside [9]. This conformation is the most energetically favorable for the double helix.

2.2 Cells of bacteria

All organisms are composed of either eukaryotic- or prokaryotic cells [10]. Eukaryotic cells have their genetic material enclosed within a nuclear membrane compared to prokaryotic cells where the genetic material is not separated from the other content in the cell. Bacterial cells do not have membrane-bound nucleus nor intracellular organelles and are therefore classed as prokaryotes. All the activities performed by the organelles in eukaryotic cells are also performed in prokaryotic cells. However, they do not take place in specialized structures in the bacteria.

Most prokaryote cells have a simple appearance and measure a few micrometers in linear dimension [11]. There are three basic shapes for individual bacteria: rod-like (bacillus), spherical (coccus), or curved (spirillum, vibrio, or spirochete) [10]. Further, bacteria often occur in characteristic aggregates (pairs, chains, tetrads, clusters, etc.) which are diagnostically useful as it can provide information that distinguishes one bacteria from another [12]. It can aid in the identification and classification of bacteria. The cell wall of the bacteria acts as a tough protective layer and it protects the inner cytoplasmic compartment [11]. Bacteria can further be divided into two primary groups: gram-negative and gram-positive bacteria [13]. The cell walls of bacteria consist of peptidoglycans, i.e. a stiff polysaccharide that provides structural strength. The main difference between gram-positive and gram-negative bacteria is the cell wall structure.

The cytoplasm of bacteria contains genetic material such as DNA and RNA, ribosomes, and proteins (see Figure 2.2) [11]. The DNA in bacterial cells is found as a long coil in the cytoplasm [10]. DNA can also appear as one or two circular chromosomes or as linear DNA. Bacteria often have plasmids, which are small and circular DNA molecules and they can carry auxiliary information.

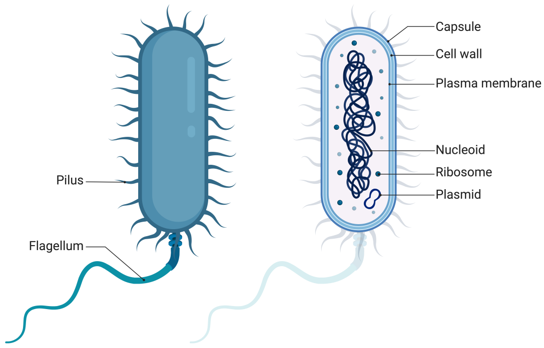

Figure 2.2: Overview of the structure of a bacteria, where features of the bacteria are marked. Created in Biorender.com

Flagellum and pili (fimbriae) are attached to the cell wall of some bacteria (see Figure 2.2) [12]. Pili are proteinaceous structures, hairlike in appearance, that play a crucial role in adhesion to host cells. They are present most commonly in Gram-negative bacteria. Flagella is a cylindrical protein filament that allows for cell movement and can be found on both Gram-positive and Gram-negative bacteria.

2.2.1 Gram-negative bacteria

The cell wall of the gram-negative bacteria consists of at least two layers. Only a small part, i.e. the inner layer of the cell wall, consists of peptidoglycans [13]. The outer membrane, or the liposaccharide layer (LPS), makes up the largest part of the cell wall. The structure of LPS varies between bacterial species.

In the LPS layer, polysaccharides and lipids create a complex that serves as a vital barrier, protecting bacteria from, for example, lipophilic antibiotics that would otherwise harm them. Several antibiotics that are effective against gram-positive bacteria, are ineffective or less effective against gram-negative bacteria due to their LPS. A crucial biological function of LPS is therefore its toxicity to organisms, where common gram-negative pathogens for humans include species of *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP).

2.2.2 Gram-positive bacteria

Whereas only a small part of the cell wall of gram-negative bacteria consists of peptidoglycans, the cell wall of gram-positive bacteria is thicker and consists of 90% peptidoglycans [13]. Many gram-positive bacteria form multiple layers of peptidoglycans that provide a strong cell wall structure. Moreover, many gram-positive bacteria have acidic molecules, teichoic acids, that are embedded in their cell wall. The teichoic acids bind to individual alcohols and are further covalently linked to peptidoglycans. An example of gram-positive bacteria is Group B *Streptococcus* (GBS) which is the most frequent cause of life-threatening bacterial infections in newborns [14].

2.2.3 Horizontal gene transfer

Horizontal gene transfer is the mechanism of how genetic information moves between bacteria and it can occur through three different mechanisms; transformation, transduction, or conjugation (See Figure 2.3) [15].

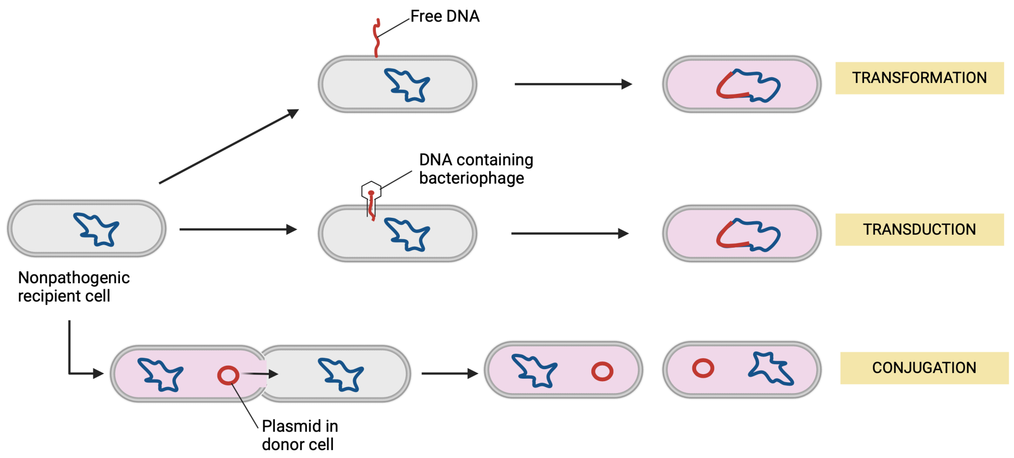

Figure 2.3: Schematic illustration of horizontal gene transfer in bacteria. The illustration shows the three main mechanisms of gene transfer: transformation (top), transduction (middle), and conjugation (bottom). Created in Biorender.com

Transformation occurs between closely related bacteria and it is the mechanism of direct absorption, incorporation, and expression of exogenous DNA. For the transformation to take place the foreign DNA needs to be transferred from the surface to the cytoplasmic membrane where it crosses through membrane channels. Transduction is the process where bacteriophages carry DNA from one bacterial cell to another cell [15]. The bacteriophage picks up a piece of the host cell's DNA and incorporates it into its genetic material before it infects a new bacterial cell and injects this DNA. Further, the injected DNA can be incorporated into the genetic material of the infected cell. This can result in cell lysis or a lysogenic cycle. During the lysogenic cycle the genome of the bacteriophage integrates into the host cell's genome [16]. Further making the host cell immune to infection from the same bacteriophage. The third mechanism of horizontal gene transfer is conjugation [15]. This mechanism needs direct contact between two cells and it results in an unidirectional transfer of genetic material from one of the cells to the other. What enables this procedure is the formation of a conjugative pore or bridge between the donor cell and the recipient cell.

2.2.4 Bacterial growth

In a controlled laboratory environment, where bacteria are cultivated in a nutrition-rich media at optimal temperature, the bacteria can obtain a continuous and balanced growth [17]. In contrast to bacteria in their natural environment where their growth is not often constant. The characteristic bacterial growth curve has four phases; lag phase, exponential growth phase, stationary phase, and death phase [17]. The lag phase is the first stage in bacterial growth, where the bacteria in nutrient-rich media adapt to the environment to be able to start cellular metabolism. The cell reproduction during the lag phase is very restricted, it starts to increase at the end of the phase when the bacteria enters the next growth phase, exponential growth. The growth rate is logarithmic as the bacteria divide by binary fission and it is characterized by the generation time. The generation time is the time for doubling the number of cells. After the exponential growth phase comes the stationary phase [17]. This occurs when the growth rate decreases and the increase of cells ceases. During the stationary phase, the number of cells is constant, it is a state of equilibrium between the dying cells and the numbers of dividing cells. When the cultivation environment no longer can maintain the bacteria they accumulate toxic products. This further results in the death phase as the number of viable cells decreases.

2.2.5 Filamentation of *Escherichia coli*

As a strategy to survive diverse environments, bacteria can transform their cell shape [18]. To survive stressful or toxic environments bacteria can form filaments. This process is called filamentation and it is characterized by a dramatic increase in cell length as a result of longitudinally division without daughter cell separation. In an environment with beta-lactam antibiotics Gram-negative bacteria generally respond by filamentation, the production of cell-wall-deficient round cells, or rapid cell lysis [19]. For example, cephalosporins, such as cefotaxime, have been shown to cause susceptible EC to form filaments when in an environment of up to 2 mg/L.

2.3 Antibiotics

Antibiotics treat and prevent bacterial infections by targeting bacteria [20]. Antibiotics are usually grouped based on their chemical structure and they differ in their mode of action; some act by suppressing the ability of bacteria to grow and some kill bacteria [21]. For example, the classes penicillins and cephalosporins kill bacteria by preventing the formation of the bacterial cell wall, while lincosamides and tetracyclines reduce bacteria's ability to form proteins which further prevent bacteria from multiplying. The class beta-lactams with increased activity are a penicillin or cephalosporin in combination with a beta-lactamase inhibitor [21]. The inhibitor has limited antibiotic activity on its own but rather works by protecting the drug it is combined with which increases the activity of the antibiotic. The carbapenem family is an example of antibiotics that are classified under the category of beta-lactams with increased activity [22].

Antibiotics can also be classified into two groups based on their effect on microbial cells through two main mechanisms; bacteriostatic or bactericidal antibiotics [23]. Whether an antibiotic is bacteriostatic or bactericidal is explained by the concept of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). MIC is the lowest concentration of an antibiotic that inhibits the visible growth of bacteria at 24 hours, while MBC is the concentration that reduces the density of bacteria by 1000-fold at 24 hours. The antibiotic is classified as bacteriostatic if the MBC to MIC ratio is greater than 4 and as bactericidal if the ratio is less or equal to 4 [24].

2.3.1 Vancomycin

Vancomycin is a tricyclic glycopeptide antibiotic that binds to the cell wall of the bacteria which causes a blockage of glycopeptide polymerization [25] [26]. This inhibits the cell wall synthesis and produces secondary damage to the cytoplasmic membrane. Vancomycin is used for infections caused by gram-positive bacteria, and has not shown clinical efficacy against gram-negative bacteria [26]. In-vitro multiple gram-positive bacteria are sensitive to vancomycin of concentration of 0.5 to 5 mg/L, while gram-negative bacteria, mycobacteria, and fungi are resistant.

2.3.2 Clindamycin

Clindamycin is an antibiotic in the class lincosamides [21]. Its mode of action is to inhibit bacterial protein synthesis by binding to the 50S subunit of the bacterial ribosome. This prevents the translocation step in protein synthesis, which effectively stops bacterial growth and replication [27]. Clindamycin is effective for infections caused by gram-positive organisms and anaerobic bacteria. This antibiotic can be both a bacteriostatic or bactericidal antibiotic depending on the organism, infection site, and the concentration of clindamycin [27].

2.3.3 Cefotaxime

Cefotaxime is a third-generation broad-spectrum bactericidal cephalosporin antibiotic [28]. Cefotaxime exerts its action by binding one or more of the penicillin-binding proteins (PBPs) which results in the inhibition of the final transpeptidation step of peptidoglycan synthesis in bacterial cell wall synthesis. There can be several mechanisms causing resistance to cefotaxime and they can co-exist [28]. Such mechanisms are the production of ESBLs, induction and/or constitutive expression of AmpC beta-lactamases, reduced outer membrane permeability, efflux pump mechanisms, or modification of target enzymes.

2.3.4 Meropenem

Meropenem is a broad-spectrum antibacterial agent of the carbapenem family [29]. Carbapenems are reserved as antibiotics of last resort as they are efficient against Gram-negative and Gram-positive drug-resistant infections [22]. WHO lists Meropenem as an essential medicine [22]. It is effective against Gram-positive, Gram-negative, and anaerobic bacteria [29]. It has also been shown that Meropenem has a broad spectrum of *in vitro* activity against ESBL-producing and AmpC-producing *Enterobacteriaceae*. Meropenem inhibits bacterial growth which leads to cell death by binding to specific PBPs, thus interfering with the synthesis of the bacterial cell wall.

2.3.5 Antibiotic resistance

Antibiotics are the cornerstone of modern medicine [30]. The spread of resistant pathogens is therefore a global threat to the ability to treat common infections and to perform life-saving procedures. This threat affects countries of all income levels but it is a certain threat for people living in low-resource settings and vulnerable populations [30]. As they are impacted by both the drivers and consequences of antibiotic resistance. In sub-Saharan Africa, antimicrobial-resistant bacterial infections were in 2019 linked to the highest infection-related mortality rates, with 99 deaths per 100,000 people in comparison with high-income countries where the numbers counted 56 deaths per 100,000 individuals due to antibiotic resistance [31]. Apart from the health-associated risks of antibiotic resistance, it is a significant economic burden [30]. An estimation performed by the World Bank states that by 2050 antibiotic resistance could result in US$ 1 trillion additional healthcare costs.

Microbes adjust to their surroundings to replicate and survive [32]. If their continued existence is threatened, the bacteria can evolve to overcome the obstacle. Antibiotic resistance is an example of such action, where the bacteria has become immune to the drug and can survive a treatment. If a microorganism develops resistance it can resist the action of certain antibiotics. There are many causes of the spread and enforcement of antibiotic resistance [32]. This involves overuse and abuse of antibiotics, improper antibiotic prescribing and inexact diagnosis, bad healthcare environments, self-medication, and widespread agricultural use of antibiotics. Lack of access to clean water, sanitation, and hygiene, poor infection, and disease prevention and control in homes are also drivers of antibiotic resistance [30].

Bacteria have developed different mechanisms to overcome the action of antibiotics [31]. This can occur through mutation of existing genes or horizontal gene transfer from other strains or species of bacteria. In addition to these mechanisms, six intrinsic resistance mechanisms can cause antibiotic resistance to one or more classes of antibiotics [31]. The first intrinsic mechanism is enzymatic modification or hydrolysis which leads to the inactivation of the antibiotic. The second mechanism is modification of the antibiotic target site which reduces the binding capacity. The third mechanism circumvents the antibiotic effect through modification of metabolic pathways. In other words, bacteria bypass metabolic pathways inhibited by antibiotics by using alternative pathways. Overproduction of antibiotic target protein can also be considered a target modification. The result of this intrinsic mechanism is that the bacteria where this occurs can reduce the effective concentration of the antibiotic that binds to each target molecule. The fifth mechanism is the reduction of intracellular antibiotic accumulation through decreased membrane permeability which prevents antibiotics from entering the bacteria or increased active efflux of the antibiotic which reduces the concentration and effectiveness of the antibiotic. The last intrinsic mechanism is that some bacteria possess the ability to form biofilms that can act as a protective barrier against antibiotics.

2.4 Healthcare-associated infections

Healtcare-associated infections (HAI), i.e. an infection that a patient acquires while staying at a healthcare facility, impact millions of patients globally [33], [34]. These infections result in extended hospital stays, long-term disability, possible rise of antibiotic resistance, and additional hospital expenses [34]. Urinary tract infections, surgical site infections, and bloodstream infections are the most common HAIs, often caused by multi-drug resistant (MDR) bacteria such as extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae*. Neonates, elderly and healthcare personnel are at risk for HAIs [33].

HAIs can be transmitted between healthcare personnel to patients, for example, due to poor hand hygiene [34]. These infections can also be transmitted by endogenous carriage among patients, i.e. when one patient transmits its infections to other patients by direct or indirect contact. Moreover, contaminated surfaces and medical devices such as stethoscopes, blood pressure machines, and catheters can also contribute to the transmission of HAIs. In developing countries, the prevalence of HAIs is higher compared to developed countries.

HAIs as well as the spread of antibiotic resistance can be prevented by proper hand hygiene by regularly washing hands with soap and running water as well or using antiseptics [34]. In healthcare settings, these hand hygiene practices should be used before, during, and after patient care.

2.4.1 ESBL-producing Enterobacterales

EC, KP, and various other *Enterobacteriaceae* are the most common pathogens in HAI [33]. Enterobacterales are a large group of different bacteria, including KP and EC. Some Enterobacterales, most commonly KP and EC, produce antibiotic degradation enzymes called extended-spectrum beta-lactamases (ESBLs). ESBLs make bacteria resistant to widely used antibiotics, such as penicillins and cephalosporins. This resistance leads to limited treatment options for infections caused by ESBL-producing bacteria. Even common infections such as urinary tract infections become complex to treat if they are caused by ESBL-producing Enterobacterales (ESBL-PE). Since the resistant bacteria are resistant to many commonly prescribed antibiotics, hospitalization of the patient with intravenous (IV) antibiotics becomes crucial. Serious infections caused by ESBL-producing Enterobacterales are often treated with Carbapenem antibiotics.

2.4.2 Carbapenem-resistant Enterobacterales

Enterobacterales are referred to as carbapenem-resistant Enterobacterales (CRE) when they become resistant to the group of antibiotics called carbapenems [35]. Carbapenems are broad-spectrum antibiotics and are used to treat MDR pathogens [36]. The definition of CRE is having developed resistance to at least one carbapenem or by carbapenemase enzyme production. Carbapenemase is an antibiotic degradation enzyme, beta-lactamase, that hydrolyzes antibiotics in the carbapenem class. Apart from possessing carbapenemase, CRE also can spread its resistance genes to other bacteria through intrinsic or acquired resistance mechanisms. Carbapenemase genes are often located on the mobile genetic elements of bacteria which facilitates their spread of, for example, resistance to beta-lactams to other bacteria. This leads to limited treatment options for carbapenem-resistant bacterial infections [37].

