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Development of novel methods for microbiological evaluation of urology products

Master of Science Thesis

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Göteborg, Sweden, 2011

THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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Abstract

Urinary tract infection (UTI) is the most common type of bacterial infection. Each year, UTIs cause morbidity, medical costs and losses in work time. For patients in long-term care settings the incidence of UTIs might be as high as 50 %. Every year, 7 million office visits are estimated to be due to UTIs at a cost to the healthcare system of over $1 billion in USA alone. It is estimated that 33% of neurogenic bladder patients have bacteriuria at any time. Nonsteroidal anti-inflammatory agents, antibiotic coatings and silver coatings have been applied to catheters in an attempt to prevent catheter associated urinary tract infections.

In this master thesis a dynamic model that simulates the lower urinary tract is developed in order to evaluate antibacterial urinary catheters. Furthermore, a novel quantitative method to analyze bacterial concentration in residual urine using the fluorescent dye resazurin has been developed.

A tailored physical glass model of the catheterized bladder was developed. Urine was supplied to the model at a rate of ~1.3 ml/min and the three different intermittent catheters tested were: Lofric Primo, Magic^3 Antibacterial and Magic^3 Antibacterial + Hydrophilic. Two different experimental protocols were evaluated. In experimental protocol 1 the catheters were contaminated with *E.coli* prior to each catheterization whereas in experimental protocol 2 catheters were contaminated only before the first catheterization. The bladder was emptied at intervals to simulate catheterization, and samples of residual urine for viable cell counts and kinetic studies with resazurin were taken. Bacteriological analysis showed no difference in inhibition of bacterial growth in the bladder in the two experimental protocols. After 22 hours of model operation the bacterial population approached ~10^8 CFU/ml in all experiments. No difference in results between the experimental protocols could be observed. Kinetic studies on resazurin reduction gave concentrations in the same range as CFU counts for samples taken after 4 hours. Concentrations were overestimated when comparing CFU counts to resazurin reduction for samples taken after 8 hours. No inhibition of bacterial growth could be shown using the antibacterial catheters (Magic^3 antibacterial) compared to the control (LoFric Primo).

Keywords: Catheter associated urinary tract infection, intermittent catheterization, surface coating, resazurin.

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1 Introduction

This introductory part of the master thesis report will begin with a description of the background to the project, where a brief theory of problems associated with catheterization will be introduced. Presentation of the problem statement and purpose of the project will be presented before ending up with a description of the scope of the study.

1.1 Background

The use of invasive medical devices is one of the most important risk factors for nosocomial infections, accounting for nearly one half of all hospital infections [1]. Device-associated infections have been shown to prolong mean length of hospital stays by 2.4 days for catheter-associated urinary tract infections (CAUTI) [2]. These infections can cause bacteremia and increase morbidity and mortality, as well as raise the cost of hospital care for affected patients [3]. Nonsteroidal anti-inflammatory agents, antibiotic coatings and silver coatings have been applied to catheters in an attempt to prevent CAUTI [4].

Patients with a neurogenic bladder are at risk for many complications, including urinary tract infection, urinary incontinence and deterioration of the upper urinary tracts with potential loss of renal function. People with this disorder can remove urine from the body through temporary placement of a catheter, i.e. intermittent catheterization [5]. Compared to other catheterization techniques, intermittent self-catheterization is associated with lower infection rate [6].

Despite the fact that intermittent catheterization is the best alternative it is not without risks as it leaves a residual volume of urine up to 50 ml in the bladder [7]. Insertion of a catheter may carry urethral organisms into the bladder, these organisms may cause infection in the lower- or upper urinary tract [8]. In order to analyze and study the bacterial content in the residual urine, an in vitro bladder model is necessary. Using such a model will facilitate simulation of repeated catheterization, a unique opportunity to study the distribution of bacterial cells and the possibility to study the potential effect of antibacterial agents. Detailed data obtained from the in vitro bladder model can contribute to creation of a mathematical model describing bacterial culture development.

Presently, the Research and Development department in Urotherapy at Astra Tech AB carry out research on comparing their standard catheter (Lofric Primo) with antibacterial catheters from other companies. Development of an in vitro bladder model will ideally provide information faster and at less cost than animal or clinical studies for Astra Tech. All information can be used for various simulations and predications, such as the optimal interval of catheterization and the potential value of antibacterial agents on intermittent catheters.

1.2 Problem Statement

It has been noted at the R&D Urotherapy department at Astra Tech AB that a new in vitro method to test the effect of antibacterial catheters is necessary. The new method should be dynamic in order to better simulate catheterization and to facilitate analysis of the growth of bacteria over a specific time interval in the bladder.

Furthermore, a quantitative method is necessary to make quick test on the bacterial content in the residual urine. Presently, to study the bacterial content a sample size is spread over a TGE-plate and incubated overnight. This process is time-consuming and by doing kinetic studies on a microplate reader, the possibility to optimize this process to just a few hours might be possible.

1.3 Aim

The purpose of this master thesis project is to develop an in vitro model of the lower urinary tract to study antibacterial catheters. The desired properties of the artificial bladder are: controllable urine flow rate, easy sampling of the residual urine, a bladder with a homogenous distribution of the content and it should be a closed system to minimize the risk of contamination. The bladder model should be robust and give reproducible results.

The project will also focus on studying the blue fluorescent dye resazurin for development of a new quantitative method to analyze bacterial concentration in residual urine. The fluorescent dye will be followed on a microplate spectrophotometer.

The aim is to provide a method which can be used for analyzing the bacterial content in the residual urine after catheterization. This can be used to determine if there are any significant differences in bacterial growth between control catheters and antibacterial catheters.

2 Theoretical background

The following sections contain the fundamental knowledge that will help understanding of neurogenic bladder management and infections of the urinary system. The sections that are covered involve urologic anatomy, urinary tract infections and complications, urinary catheters and surface coatings, and resazurin.

2.1 Urologic anatomy

The urinary system is a system that maintains the volume and composition of body fluids within normal limits. Its function are e.g. to eliminate waste products from the body that accumulate as a result of cellular metabolism, regulate the concentrations of various electrolytes on the body fluids and maintain normal pH of the blood [9]. Besides maintaining fluid homeostasis in the body, the urinary system secretes the hormones erythropoietin and rennin to control red blood cell production and maintain normal blood pressure, respectively [9, 10].

The urinary system consists of the kidneys, ureters, urinary bladder and urethra, see figure 2.1. The function of each organ can be summarized as: the functional units in the kidneys form the urine, the ureters carry the urine away from kidneys to the urinary bladder which is a temporary reservoir and the urethra transports the urine from the urinary bladder to the outside [10].

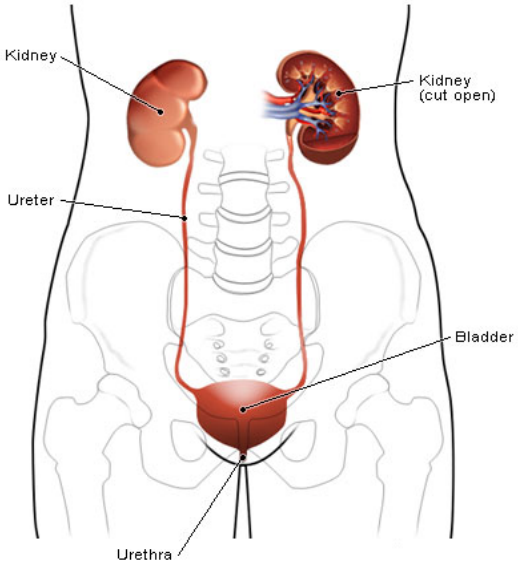


Figure 2.1 The structure of the main components of the urinary system [12].

The main organs of the urinary system are the kidneys, which lies on either side of the vertebral column. The kidneys filter the blood, remove the end products of metabolism and excrete the wastes in the urine [10]. Each kidney is approximately 11 cm long, 6 cm wide and 3 cm thick. On the concave surface of the kidneys lies the hilus from which the ureter, the main blood vessels and nerves access the kidney [9].

The peristaltic contractions of the ureters transport urine from the kidneys to the urinary bladder [11]. To prevent backflow of urine when pressure builds up in the bladder during urination, the ureters pass under the urinary bladder for a few centimeters, causing the bladder to compress the ureters [10]. Each ureter is 25 – 30 cm in length and is thick walled consisting of three layers: mucosa, smooth muscle cells and areolar connective tissue. The diameter is approximately 3 mm and is slightly less at its junction with renal pelvis [11].

The urinary bladder is a reservoir and varies in shape, position, size and relations, according to its content and the state of neighboring internal organs [11]. It is a hollow muscular organ positioned directly in front of the rectum in males and in front of the vagina. When empty, it is somewhat tetrahedral and it becomes spherical when slightly stretched as urine volume increases. The capacity of the urinary bladder is approximately 700 – 800 ml; due to anatomical differences it is smaller in females [10, 11].

The male urethra is 18 – 20 cm long and extends from the floor of the urinary bladder to the external opening, see figure 2.2. It can be divided into two parts, with a relatively long anterior urethra and a relatively short posterior urethra [11].

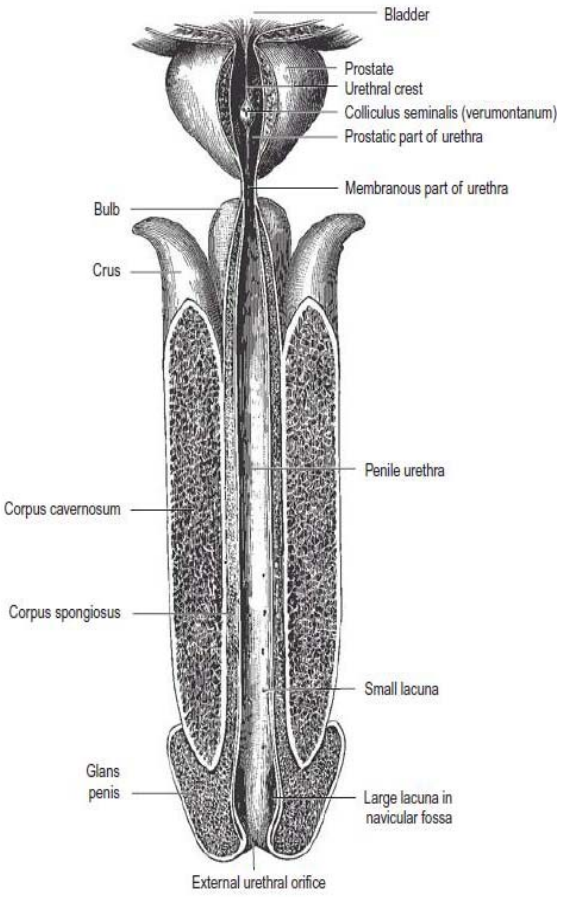


Figure 2.2 Structure of the male urethra [11].

The posterior urethra is divided into preprostatic, prostatic and membranous part. The preprostatic urethra extends from the bladder to the superior verumontanum, see figure 2.2. The prostatic urethra is approximately 3 – 4 cm in length and goes through the prostate. This part of the urethra is continuous with the preprostatic urethra. The membranous part of the urethra is the shortest (~1.5 cm), least dilatable and the narrowest part of the urethra. The wall of the membranous urethra consists of a muscle coat, which in turn consists of thin layers of smooth muscle cells. The muscle coat has an outer layer of circularly oriented striated muscle fibers, which form the urethral external sphincter [10, 11].

The anterior part of the urethra lies within the corpus spongiosum penis and it is approximately 15 cm long when the penis is flaccid. It extends from the end of the membranous urethra to the external urethral orifice on the penis glands. The anterior urethra has the widest part of the urethra and has a diameter of ca 6 mm when passing urine [11].

The female urethra is about 4 cm in length and 6 mm in diameter, see figure 2.3. It lies behind the pubic symphysis and is embedded in the front wall of the vagina.