2.5 Neonatal ward at Muhumbili National Hospital

The neonatal unit at MNH in Dar es Salaam, Tanzania, offers level three Neonatal Intensive Care and is the largest neonatal ward in Tanzania [38], [39]. During peak season, the bed capacity can accommodate up to 200 neonates [38]. The healthcare personnel comprises three neonatologists along with pediatricians and nurses from the hospital and the university [39]. The unit has a large patient load which leads to staffing challenges for nurses and doctors [38]. The nurse-to-patient ratio is 1:5-10 which is low compared to high-income settings where the ratio is 1:2-3 in neonatal intensive care [39]. Additionally, there is a shortage of expertise that is necessary for a Neonatal Intensive Care Unit [38].

A screening of 53 neonates was conducted at the neonatal unit at MNH in 2022 in collaboration with Karolinska Institute and CTH [40]. The findings revealed that 42 out of 53 neonates were colonized with ESBL-producing EC/KP. The neonates get colonized from vertical transmission from the mother at birth, horizontal spread from healthcare staff to patients, and horizontal spread between neonates when sharing cots [39]. As a result, the unit emphasized the importance of not letting neonates share or be moved between cots [40]. The findings of this study were used to stress the need for proper hand hygiene to prevent the spread of infection and HAIs. Consequently, the healthcare staff at the unit were required to wash their hands before and after touching a patient and to use alcohol rub.

2.6 FISH

Fluorescence *in situ* hybridization (FISH) is typically based on the hybridization of an oligonucleotide probe to a sequence of interest. For bacterial detection one often targets a part of the 16S rRNA [8]. FISH is used to visualize DNA sequences or messenger RNA (mRNA) transcripts in for example cultured cells or tissue sections [41]. By attaching a fluorophore to the probe, the FISH sample can be visualized with fluorescence microscopy. The fluorophore can either be attached straight to the probe or linked to an antibody that is further linked to an antigen that is attached to the probe.

Most traditional FISH protocols use DNA probes which a short single-stranded DNA sequence or a mixture of multiple single-stranded DNA sequences, each covering a part of the target's length [41], [42]. However, these probes show limitations by having low affinity to their target, low robustness, and being prone to enzymatic degradation [42]. These limitations can be overcome by using nucleic acid mimics (NAMs) such as Peptide Nucleic Acid (PNA), Locked Nucleic Acid (LNA), and 2'-O- methyl-RNA (2'OMe). The target sequence of the genome, which the probe is designed to hybridize, can be composed of DNA, cDNA or RNA [41]. Each probe can have fluorophores attached to either the 3' terminus, 5' terminus, or both. There is a wide selection of fluorophores including, Cy3, Cy5, Alexa fluor and Quasar. The probes vary in length from 20 bases to over 1500 bases.

2.6.1 The FISH protocol

The FISH protocol generally consists of three steps; fixation and permeabilization, hybridization and post-hybridization treatments [41].

The fixation and permeabilization step of the FISH protocol is crucial to ensure proper probe penetration [41]. Most commonly 4% formaldehyde or paraformaldehyde (PFA) in phosphate-buffered saline (PBS) are used as fixative agents, where formaldehyde crosslinks lipids, peptides, and DNA to make the sample stable and minimize enzymatic degradation. The FISH samples are generally also permeabilized with detergents such as Tween-20 and Triton X-100. This allows the hybridization reagents to penetrate the sample.

The efficiency of the following hybridization step depends on the affinity of the probe, hybridization time and hybridization temperature [8]. Hybridization time is connected to the kinetics of the process. This includes the probe getting into the cell, binding to the complementary sequence, and unfolding rRNA structures. Hybridization temperature, i.e. the temperature at which the hybridization occurs, is connected to the probe's affinity to the target. The optimal hybridization temperature is specific for each probe and depends on the length and composition of the probe, where longer probes require higher hybridization temperatures than short probes [41]. A denaturant, typically formamide, is used to lower the hybridization temperature [8]. Formamide acts by lowering the thermal stability of double-stranded polynucleotides. This enhances accessibility to the rRNA target. Additionally, formamide competes for hydrogen bonding which further helps in separating the double-strands. It has therefore been assumed that the concentration of formamide together with the hybridization time and temperature are crucial variables in regulating the process stringency, i.e. how closely the probe binds to its target sequence. Other crucial conditions to be considered include salt concentration and pH [41].

The last step of the FISH protocol is a post-hybridization treatment, more specifically a washing step that is performed at the optimal hybridization temperature [41]. The purpose is to wash away nonspecific and unstable hybrids, i.e. when the probe binds to non-targeted sequences. This will allow only stable and specific hybrids (RNA:RNA or DNA:DNA) to remain. The washing step also removes unbound fluorescent probe molecules to reduce the background signal when analyzing the sample.

2.6.2 PNA-FISH

In PNA-FISH, Peptide Nucleic Acid (PNA) probes are used. PNA is a DNA mimic with an uncharged polyamide backbone that consists of repetitive units of *N*-(2-aminoethyl) glycine [43]. It was developed in the early 1990s and has been studied in multiple research and diagnostic applications since. For example, The Azevedo group at the University of Porto used a PNA-FISH assay year 2013 for the detection of *Lactobacillus* species and *Gardnerella vaginalis* which are associated with bacterial vaginosis, a common vaginal infection in women of reproductive age [44].

The uncharged backbone of PNA reduces the electrostatic repulsion between the PNA sequence and the target sequence, such as rRNA [8]. The reduced repulsion allows PNA to hybridize more efficiently with rRNA. The fact that PNA lacks charged phosphate groups also makes its hybridization independent from salt concentration. FISH with DNA probes requires higher salt concentrations to stabilize the duplex formed between the probe and the target rRNA sequence. The PNA probe's independence of salt concentration gives it greater affinity compared to DNA probes.

Because of their high affinity, PNA probes tend to be shorter than DNA probes (approximately 15 bases) [8]. The short probes have higher specificity since a single-base mismatch leads to a great destabilizing effect of the probe. Longer probes may tolerate such mismatches without getting destabilized. PNA probes also show a resistance to ubiquitous enzymes, such as proteases and nucleases, making them more biostable and suitable for diagnostic applications than DNA.

There are several alternative reagents in a hybridization solution to create an optimal environment for the probe to penetrate the sample [41]. By adding dextran sulfate, the hybridization rate will accelerate, and higher concentrations of dextran sulfate lead to an increased probe gradient during the membrane diffusion through the peptidoglycan layer [45]. Therefore, an increased concentration of dextran sulfate is favorable when it comes to gram-positive bacteria and their thick peptidoglycan layer. Contrariwise, a decreased concentration of dextran sulfate is favorable when targeting gram-negative bacteria. Additionally, having dextran sulfate in the hybridization solution has been shown to reduce the background signal [41].

A PNA-FISH assay can be used to detect different bacteria by varying the PNA-sequence [45]. A fluorescently labeled PNA probe enables the study of the distribution of bacteria within a sample using fluorescence microscopy (see Figure 2.4) [43].

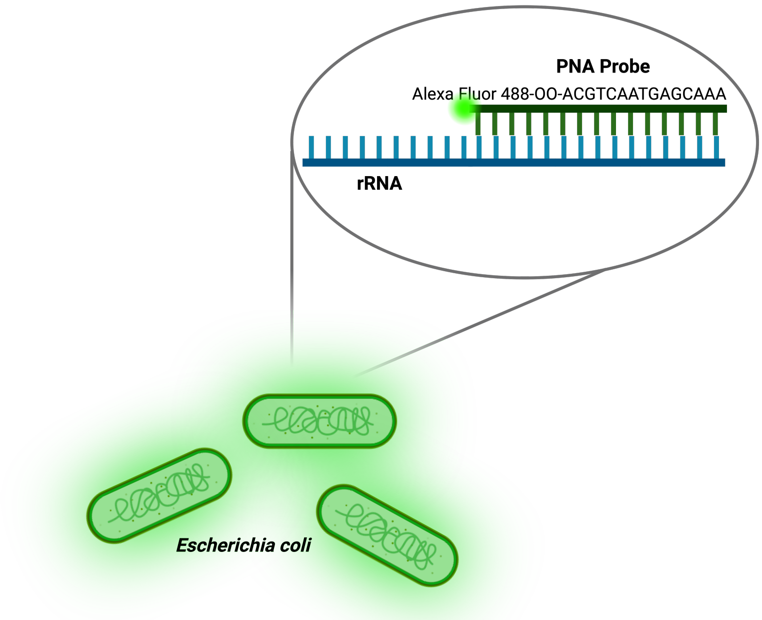

Figure 2.4: Detection of bacterial rRNA with a fluorescent PNA probe. Created in Biorender.com

2.6.2.1 Targeting ribosomal RNA for microbial identification

Differences in the sequences of ribosomal DNA among closely related species have facilitated the design of species-specific probes [43]. These probes provide a valuable tool for accurate identifications of microbial species by shifting the focus from relying on phenotypic characteristics to a single molecular target.

Another reason for using rRNA as a target for microbial identification is its cellular abundance of it [43]. The amount of rRNA relates to an organism's growth rate and EC can have as many as 10^4 - 10^5 molecules.

Since the hybridization of PNA probes is independent of the salt concentration, the hybridization can be performed under low concentrations which will destabilize the secondary structures of rRNA. This condition will enhance the probe's ability to attach to the highly structured target sequences. DNA probes have difficulty in accessing these regions. A valuable attribute of PNA probes is therefore that they can be designed with no consideration to the secondary structures of rRNA.

2.7 Fluorescence and Microscopy

Fluorescence is the light emitted by some molecules after absorbing energy. Incoming photons of a particular wavelength are absorbed by the molecule, causing the molecule, or so-called fluorophore, to excite to an increased energy state. When the molecule returns to its ground state, it emits photons with a longer wavelength than the absorbed ones. This is due to a certain loss of energy during the process [46].

There are several types of microscopes, yet their resolution is constrained by optical diffraction. A cause of this limitation is that one cannot, with optical microscopy, measure objects smaller than approximately 200 nm [47]. A fluorescence microscope is instead adapted to study samples that emit fluorescence. For the Zeiss fluorescence microscope, *Primo Star iLED* the incoming light source is a white LED diode with a peak intensity of 455 nm. Light passes through a bandpass excitation filter, that allows light with a wavelength between 450-470 nm to pass through. The light then hits a dichroic mirror that reflects light shorter than 477 nm down toward the sample, see figure 2.5, while longer wavelengths pass through [48]. The reflected light excites the sample through the objective. As a result of the energy loss, the emitted light has a longer wavelength and can then pass through the mirror to an emission filter, which filters out light below 485 nm. Finally, the light continues toward the eyepiece [46]. *Primo Star iLED* can also be used as a light microscope by changing the channel. The light then passes through the sample and a contrast is formed by the level of absorption as a result of the different components of the sample [49].

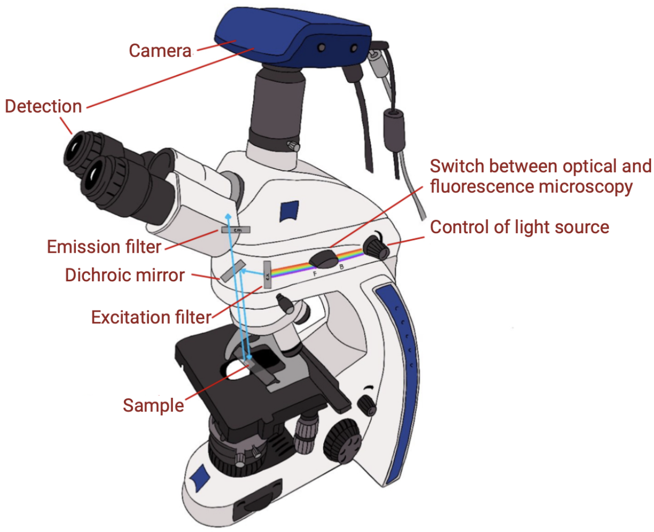

Figure 2.5: Schematic image of the *Primo Star iLED* microscope and the components. Created in Biorender.com.

2.7.1 Autofluorescence

Autofluorescence is when biological materials due to various internal components like flavins, nicotinamide adenine dinucleotide (NAD), amino acids, and collagen, emit light in response to being excited by light at specific wavelengths, covering the UV-visible to near-infrared range. [50] [51]. This spectrum of autofluorescence can overlap with the fluorescence of externally added markers, complicating fluorescent microscopy.

3

Materials & Methods

This chapter describes the different methods and materials used. The chapter is divided into several sub-parts starting with the culturing of EC, the preparation of solutions for PNA-FISH, followed by the general workflow for the PNA-FISH procedure, and modifications of the protocol. This section also covers the PNA-FISH procedure for patient samples.

3.1 Culturing of *Escherichia coli* for PNA-FISH optimization

The preparation of Luria-Bertani (LB) Agar plates (MP Biomedicals) was done by mixing 10.5 g of agar with 300 mL of MQ water. The mixture was autoclaved at 121°C for 2.5 hours. The solution was then poured onto agar plates (Ø90x15 mm, MFLAB Medical) until the bottom was covered. The plates cooled down for 10 minutes, the condensation was wiped off. Then the agar plates were stored at 4°C until use.

A non-pathogenic EC strain DH5α was inoculated on pre-heated (37°C) agar plates incubated overnight (16 hours) at 37°C. The plates were stored at 4°C after incubation.

Following the incubation, sub-culturing was conducted on the inoculated agar plate. An agar plate was pre-heated to 37°C. A single colony from the overnight plate was collected and suspended in 200 μL of super optimal broth with catabolite repression (SOC) medium (Thermo Fisher Scientific) in an Eppendorf tube. The tube was incubated with shaking (225 rpm) for 1 hour and 20 minutes at 37°C. Subsequently, the contents of the tube were transferred to the preheated agar plate. The medium was evenly spread using circular motions and a T-shaped bacterial cell spreader until fully absorbed by the agar. The plate was incubated at 37°C for approximately 16 hours, then stored at 4°C. The workflow for the culturing and sub-culturing of the EC DH5α is shown in Figure 3.1.

Note that the EC strain DH5α was used in all procedures for PNA-FISH optimization, including the initial tests of the PNA-probe for detection of EC, as well as to conduct tests with varying hybridization temperature and hybridization time. The KP probe was also tested with a fixated pure bacterial culture of KP (Gifted from Azevedo group, University of Porto).

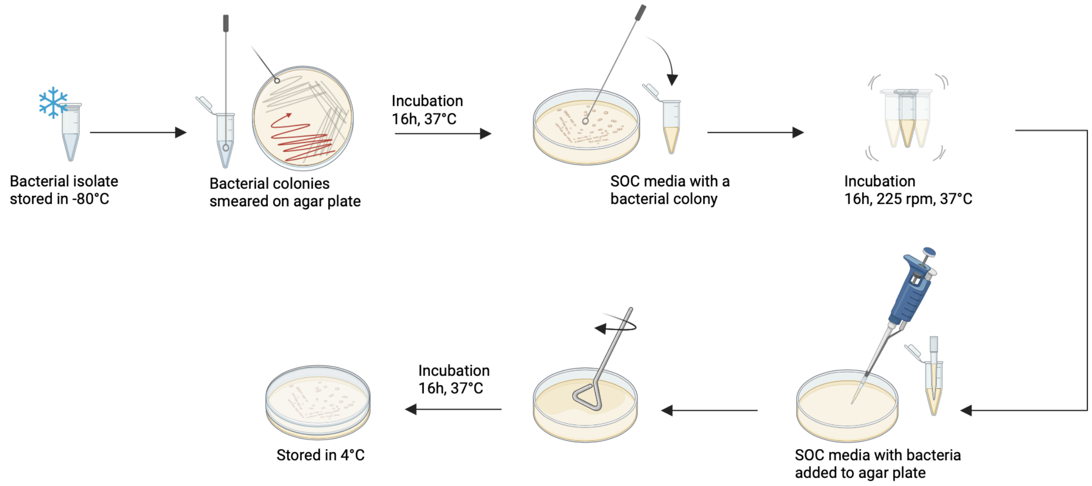

Figure 3.1: Schematic workflow for culturing and sub-culturing of EC, strain DH5α. Created in Biorender.com.

3.1.1 Fixation and permeabilization of *Escherichia coli* in suspension

By fixating and permeabilizing the cultivated EC in suspension, the samples could be stored for up to 6 months long periods in a freezer for a subsequent continuation of PNA-FISH.

The schematic workflow is depicted in Figure 3.2. To prepare an overnight-grown cell inoculum, a substantial amount of LB media was added to a 50 mL Falcon tube, in which EC DH5α (see Section 3.1) was suspended. The inoculum was placed in a shaking incubator at 37°C for 16 hours. Subsequently, the inoculum was diluted 1:10 in fresh LB media and incubated in a shaking incubator at 37°C for 2.5 hours. Next, the suspension was centrifuged at 10,000 x g at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of MilliQ water. The 1 mL liquid inoculum was then centrifuged at 10,000 x g at 4°C for 10 minutes, and the supernatant was discarded. The pellet was suspended in 400 µL 4% (vol/vol) paraformaldehyde in PBS (phosphate-buffered saline) (Thermo Fisher Scientific) and incubated for 1 hour. The liquid inoculum was then centrifuged at 10,000 x g at 4°C for 5 minutes, and the supernatant was discarded. The pellet was subsequently suspended in 500 µL 50% (vol/vol) ethanol and incubated at -20°C for at least 30 minutes.