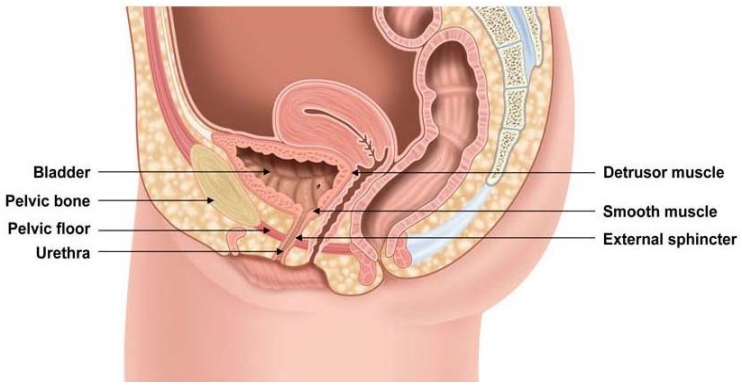


Figure 2.3 The lower urinary system in females [13].

The external urethral orifice lies between the clitoris and vaginal opening. Around the opening to the urethra is an internal urethral sphincter composed of smooth muscle, which opens and closes involuntary [10, 11].

2.2 Micturition

Micturition is the process of emptying the urinary bladder. This process requires a combination of involuntary and voluntary muscle contractions [10]. Two processes are involved in micturition, the first is gradually filling of the bladder until a critical value of pressure is reached, and the second is a neuronal reflex called micturition reflex, which empties the bladder. Micturition reflexes are triggered by nerve impulses transmitted from stretch receptors in the bladder wall into the spinal cord [14]. Filling of the bladder stretches the bladder wall and cause it to contract, then stretch receptors in its wall transmit nerve impulses into the spinal cord and return directly to the bladder through parasympathetic fibers [11]. Nerve signals from the spinal cord cause relaxation of the internal urethral sphincter muscle and contraction of the detrusor muscle, see figure 2.3 for detrusor muscle. At the same time, the spinal cord inhibits somatic motor neurons and cause relaxation of the muscles in the external urethra. When the urinary bladder wall contracts and the sphincters relax, urination takes place [11, 14].

The inability to prevent micturition is called incontinence. Incontinence is normal until two years of age, when the neurons to the external urethral sphincter muscle are not completely developed. Stress incontinence occurs when physical stresses such as coughing, sneezing, laughing, exercising, or simply walking cause leakage of urine from the urinary bladder [10]. Incontinence can also be related to defects in the nervous system.

2.3 Neurogenic bladder

Leu *et al* [15]. describes neurogenic bladder dysfunction (NBD) as a disorder with many different characteristics. The symptoms are as varied as the conditions that cause them, varying from no bladder function at all to severe overactivity. Long-term consequences of the disorder cover also a broad spectrum, ranging from no consequences for the patient to even death. The dysfunction of the bladder is a result of broken nerve signal communication between urinary bladder and the nervous system that controls the bladder function [15].

The extent of bladder dysfunction depends on whether the injury on the spinal cord is complete or incomplete, i.e. if all the neural paths are broken or if some are still in function. The location of the injury is also a factor that affects the dysfunction of the bladder [16], see figure 2.4.

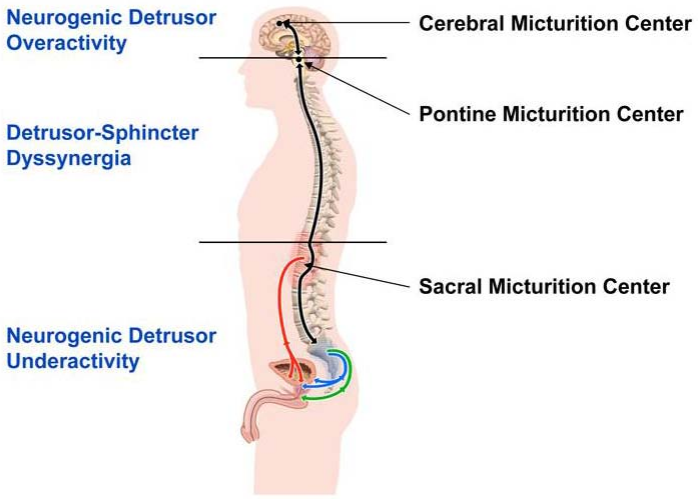


Figure 2.4 Illustration of injury sites in the spinal cord that cause neurogenic bladder [17].

Damage occurring in the cerebral micturition center, due to e.g. brain tumor or stroke, leads to inability to urinate at will and uncontrolled contractions in the bladder, which will lead to incontinence [16]. If the damage has occurred above the sacral micturition center, see figure 2.4, the experienced symptoms are cramps in the bladder wall and in the urethra's outer sphincter muscle. Finally, if the injury is at or below the sacral micturition center, the connection to central nervous system will be broken. This will result in e.g. inability to empty the bladder and no urge of emptying a full bladder [16].

For patients with spinal cord injury the quality of life is reduced, this is often affected by the ability to work or attend to school. Urinary factor are a large part of this and if neurogenic bladder is poorly managed, the shame of accidents can lead to withdrawal from social contact [6]. Studies have shown that compared with assisted IC, intermittent self-catheterization is associated with reduced depression [18]. Problem with having intimate relationship are also a factor affecting quality of life for NBD patients. Successfully performed bladder management can make a big difference in patient's daily life and thereby quality of life [6].

2.4 Urinary catheters

A urinary catheter is a medical device used for emptying the bladder of patients suffering from urine retention. A catheter is a thin and flexible tube composed of a polymeric material, see figure 2.5. The structure of the surface is of importance and is designed to minimize damage to the urethra [19]. Urine retention can depend on several factors such as spinal cord injury, multiple sclerosis or prostatic [20]. Catheter left inside the body are referred to as an indwelling catheter and temporary placement of a catheter to remove urine from the body are called intermittent catheterization.

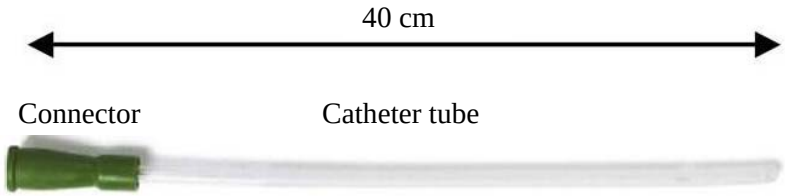


Figure 2.5 An intermittent catheter used for drainage of the urinary bladder.

An indwelling catheter or a Foley catheter is used for continuous drainage of the bladder, see figure 2.6. To keep the catheter in place there is a balloon filled with sterile water at the end of each foley catheter and the urine drains into a bag [21].

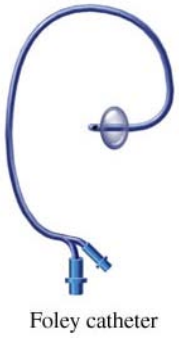


Figure 2.6 A foley catheter for continuous drainage of the bladder [22].

Catheters are measured in the french scale system Charriere unit, abbreviated as CH. A size-one Charriere catheter has a diameter of one-third millimeter. The most commonly used male intermittent catheter size are CH 12 – 14 and size CH 14 – 16 for females.

2.4.1 Material used in urinary catheters

Materials used in medical devices are called biomaterials and is defined as any natural or synthetic substances that interfaces with tissue. Biomaterials have been used in the urinary tract for centuries and have advanced from metal tubes to complex surface modified polymeric materials [23]. Metallic biomaterials are used for stenting the urethra and ureter; these devices are composed of stainless steel, titanium or other mixed alloys. The stents are intended for permanent use and are surface modified to minimize the risk of any immunological response [24].

Plastic materials that have dominated the market in production of hydrophilic urinary catheters are polyvinylchloride (PVC) and thermoplastic polyurethane (TPU). PVC can in certain conditions be a potential risk factor since it contains chloride and plasticizers [19]. Sterilization techniques on medical devices such as ethylene oxide are not ideal

from an environmental perspective on catheters composed of PVC and its plasticizers [25].With a concern of environmental impact from intermittently used medical devices, special environmental requirements have to be fulfilled. This has led to an attempt to find polymeric materials with better life cycle assessment [19].

Catheters composed of silicone layers on top of latex is an alternative for indwelling catheters [26]. Latex is a flexible and cheap polymer but is prone to infection. The silicone layer usually gets damaged after a while and the underlying latex come into contact with the urothelium, which limits the products life-span. All-silicone catheters have been produced but are more expensive. They are more rigid and have a longer life, up to 3 months. A disadvantage is that the balloon at the tip of the catheter tends to empty by loss of water due to semimembranous effect and has to be refilled periodically [27].

The fundamental properties of a polymeric material used in hydrophilic urinary catheters are that it must be flexible, have acceptable mechanical strength, the material must tolerate a certain sterilization process and it has to be compatible with the chemical coating process. Currently at Astra Tech AB conversion from PVC catheters to a new polymeric material is in progress. Polyolefin-based elastomer (POBE) is the new material of choice, which is a PVC-free copolymer. This new material is believed to fulfill all the technical and medical constraints, while at the same time being an environmentally good alternative to PVC and TPU [19].

2.4.2 Catheter coating

2.4.2.1 Hydrophilic catheters

Hydrophilic catheters have been developed to reduce the urethral friction, thus minimizing complications. The coating is activated by addition of water before use and compared with non-coated catheters used with addition of hydrogel, these catheters are associated with less microhematuria and pain [6]. The slippery surface of the intermittent catheter Lofric® from Astra Tech consists of a combination of polyvinyl pyrrolidone (PVP) and sodium chloride, see figure 2.7 [25].

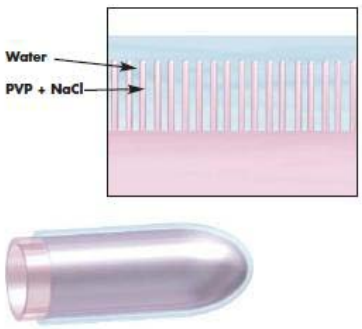


Figure 2.7 The hydrophilic and isotonic surface layer makes catheters slippery [28].

The Lofric® catheter is the first catheter with an urotonic surface technology. The chemical process of the surface technology makes the surface layer isotonic to urine, i.e. the salt concentration of the catheter's surface is the same as in the urine. This technology makes water in the hydrophilic layer stay intact during catheterization [28]. The friction from Lofric against the urethra is up to 95 % lower compared with conventional plastic catheter, see figure 2.8 for the mechanism of the surface layer technology.

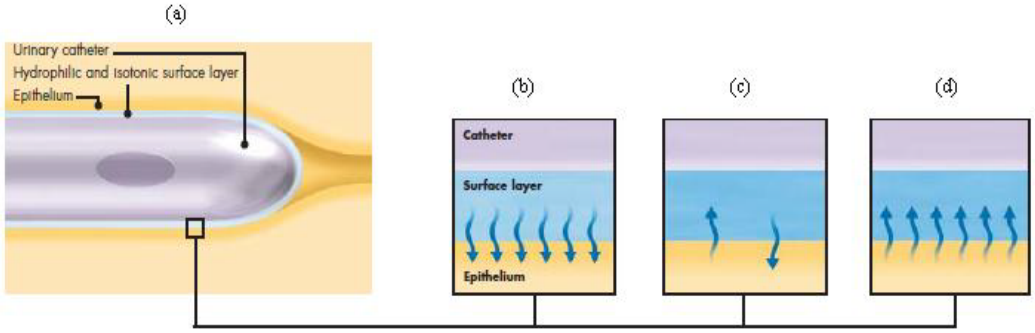


Figure 2.8 The mechanism of the urotonic surface layer. (a) Urethra in cross-section. (b) Hypotonic in relation to urine, the catheter might dry out and stick. (c) Isotonic to urine and no risk that the catheter dries out. (d) Hypertonic in relation to urine, risk of increased pain [28].

2.4.2.2 Antimicrobial coating

Antimicrobial coatings that have been directed against urinary tract infections are ciprofloxacin, silver, nitrofurazone, minocycline and rifampin [23]. Commercially available antibacterial catheters are coated with e.g. silver, chitosan and nitrofurazone. The coatings have varied from simple dipping of catheters in an antibiotic solution to development of antimicrobial composite polymers [29]. Direct application of antibiotics in medical devices promotes antibiotic resistance, which is not acceptable in clinic [30].

Urinary catheter composed of antimicrobial composite polymers releases antimicrobial agents through the natural degradation of the polymer. Degradation is accelerated in the presence of inflammatory enzymes such as cholesterol esterase [31]. If the urinary infection leads to an inflammatory response and increase the concentration of enzymes, then more antibiotics releases from the catheter. The increase in antimicrobial agent from the surface would thus help to eliminate organisms in the planktonic form before they establish a biofilm in indwelling catheters [29].

The oldest surface coating used to prevent colonization by microorganisms is silver. Different attempts to develop urinary catheters with silver coating have been done, such as inclusion of metallic silver, silver salts or silver sulfadiazine in the coatings that release silver ions close to the surface of the catheter [32]. Some studies have shown that silver-coated catheters reduce bacterial colonization on the surface of indwelling catheters [33]. In vitro studies on silver-coated peritoneal dialysis catheters have shown no significant reduction on bacterial adhesion on the catheter surface. It has later been shown that silver alloy catheters were significantly more protective than silver oxide

catheters [29]. The introduction of silver nanoparticles as coating on medical devices is believed to be the new strategy to reduce bacterial growth [32]. Silver particles with a diameter of about 1 – 10 nm have a direct interaction with bacteria, resulting in a stronger bactericidal activity compared to particles with greater diameter. The majority of studies on the cytotoxicity of silver nanoparticles have showed to be nontoxic under specific concentrations [34]. Studies on human skin carcinoma and fibrosarcoma cells exposed to silver nanoparticles at concentrations up to 6.25 µg/ml showed no changes in cell morphology. But at concentrations 6.25 – 50 µg/ml cells became less polyhedral, more fusiform and shrunken [35]. In figure 2.9 the mechanism of antibacterial agents is illustrated.

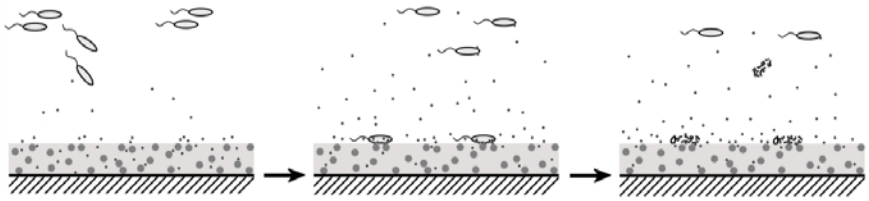


Figure 2.9 The antibacterial mechanism of e. g. silver. Nanoparticles slowly releases active agents into the coating layer and successive to the solution. Bacterial membrane and proteins will bind to antibacterial agents and eventually die [32].

Long-term use of silver can lead to bacterial resistance, but the broad range of silver targets in the cell makes evolution of silver resistance slow [32].

Chitosan is a natural biocompatible cationic polysaccharide used as antibacterial coating on intermittent catheters. It has an antibacterial activity due to the presence of amine functions [30, 36]. Studies with chitosan have shown promising result on preventing biofilm formation and the exact mechanism of antibacterial action is still unknown. A proposed mechanism is that the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular components, causing cell death [36]. It has not yet been clinically demonstrated that chitosan coated intermittent catheters reduce CAUTI.

Nitrofurazone is an agent useful against a broad spectrum of Gram-positive and Gram-negative bacteria. The antibacterial mechanism of nitrofurazone act by inhibition of bacterial enzymes involved in DNA and RNA synthesis, carbohydrate metabolism and other metabolic enzyme proteins [37]. Nitrofurazone impregnated catheters have been developed in an attempt to reduce UTI. Foley catheters impregnated with nitrofurazone have shown reduced number of catheter associated bacteriuria [38], but no clinical evidence on the efficiency of nitrofurazone impregnated intermittent catheters have been shown.

2.4.3 Intermittent catheterization

Intermittent catheterization was introduced in the 1940s by Sir Ludwig Guttman to empty the bladder in neurogenic bladder dysfunction (NBD) patients during the spinal

shock phase [39]. In the 1970s clean intermittent catheterization (CIC) was suggested as treatment for neurogenic bladder. Intermittent catheterization mimics normal emptying of the bladder, eliminates the indwelling catheters as a persistent foreign body and is associated with increase in quality of life [40]. To find the bladder storage capabilities and to select the optimal catheterization interval urodynamic evaluation is necessary. Catheterization is ideally performed at intervals of 3 - 4 hours. The fluid intake in NBD patients should not be more than 2000 ml per day and catheterization should take place before the amount of urine in the bladder reaches 500 ml [39]. Self-catheterization can be performed in different positions: supine, sitting or standing. Female patients can use a mirror to visualize the meatus [41]. The urine can be drained directly in the toilet, in a urinal, plastic bag or in other reservoir. After properly executed catheterization, the residual urine should be maximum 6 ml [39], but studies have shown that residual urine could sometimes exceed 50 ml or even 100 ml [41].

Complications can rise by placing a catheter in the bladder several times a day. In new patients urethral bleeding is seen and occurs regularly in one-third on a long-term basis [42]. With proper management in the acute stage of spinal cord injury urine can be kept sterile for 15 - 20 day without antibiotic prophylaxis. The bacteria found are mostly *E.coli, Proteus, Citrobacter, Pseudomonas, Klebsiella* and *Staphyllococcus* [43]. In several studies *E.coli* is considered to be the dominant species [43, 44]. Prevention of UTI in intermittently catheterized patients includes factors such as using noninfecting techniques, nursing education and emptying the bladder completely since residual urine plays a role in the infection [42].

2.5 Urinary tract infection

Urinary tract infection (UTI) is one of the most common bacterial infection of any organ system [43]. Each year UTIs causes losses in work time, morbidity and medical costs. For people living in long-term care settings the incidence of UTIs might be as high as 50 % [44]. 7 million office visits per year are estimated to be due to UTIs at a cost to the healthcare system of over $1 billion in USA alone [45]. It is estimated that 33% of neurogenic bladder patients have bacteriuria at any time, and the major cause of fever in spinal cord patients are due to UTIs [20].

Bacteriuria is the presence of bacteria in the urine and it is well known that urine is normally free of bacteria. Bacteriuria can be symptomatic or asymptomatic and it does not necessarily mean a urinary tract infection. The majority of pathogens that cause UTI are either coliforms or enterococci [44]. Coliforms include the Enterobacter family members of *E.coli*, *Enterobacter* and *Klebsiella*. *E.coli* is a bacteria inhabitant in the intestines that usually live in humans without any problem. It makes no distinction for age and accounts for the majority (80 – 90 %) of uncomplicated infections in young and old [44, 46]. *Staphylococcus saprophyticus* is another bacterium that accounts for 10 – 20 % of uncomplicated UTIs [42]. Bacteriuria can be asymptomatic; it is when a significant number of bacteria occur in the urine without any symptoms such as burning during urination or fever. Treatment is usually not necessary for patients with asymptomatic bacteriuria [47].

Microbiological examination of the urine is often performed to confirm the presence of infection in the urinary tract. Treatment is considered necessary when growth of more than 10^5 colony forming units (CFU) per ml of an organism or strain is observed from a midstream specimen or catheter specimen of urine [46]. For patient on clean intermittent catheterization, growth of more than or equal to 10^2 CFU/ml are considered to be significant [20]. But some patients with symptomatic UTI may not have a significant bacteriuria even when bacteria can be seen from their bladder mucosa. This can be due to hematogenous infections which can be present without bacteriuria. Hematogenous UTI are infections where organisms are delivered to the urinary system by the bloodstream [43].

There are three ways for bacteria to enter the bladder: ascension through the urethra, the hematogenous route and the lymphatic channels. The most common route is the ascent of bacteria from the urethra into the bladder [43, 44]. The anatomical structure of the female lower urinary tract (relatively short and straight urethra), makes it more susceptible to colonization of the urethra and to bacterial cystitis.

Urinary tract infections can be divided into two categories: complicated and uncomplicated UTIs. Uncomplicated UTIs occur usually in healthy people, where the hosts have normal urinary tract and do not have systemic diseases subjecting them to bacterial infections. Complicated UTIs affect people with problematical urinary tracts i.e. not functioning as it should due to anatomic or functional defects [43]. The second group affiliated with complicated UTIs is hosts generally susceptible to infections, for instance patients with immunosuppression. Catheter associated urinary tract infections are also considered as complicated infections due to presence of a foreign body. Characteristics of complicated UTIs are that the spectrum of organisms is broader and that antibiotic treatment may not have an effect [44].

Nosocomial UTIs are infection acquired in connection with hospital stays. The common pathogen species are *E.coli* or *Staphylococcus* [43]. People with spinal cord injury are susceptible of colonization of *Pseudomonas* species, particularly if they are wearing external urinary catheters or using intermittent catheterization [44]. The high frequency of UTIs has led to an increasing resistance to antibiotics of many bacterial strains, which have become a major concern in healthcare [43].

2.5.1 Lower- and upper urinary tract infections

Lower urinary tract infections include complications in the bladder and the organs below it. It might be experienced with symptoms including dysuria, suprabubic pain and hematuria [44].

Cystitis is the most common lower urinary tract complication of neurogenic bladder. The primary bladder defenses against infections are the flow of urine through the urinary tract and voiding. NBD patients are at risk for recurrent attacks of cystitis, since they usually have residual urine in their bladder. The symptoms that are experienced for

an NBD patient are back or abdominal pain, complain of fever, leakage between catheterization and cloudy urine [20].

According to Ghoniem *et al* [48] there are two major risk factors contributing to upper urinary tract infection among NBD patients. The first one is recurrent lower urinary tract infections which interfere with the antireflux mechanism, causing infected urine reaching the kidney. Secondly, functional infravesical obstruction that slows down or stops the flow of urine and leads to high intravesical pressure, which creates a risk for reflux of bacteriuria. Studies on patients with detrusor external sphincter dyssenergia [48], which is a complication that creates a buildup of urinary pressure as a result of not emptying the bladder completely [49], have shown that 50 % of men will develop significant complications such as upper tract deterioration, urosepsis and ureterovescial obstruction [48].

Since neurologic patients is often absent of sensation, the main symptoms of acute upper UTI is fever up to 40°C. Acute infection in the upper urinary tract requires hospitalization of NBD patients. The treatment duration is approximately two weeks and is initiated with intravenous antibiotics and completed with antibiotics taken orally. In severe cases, where the symptoms persist beyond 72 hours radiologic investigation with CT are performed to analyze the possibility of internal abscesses, urinary tract abnormalities or obstruction [48].

2.5.2 Catheter associated urinary tract infection (CAUTI)

CAUTI is defined as a symptomatic infection in a person with an indwelling urinary catheter and it is the most frequent healthcare associated infection [43, 50]. Treatments with long-term urethral catheterization in patients have indicated that *P. mirabilis* urease is correlated with development of catheter encrustation, including blockage of the catheter [51]. This may block the urine flow and result in reflux, causing infection of the upper urinary tract [44].

Depending on the insertion technique, gender and state of the patient's health, bacteriuria might occur in less than 2 days after catheterization. Studies show that bacteria initiate infection by entering the bladder via migration from the catheter-urethral-meatus interface along the external surface of the catheter [51]. Biofilm formation covers and secures bacteria against mucosal surface. Mechanical flow of urine, host defenses and even antibiotics doesn't seem have a big impact on organisms contained within a biofilm [52]. Slow and variable growth rate influenced by the availability of nutrients is characteristics of microorganism deep within the biofilm. These organisms may develop resistance to anti-microbial therapy, making treatment of patients with indwelling catheters complicated [53].