Before performing FISH with the fixated EC, the fixated EC is heavily diluted with MilliQ water. The FISH procedure is next proceeded according to the general workflow of PNA-FISH (see Section 3.3.3), except that the fixation step only consists of fixating the bacterial smear with heat.

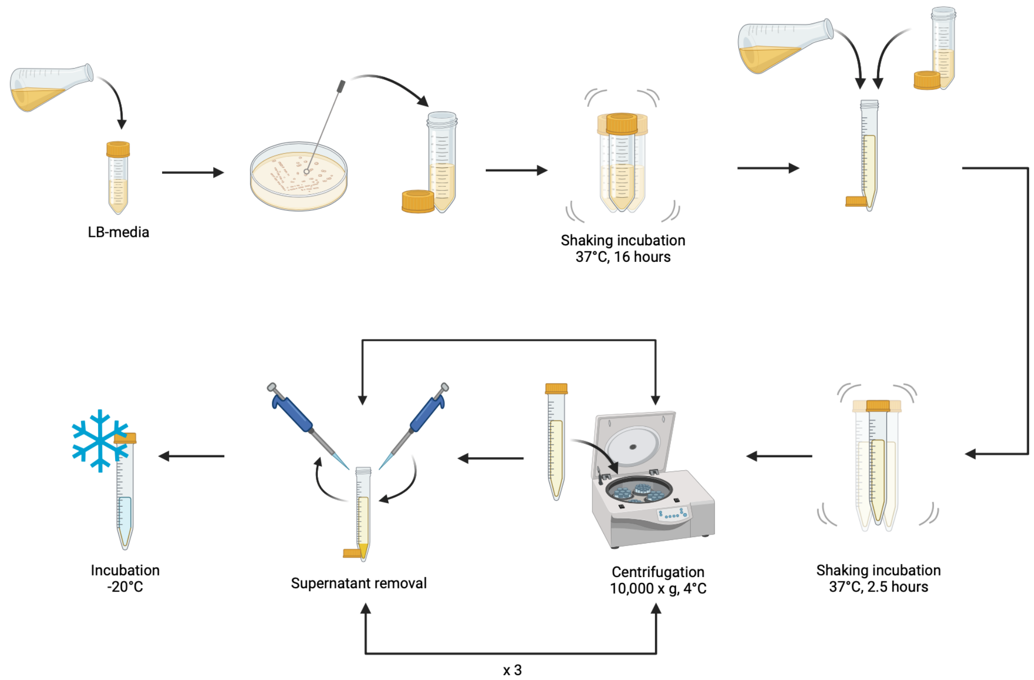

Figure 3.2: Schematic workflow for culturing, fixation, and permeabilization of bacteria. Created in Biorender.com.

3.2 Preparation of solutions and probe for PNA-FISH

The PNA-FISH assay of this project is based on the assay developed by the Azevedo Group, University of Porto, Portugal [52]. Two solutions are used in the PNA-FISH assay. The following section describes the preparation of the solutions for the PNA-FISH assay and their optimal storage conditions.

Stock solutions of Ethylenediaminetetraacetic acid disodium salt dihydrate (Disodium EDTA) (Sigma-Aldrich), Sodium pyrophosphate tetrabasic decahydrate (Sigma-Aldrich), Polyvinylpyrrolidone (Sigma-Aldrich), Ficoll® (Sigma-Aldrich), Trizma® base (Sigma-Aldrich) and Sodium chloride (Sigma-Aldrich) were prepared to simplify the preparation of the PNA-FISH solutions (see Appendix A.2).

3.2.1 Hybridization solution

The hybridization solution for the PNA-FISH procedure was prepared in a 50 ml falcon tube; all chemicals and their final concentrations can be seen in Table 3.1.

Table 3.1: Chemicals and concentrations of components in the hybridization solution used for the PNA-FISH assay

Hybridization solution	
Chemical	**Concentration**
NaCl	0.01 M
Disodium EDTA	0.005 M
Tris-HCl	0.05 M
Urea	4 M
Dextran sulfate	10%(wt./vol)
Sodium pyrophosphate	0.1%(wt./vol)
Polyvinylpyrrolidone	0.2%(wt./vol)
Ficoll	0.2%(wt./vol)
Triton X-100	0.1%(vol/vol)
Ultrapure water	

To prepare 50 ml of hybridization solution 5 g Dextran sulfate sodium salt (Sigma-Aldrich), 12.012 g Urea 99.5 % (Thermo Fisher Scientific), and 2.5 mL Trizma® hydrochloride solution (Sigma-Aldrich) was dissolved in 20 ml of UltraPure Distilled Water (Invitrogen). Next, the stock solutions (see Appendix A.2) were added, 5mL disodium EDTA, 2.5 mL sodium pyrophosphate, 0.80 mL polyvinylpyrrolidone, 1.33 mL Ficoll® and 0.5 mL NaCl. The final volume was adjusted to 50 ml with UltraPure water. The pH was adjusted to 7.5 using HCl. To sterilize the solution and remove any particles the solution was filtered through a 0.2 μm porosity acrodisc into a new 50 ml falcon tube. The hybridization solution was stored in 4°C.

3.2.2 Washing solution

The washing solution for the PNA-FISH procedure was prepared in heat-resistant glassware, see components and concentrations in Table 3.2. The washing solution should be as fresh as possible and was therefore stored for maximum one week.

Table 3.2: Chemicals and concentrations of components in washing solution used for the PNA-FISH assay

Washing solution	
Chemical	**Concentration**
Tris base	0.005 M
NaCl	0.015 M
Triton X-100	0.1%(vol/vol)
Distilled water	

To prepare 1 L of washing solution 50 mL of Trizma® Base stock solution and 15 mL NaCl stock solution were added (see Appendix A.2 for details regarding the stock solutions) together with 1 mL Triton™ X-100 (Sigma-Aldrich). Distilled water was used to reach the desired volume of 1 L. The solution was autoclaved for 15 minutes at 121°C. The washing solution was allowed to cool down to room temperature and was then stored at 2-8 °C.

3.2.3 Stock aliquots and working solution of PNA-probe

The preparation and handling of the PNA probe were performed in low-light conditions to avoid bleaching the fluorophores. The probes were received in lyophilized form and stored at -20°C. Both the PNA probes for the detection of EC and KP are composed of a PNA sequence and are labeled with the fluorophore Alexa Fluor 488 (see Table 3.3) and both probes were prepared in the same way. Alexa Fluor 488 emits light in the green region of the visible spectrum. The Azevedo Group at the University of Porto conducted tests on hybridization temperature and time to measure the effectiveness of both probe's binding. For both the EC and KP probes, a hybridization temperature of 50°C for 60 minutes achieved the most optimal signal. The tubes containing the lyophilized probes were centrifuged for 10 minutes (5500 rpm). After that, the 4 µM stock aliquots were prepared by adding 1 ml of ultrapure water to the lyophilized probe. The dissolved probe was incubated at room temperature for 30 minutes to ensure a homogeneous solution before further use. The working solution was prepared by diluting the stock aliquots with a hybridization solution in a 5:95 ratio. The stock aliquots, as well as the working solution, were stored in -20°C wrapped in aluminum foil.

Table 3.3: Target bacterial species and the corresponding probe sequences.

PNA-FISH Probes		
Strain	**Probe (5'-3')**	**Emitted fluorescence**
EC	Alexa Fluor 488-OO-ACGTCAATGAGCAAA	Green
KP	Alexa Fluor 488-OO-CTACACACCAGCGT	Green

3.3 PNA-FISH general workflow

The PNA-FISH assay is divided into three parts: fixation and permeabilization, hybridization, and washing. The samples are thereafter visualized using the Zeiss® Primostar iLED microscope. Additionally, instruction manuals of the PNA-FISH assay were composed and two persons from the staff at the Bacteriology department of MNH were instructed and trained to perform the assay.

3.3.1 Fixation and permeabilization

FISH was carried out either on epoxy diagnostic microscope slides (Thermo Fisher Scientific) with three wells or regular microscope slides (Thermo Fisher Scientific). Microscope slides were prepared one for the sample and one for the negative control, by marking the working area with a circle on the backside of the slide using a permanent marker. If diagnostic epoxy slides were used, there was no need to mark the working area. The bacterial solution was prepared using non-pathogenic EC, strain DH5α. This was performed by suspending approximately two colonies from a sub-culture plate in 500μL MQ-water in an Eppendorf tube. The concentration should be approximately 10^7 cells/mL. The bacterial solution was homogenized by vortexing for approximately one minute. 50μL of the bacterial solution was added to the designated working area of the microscope slides or the wells of epoxy slides. For each sample, two replicates were done. The slides were incubated in 55°C until complete drying was achieved. The bacterial smear on each slide was fixated by using a lighter beneath the slide for 10 seconds and the black soot was wiped off from the slides. Further, the bacterial smear on each slide was first covered with 50-100μL (the volume depends on the size of the well or marked working area) 4% (vol/vol) paraformaldehyde in PBS (Thermo Fisher Scientific) and incubated at room temperature for 10 minutes before the excess paraformaldehyde was removed with a KIMTECH tissue. Secondly, the smears were covered with 50-100μL (the volume depends on the size of the well or marked working area) 50% (vol/vol) ethanol (Thermo Fisher Scientific) and incubated at room temperature for 10 minutes before the excess was removed with a KIMTECH tissue. The microscope slides were left to air-dry for a few minutes.

3.3.2 Hybridization

The hybridization step was initiated by heating the incubator to the appropriate hybridization temperature. Two Coplin jars (Avantor™), one for the sample with probe and one for the negative control, were prepared by filling them with washing solution and placing them in the heated incubator to pre-heat. Simultaneously, 20μL of hybridization solution was added to the negative control slide and 20μL working solution (hybridization solution with PNA-probe) was added to the other slide. Thereafter, a coverslip (Thermo Fisher Scientific) (26x60 mm) was added on top of each slide and the slides were placed in a closed container. Damp tissues were placed in the container to keep the environment humid during the incubation. The slides were incubated for 60 minutes at the appropriate hybridization temperature.

3.3.3 Washing

After the hybridization incubation, the coverslips were removed from the slides. If regular glass slides were used, a tissue with ethanol was used to remove the circular outline on the back of the slides marking the working area. Then the slides were placed in separate Coplin jars with the pre-heated washing solution. Thereafter, the coplin jars with slides were incubated at the hybridization temperature for 30 minutes. Subsequently, the slides were taken out of the Coplin jars and left to dry in a dark area before analysis.

3.4 Microscopy

The PNA-FISH slides were analyzed using the Zeiss® Primostar iLED microscope. Before microscopy, one drop of immersion oil (Zeiss®) was placed on the sample area of the slide and covered with a coverslip (26x60 mm). The coverslip was gently pressed to the microscope slide. Each slide was analyzed using a 100x oil immersion objective after placing another drop of immersion oil on top of the coverslip. The documentation of the samples was performed using a Samsung S21 camera, see settings in Appendix A.3. A smartphone holder was attached to the center lens of the camera and put on the ocular of the microscope. Approximately five pictures were taken on each replicate.

To convert a measurement in pixels to µm, a 10 μm ruler was photographed with the Samsung S21 mounted on the microscope *Primo Star iLED* (see Figure 3.3).

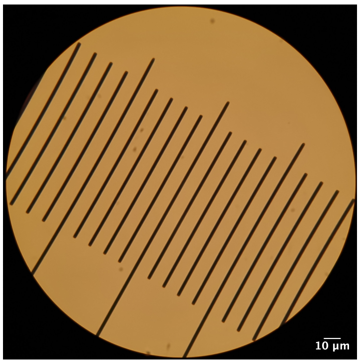

Figure 3.3: 10 μm ruler on microscope glass slide photographed with a Samsung S21 through the ocular of the microscope *Primo Star iLED* from Zeiss.

The image of the ruler was opened in the image processing program ImageJ and measurements were taken between the marks. The conversion factor for the Samsung s21 camera used to capture images to document the results was 1 pixel equals 0.079 μm.

3.5 Modifications to PNA-FISH protocol

To evaluate the impact of the hybridization temperature and time, the PNA-FISH assay was performed with varying conditions. Additionally, the PNA-FISH solutions were simplified by removing certain components. Each test was conducted twice. Note that the EC strain DH5α, fixated and permeabilized as described in Section 3.1.1, was used for all tests. The Alexa Fluor 488 probe for EC at a concentration of 5:95 (probe stock aliquot solution: hybridization solution) was used for all tests.

3.5.1 Hybridization time and temperature

To evaluate the influence of the hybridization temperature the PNA-FISH assay (see Section 3.3.3) was performed at 22 (room temperature), 45, 55, and 65 degrees. In these experiments, EC strain DH5α was used. All tests were conducted according to the PNA-FISH assay except for the changed hybridization temperature.

To evaluate the impact of hybridization time the PNA-FISH assay (see Section 3.3.3) was performed with a hybridization time of 15, 30, 45, and 60 minutes. All tests were conducted according to the PNA-FISH assay, at 50°C, except for the modified hybridization time.

3.5.2 Simplification of PNA-FISH solutions

A simplified hybridization solution was prepared according to Section 3.2.1, but only containing urea, dextran sulfate, NaCl, disodium EDTA, and Tris-HCl. The remaining chemicals (see Table 3.1) were replaced with ultrapure water. The simplified hybridization solution was also used to prepare a probe working solution according to Section 3.2.3. The PNA-FISH assay was next performed according to Section 3.3.3.

A simplified washing solution was prepared according to Section 3.2.2, excluding Triton X-100 that was exchanged with MilliQ-water. The PNA-FISH assay was next conducted according to Section 3.3.3.

3.6 Field studies at MNH, Dar es Salaam, Tanzania

Field studies were conducted from mid-January to early June 2024. The preparatory phase, from February to May, involved detailed planning for the establishment of PNA-FISH at the Bacteriology Department of MNH. This included extensive communication with the staff at the department, as well as coordination with suppliers needed to ensure the availability of the necessary laboratory materials. The PNA-FISH protocol was optimized to suit the capabilities of the lab at the Bacteriology department. During the optimization phase, PNA-FISH was performed on pure bacterial cultures to establish and validate the assay in the local environment, before commencing the actual screening at the neonatal ward at MNH.

3.6.1 Study duration, area and inclusion criteria

The study was conducted at the neonatal unit at MNH, Dar es Salaam, Tanzania. All admitted and discharged neonates (0-28 days) from the unit were included in the study, starting from the 28th of May and continuing for one month. Neonates who died within 12 hours of admission were excluded from the study. Due to time constraints, the results of this project will be based on the samples collected from the 28th of May to the 1st of June.

3.6.2 Data collection

The data collection was performed by the staff of the neonatal unit. Antenatal history such as febrile illness prior to birth, mode of delivery, malaria/HIV status, and use of antibiotics (broad-spectrum>4 h prior birth, GBS spectrum >2 h prior birth, no antibiotics or any antibiotics < 2h prior to birth), was recorded. Rectal swabs were taken from all neonates (0–28 days) upon admission and discharge from the neonatal unit.

3.6.3 Microbiological procedures

Two rectal swabs were collected for each neonate, both at admission and discharge, using sterile cotton swabs. The cotton swabs were placed in vacuum blood collection tubes (Revital) containing 2 mL TSB as a transport medium for the shipment from the neonatal unit to the Bacteriology lab. The content of one of the tubes was used for PNA-FISH to determine the colonization status of ESBL-producing EC (see Section 3.8.1). The second tube was inoculated on ESBL-selective chromogenic agar plates (Liofilchem® Chromatic ESBL) for detection of ESBL-producing EC and KP.

3.6.4 Ethics approval

This study obtained clearance from the National Institute for Medical Research (NIMR), Dar es Salaam, Tanzania. Written informed consent was obtained from parents of all neonates participating in the study (See Appendix A.1).

3.7 Preparatory work for screening

Tryptone Soya Broth (TSB) (Thermo Fisher Scientific) was prepared by adding 40 g/L powder to distilled water followed by autoclaving for 15 minutes at 121°C. To control the sterility of the media 100 μL was inoculated on a MacCONKEY AGAR (Thermo Fisher Scientific) plate and incubated at 37°C for 16-24 hours.

After the incubation, the plate was checked for growth, if no growth the medium was considered sterile. 2 mL of the TSB was added to separate plain vacuum blood collection tubes (Revital), and the tubes were sent to the neonatal unit where the samples were collected.

3.7.1 Preparation of ESBL-selective chromogenic agar plates

The chromogenic ESBL agar plates were prepared by adding 59.2 g of the Chromatic ESBL Agar Base (Liofilchem®) to 1 L destilled water. The solution was autoclaved at 121°C for 15 minutes. When the agar solution had cooled down to 50°C two vials of Chromatic ESBL supplement (Liofilchem®) were added. The solution was gently stirred before being distributed to agar plates. The plates were allowed to solidify and then stored at 2-8°C pending inoculation.

3.7.2 Milk media preparation

For long-term storage of bacterial isolates of EC and KP, picked from the chromatic ESBL Agar plates, a milk media was used. TSB, alpha-D-Glucose pentaacetate 98 (Thermo Fisher Scientific), Skim milk (Liofilchem®), Glycerol (Sigma-Aldrich), and distilled water was mixed according to Table 3.4. The solution was stirred until achieving a homogeneous solution.

Table 3.4: Chemicals and their volumes for the preparation of milk media.