The most significant difference between UTI and CAUTI in respect of microbial pathogenesis is the increase in resistance to antimicrobial therapy. The introduction of a foreign body, such as a catheter, into the host provides a surface for attachment and growth of bacteria as a biofilm. The physiology and growth rate of organisms growing

in planktonic culture are different from the same organism growing in a biofilm mode [29]. Organism in a biofilm can have a resistance to antimicrobial agent up to 20 to 50-fold compared to their planktonic counterparts [54]. As long as the catheter remains in place CAUTI gets very difficult to eliminate [29].

The most important risk factor for the development of catheter associated bacteriuria is the duration of catheterization. Most of the catheterized patients are bacteriuric by the end of 30 days, which is the dividing line between short-term and long-term catheterization [52]. 15 to 25 % of patients in general hospitals might have a catheter in place during their stay and most of them are in place for a short time. In short-term catheterization most cases of bacteriuria are asymptomatic. Whereas, long-term catheterization is indicated by complications such as urinary incontinence and bladder outlet obstruction. This type of bacteriuria is associated with complications that fall into two categories. The first includes symptomatic UTIs which cause fever, bacteremia and acute upper UTI. The second group is complications associated with catheterization such as obstruction, urinary tract stones, chronic upper UTI, and with prolonged use, bladder cancer [52, 55].

Intermittent catheterization carries the risk of introducing bacteria into the bladder. A new episode of bacteriuria occurs every 1 to 3 weeks in neurogenic bladder patients doing four catheterizations per day. Bacteriuria develops in about 1 to 5 % of patients, with an increase of risk in diabetics, elderly and debilitated patients [43, 44].

2.6 Prevention of catheter associated urinary tract infection

The best way to prevent CAUTI is to avoid the use of indwelling catheters and to keep the duration of use to an absolute minimum when catheters cannot be avoided [43]. Since 1940s intermittent catheterization has become the standard and optimal way of care for neurogenic bladder patients. Intermittent catheterization is believed to be an improvement over indwelling catheters in respect to periurethral infections, bacteremia, bladder and renal stones, and deterioration of renal function. Studies have shown that there is a fivefold incidence of UTI when intermittent catheterization was performed 3 times a day compared with 6 times [20]. Comparing clean against sterile catheters has shown that there is no significant difference in symptomatic UTI, but the study did show that clean catheterization was associated with reduced cost [56]. To postpone bacteriuria for short periods in intermittently catheterized patients oral antibiotics and methenamine can be used, but if such practice are beneficial over long-term has not yet been proved [52].

2.7 Urine

The volume of urine produced per day in a healthy person can vary from 300 ml if no water is ingested or up to a maximum of 23 l in cases of excessive fluid intake if there is an excessive water loss from the body. To excrete the daily load of toxic waste

products, the minimum urine output cannot drop below 300 ml/day in a healthy person. The average urine output per day is approximately 1500 ml [9].

The amount of solutes to be excreted by the kidneys each day is much less variable, even if the volume of urine can vary over a wide range [9]. Thus, the kidney must have the ability to concentrate or dilute the urine, in order to excrete a fairly fixed volume of solutes each day in a very variable volume of water. This ability of the kidney is essential to maintain a constant body osmolality. Mechanism that controls the concentration or dilution of urine in the kidney is most often affected early in renal disease, making it difficult to control both body fluid volume and osmolality [9].

The elements of urine are varied and include a range of substances such as:

- Ions: ammonium, phosphate, bicarbonate, chloride, magnesium, calcium, potassium and sodium
- Metabolic waste: uric acid, creatinine and urea
- Drug metabolites: after detoxification of pharmacological agents in the liver, excretion of metabolites from the body occurs through the kidneys
- Products of normal metabolism: with suitable assays metabolites of hormones can be detected in the urine

Depending on the concentration, normal urine may vary in color from colorless to yellowish-brown. Urine can have a pH between 4.0 - 8.0 under special circumstances of acidosis or alkalosis, although normal urine is to some extent acidic with a pH around 6 [9].

2.7. 1 Bacterial growth in urine

Bacteria in urine multiply in four phases – lag phase, logarithmic phase, stationary phase and decline phase, see figure 2.10. In the lag phase the bacteria are adapting to the new environment instead of increasing in cell mass or number. The logarithmic phase begins when the bacteria have adapted and started to grow at a constant growth rate until inhibition of growth occurs due to reduced nutrients or increased toxic waste. For viable *E.coli* cells the time to double in number can be as short as 12.5 minutes [44]. The stationary phase is reached at the same time as the resources in the urine decline. Lack of nutrients in combination with increase of toxic waste results in the start of the decline phase and occurs when cells die faster than they are replaced, but in the urinary bladder this phase is never reached due to continuous supply of urine. The properties of urine such as pH, osmolality, organic acids, glucose content and urea can promote or inhibit the growth of bacteria. The frequency of bladder emptying, high urinary flow and the amount of residual urine is also factors that contribute to the presence or absence of bacteria in the urine [40].

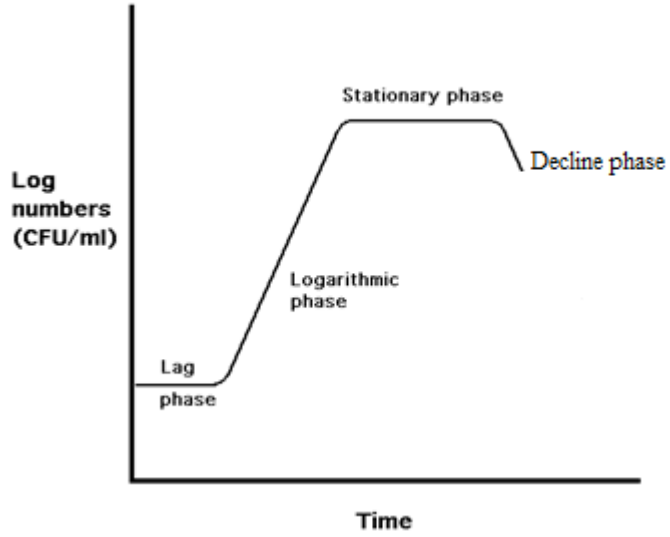


Figure 2.10 Bacterial growth curve illustrating the four different phases.

2.8 Resazurin

Resazurin is a redox dye used as an indicator of chemical cytotoxicity in cultured cells [57]. It becomes fluorescent when reduced by oxidoreductases within viable cells to resorufin. It is stable in culture medium and non-toxic to cells. Agents that damage cell proliferation and viability also affects the ability to reduce resazurin, thus is the rate of dye reduction directly proportional to the number of viable cells. Therefore, measurement of resazurin reduction may provide an index of cell proliferation [58]. In this thesis the resazurin reduction was studied by absorbance measurements at 600nm. While resazurin absorbs light at 600 nm resorufin, with absorptions peak at 575 nm [59], does not. Thus, by performing absorbance measurements over time at 600 nm the kinetics of color change of resazurin reduction can be followed.

3 Materials and Methods

After many different constructions and bacteriological analysis of in vitro bladder models a promising construction was made. The experimental data can provide information that proves or disproves the theory that antimicrobial catheters have a significant effect on inhibiting bacterial growth . The absorbance measurements on microplate reader are compared with reference tests and counting colony forming units on agar plates. Analysis of the bacterial content will inform if the bacteriuria are considered significant.

3.1 Growth medium

The growth of *E.coli* is dependent on having an energy source, a source of carbon and other reuqired nutrients. The artificial urine as growth media was prepared according to the recipe shown in table 1.

Table 1. List of ingredients for the artificial urine.

A: Stock solution		**B: Urea – glucose solution**	
Compound	Amount	Compound	Amount
NaCl	8.77 g	Urea	36 g
K_2HPO_4	3.48 g	Glucose	0.18 g
$NaH_2PO_4 * 2H_2O$	1.56 g	Dilute with H_2O Milli-q to	100 ml
NH_4Cl	2.67 g	**C: Cation – solution**	
Na_2SO_4	2.84 g	Compound	Amount
Lactic acid	0.37 ml=0.45 g	$MgCl_2 * 6H_2O$	6.1 g
Yeast extract	4.0 g	$CaCl_2 * 2H_2O$	4.4 g
Dilute with distilled H_2O to	1000 ml	Dilute with H_2O Milli-q to	100 ml

The solutions A and C were sterilized through autoclaving, and solution B was sterilized through filters with pore size of 0.22 µm. All solutions were mixed according to table 2 and the pH had to be around 6.60. Osmolality were analyzed and compared with earlier measurements in order to be in the same range, around 800 mOsm/kg. The volume of artificial urine can vary depending on the needed amount, but the mixing ratio had to be the same, see table 2.

Table 2. The mixing ratio of the solutions for the artificial urine.

A: Stock solution	940 ml
B: Urea – glucose solution	50 ml
C: Cation – solution	10 ml

3.2 Tested catheters

The tested catheters are shown in table 3. Rochester antibacterial catheters contained 10.2 +/- 2.0 µg nitrofurazone (5 nitro-2-furaldehyde semcarbazone) per mm^2**.**

Table 3. Intermittent catheters (IC) were used in the experiments.

Catheter Name	Material	Type	Antimicrobial coating
Lofric® Primo	POBE	IC, CH14	None
Rochester Magic^3 Antibacterial Catheter	Silicone	IC, CH14	Nitrofurazone
Rochester Magic^3 Antibacterial+Hydrophilic Catheter	Silicone	IC, CH14	Nitrofurazone

Rochester Magic^3 Antibacterial + Hydrophilic Catheter packages contained sterile water in a foil packet which was released before usage, making the surface slippery. To activate the Urotonic™ surface prior to use, Lofric® Primo also contained an activation solution.

3.3 Instrumentation

The spectrophotometer SPECTRAmax 340PC enables quick analysis on the bacterial concentration of the residual urine by following the kinetics of resazurin reduction. This was performed by measuring the optical density (OD) over a specified time interval. In the experiments absorbance measurements were performed at 600nm and the background from the cells was corrected by subtracting the value for samples without resazurin to samples with resazurin. The result was then analyzed to study the inhibiting effect of antibacterial coated catheters.

Standard 96-well micro plates were used in the SPECTRAmax 340PC. The content of the wells in the micro plate were mixed automatically by shaking before each read cycle and the chamber temperature were set to 37 ºC.

The pH of the artificial urine was measured with Metrohm pH-Meter 744 and the osmolality was measured using Osmometer 15. The measurements were performed after each prepared batch.

3.4 Growth of *E.coli*

The *E.coli* strains 24T, 10979 and 17620 was purchased from the Culture Collection of the University of Gothenburg. The strains were stored and maintained in stock solutions at -80°C. Petri dishes with trypton glucose extract agar (TGE) were prepared and a loopful of *E.coli* culture was scooped with a bacteria loop from the stocks. It was spread across the agar plate and incubated at 37 ºC for 24 hours in an inverted position. Bacterial colonies were formed and a single colony was taken from the plates to tubes with LB-broth. The tubes were incubated at 37 ºC and the bacterial concentration was estimated to be ~$1*10^8$ CFU/ml.

3.5 Artificial sphincter

The urinary sphincter acts as a valve from an engineering point of view. It opens for a short period of time to pass urine and is otherwise usually closed. Pressure on the urethra along a certain length has to be applied, in order to close the urethra against the bladder pressure. If the pressure is to low it will lead to incontinence [60]. The sphincter surrounds the urethra and expansion of the sphincter muscle opens the urethra. In the experiments a check valve composed of silicone was used as an artificial sphincter, see figure 3.1. It has the strength to withstand the pressure from the bladder without leakage and has appropriate dimensions. When a catheter is passed through the check valve it surpasses the valve and the urine can pass the valve.