Chemical	**Volume**
Skim milk powder	8 g
Glucose	2 g
TSB	12 mL
Glycerol	40 mL
Destilled water	400 mL

Aliquotes with 1 ml milk media were prepared in 2 ml microctubes (Sanifico) and autoclaved at 121°C, for 15 minutes. The milk media tubes were stored at 2-8°C until bacterial isolates were added and transferred to -80°C for long-term storage.

3.7.3 Antibiotics supplemented TSB media

TSB was prepared according to Section 3.7. Clindamycin (Tidact), Cefotaxime (Alkem), and Vancomycin (Swiss Parenterals) were measured according to Table 3.5 and each antibiotic was dissolved in 1 mL distilled water, before being added to the room temperatured TSB media.

Table 3.5: Antibiotics and their concentration in TSB media used for cultivation of ESBL producing EC.

Antibiotic	**Concentration (mg/L)**
Cefotaxime	12
Vancomycin	10
Clindamycin	8

To check that the antibiotics-supplemented TSB media was sterile and potent enough to inhibit or select specific bacterial strains as intended, quality control was performed. The media was designed to select ESBL-producing bacteria. For quality control, an ESBL-positive strain of KP isolated from a patient sample from MNH was used. This strain was confirmed, by staff at the MNH, as resistant against cefotaxime. An ESBL negative strain, the non-resistant EC strain (ATCC 8739), was also used. The quality control was performed by adding 1 mL of TSB to three separate falcon tubes. Approximately two colonies of the ESBL-positive strain were added to one of the tubes, and to another tube, two colonies of the ESBL-negative strain were added. One tube was left with only the supplemented TSB. All tubes were incubated for 5 hours at 37°C and vortexed every hour. After the incubation, the sterility of the supplemented TSB was controlled by spreading 100 μL of the TSB with a sterile cotton swab to a MacCONKEY AGAR (Thermo Fisher Scientific) plate. To control the selectivity of media, the ESBL negative strain was inoculated on both a MacCONKEY AGAR (Thermo Fisher Scientific) plate and a Chromatic ESBL Agar Base (Liofilchem®) plate and the ESBL positive strain was inoculated on a Chromatic ESBL Agar Base (Liofilchem®) plate. The procedure for both strains was to spread 100 μL of the supplemented TSB with each strain after the 5-hour incubation using a sterile cotton swab. All plates were incubated at 37°C for 16-24 hours and controlled for bacterial growth. For a passed quality control there should be no growth on the sterility test and the ESBL negative plates and there should be growth on the ESBL positive plate. When the supplemented TSB media passed the quality control it could be used for PNA-FISH as the selective media for cultivation in a solution of fecal samples (see Section 3.8.1).

3.8 Screening workflow

When the neonatal fecal samples arrived to the lab at MNH, one swab was used for PNA-FISH, and the other for inoculation on a chromogenic ESBL agar plate. The selective plates were used as a verification step to compare the PNA-FISH results. The workflow of the screening is illustrated in Figure 3.4.

The chromogenic ESBL agar plates were pre-heated to 37°C. The swab from the sample tube was used to add a drop of the transport media onto the plate and an inoculation loop was used to spread the sample. This was followed by a 16-24 hour incubation at 37°C. The plate was observed and details of the eventual colonies were noted.

For each patient where bacterial growth was visualized on the chromatic ESBL agar plate, blue colonies (KP) and pink colonies (EC) were picked and transferred to separate milk media tubes, two tubes for blue colonies and two tubes for pink colonies (see Figure 3.4). One pair of tubes was stored at MNH at -80°C for long-term storage, and the other pair was sent to the Karolinska Institute for further analysis.

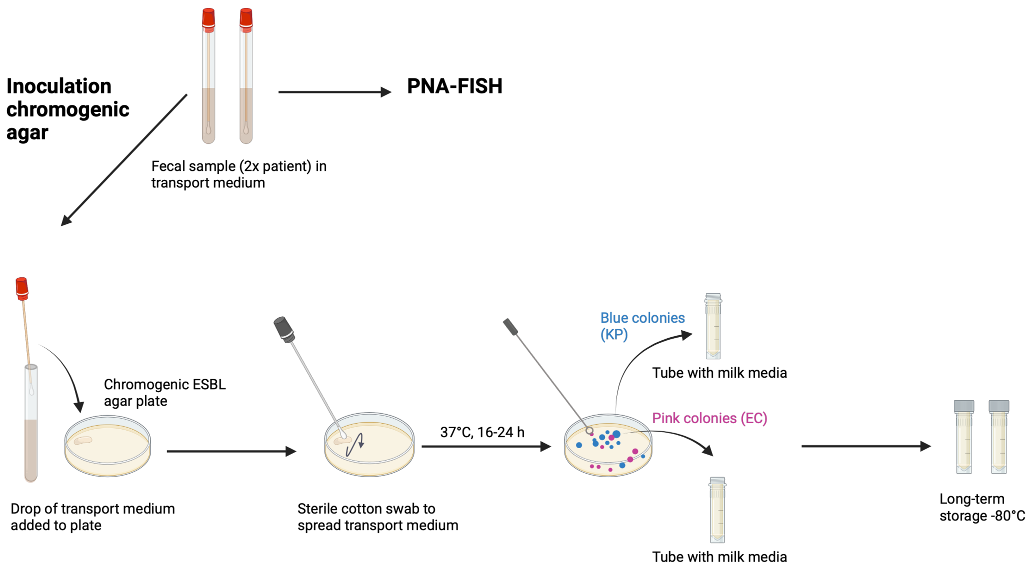

Figure 3.4: Schematic workflow from when patient samples were received to long-term storage of bacterial isolates. Created in Biorender.com.

3.8.1 Selective culture of fecal samples for PNA-FISH

The selective culture was conducted using separate vacuum blood collection tubes (Revital) for each sample. To each tube, 1 mL of patient fecal sample transport media was added with 3 mL TSB supplemented with antibiotics according to Table 3.5. The lid of the tubes was opened to ensure the availability of oxygen for the bacteria and next incubated at 37°C for 5 hours. The tubes were vortexed for 1 minute once every hour during the incubation period.

After the incubation, to extract the bacteria from the supplemented TSB broth, the tubes were centrifuged for 10 minutes at 5,500 rpm to get a pellet. The supernatant was carefully removed and discarded, leaving only the bacterial pellet in the tube. The bacterial pellet was re-suspended in 2 mL of distilled water to create a liquid inoculum. The liquid inoculum was used to perform PNA-FISH according to Section 3.3.3. After the microscopy analysis (see Section 3.4), the results of the PNA-FISH procedure for each patient were compared to the result of the inoculation on the chromatic agar plate.

4
Results and Discussion

The results obtained from the project are presented in the following chapter, starting with the results of the first evaluation of the PNA-probes for the detection of EC and KP as well as the results of the modifications implemented to the PNA-FISH protocol. Next, the results of the field studies at MNH are presented, including the PNA-FISH results as well as the results of the verification step with ESBL-selective chromogenic agar plates.

Note that all images that are presented in the following chapter are captured with a Samsung S21 phone that is attached to the ocular of a Zeiss® Primostar iLED microscope.

4.1 First evaluation of EC and KP probe

A PNA-FISH assay was performed at CTH according to Section 3.3.3 with EC DH5α (see Section 3.1.1) to test the probe for detection of EC. Figure 4.1a shows the sample to which the PNA-probe for detection of EC was added. The PNA-probe for EC showed expected hybridization efficiency where clearly fluorescent EC could be seen. Figure 4.1b shows the negative control to which the PNA-probe was not added. This image is dull and no fluorescence intensity, apart from the auto-fluorescence of EC, could be detected. This implies that the PNA-probe for detection of EC worked as expected.

a) b)

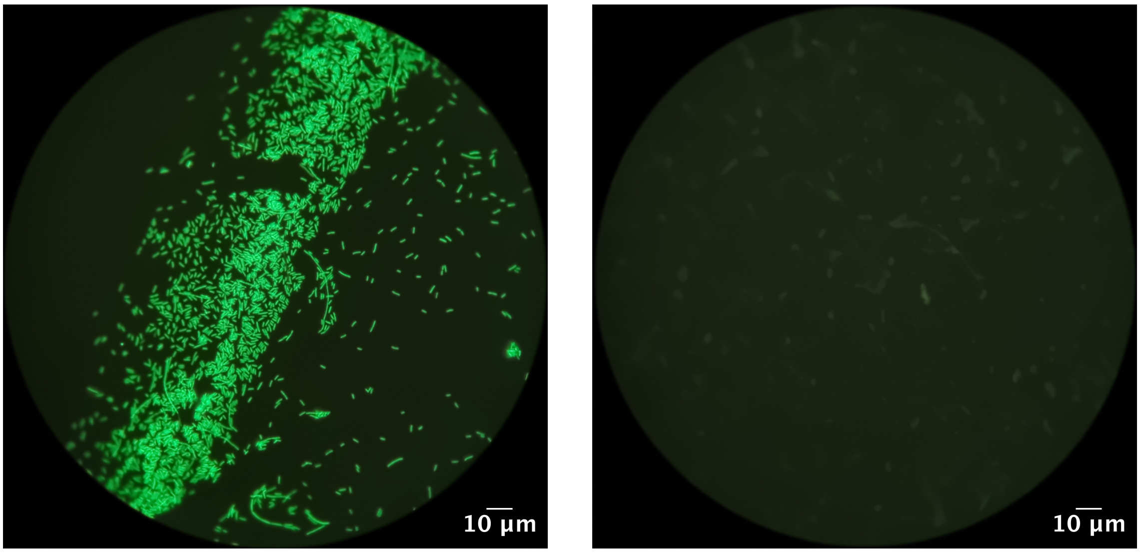

Figure 4.1: First evaluation of EC probe with Alexa Fluor 488 (EC strain DH5α): a) Sample (PNA-probe added), b) Negative control (no PNA-probe added).

An initial PNA-FISH procedure was also conducted with pure KP cultures (Gifted from Azevedo group, University of Porto). Figure 4.2a presents the sample to which the PNA-probe for the detection of KP was added. Comparing Figure 4.2 a-b, the sample to the negative control, it could be concluded that the PNA-probe for detection of KP did not show any hybridization efficiency since no fluorescent KP was seen. The project proceeded therefore solely focusing on the PNA-probe for EC.

a) b)

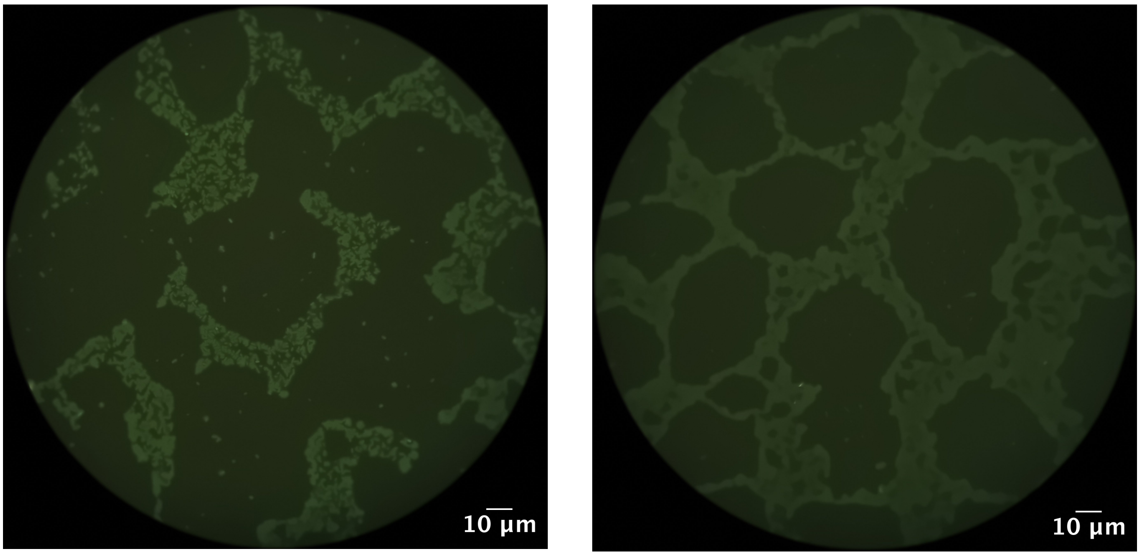

Figure 4.2: First evaluation of KP probe with Alexa Fluor 488: a) Sample (PNA-probe added), b) Negative control (no PNA-probe added).

4.2 Modifications to PNA-FISH protocol

Modifications were made to the PNA-FISH protocol by varying the hybridization time and temperature. Additionally, the PNA-FISH solutions were simplified by removing certain components. The differences in fluorescence intensity between the different tests were determined by visually analyzing the images. While only one microscopy image is presented in all cases, it is important to acknowledge that it does not represent the full scope of reality. To draw conclusions, multiple images from various areas of the sample was thoroughly analyzed visually.

4.2.1 Modified hybridization temperature

To evaluate the impact of the hybridization temperature, both higher and lower temperatures than the optimal 50°C for EC were tested. The hybridization temperatures tested were 65°C, 50°C, 45°C and 22°C (room temperature).

When performing PNA-FISH at 65°C, no to very low fluorescence could be seen (see Figure 4.3 a-b). This is to be compared with the normal case (see Figure 4.3 c-d) where hybridization occurs at 50°C and visibly fluorescent EC is obtained. Thus, it was concluded that PNA-FISH with the EC probe should not be performed at 65°C or above.

However, when performing FISH at 45°C, the probe did successfully bind to the bacteria despite conducting FISH at a lower temperature than optimal (see Figure 4.3 e-f). Comparing the results of FISH performed at 45°C with those following the standard protocol suggests no visible difference in fluorescence intensity.

When performing FISH at room temperature, 22°C, the probe showed effective binding to the bacteria (see Figure 4.3 g-h). It could be further concluded that PNA-FISH can be carried out at a minimum of 20 degrees lower than the optimal temperature without compromising the quality of the results.

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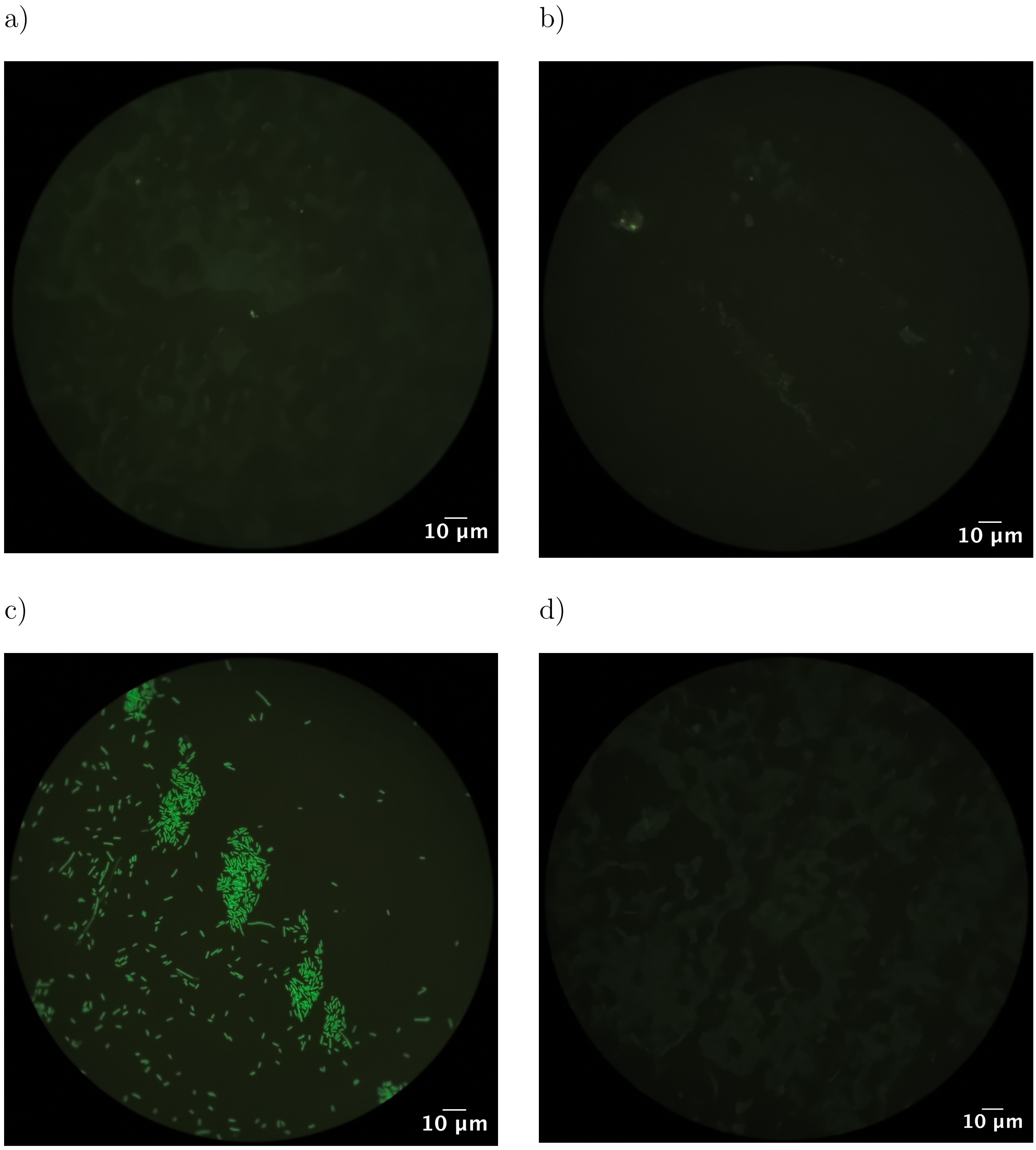

Figure 4.3: Varying hybridization temperatures (EC strain DH5α, Alexa Fluor 488): a) 65°C, b) 65°C negative control, c) 50°C, d) 50°C negative control.

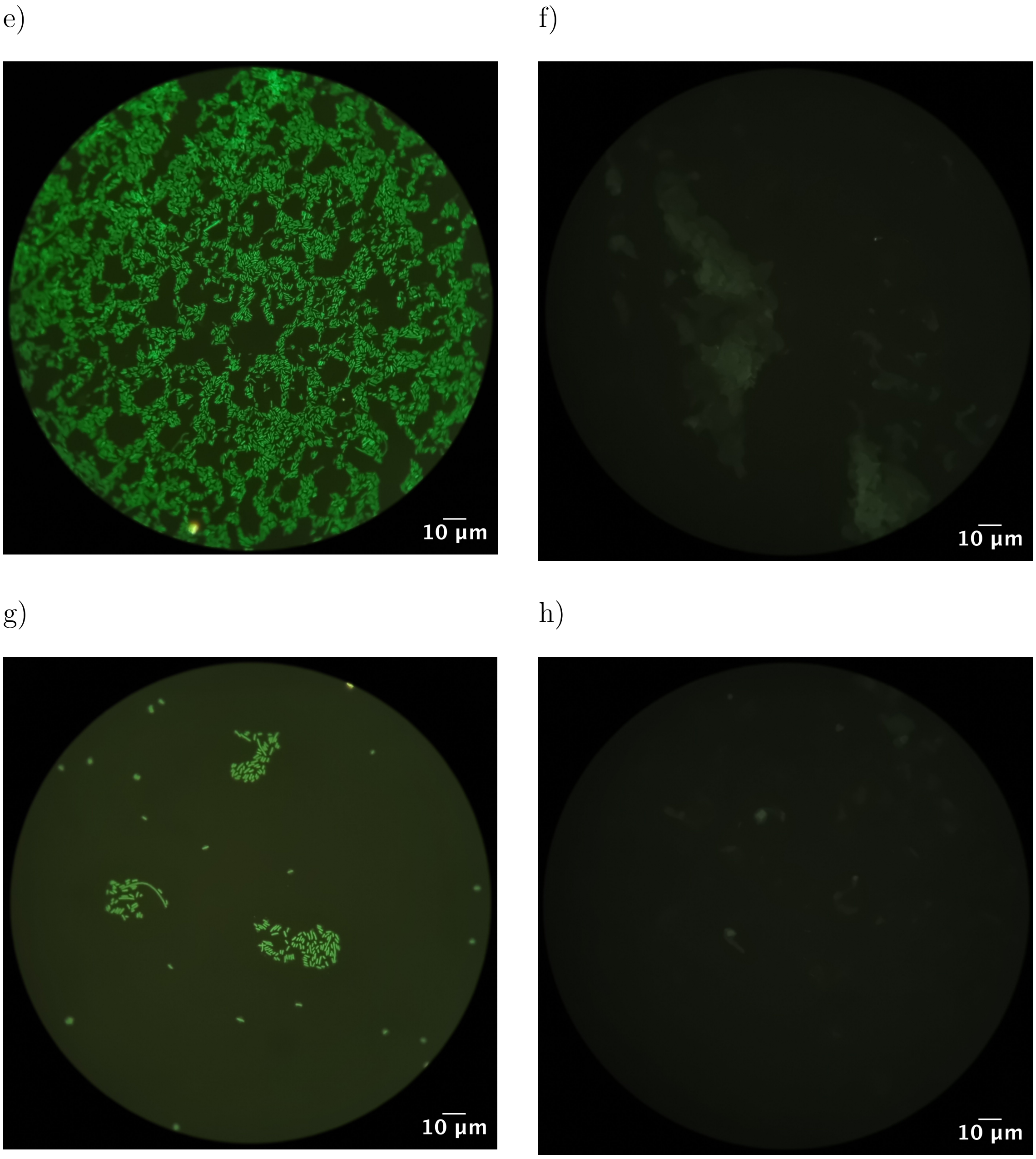

Figure 4.3: Varying hybridization temperatures (EC strain DH5α, Alexa Fluor 488): e) 45°C, f) 45°C negative control, g) 22°C, h) 22°C negative control.

4.2.2 Modified hybridization time

The optimal time of hybridization for detection of EC with PNA-FISH is 60 minutes, making it the most time-consuming step of the assay. To evaluate the impact of the hybridization time, the PNA-FISH assay was conducted with reduced times starting from the optimal hybridization time. The hybridization times tested were 60, 45, 30, and 15 minutes.

It is evident that fluorescent bacteria were still achieved when comparing the results of FISH with 45 minutes of hybridization (see Figure 4.4 c-d) to the optimal case with 60 minutes of hybridization (see Figure 4.4 a-b). This could be concluded due to the negative control showing sharp contrast to the sample, indicating that the assay is working efficiently. When shortening the hybridization time to 30 min, fluorescent bacteria were still achieved, and the negative control further confirmed that the assay worked (see Figure 4.4 e-f). Moreover, shortening the hybridization time to 15 min also resulted in clearly fluorescent bacteria (see Figure 4.4 g-h). Therefore, it could be concluded that shorter hybridization times did not affect the ability of the probe to bind to the bacteria. In all cases, clearly fluorescent bacteria with low to no difference in intensity could be achieved. This further implies that PNA-FISH can be conducted in a more time-efficient manner without having to compromise on the quality of the results.

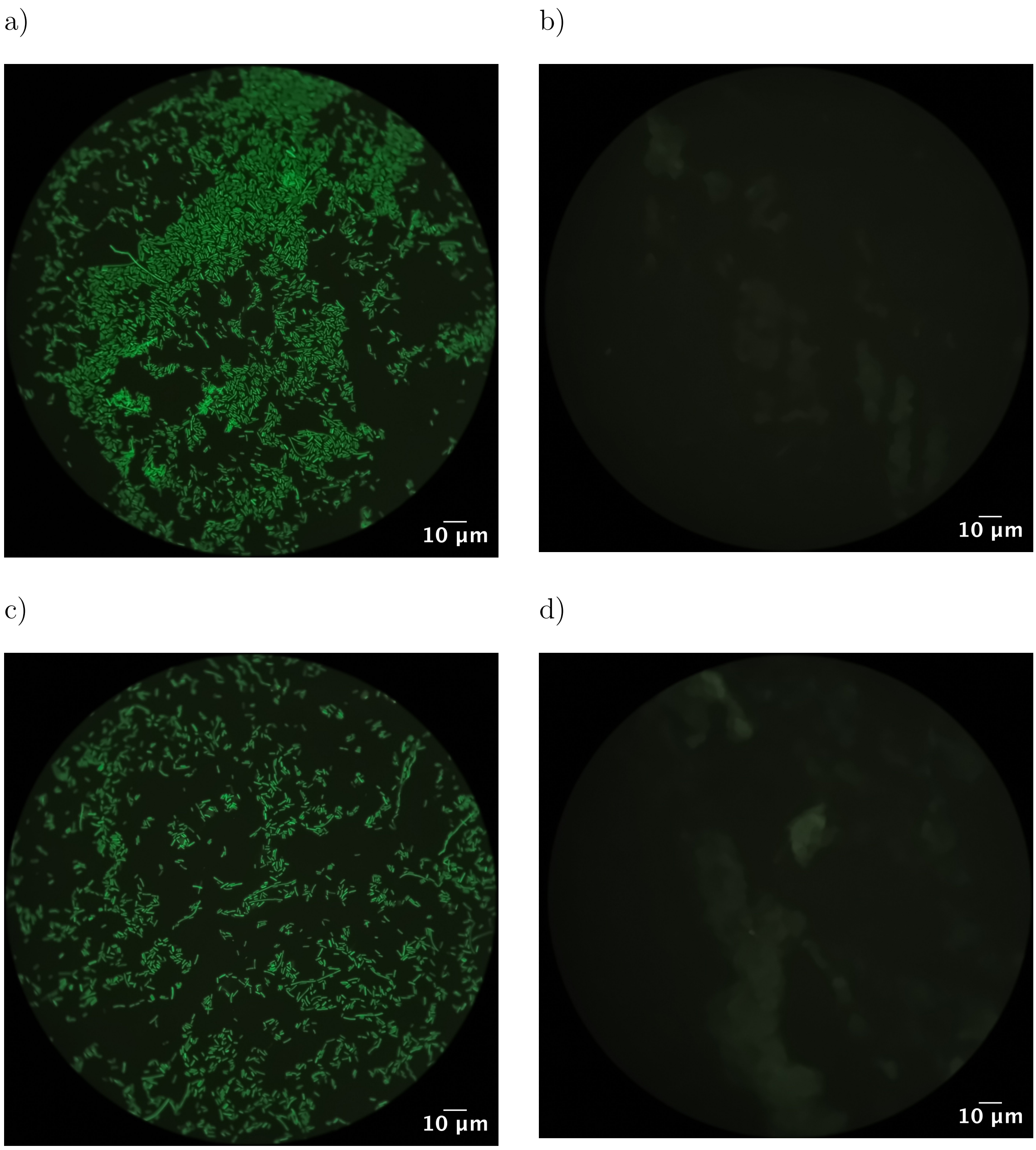

Figure 4.4: Varying hybridization times (EC strain DH5α, Alexa Fluor 488): a) Normal: 1 hour, b) 1 hour, negative control, c) 45 min, d) 45 min negative control.

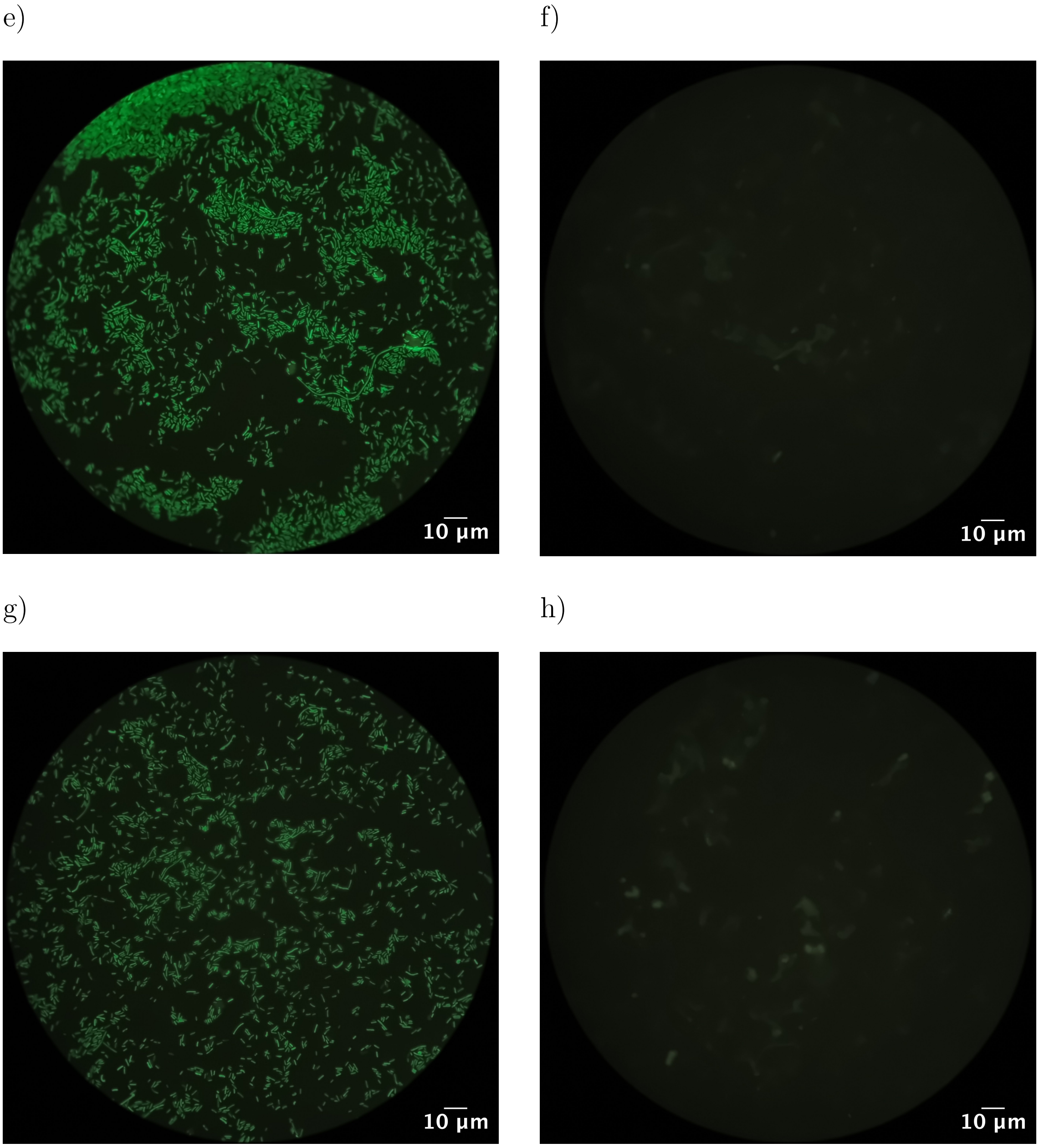

Figure 4.4: Varying hybridization times (EC strain DH5α, Alexa Fluor 488): e) 30 min, f) 30 min negative control, g) 15 min, h) 15 min negative control.

4.2.3 Simplification of PNA-FISH solutions

Since the project aims towards low-resource settings, it was of interest to evaluate whether the PNA-FISH procedure could be simplified. Therefore simplifications of the FISH procedure solutions, washing solution and hybridization solution, were done and tested in the assay. The goal was to identify the crucial components for the PNA-FISH assay to function properly.

When testing the simplified hybridization solution, the results were compared to the results of the normal hybridization solution (according to Section 3.2.1). When visually comparing the fluorescence intensity of the EC in the case where the simpler hybridization solution was used (see Figure 4.5 c-d) to when the normal hybridization solution was used (see Figure 4.5 a-b), no difference could be seen.

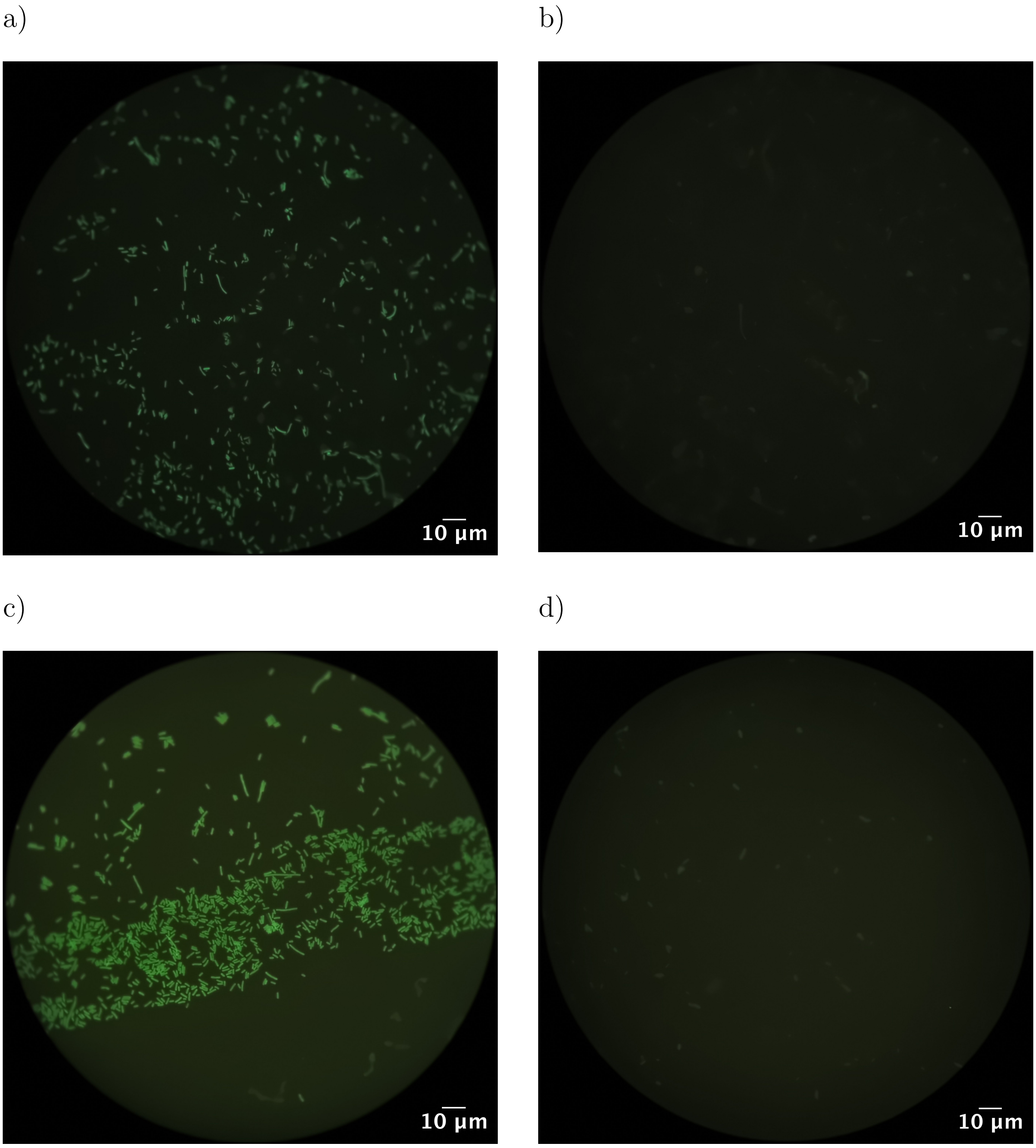

Figure 4.5: PNA-FISH with normal and simpler hybridization solution (EC strain DH5α, Alexa Fluor 488): a) Normal hybridization solution, b) Normal hybridization solution, negative control, c) Simpler hybridization solution, d) Simpler hybridization solution, negative control.