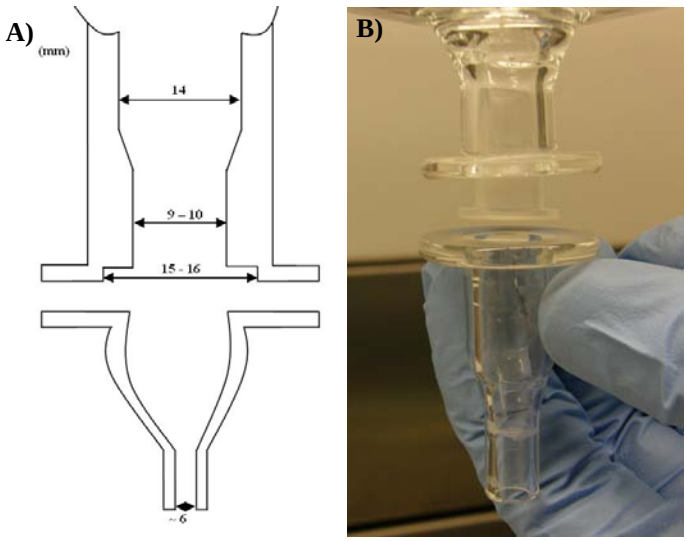
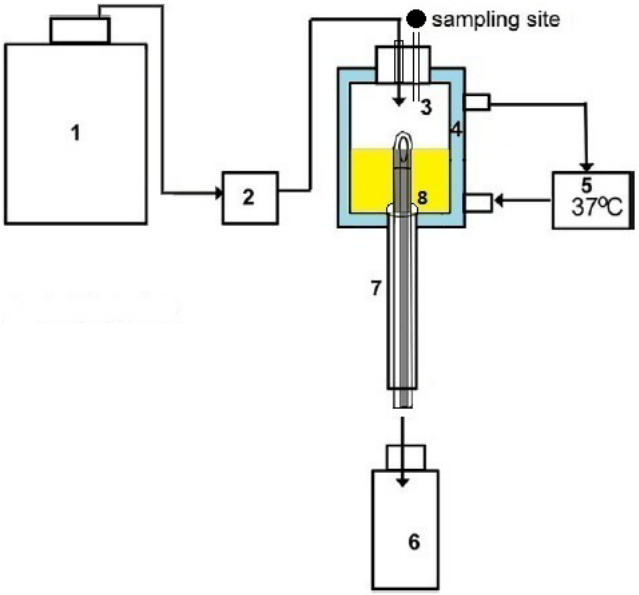


Figure 3.1 A) shows the base of the glass chamber in cross section. In B) the position of silicone check valve is observed. The O-ring joint is held together with a pinch clamp.

3.6 In vitro bladder model

The model consist of a custom made glass chamber (800 ml) from Glahi HB maintained at 37 ºC by a water jacket, see figure 3.2. The model is sterilized by autoclaving and urinary catheters were inserted into the glass chamber through a section of silicone tubing, mimicking the urethra, attached to a glass outlet at the base. The chamber lid and the glass section at the base of the glass chamber were held together with clamps.

A)



B)

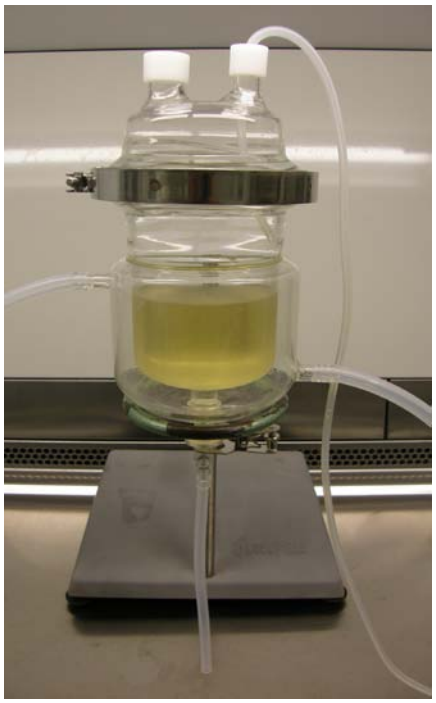


Figure 3.2 A)Scheme of the in vitro bladder model assembly: 1, artificial urine reservoir; 2, peristaltic pump; 3, glass artificial bladder; 4, heating jacket; 5, water bath; 6, collection bottle; 7, artificial urethra; 8, artificial sphincter. B) The catheterized in vitro bladder model.

Sterile artificial urine was supplied to the bladder via a peristaltic pump with a flow rate of 1.3 – 1.5 ml/min. Residual urine of 25 – 40 ml was left in the bladder and samples were taken for determining the bacterial concentration. Absorbance measurements and quantification of colony forming units on agar plates were performed to determine the number of bacteria in the sample.

3.7 Experimental protocol

Two different experimental protocols were evaluated. In experimental protocol 1 the catheters were contaminated with 10^6 CFU/ml *E.coli* in 60 seconds prior to each catheterization whereas in experimental protocol 2 catheters were contaminated only before the first catheterization

Aseptic technique was used to reduce the risk of introducing additional organisms. All components of the in vitro bladder model were sterilized through autoclaving, which included silicone tubing, artificial urine and laboratory equipment for ensuring sterile conditions. The three *E.coli* strains were pooled and the tip (6cm) of the catheters was dipped in artificial urine with a bacterial concentration of 10^6 CFU/ml before catheterization. Residual urine of 20- 40 ml were left in the bladder. Urine was supplied to the model with a flow rate of 1.3 – 1.5 ml/min. The sample volume was 1 ml and appropriate dilution series were made for plating and viable cell counts. Sampling was repeated after each catheterization; immediately after the first catheterization, after 4, 8, 12 and 22 hours. 200 µl of the sample were mixed with 30 µl resazurin in the micro titer plate for absorbance measurements.

4 Results

In this chapter results from the developed lower urinary tract model are presented. First the analysis of bacterial growth acquired from the in vitro bladder model is presented, followed by antibiotic sensitivity tests and spectrophotometric measurements of resazurin reduction. Conducted experiments are compared to reference experiments.

4. 1 Bacteriological analysis of the in vitro bladder

The experiments with Lofric® Primo and Magic[3] catheters impregnated with the antibacterial agent nitrofurazone were performed in duplicates. Figure 4.1 and 4.2, shows the representative result out of two replicates from experimental protocol 1 and experimental protocol 2, respectively.

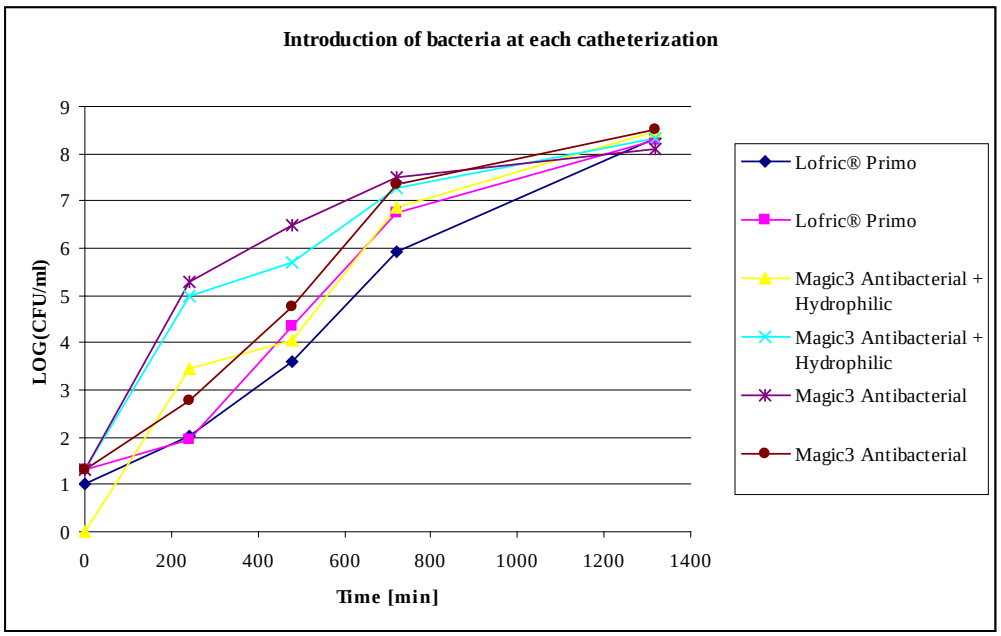


Figure 4.1 Bacteria were introduced into the bladder at each catheterization. No significant difference could be seen in inhibition of bacterial growth between the catheters. After 22 hours (1320 min) the bacterial population approached over 10^8CFU/ml in all experiments.

The initial volume of artificial urine in the bladder was 350 ml and 6 cm of the catheter tip was contaminated with *E.coli* before the bladder was drained. The concentration of *E.coli* in the residual urine after the first catheterization varied between 0 - 1.30 $\log_{10}$ CFU/ml. After 22 hours the bacterial concentration was in the range of 8.2 – 8.6 $\log_{10}$

CFU/ml in the experiments. No inhibition of bacteria growth after catheterization with antibacterial catheters could be observed. The pH and osmolality of the urine were within the range of 6.55 – 6.65 and 770 – 830 mOsm/kg, respectively.

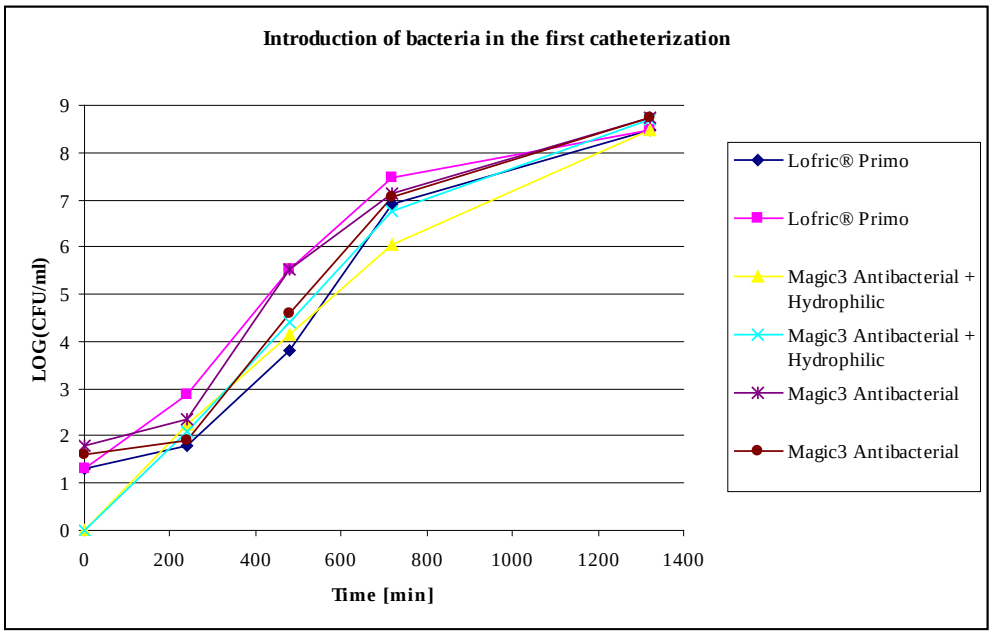


Figure 4.2 Bacteria were introduced into the bladder only in the first catheterization. No significant differences in inhibition of bacteria growth could be seen between the catheters. After 22 hours (1320min) the population approached over 10^8CFU/ml in all experiments.

Figure 4.2 illustrates the introduction of *E.coli* into the bladder at time 0 and the growth of it over 22 hours. The concentration of bacteria after the first catheterization is in the range of 0 - 1.78 $\log_{10}$ CFU/ml. After 22 hours the concentration are between 8.3 – 8.75 $\log_{10}$ CFU/ml for the different experiments. No significant difference between inhibition of bacterial growth was observed ($p>0.05$). In figure 4.3 insertion of Magic^3 Antibacterial + Hydrophilic catheter into the bladder are shown.