Although the simpler hybridization solution included fewer chemicals, it still allowed the PNA-probe to hybridize to the EC. It could therefore be concluded that the PNA-FISH assay could be performed using a simpler hybridization solution for detection of EC DH5α.

When testing the simplified washing solution (excluding Triton X-100), the results were compared to the results of a PNA-FISH procedure using the normal washing solution (including Triton X-100). When visually comparing the results of using the simpler washing solution (see Figure 4.6 c-d) to the results of using the normal washing solution (see Figure 4.6 a-b), no clear differences in fluorescence intensity could be seen.

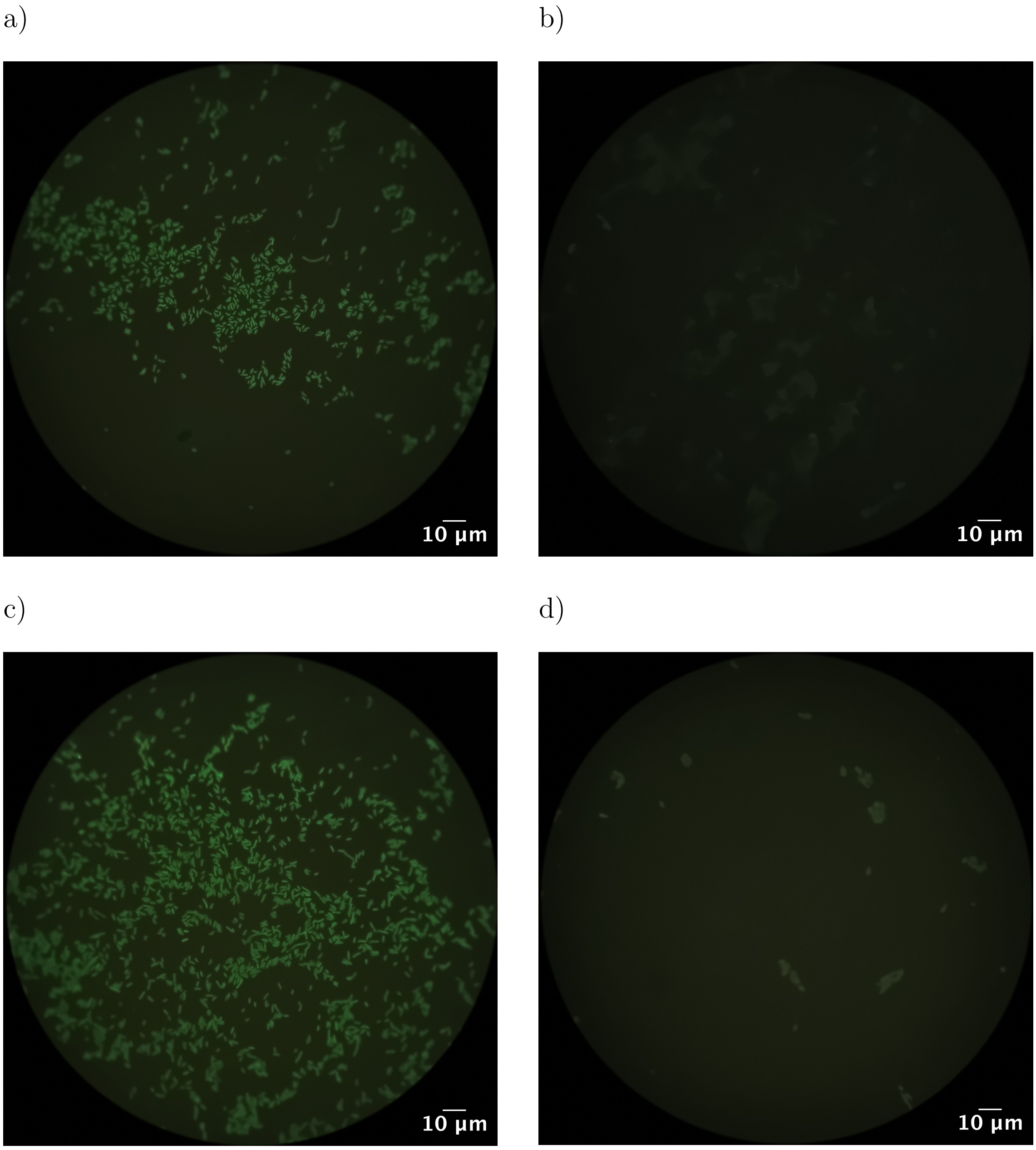

Figure 4.6: PNA-FISH with normal and simpler washing solution (EC strain DH5α, Alexa Fluor 488): a) Normal washing solution, b) Normal washing solution, negative control, c) Simpler washing solution, d) Simpler washing solution, negative control.

Triton X-100 is a detergent included in the washing solution to primarily wash away unbound fluorescent probe. Therefore, the background noise was expected to increase when it was excluded. Despite some images showing slightly brighter EC, the difference was too minor to conclude that it did have an impact on the results. It could therefore be concluded that a simplified washing solution could be used in the washing step of the PNA-FISH assay for detection of EC DH5α.

4.3 Field studies at MNH, Tanzania

Following section presents the results acquired from the screening at the neonatal unit of MNH including the demographic and clinical characteristics of the patients, the PNA-FISH results as well as the results from the ESBL-selective chromogenic agar plates.

4.3.1 Demographic and clinical characteristics of patients

Admission samples were collected from 18 patients. Discharge samples were obtained from 10 of the 18 patients. The 10 neonates were discharged from the neonatal unit after an average of five days after admission. Table 4.1 summarizes the demographic and clinical characteristics of all patients such as the neonate's body weight, sex, admission date, discharge date (if any) and current status.

Table 4.1: Demographic and Clinical Characteristics of Patients.

Patient ID	Bwt (g)	Sex (M/F)	Adm. Date	Disch. Date	Status
1	4200	M	28.5		
2	1970	F	28.5	29.5	
3	2380	F	28.5	30.5	
4	2585	F	28.5	29.5	
5	2850	M	28.5	31.5	
6	2300	F	28.5	31.5	
7	2400	F TWIN	29.5		Missed
8	1800	M TWIN	29.5		Missed
9	2080	M	29.5	10.6	
10	2500	M	29.5		Missed
11					Tracing
12	1625	F	29.5	4.6	Admitted
13	1350	F	29.5	9.6	Admitted
14	1150	M	29.5	6.6	Admitted
15	700	F	29.5		Died
16	2300	M	30.5		Missed
17	1700	M	30.5		Missed
18	4000	F	30.5	31.5	
19	3650	F	30.5		Admitted

BWT = Body weight.
Disch. Date = Discharge date.
Adm. Date = Admission date.
Status = Current patient condition/outcome.
Admitted = Patient has been admitted to the neonatal unit.
Missed = Clinicians missed to take the discharged sample.
Tracing = The neonate's mother was too unwell for consent.
Died = Patient died >12 hrs after birth.

4.3.2 FISH Results for ESBL-producing EC

FISH was performed on all 18 admission samples and on three of the discharge samples. Note that PNA-FISH was not performed on all discharged patients from Table 4.1 due to time constraints. Table 4.2 summarizes the results, indicating whether each patient was colonized with ESBL-producing EC or not when performing PNA-FISH. "-" indicates that FISH was not performed on the patient's discharge sample or that the discharge sample was not available for that patient.

Table 4.2: PNA-FISH results of admission and discharge samples.

Patient ID	**Admission: ESBL-Producing EC**	**Discharge: ESBL-producing EC**
1	No	-
2	Yes	No
3	No	Yes
4	Yes	Yes
5	No	-
6	No	-
7	No	-
8	No	-
9	No	-
10	No	-
11	-	-
12	No	-
13	No	-
14	No	-
15	Yes	-
16	No	-
17	No	-
18	No	-
19	Yes	-

Out of the 18 patients, four were found to be colonized with ESBL-producing EC upon admission, accounting for approximately 22%. One out of three discharged patients had acquired ESBL-producing EC upon discharge. Patient 2 showed unexpected results with no ESBL-producing EC detected in the discharge sample although the patient's admission sample showed ESBL-producing EC. These results corresponded to the result of the inoculation on ESBL-selective chromogenic agar where no growth of EC colonies could be seen in the discharged sample, compared to the admission sample where growth of EC was present. There are two possible reasons for this. Patient 2 might have shown infection symptoms due to colonization with ESBL EC and was prescribed antibiotics, which eliminated the ESBL EC. However, no information regarding prescribed antibiotics was given to the laboratory. This outcome could also been due to the discharged sample of Patient 2 looking too dense prior to sample preparation. This may hinder proper hybridization in

PNA-FISH and growth on the chromogenic agar, therefore affecting the accuracy of the results.

Following images were considered to be representative of the study's findings. Figure 4.7 presents the FISH images for Patient 3 at admission. Figure 4.7 a-c are the samples to which the fluorescent probe was added; none of these pictures show fluorescent EC. The negative control (see Figure 4.7 d-f) also showed no fluorescent EC, indicating the absence of ESBL-producing EC.

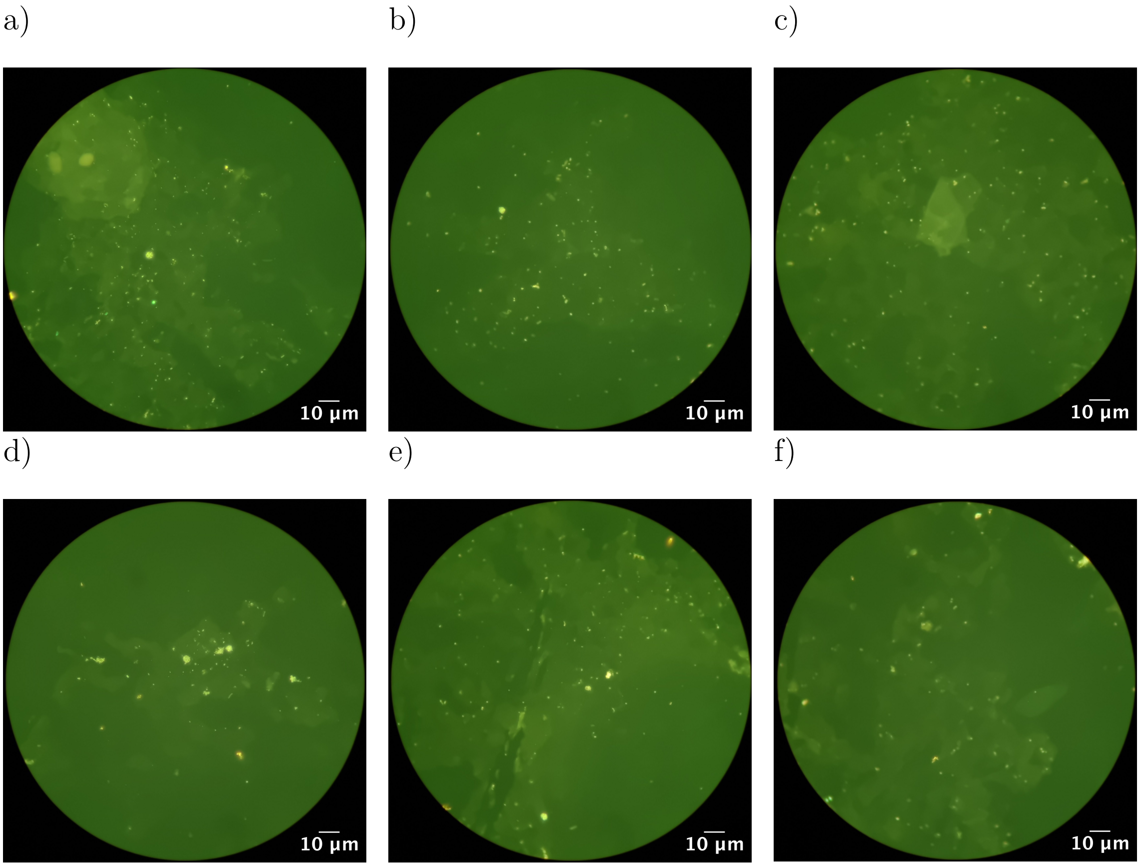

Figure 4.7: FISH images for Patient 3 at admission: a-c is the sample (PNA-probe added), d-f are the negative control (no PNA-probe added).

Figure 4.8 presents the FISH images for Patient 3 at discharge. Figure 4.8 a-c is the sample to which the fluorescent probe was added; showing fluorescent EC. The negative control (see Figure 4.8 d-f) shows no fluorescence (except the natural autofluorescence of the bacteria), indicating the presence ESBL-producing EC. When comparing the FISH results from Patient 3 at admission with the results of the discharge sample (Figure 4.7 to Figure 4.8) it can be concluded that Patient 3 acquired ESBL-producing EC during its stay at the neonatal ward.

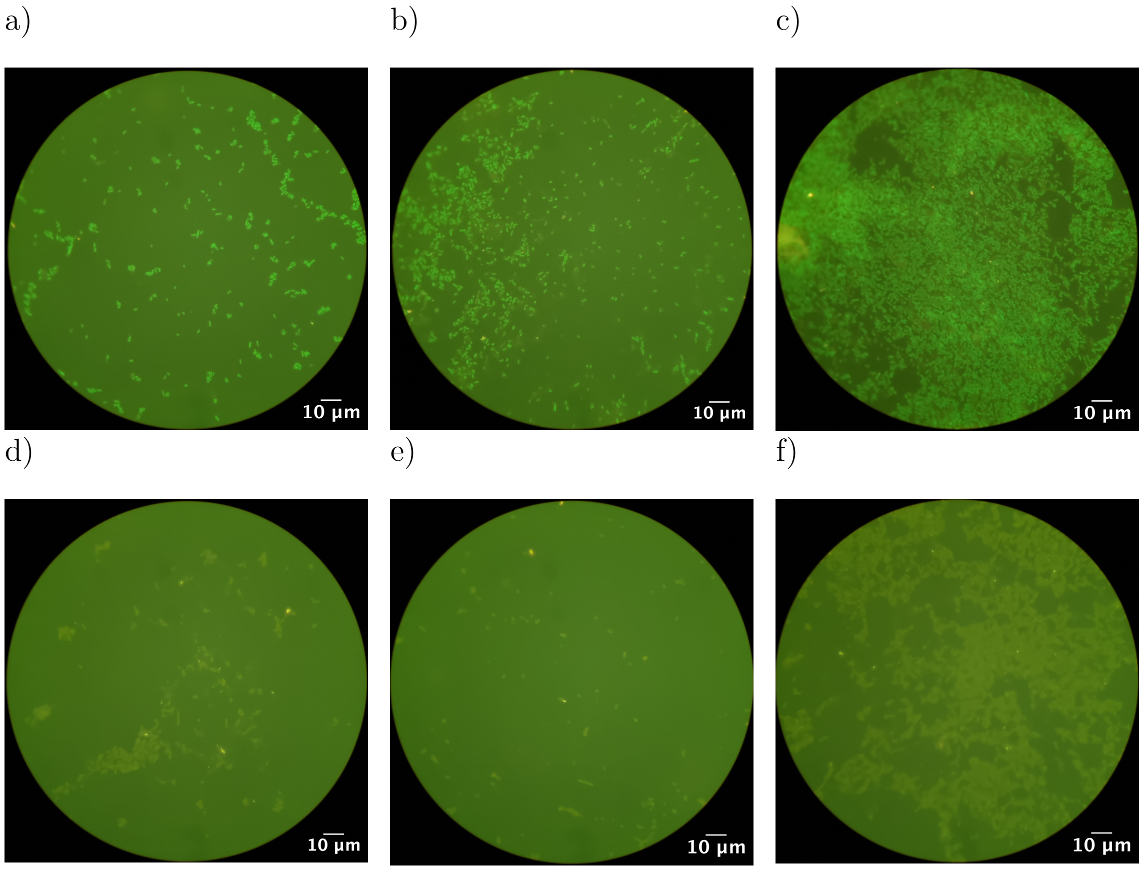

Figure 4.8: FISH images for Patient 3 at discharge: a-c is the sample (PNA-probe added), d-f are the negative control (no PNA-probe added).

Figure 4.9 presents the FISH images for Patient 5 at discharge. The upper row (see Figure 4.9 a-c) is the sample to which the fluorescent probe was added. The sample shows no fluorescent EC. The negative control (see Figure 4.9 d-f) also shows no fluorescent EC, indicating the absence of ESBL-producing EC.