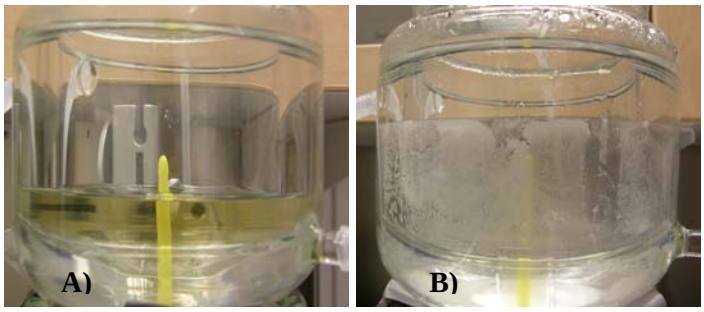


Figure 4.3 The catheters were contaminated with *E.coli* prior to each catheterization. In A) the first catheterization is observed, whereas in (B) the last catheterization is shown.

In the experiment shown in figure 4.3 the catheters were contaminated prior to each catheterization and after 22 hours bacterial growth could be observed on the wall of the bladder. In figure 4.4 – 4.6 the difference of bacterial growth between the experimental replicates are shown, and in table 4 the growth rates of E.coli are presented at different time points.

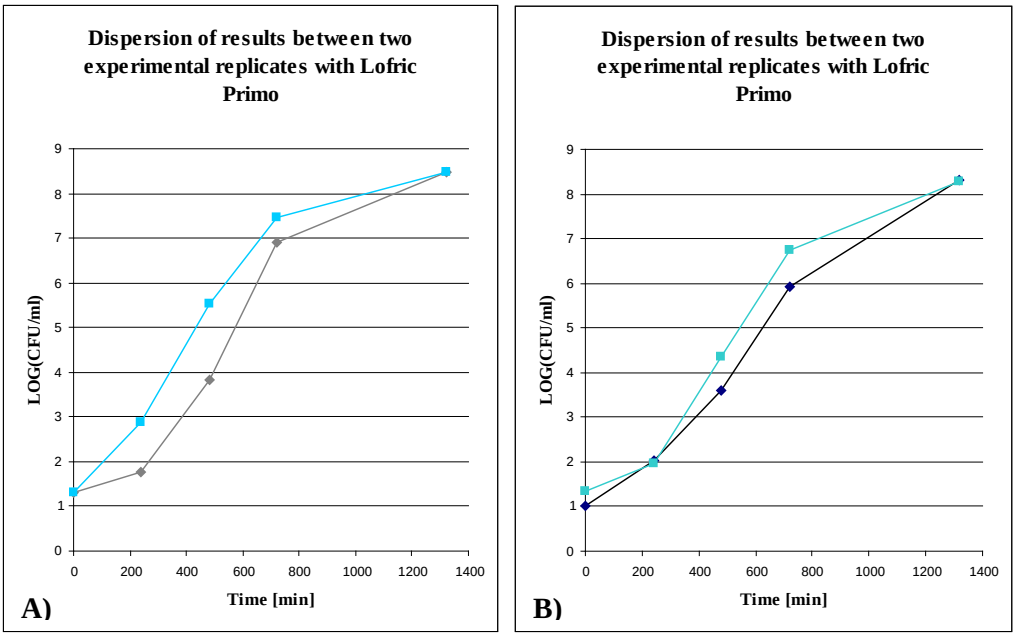


Figure 4.4 The differences in bacterial concentration within the experimental replicates performed with Lofric Primo catheter. A) Catheters are contaminated with *E.coli* before the first catheterization. B) Catheters are contaminated with *E.coli* before each catheterization.

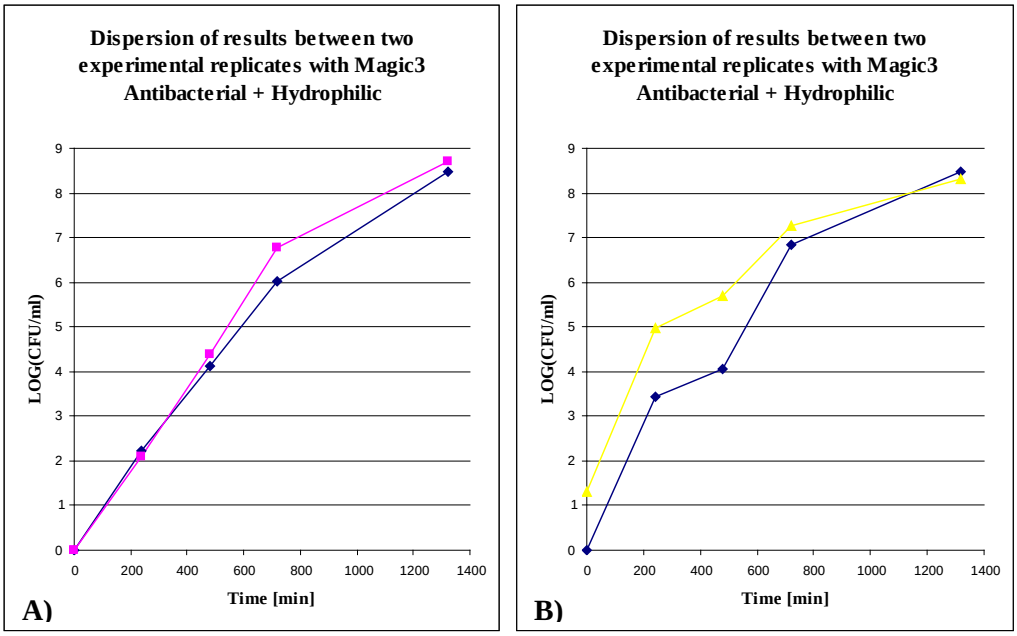


Figure 4.5 The differences in bacterial concentration within the experimental replicates performed with Magic[3] Antibacterial + Hydrophilic catheter. A) Catheters are contaminated with *E.coli* before the first catheterization. B) Catheters are contaminated with *E.coli* before each catheterization.

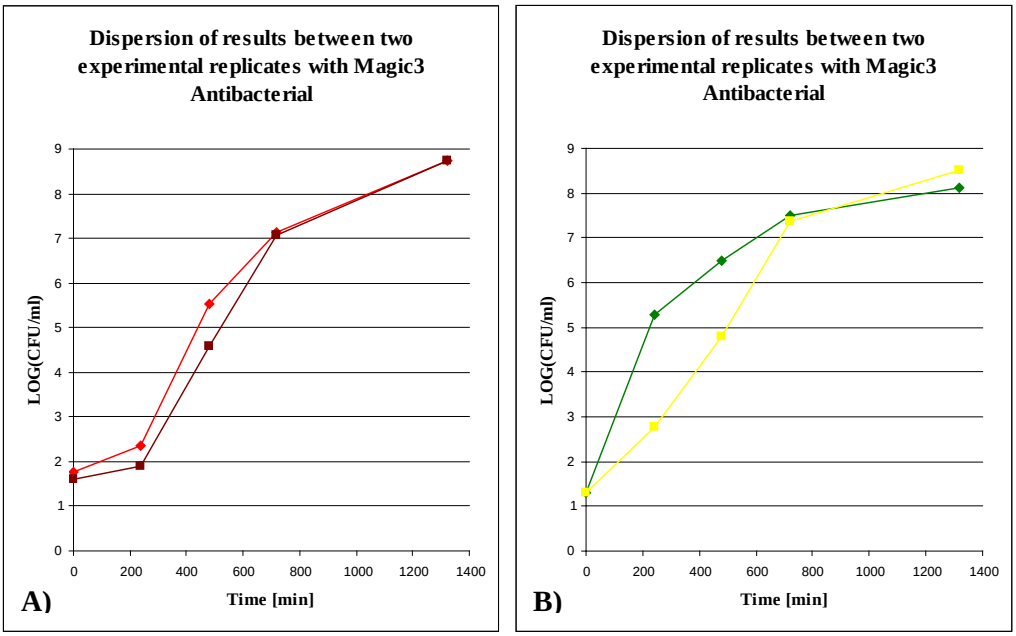


Figure 4.6 The differences in bacterial concentration within the experimental replicates performed with Magic^3 Antibacterial catheter. A) The catheter is contaminated with *E.coli* before the first catheterization. B) The catheter is contaminated with *E.coli* before each catheterization.

Table 4. The growth rate of *E.coli* after catheterization with Lofric Primo and Magic^3 antibacterial at different time points. The results shows the differences between the replicates performed according to experimental protocol 1.

	Lofric Primo			**Magic3 Antibacterial**	
Time points	Growth rate [logCFU/h]		Time points	Growth rate [logCFU/h]	
0-4h	0,12	0,40	0-4h	0,37	0,15
4-8h	0,51	0,66	4-8h	0,50	0,79
8-12h	0,78	0,48	8-12h	0,65	0,41

The figures 4.4 – 4.6 demonstrate the uneven characteristic of bacterial concentration at different time points. As shown in table 4 the growth rate of bacteria differs from each replicate, and reaches a concentration of 8.2 – 8.7 $\log_{10}$ CFU/ml after 22 hours.

4.2 Kirby – Bauer test

To analyze the antibacterial effect of Rochester Magic^3 catheters a Kirby – Bauer test was conducted. 10^6 CFU/ml *E.coli* were spread over an agar plate with catheters inserted in it, see figure 4.7. The incubation time was 24 hours in 37 °C.

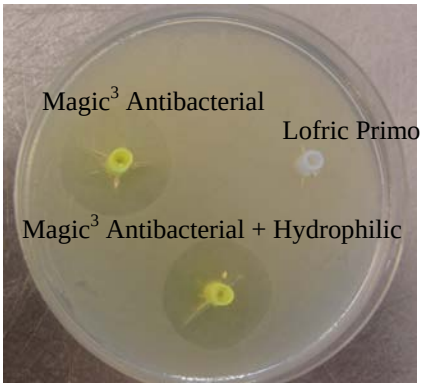


Figure 4.7 The activity of nitrofurazone treated catheter against *E.coli*. An inhibition area of ~3.14 cm^2 could be observed on agar plates with an *E.coli* concentration of ~10^6 CFU/ml. The experiment was performed in duplicates.

The $Magic^3$ catheters were impregnated with 10.2 +/- 2.0 µg nitrofurazone/mm^2 and both of the catheters showed an inhibition area in the same size, ~3.14 cm^2. Lofric Primo acted as the control and no inhibition of bacteria could be observed around this catheter.

4.3 Absorbance measurements with SPECTRAmax 340PC

The relationship between resazurin reduction time and bacterial concentration was studied for *E.coli* cells. When metabolically active cells reduce the blue dye resazurin to the pink fluorescent resorufin a shift in the OD occurs from 1.3 to 0.1. In figure 4.8 resazurin reduction of *E.coli* cells in stationary phase is shown. The start concentration of *E.coli* was 8.1*10^8 CFU/ml and sequential 10-fold dilution of the bacterial culture was done.

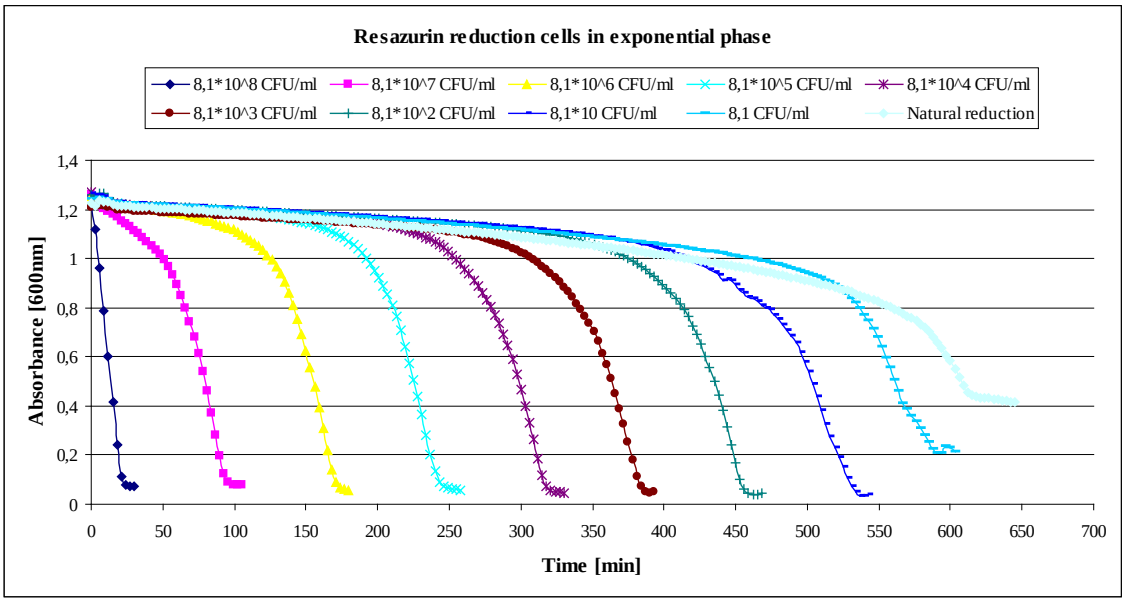


Figure 4.8 The plot shows the absorbance at 600 nm as a function of time with different bacterial concentrations when the cells are in exponential phase. The shift in OD from 1.3 to 0.1 is the result of resazurin reduction. The plot is an average of 3 measurements.

The highest cell concentration, $8.1*10^8$ CFU/ml, reduced resazurin to resorufin within 18 min, whereas the cell concentration 8.1 CFU/ml reduced resazurin in 580 min. The time required for each concentration to shift to OD 0.4 was recorded and plotted against the bacterial counts, see figure 4.9.

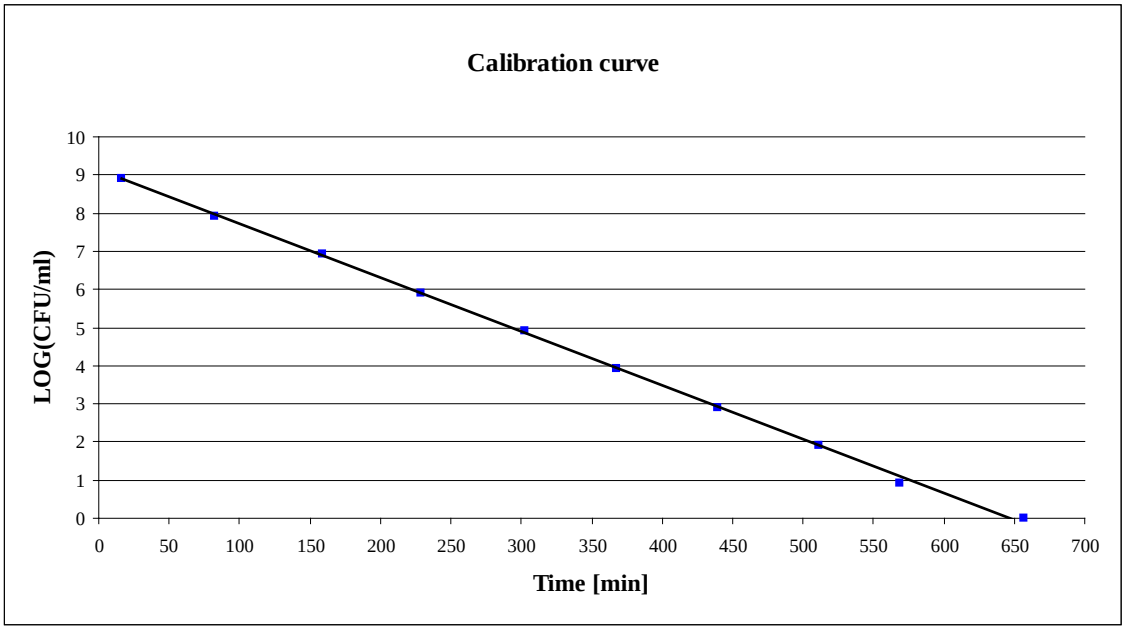


Figure 4.9 Correlation between bacterial concentration and the time required to reach OD value 0.4. Linear regression analysis gives the following equation that describes the bacterial concentration over time: y = -0.0141x + 9.1187 (R2 = 0.9992) for cells in stationary phase.

The reduction of resazurin to resorufin for cells in the stationary phase can be described with the formula:

$$y = -0.0141x + 9.1187, \qquad \text{(eq.1)}$$

where y is the cell concentration in logCFU and x is time in minutes. The time required to reach the threshold OD showed a good linear relations to the bacterial concentration, R= 0.99.

Kinetic studies with resazurin on samples from the bladder were performed, see figure 4.10 and 4.11. The time required to reach OD – value 0.4 were recorded, and inserted in the formula obtained from the calibration curve. The calculated bacterial concentration where then compared to viable cell counts, see table 4 and 5. In figure 4.11 absorbance measurements of resazurin reduction are observed for the experiments where bacteria were introduced at each catheterization, one experiment per catheter is shown.

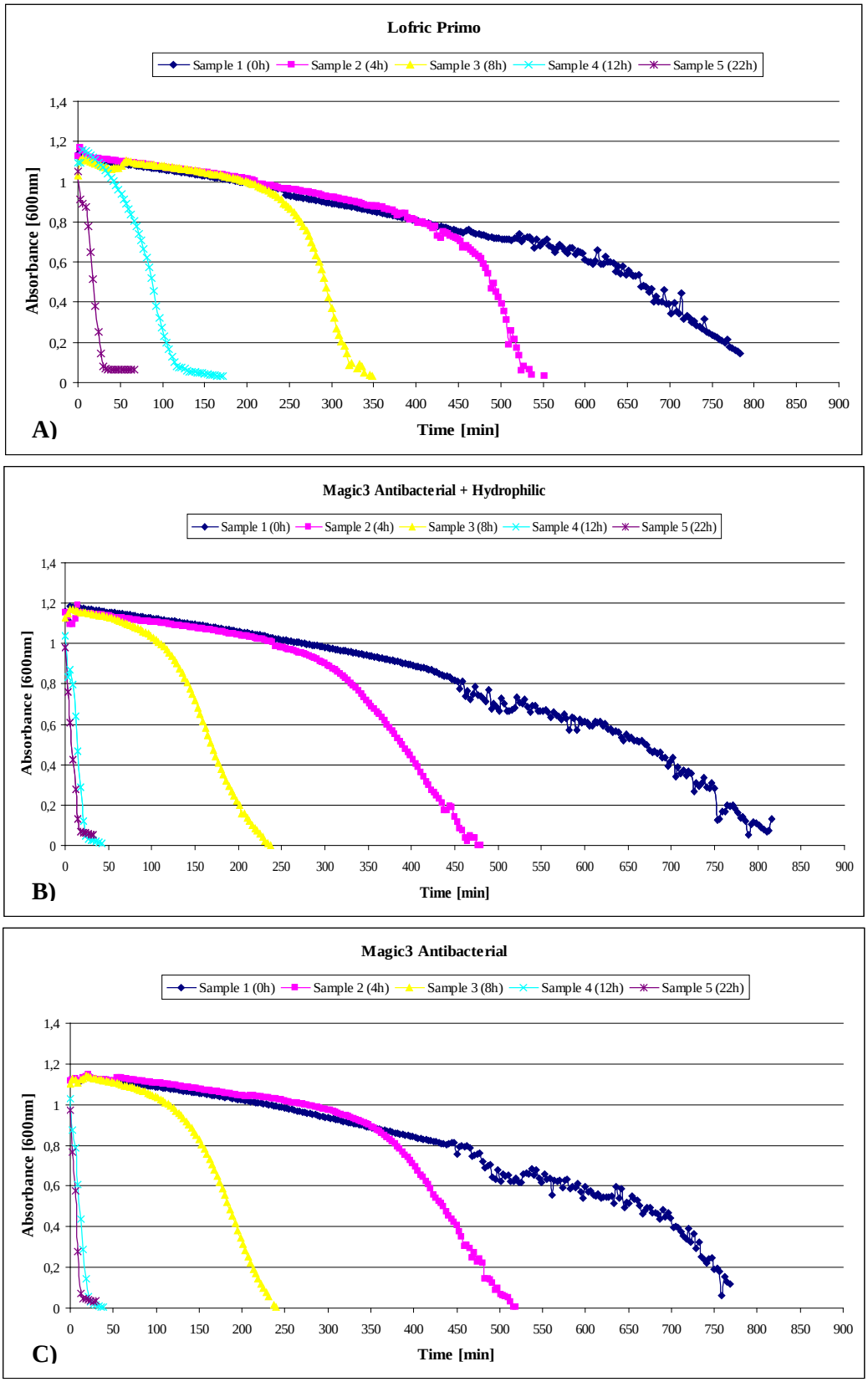


Figure 4.10 The plot shows the absorbance at 600 nm as a function of time for each sample. The bladder was catheterized with Lofric Primo in chart A, with Magic^3 Antibacterial + Hydrophilic in chart B and with Magic^3 Antibacterial in chart C. The shift in absorbance from 1.2 to 0.1 is the result of resazurin reduction. The plot is an average of 3 measurements.

Table 4. Comparing the estimated concentration from viable plate counts (LogCFU) with the calculated concentration from the absorbance measurements using equation 1 (Calculated LogCFU).

	Lofric Primo		**Magic3 Anti.+ Hydro.**		**Magic3 Anti.**	
	Log (CFU)	Calculated log(CFU)	Log (CFU)	Calculated log(CFU)	Log (CFU)	Calculated log(CFU)
Sample 1 (0h)	1,30		0		1,30	
Sample 2 (4h)	1,95	2,05	3,37	3,41	2,78	2,63
Sample 3 (8h)	4,34	4,90	4,05	6,62	4,78	6,37
Sample 4 (12h)	6,75	7,83	6,85	8,88	7,36	8,77
Sample 5 (22h)	8,30	8,82	8,46	8,98	8,51	8,98

In figure 4.10 no distinct shift in absorbance for Sample 1 (0h) is observed. In table 4 the calculated logCFU for Sample 2 (4h) are in the same logarithmic level as for the viable plate counts. For Sample 3 – 5 the calculated logCFU overestimate the concentration of bacteria with 0.4 – 2 logCFU compared to viable plate counts.

In figure 4.10 absorbance measurements of resazurin reduction are shown for the experiments where bacteria were introduced in the first catheterization, one experiment per catheter is shown.