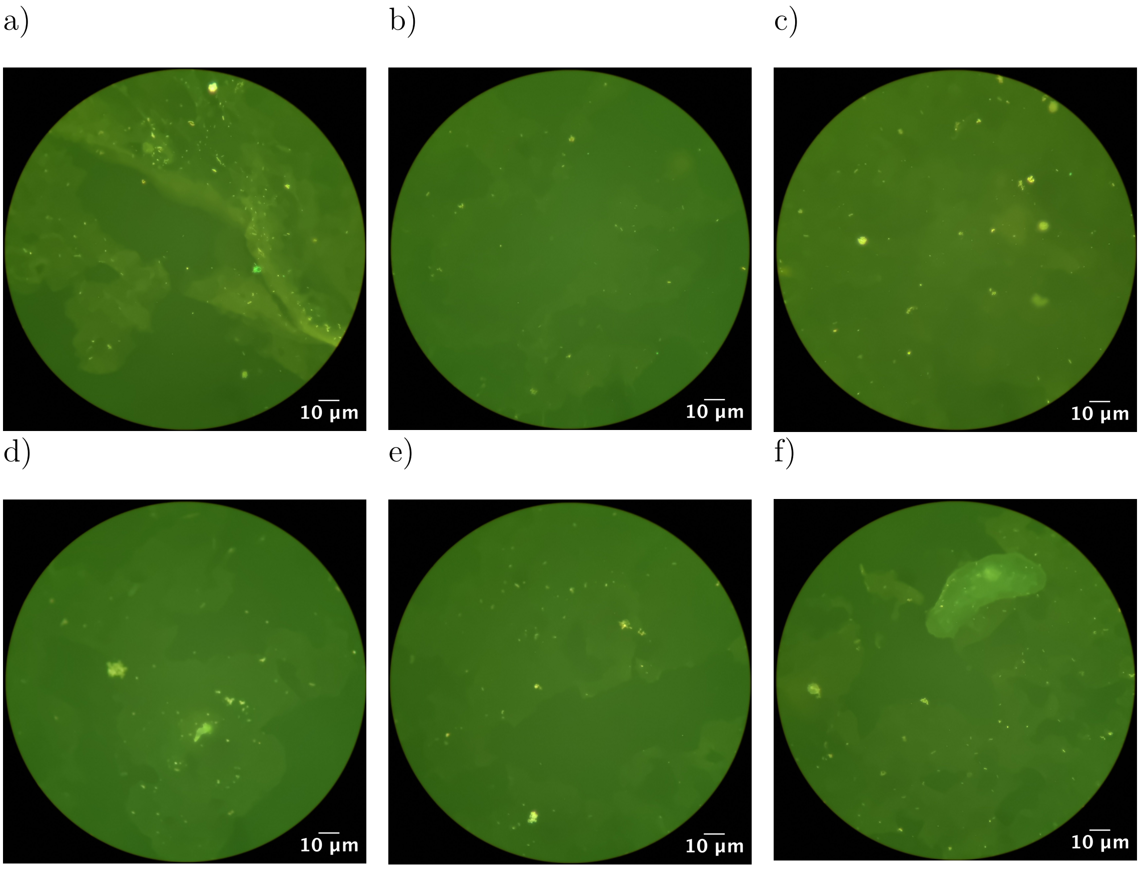

Figure 4.9: FISH images for Patient 5 at admission: a-c is the sample (PNA-probe added), d-f are the negative control (no PNA-probe added).

Figure 4.10 presents the FISH images for Patient 19 at admission. The upper row presents the sample to which the fluorescent probe was added (see Figure 4.10 a-c). The sample shows plenty of fluorescent EC. The negative control (see Figure 4.10 d-f) shows no fluorescent bacteria and the contrast to the sample indicates therefore that Patient 19 was colonized with ESBL-producing EC at admission to the ward. Figure 4.10g is a close-up of Figure 4.10a that shows EC filaments. As described in Section 2.2.5, EC forms filaments in the presence of high concentrations of cefotaxime.

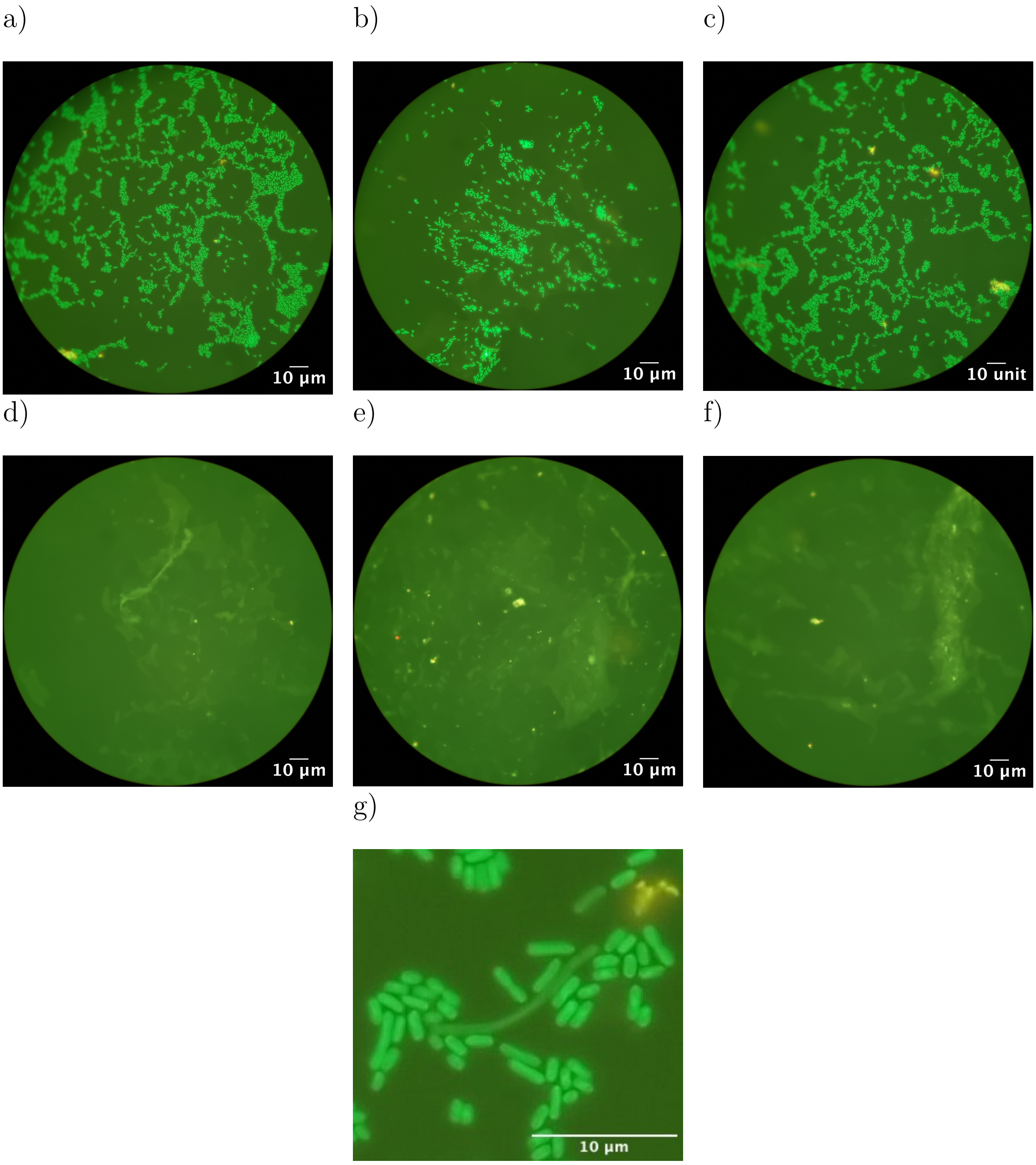

Figure 4.10: FISH images for Patient 19 at admission: a-c is the sample (PNA-probe added), d-f are the negative control (no PNA-probe added), g is a close-up representing the EC filaments of figure a.

4.3.3 Results of selective chromogenic agar plates

The chromogenic ESBL agar plates were used as a verification step for PNA-FISH. The data, such as the presence of KP, EC, and the colony morphology, acquired from visually analyzing the chromogenic plates is summarized in Table 4.3 for all admission samples of 18 patients.

Out of 18 patients, four patients were colonized by ESBL-producing EC upon admission, accounting for approximately 22% of the patients. This corresponded to the results of PNA-FISH. Two out of 18 admitted patients were colonized with ESBL-producing KP, representing around 11%. Lastly, two patients were colonized with both ESBL-producing EC and KP upon admission, accounting for approximately 11%.

Table 4.3: Chromogenic ESBL agar plates results: Admission Samples.

Patient ID	EC Colonies	KP Colonies	Colony morphology
1	No	No	No bacterial growth.
2	Yes	Yes	1) Small, raised, wet, blue. 2) Small, raised, wet, creamy.
3	No	No	No bacterial growth.
4	Yes	No	Large, raised, wet, pink.
5	No	No	No bacterial growth.
6	No	No	No bacterial growth.
7	No	No	No bacterial growth.
8	No	No	No bacterial growth.
9	No	No	No bacterial growth.
10	No	No	No bacterial growth.
11	-	-	-
12	No	No	No bacterial growth.
13	No	No	No bacterial growth.
14	No	No	No bacterial growth.
15	Yes	Yes	1) Small, raised, wet, pink. 2) Large, raised, wet, blue.
16	No	No	No bacterial growth.
17	No	No	No bacterial growth.
18	No	No	No bacterial growth.
19	Yes	No	Small, raised, wet, pink.

The data acquired from visually analyzing the chromogenic plates for all discharged patients is summarized in Table 4.4. Among the four discharged patients, one patient had acquired both ESBL-producing EC and KP upon discharge, which was not present at admission. Three out of four discharged patients were colonized with ESBL-producing KP, where two of the patients were not colonized at admission. This implies that the patients acquired ESBl-producing KP during their hospital stay.

Table 4.4: Chromogenic ESBL agar lates results: Discharge Samples.

Patient ID	**EC Colonies**	**KP Colonies**	**Colony morphology**
2	No	Yes	Large, raised, wet, blue.
3	Yes	Yes	1) Medium, raised, wet, pink. 2) Medium, raised, wet, blue.
4	Yes	Yes	1) Medium, raised, wet, pink. 2) Medium, raised, wet, blue.
18	No	No	-

5
Conclusion

The tests conducted at CTH aimed to evaluate if PNA-FISH could be performed more efficiently regarding the chemicals used for the PNA-FISH solutions as well as the hybridization time and temperature. Simplifying the PNA-FISH solutions and the reduction of the hybridization time to 15 minutes did not compromise the assay's effectiveness. As for the hybridization temperature, a range from 65°C to 22°C (room temperature) were tested, revealing that the PNA probe does not hybridize above 65°C but is effective at room temperature. These results imply that PNA-FISH can be conducted more time- and resource-efficient without compromising the quality of the results, making the assay more feasible for routine clinical application in low-resource settings. For instance, conducting the hybridization at 37°C would allow the use of one incubator for the entire procedure, including the cultivation of the patient sample. These experiments were performed on a single strain of EC (DH5α), and to implement the same simplifications for patient samples, similar evaluations must be conducted for those types of samples. Nonetheless, these results still demonstrate the potential that PNA-FISH possesses for efficient bacterial detection.

All PNA-FISH results achieved from the field studies correspond to the results of the chromogenic ESBL agar plates which further strengthens the accuracy of using PNA-FISH for the detection of ESBL-producing bacteria. From the collected samples one can conclude that some patients enter the hospital already carrying ESBL-producing bacteria, while others acquire it during their stay. However, definite conclusions can not be drawn until the full sample size of the screening is collected.

This study has proved that neonates acquire ESBL-producing bacteria during their hospital stay. The results of whether the neonate is colonized by ESBL-producing EC or not have been determined within the same working day. This acquired knowledge may create more awareness of sources of transmission, making the healthcare staff at the neonatal unit more aware of how to prevent bacterial transmission from themselves to the neonates, but also between the neonates themselves. A future perspective of this study would be to only conduct PNA-FISH on neonates showing signs of infection. By doing so, the underlying cause of infection can be concluded within 7-8 hours. This will help the clinicians to give the correct treatment and by doing so the misuse and overuse of antibiotics will be reduced; which is crucial to tackle the AMR challenge.

It is of great importance to ensure the continuation of the project, which involved training two staff members of the Bacteriology unit of MNH, as well as writing instruction manuals of the PNA-FISH procedure. The PNA-FISH was adapted to suit the lab of MNH. This was ensured by utilizing existing microscopy equipment, locally available chemicals, or those for which MNH has established suppliers. By doing this, the likelihood of a successful continuation is increased. This research not only raised awareness of infections caused by ESBL-producing bacteria at MNH but also stressed the importance of developing simple tools by utilizing existing equipment to detect antibiotic-resistant bacteria in low-income countries, where it is most needed.

5.1 Future perspectives

Initial tests were done with the KP probe but further evaluation must be done to make it work equivalently to the EC probe. A future perspective would be to test whether two ESBL-producing bacteria of clinical relevance, such as EC and KP, could be detected in the same sample. This may be possible by labeling the EC and KP probes with different fluorophores allowing for their simultaneous detection using the simple fluorescence microscope available in many low-income countries. The Zeiss® Primostar iLED microscope only has one excitation wavelength and one emission filter. The emission filter is a cut of filter letting all light above 485 nm through. Thus the challenge is to find two probes that can be excited by the same light source but emit different colors so that the probes can be detected at the same time.

Another bacteria of clinical relevance is the gram-positive bacteria, GBS. If a woman carries GBS during delivery the child might become infected [53]. While the mother might not have any symptoms, a GBS infection in the newborn may cause meningitis, pneumonia, or sepsis. There is a GBS probe now being tested by the Azevedo group and it would be of interest to develop an assay to use this probe to test for GBS in urine samples from pregnant women before birth. By screening the mothers, treatment for the infection can be started early to prevent the transfer of GBS from the mother to the neonate.

By varying the antibiotics used for the cultivation of the patient samples, PNA-FISH can potentially be used for the identification of other resistant bacteria than ESBL-producing. Carbapenems are reserved as antibiotics of last resort as they are efficient against Gram-negative and Gram-positive drug-resistant infections and are therefore of clinical relevance. It would be of interest to investigate the feasibility of using PNA-FISH to detect carbapenemase-producing bacteria, given the limited treatment options for such infections.

Bibliography

1. World Health Organization. Newborn Mortality. 2022 Jan. Available from: https://www.who.int/news-room/fact-sheets/detail/levels-and-trends-in-child-mortality-report-2021
2. World Health Organization. Newborn Infections. Available from: https://www.who.int/teams/maternal-newborn-child-adolescent-health-and-ageing/newborn-health/newborn-infections
3. Gajul SV, Mohite ST, Mangalgi SS, Wavare SM, and Kakade SV. Klebsiella Pneumoniae in Septicemic Neonates with Special Reference to Extended Spectrum β-lactamase, AmpC, Metallo β-lactamase Production and Multiple Drug Resistance in Tertiary Care Hospital. Journal of Laboratory Physicians 2015 Jan; 7:32–37. DOI: 10.4103/0974-2727.151689
4. Nordberg Yao V. Personal communication. 2022 Oct
5. Lohr IH, Rettedal S, Natas OB, Naseer U, Oymar K, and Sundsfjord A. Long-term faecal carriage in infants and intra-household transmission of CTX-M-15-producing Klebsiella pneumoniae following a nosocomial outbreak. Journal of Antimicrobial Chemotherapy 2013 May; 68:1043–8. DOI: 10.1093/jac/dks502
6. Centers for Disease Control and Prevention. ESBL-producing Enterobacterales in Healthcare Settings. 2019 Nov. Available from: https://www.cdc.gov/hai/organisms/ESBL.html
7. Murray CJL et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet 2022 Feb; 399:629–55. DOI: 10.1016/S0140-6736(21)02724-0
8. Santos RS, Guimarães N, Madureira P, and Azevedo NF. Optimization of a peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) method for the detection of bacteria and disclosure of a formamide effect. Journal of Biotechnology 2014 Oct; 187:16–24. DOI: 10.1016/j.jbiotec.2014.06.023
9. Alberts B et al. Molecular Biology of the cell. 6th ed. 2015 :174–236
10. Kadner RJ and Rogers K. Bacteria. 2023 Sep. Available from: https://www.britannica.com/science/bacteria
11. Alberts B et al. Cells and Genomes. *Molecular Biology of the cell.* 6th ed. New York: Garland Science, 2015. Chap. 1:1–42
12. Salton MR and Kim KS. Bacteriology. *Medical Microbiology.* Ed. by Baron S. 4th ed. Galveston (TX): University of Texas Medical Branch at Galveston, 1996. Chap. 2. Available from: https://www.ncbi.nlm.nih.gov/books/NBK8477/

13. Bender M, Buckley, Sattley, and Stahl. Brock Biology of Microorganisms FIFTEENTH EDITION. Tech. rep.
14. Rajagopal L. Understanding the regulation of Group B Streptococcal virulence factors. Future Microbiology 2009 Mar; 4:201–21. DOI: 10.2217/17460913.4.2.201
15. Bello-López JM et al. Horizontal Gene Transfer and Its Association with Antibiotic Resistance in the Genus Aeromonas spp. Microorganisms 2019 Sep; 7:363. DOI: 10.3390/microorganisms7090363
16. Erez Z et al. Communication between viruses guides lysis-lysogeny decisions. Nature 2017 Jan; 541:488–93. DOI: 10.1038/nature21049
17. Ughy B et al. Reconsidering Dogmas about the Growth of Bacterial Populations. Cells 2023 May; 12. DOI: 10.3390/cells12101430
18. Tran TD, Ali MA, Lee D, Félix MA, and Luallen RJ. Bacterial filamentation as a mechanism for cell-to-cell spread within an animal host. Nature Communications 2022 Feb; 13:693. DOI: 10.1038/s41467-022-28297-6
19. Kjeldsen TS, Sommer MO, and Olsen JE. Extended spectrum β-lactamase-producing Escherichia coli forms filaments as an initial response to cefotaxime treatment. BMC Microbiology 2015 Dec; 15:63. DOI: 10.1186/s12866-015-0399-3
20. Patel P, Wermuth HR, Calhoun C, and Hall GA. Antibiotics. 2023 May. Available from: https://www.ncbi.nlm.nih.gov/books/NBK535443/
21. National Center for Emerging and Zoonotic Infectious Diseases. Antibiotic Class Definitions. Available from: https://arpsp.cdc.gov/resources/OAU-Antibiotic-Class-Definitions.pdf
22. Armstrong T, Fenn SJ, and Hardie KR. JMM Profile: Carbapenems: a broad-spectrum antibiotic. Journal of medical microbiology 2021 Dec; 70. DOI: 10.1099/jmm.0.001462
23. Bernatová S et al. Following the Mechanisms of Bacteriostatic versus Bactericidal Action Using Raman Spectroscopy. Molecules 2013 Oct; 18:13188–99. DOI: 10.3390/molecules181113188
24. Pankey GA and Sabath LD. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. Clinical Infectious Diseases 2004 Mar; 38:864–70. DOI: 10.1086/381972
25. Wilhelm MP. Vancomycin. Mayo Clinic proceedings 1991 Nov; 66:1165–70. DOI: 10.1016/s0025-6196(12)65799-1
26. Vancomycin Hydrochloride for Injection USP 500 mg. Available from: http://www.efda.gov.et/wp-content/uploads/2024/03/Vancomycin-Hydrochloride-for-Injection-USP-500-mg_Vanco-500_SWISS-PARENTERALS-LTD.pdf
27. Murphy PB, Bistas KG, Patel P, and Le JK. Clindamycin. 2024 Feb. Available from: https://www.ncbi.nlm.nih.gov/books/NBK519574/
28. CEFOTAXIME FOR INJECTION USP 1g. Available from: https://www.tmda.go.tz/uploads/1709024548-T19H0387SmPCV1.pdf
29. Baldwin CM, Lyseng-Williamson KA, and Keam SJ. Meropenem. Drugs 2008; 68:803–38. DOI: 10.2165/00003495-200868060-00006
30. Antimicrobial resistance. 2023 Nov. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