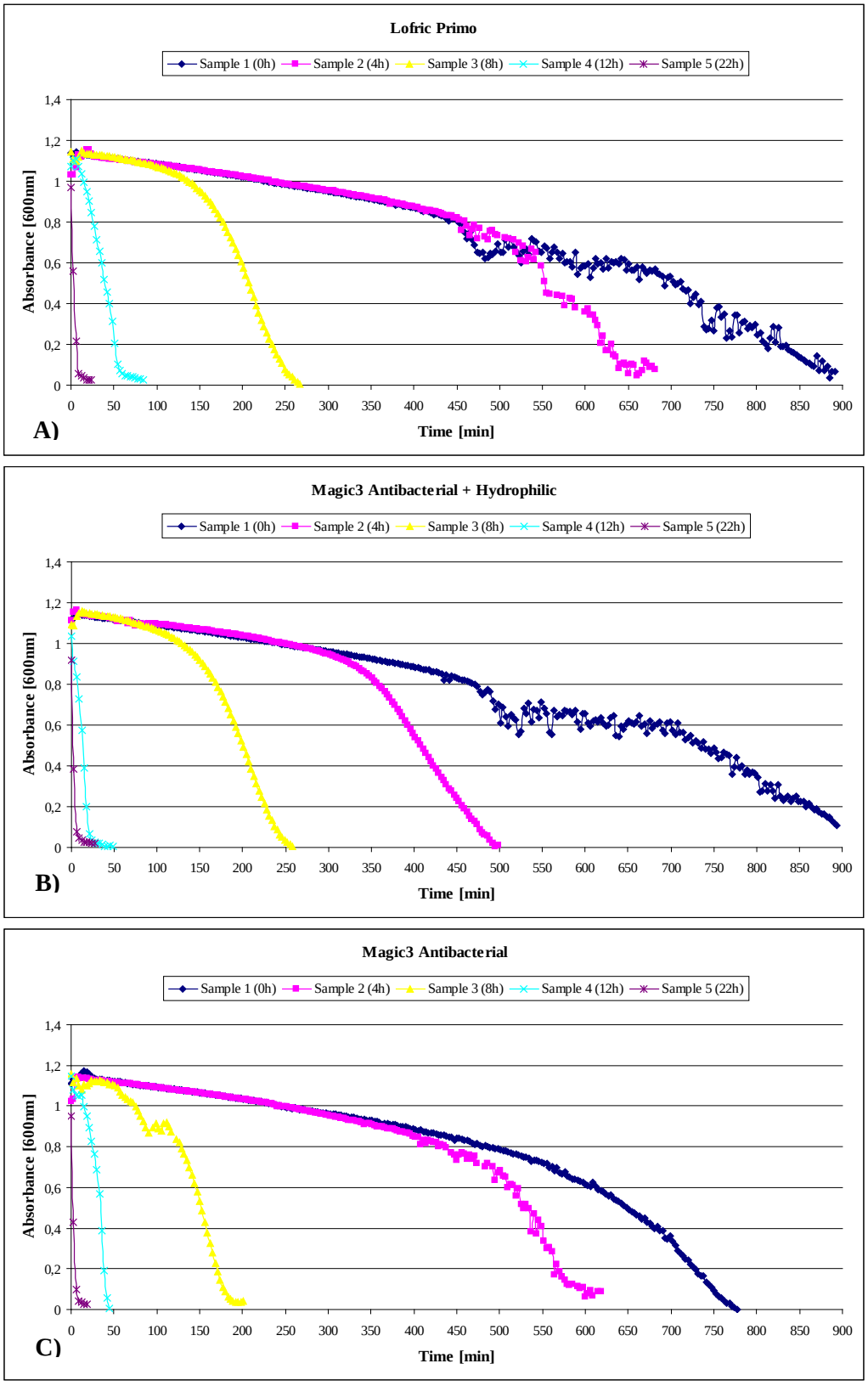


Figure 4.11 The plot shows the absorbance at 600 nm as a function of time for each sample. The bladder was catheterized with Lofric Primo in chart A, with Magic[3] Antibacterial + Hydrophilic in chart B and with Magic[3] Antibacterial in chart C. The shift in absorbance from 1.2 to 0.1 is the result of resazurin reduction. The plot is an average of 3 measurements.

Table 5. Comparing the estimated concentration from viable plate counts (LogCFU) with the calculated concentration from the absorbance measurements using equation 1 (Calculated LogCFU).

	Lofric Primo		**Magic3 Anti.+ Hyd.**		**Magic3 Anti.**	
	Log (CFU)	Calculated log(CFU)	Log (CFU)	Calculated log(CFU)	Log (CFU)	Calculated log(CFU)
Sample 1(0h)	1,30		0		1,78	
Sample 2(4h)	1,78	0,80	2,23	3,15	2,36	1,51
Sample 3(8h)	3,81	6	4,14	6,2	5,52	6,87
Sample 4(12h)	6,92	8,42	7,03	8,89	7,15	8,56
Sample 5(22h)	8,46	8,92	8,48	9,08	8,75	9,05

In figure 4.11 the absorbance measurements for Sample 1 (0h) shows a steady decrease of OD with time. No distinct shift is observed and the calculated concentration is therefore excluded from table 4 and 5. Comparing the concentration for calculated and viable plate counts for Sample 2 (4h) shows difference in the range of 0.7 – 1 logCFU. For sample 3 – 5 an overestimation of the calculated concentration is observed. It is 0.4 – 2.3 logCFU greater than the concentration obtained from viable plate counts.

5 Discussion

Despite the fact that commercially available antibacterial intermittent catheters carry the risk of introducing bacteria at each catheterization, there has not been a method to evaluate the efficiency of the catheters. In this study a novel method was developed to study the antibacterial effect of intermittently used catheters impregnated with nitrofurazone.

5.1 Catheter analysis

The differences in colony forming unit (CFU) counts in figure 4.1 and 4.2 might reflect the inability of antimicrobial treated catheters to inhibit bacterial growth in the bladder. The lack of noticeable difference in CFU counts during the 22 hours of model operation for the catheters with nitrofurazone impregnation might have resulted from the dilution of the released antimicrobial agents by the flowing urine in the bladder. Results presented in figure 4.1 shows experiments where bacteria were introduced at each catheterization and significant bacteriuria ($>10^2$ CFU/ml) were observed after 4 hours, except for Lofric Primo which had a concentration of 90 CFU/ml. The introduction of bacteria at each catheterization makes the comparison between the catheters complicated. Even if the catheters are contaminated with the same concentration of bacteria, the same amount might not be introduced into the bladder. Different materials and surface technologies are used in the catheters which may affect the attachment of bacteria to it during the contamination step. Thus, the antimicrobial effect of nitrofurazone impregnated catheters cannot be excluded during the first 12 hours.

Experiments in which bacteria were introduced only in the first catheterization did not show any significant difference in inhibition of bacteria growth. The introduced amount of bacteria might be higher, than the antibacterial effect of nitrofurazone. It can also be that the diffusion rate of antimicrobial agents on the catheter are to low and are not released into the urine during the 90 seconds of catheterization. The experiments were also characterized by a large dispersion of results, which is shown in figure 4.4 – 4.6. Since the amount of bacteria introduced into the bladder differs from each time, and it is not certain whether antibacterial catheters have any effect on inhibiting bacterial growth in the bladder, such a dispersion of results may be expected. This may also depend on how fast the catheter is pulled in the contamination step from artificial urine with 10^6 CFU/ml *E.coli*. Release of antimicrobial agents or bacteria attachment to the catheter may vary in this step, thus influence the final result.

In figure 4.3 the observed layer on the bladder wall drained with Magic^3 Antibacterial + Hydrophilic catheter may have resulted from free additives incorporated into the

surface. These were released in the bladder and in combination with bacteria growth it aggregated and resulted in a layer. The same outcome was observed with Lofric Primo, but not with Magic^3 Antibacterial. This is a limitation in the model, since most of the lubricous hydrophilic coating on intermittent catheters is released in the urethra and not in the bladder.

Kirby – Bauer test showed that Magic^3 catheters had an inhibiting effect against *E.coli* compared to Lofric Primo. The catheters were incubated for 24 hours, which gave the antibacterial agents more time to diffuse from the surface coating and inhibit bacteria growth. It is also noteworthy to mention that the inhibition zone does not necessarily indicate that bacteria have been killed by nitrofurazone; it may only have been prevented from growth.

5.2 Absorbance measurements

The even distance between the curves in figure 4.8 indicates that the serial dilution was successfully performed. The calibration curve was done with cells in stationary phase, whereas cells in the residual urine were in exponential phase. The correlation between bacterial numbers in stationary phase and the time required to reach OD 0.4 was used to estimate the concentration of samples from the bladder. OD – value 0.4 was chosen as the threshold because cells are in the logarithmic growth phase at that point. The bacterial estimation was less accurate because cells were in different physiological state. Since cells in the calibration curve are in the stationary phase, the resazurin reduction might take longer time compared to samples from the in vitro bladder model due to adaption to the new environment. This will then contribute to an overestimation of the bacterial concentration when performing the analysis.

Concentrations obtained from the spectrophotometric method and viable plate count did not always correlate (table 5 and 6). The micro titer plate with the samples was at 37 °C uncovered during the absorbance measurement which lead to evaporation of the samples over time. This trend can be seen in Sample 1 (0h) where the measurement takes over 10 hours with an increase in noise over time. Compared to controls (data not shown), resazurin reduction in Sample 1 (0h) shows spontaneous reduction. Measurements that last for 9 – 10 hours are therefore not time-efficient compared to CFU counts, since samples are analyzed the day after the measurements.

The overestimation of bacteria counts by the spectrophotometric method can also be due to the different mediums that were used in the measurements. Cells in the calibration curve were in LB-broth compared to cells from the bladder that was measured in synthetic urine. Overnight cultures to the calibration curve were done with LB-broth, since it is a more nutritionally rich and stable medium than synthetic urine. But differences in osmolality and pH between the mediums may have affected the growth rate causing differences in metabolic activity. This might affect the comparative analysis of the curves, making them less accurate. The synthetic urine contained different forms of salts, and it has been shown that the metabolic activity of *E.coli* cells is affected when the cells are exposed to salt stress [61]. Further studies have to be

performed to investigate if the salt concentration is high enough to affect the metabolic activity and induce salt stress.

The plate counting method might not be completely accurate, since plating and dilution are variables that can be affected by the human factor. The variability in plate counts could have been studied by performing duplicates or triplicates of the plates. Even if the deviation between replicates in this method are not expected to be big, possible confounding factors cannot be excluded.

5.3 Clinical validity of the in vitro model

It is very difficult to predict the clinical validity of the model before the results obtained in the model are tested clinically. Such a comparison will determine how valuable the lower urinary tract model is in therapeutic terms. Ideally the in vitro bladder model should give information faster and at less cost than clinical studies, and provide data on the potential value of antibacterial agents on intermittent catheters. From the experiments it can be concluded that the nitrofurazone dose is too low to inhibit the growth of bacteria, which should be further studied. More experiments have to be performed with lower bacteria concentration to statistically confirm the positive effect of antibacterial catheters.

Artificial urine used in the experiments was not stable in 37 °C after 3 hours model operation and formation of different precipitates could be observed. The effect of antibacterial agents may have been affected by this, since bacterial cells and the precipitate may have aggregated. The precipitate formation can be diminished by decreasing the temperature to 25 °C. But this will negatively affect the bacterial growth rate and not reflect the human body temperature. Alternatively, the artificial urine can be substituted to other developed mediums suitable for replacement of normal urine. In a study published by Brooks *et al* [62], artificial urine was developed which showed good conditions for the growth of many urinary pathogens and experiments were conducted at 37 °C with reproducible results. The results provide an alternative for the currently used artificial urine.

5.4 Future work

Since no inhibition of bacteria growth could be observed it would be of great importance to study the effect of the catheters by contaminating them in lower bacteria concentrations before catheterization. These experiments will give a deeper insight into the antibacterial effect of the catheter and prove or disprove the effect suggested by the manufacturer. Additionally, an improvement of the currently used urethra model is necessary. In the experiments the silicone tube mimicking the urethra was not sufficiently tight when passing a catheter through to resemble a human urethra. The new urethra model should also have a bend according to the anatomical structure of the

lower urinary tract. Furthermore, it would be of interest to study the bacterial colonization in the urethra.

The calibration curve could in the future be performed with cells in the exponential phase and measured in synthetic urine. This might give a better comparative analysis.

Instead of introducing bacteria in the bladder with a catheter, the bladder could be contaminated with a known concentration bacteria and then drained with catheters in intervals. This will give a model that is more controlled and make the comparison of the catheters equitable.

6 Conclusion

A novel method to study urinary catheters was developed in this thesis. The purpose of this study was to develop an experimental model of the lower urinary tract and a novel quantitative method to analyze bacterial concentration in residual urine using the fluorescent dye resazurin.

The results obtained from the experimental urinary model suggest that antibacterial catheters have no inhibition of bacterial growth compared to the control. The introduced bacteria number is too high for antibacterial catheters to have an effect and additional experiments with lower concentration bacteria can maybe provide more information about the antibacterial effect. Kirby – Bauer tests with antibacterial catheters showed inhibition of bacteria growth on agar plate, but the diffusion time for antibacterial agents was 24h compared to 90 seconds in the in vitro bladder model.

The quantitative method to analyze the bacterial concentration in the residual urine overestimated the concentration compared to plate count method, probably due to a biased calibration curve. The studied method can be applied for bacterial concentration estimation in various applications, and is not limited to determination of bacterial concentration in residual urine.

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