31. Sartelli M et al. Ten golden rules for optimal antibiotic use in hospital settings: the WARNING call to action. World Journal of Emergency Surgery 2023 Oct; 18:50. DOI: 10.1186/s13017-023-00518-3
32. Uddin TM et al. Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. Journal of Infection and Public Health 2021 Dec; 14:1750–66. DOI: 10.1016/j.jiph.2021.10.020
33. Centers for Disease Control and Prevention. ESBL-producing Enterobacterales in Healthcare Settings. Available from: https://www.cdc.gov/hai/organisms/ESBL.html
34. Rayson D, Basinda N, Pius RA, and Seni J. Comparison of hand hygiene compliance self-assessment and microbiological hand contamination among healthcare workers in Mwanza region, Tanzania. Infection Prevention in Practice 2021 Dec; 3:100181. DOI: 10.1016/j.infpip.2021.100181
35. Centers for Disease Control and Prevention. Carbapenem-resistant Enterobacterales (CRE). Available from: https://www.cdc.gov/hai/organisms/cre/index.html
36. Smith HZ, Hollingshea CM, and Kendall B. Carbapenem-Resistant Enterobacterales. Available from: https://www.ncbi.nlm.nih.gov/books/NBK551704/
37. Jiménez-Belenguer AI, Ferrús MA, Hernández M, García-Hernández J, Moreno Y, and Castillo MÁ. Prevalence and Characterization of Beta-Lactam and Carbapenem-Resistant Bacteria Isolated from Organic Fresh Produce Retailed in Eastern Spain. Antibiotics 2023 Feb; 12:387. DOI: 10.3390/antibiotics12020387
38. Pediatric Health Initiative. Neonatal collaboration. Available from: https://pediatrichealthinitiative.org/muhimbilineo
39. Personal communication with Viveka Nordberg
40. Viveka Nordberg. Powerpoint: Neonatal Sepsis - Aspects on infection prevention and antibiotic resistance
41. Young AP, Jackson DJ, and Wyeth RC. A technical review and guide to RNA fluorescence in situ hybridization. PeerJ 2020 Mar; 8:e8806. DOI: 10.7717/peerj.8806
42. Teixeira H, Sousa AL, and Azevedo AS. Bioinformatic Tools and Guidelines for the Design of Fluorescence In Situ Hybridization Probes. 2021 :35–50. DOI: 10.1007/978-1-0716-1115-9{_}3
43. Stender H, Fiandaca M, Hyldig-Nielsen JJ, and Coull J. PNA for rapid microbiology. Journal of Microbiological Methods 2002 Jan; 48:1–17. DOI: 10.1016/S0167-7012(01)00340-2
44. Machado A et al. Fluorescence in situ Hybridization method using Peptide Nucleic Acid probes for rapid detection of Lactobacillus and Gardnerella spp. BMC Microbiology 2013 Dec; 13:82. DOI: 10.1186/1471-2180-13-82
45. Oliveira R, Azevedo AS, and Mendes L. Application of Nucleic Acid Mimics in Fluorescence In Situ Hybridization. 2021 :69–86. DOI: 10.1007/978-1-0716-1115-9{_}5
46. Sanderson MJ, Smith I, Parker I, and Bootman MD. Fluorescence Microscopy. Cold Spring Harbor Protocols 2014 Oct; 2014. DOI: 10.1101/pdb.top071795

47. Herbert S, Soares H, Zimmer C, and Henriques R. Single-Molecule Localization Super-Resolution Microscopy: Deeper and Faster. Microscopy and Microanalysis 2012 Dec; 18:1419–29. DOI: 10.1017/S1431927612013347
48. Ockenga W. Fluorescence in Microscopy. 2011 Apr. Available from: https://www.leica-microsystems.com/science-lab/life-science/fluorescence-in-microscopy/
49. Wang G and Fang N. Detecting and Tracking Nonfluorescent Nanoparticle Probes in Live Cells. 2012 :83–108. DOI: 10.1016/B978-0-12-391857-4.00004-5
50. Surre J, Saint-Ruf C, Collin V, Orenga S, Ramjeet M, and Matic I. Strong increase in the autofluorescence of cells signals struggle for survival. Scientific Reports 2018 Aug; 8:12088. DOI: 10.1038/s41598-018-30623-2
51. Croce A and Bottiroli G. Autofluorescence spectroscopy and imaging: a tool for biomedical research and diagnosis. European Journal of Histochemistry 2014 Dec. DOI: 10.4081/ejh.2014.2461
52. Oliveira R, Azevedo AS, and Mendes L. Application of Nucleic Acid Mimics in Fluorescence In Situ Hybridization. 2021. Chap. Chapter 5:69–86. DOI: 10.1007/978-1-0716-1115-9{_}5
53. Nguyen Phu T. Group B Strep and Pregnancy. 2022 Jul. Available from: https://kidshealth.org/en/parents/groupb.html

A
Appendix

A.1 Informed Consent Form

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

Consent forms

Informed consent form (English version)

Title: Identification of ESBL-producing bacteria in the neonatal unit at Muhimbili National Hospital using PNA-FISH assay and smartphone-based fluorescent microscopy and Optical DNA mapping
Muhimbili University of Health and Allied Sciences. Directorate of research and publications, MUHAS.

Identification Number.......................................
Greetings,
You are requested to participate in a research study conducted by -------------,
Your participation in the study is entirely voluntary. You should read the information below and ask questions to clarify any doubts, before deciding to agree to participate. You are being asked to participate in the study because your baby is admitted in the neonatal care unit of Muhimbili National Hospital.

Study Purpose

The purpose of this study is to evaluate the use of two assays, based on simple fluorescence microscopy, for surveillance and diagnostics of ESBL producing bacteria at MNH.
The findings obtained from the study will be dedicated to make improvements of the treatment of neonatal bacterial infections by arelevant change in infection prevention and control protocols in the NICU at MNH.

Participants and methods

If you agree to join the study, you will be interviewed using questionnaire, detailed information on social demographic characteristics, your medical history will also be requested. Swabs will be taken from your babies rectum from admission and the other sample will be taken on discharge. Also if your baby is found to have any of the following signs including temperature instability, lethargy and pallor and/or mottling, apnea and/or bradycardia, jaundice, abdominal distension, inability to feed, a blood culture sample will be taken. For blood culture about 1-3 mls of blood will be drawn from your baby.

Confidentiality

Identification number will be used on all collected information instead of names. The principle investigator, research assistance, supervisor and MUHAS at large are obliged to maintain confidentiality of all data or information collected from you. No unauthorized persons will have access to the data collected.

Benefits

If you agree for your baby to participate in this study your baby will be treated according to the current standard of care in Tanzania.
The benefits may be direct or indirect. You will benefit by knowing the result of bacterial culture of your baby, which will assist with your baby´s treatment accordingly. This information will also be helpful to others as the hospital will also be aware of the bacteria with resistance to antibiotics This would provide a guideline for the clinicians to prescribe the effective drugs empirically and intensify infection control.

Risks and discomfort

No harm is expected to happen to your baby because of your participation in this study. Experienced personnel will prevent pain and comfort your baby during bloodsampling. We will take appropriate measures to ensure no major injury occur as a result of participation in this research.

Compensation

No payment will be given to you as a fee to participate in the study.

Rights to withdraw

Participation in this study is completely voluntary. You can decide for your baby to participate or not, and you are allowed to stop participating in this study at any stage, even if you have already given your consent. If you do not wish to participate in the study, it will not affect your treatment or your baby's treatment in this hospital in any way.

Who to Contact

If you have any questions you may ask them now, or anytime later. If you wish to ask questions at any time, you may contact any of the following**: [--------------, Department of -------------- MUHAS**, **P. O. Box.** ***65001, Telephone no:*** **-----------,** ***Email:***

This proposal has been reviewed and approved by the MUHAS Institutional Review Board and the National Health Research Ethics Review Committee, the committees whose task it is to make sure that research participants are protected from harm. If you wish to find more about the IRB, please contact **Dr Bruno Sunguya**, the Director of Research and Publications Committee – MUHAS [P. O. Box 65001, Dar es Salaam, Telephone no: +255 22 2150302-6]

I ……………………………………………….have read and understand the contents in this form. My questions have been answered. I agree to participate in this study / I agree that my child can participate in this study.

Signature of the participant/the responsible

Parent ……………………………..Date………………………………..

Witness……………………………..Date…………………………………..

Signature of the researcher ………………..……...Date ……………………

Informed Consent Form (Swahili Version)

Form ya ridhaa ya kushiriki

Kichwa cha habari: Utambuzi wa vimelea vyenye uwezo wa kuzalisha usugu (ESBL) kwa watoto katika hospitali ya Taifa Muhimbili kwa kutumia kipimo cha maabara (PNA-FISH assay) na simu iliyoungwanishwa kwenye darubini

Chuo Kikuu cha Sayansi na Tiba ya Afya Muhimbili, Kurugenzi ya Utafiti na Machapisho

Numba ya utambuisho......................................

Salaam,

Unaombwa kushiriki katika utafiti huu unaofanywa na ………………………

Ushiriki wako katika utafiti huu ni kwa hiari kabisa. Unatakiwa kusoma maelezo kuhusu utafiti huu kama ilivyoainishwa hapo chini na kuuliza maswali pale ambapo hapajaeleweka vizuri ili uweze kufanya maamuzi sahihi kabla ya kushiriki. Unaombwa kushiriki katika utafiti huu kwa sababu mtoto wako amelazwa katika wodi ya watoto wachanga katika Hospitali ya Taifa Muhimbili.

Lengo la utafiti

Lengo la utafiti huu ni kuangalia uwepo na kiwango cha vimelea vyenye uwezo wa kuzalisha usugu (ESBL) kwa kutumia kipimo cha maabara (PNA-FISH assay) na simu iliyoungwanishwa kwenye darubini kwa watoto wachanga katika Hospitali ya Taifa Muhimbili. Majibu yatakayopatikana kutokana na utafiti huu yatatumika kuboresha na kuleta mabadiliko muhimu katika kuzuia magonjwa ya kuambukiza katika wadi za watoto mahututi katika hospital ya Taifa Muhimbili.

Ushiriki na jinsi ya kushiriki

Ukikubali kushiriki katika utafiti huu, utasailiwa na kuhojiwa kwa kutumia dodoso lililoandaliwa ili kupata taarifa zako, na pia taarifa za matibabu. Utaulizwa kuhusu masuala yanayomhusu mwanao na historia ya matibabu ya aina yoyote aliyowahi kupatiwa. Pia utaombwa sampuli ya kinyesi cha mtoto. Sampuli ya kinyesi kutoka kwa mtoto wako kitachukuliwa wakati wa mtoto kulazwa hospitalini na sampuli nyingine wakati atakaporuhusiwa kwenda nyumbani. Na pia kama mtoto wako ataonekana kuwa na dalili kama vile, homa au kupanda na kushuka kwa joto la mwili, kulegea, dalili za kupungukiwa damu, kupumua kwa shida, njano, tumbo kujaa au kushindwa kula basi, mtoto wako atatolewa sample ya damu kwa ajili ya kuotesha maabara. Kiasi kidogo cha damu (kama kijiko cha chai) kitatolewa kwa mtoto wako kwa ajili ya utokeshaji maabara.

Usiri

Tutatumia namba kuwakilisha taarifa zitakazokusanywa kutoka kwako na siyo jina. Mtafiti Mkuu, na watafiti wasaidizi na wale kutoka Chuo Kikuu cha Sayansi na Tiba na afya Muhimbili watahusika na utunzaji wa taarifa zote zilizokusanywa toka kwako kwa usiri mkubwa. Hairuhusiwi kwa mtu asiyeidhinishwa kupata taarifa zilizokusanywa kutoka kwako.

Faida

Ukikubali mtoto wako kushiriki katika utafiti huu mwanao, atahudumiwa kulingana na taratibu zote za matibabu katika nchi yetu ya Tanzania. Utafaidika kwa kupata majibu ya vipimo vya uoteshaji wa damu kutoka maabara vitakavyomwezesha mtoto kupatiwa matibabu sahihi. Hii pia itaisaidia hospitali kwa ujumla kujua aina ya vimelea, usugu wa dawa na antibiotiki sahihi ya kutibu vimelea hivyo. Faida zaweza kuwa za moja kwa moja kwako au kwa wengine. Taarifa hizi zitasaidia kwa watoa huduma wa afya wa hospitali ya taifa ya Muhimbili kwa kujua aina ya bakteria wa aina hii ambao wapo katika mzunguko kwa watoto wachanga na aina ya antibiotiki zinazoweza kutibu vimelea hivyo. Hii pia itawawezesha madaktari kutoa matibabu sahihi kwa wagonjwa na pia kuzuia usaambaaji wa magonjwa hayo.

Athari

Hakuna madhara yoyote yanayoweza kutokea kwa mtoto wako kwa sababu ya kushiriki katika utafiti huu. Ila, maumivu kidogo yanaweza kutokea wakati wakuchukua sampuli ya damu kwa mtoto wako. Hata hivyo sampuli zitatolewa na wataalamu wenye uzoefu na tahadhari zote zitachukuliwa wakati wa kutoa sampuli ili kuhakikisha kuwa hakuna madhara yanatokea kutokana na ushiriki wako.

Fidia

Hakuna malipo yoyote utakayopewa kwa kushiriki kwako katika utafiti huu.

Haki ya kujitoa katika utafiti

Ushiriki wako katika utafiti huu ni wa hiari yako kabisa. Unaweza kuamua mtoto wako kushiriki au kutokushiriki na kumsimamisha mwanao katika kushiriki kwenye utafiti huu katika hatua yeyote hata kama ulishatoa ridhaa. Kukataa kushiriki au kujitoa katika ushiriki hautaadhiri matibabu yako au matibabu ya mwanao hapa hospitalini kwa njia yoyote ile.

Wakuwasiliana naye

Endapo utakuwa na swali lolote kuhusu utafiti huu unaweza kuuliza sasa au wakati wowote baadae. Ikiwa utataka kuuliza swali lolote baadae unaweza kuwasiliana na Dr Helga Naburi simu namba +255754283622 au Dr Joachim simu +255717874791 wa Chuo Kikuu Kishiriki cha Sayansi za Afya Muhimbili, S. L. P 65001, Dar es Salaam. S.L.P 65001, Dar es Salaam au unaweza pia wasiliana na Mwenyekiti wa Utafiti, Dr Bruno Sunguya, S.L.P 65001, namba ya simu: +255 22 2150302-6] Dar es Salam. Utafiti huu umehakikiwa na kupitishwa na kamati ya maadili ya utafiti ya Chuo Kikuu cha Sayansi za Afya Muhimbili na ile ya Hospitali ya Taifa Muhimbili ambao jukumu lake ni kuhakikisha utafiti unafanyika katika hali ya usalama na bila madhara kwa mshiriki.

Makubaliano ya mshiriki

Mimi ………………………………… nimesoma na kuelewa yaliyomo katika fomu hii. Maswali yangu yamejibiwa. Ninakubali kushiriki /mtoto kushiriki katika utafiti huu.

Sahihi ya mshiriki/mzazi……………………………. Tarehe………………………
Sahihi ya shahidi…………………………………Tarehe……………………….
Sahihi ya mtafiti …………………………………Tarehe………………………..

A.2 Stock solutions for PNA-FISH

Table A.1: Stock solutions for PNA-FISH

Stock solutions			
Chemical	Concentration	Mass [g]	Volume MQ/distilled water [mL]
Disodium EDTA	0.05 M	0.9306	50
Sodium pyrophosphate	2 (wt./vol)	1	50
Polyvinylpyrrolidone	12.5 (wt./vol)	1.25	10
Ficoll®	7.5 (wt./vol)	0.75	10
NaCl	1 (wt./vol)	2.922	50
Trizma® Base	0.1 M	0.6057	50

A.3 Settings for Samsung S21 camera

Table A.2: Settings for the Samsung camera used when documenting the samples after PNA-FISH.

Settings Samsung S21	
ISO	A50
Speed	1
EV	0.0
Focus	Manual
WB	A4100K

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