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EVALUATION OF HB&L UROQUATTRO AUTOMATED SYSTEM FOR RAPID DIAGNOSIS OF URINARY TRACT INFECTIONS

DISSERTATION SUMMARY

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The dissertant is a full-time PhD student at the Department of Microbiology and Virology of MU - Varna and a doctor - microbiologist at SMDL Laborexpress 2000 - Varna.
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The defense materials are published on the website of Medical University "Professor Dr. Paraskev Stoyanov"- Varna and are available in the Scientific Department of Medical University - Varna.

CONTENTS

ABBREVIATIONS

AST – antibiotic susceptibility test

CAUTI - Catheter Associated Urinary Tract Infections

CFU – colony forming units

DDST – double - disk synergy test

ESBL – extended – spectrum beta – lactamase

FISH – Fluorecsent in situ hybridisation

GHLAR – gentamycin high level aminoglycoside resistance

HAI – Hospital Acquired Infections

ICU – intensive care unit

ME – major error

mE – minor error

MALDI-TOF MS - Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry

MRSA – methicillin – resistant *Staphylococcus aureus*

MSSA – methicillin – sensitive *Staphylococcus aureus*

NGS – Next Generation Sequencing

NPV – negative predictive value

PCR – Polymerase chain reaction

PPV – positive predictive value

RAA – residual antibiotic activity

TGS – Third generation sequencing

VRE – vancomycin- resistant enterococcus

VME – very mjor error

1. INTRODUCTION

Urinary tract infections (UTIs) affect a large group of patients and can be considered a socially significant disease. They have become a model for studying the pathophysiology of infections, such as host-pathogen interactions and the evolutionary mechanisms of infection, as well as for the development of new antibiotic drugs (*Öztürk R, 2020*).

As an important health issue at all stages of life, from newborns to elderly patients, their prevalence changes depending on gender and age (*Foxman B, 2003*). Various predisposing factors include congenital anomalies, diabetes, surgeries, neurological disorders, and pregnancy (*Flores-Mireles AL, 2015*). Their clinical presentation spans a wide range, from simple cystitis to life-threatening urosepsis and septic shock (Wagenlehner F, 2015). The collection of epidemiological data provides a basis for rational decisions on initiating treatment and ensuring prevention and control of these infections (*Tandogdu Z, 2016*).

In addition to frequently used classifications for urinary infections based on recommendations from urological and nephrological guidelines, those according to the site of infection, episodes, symptoms, and complicating factors are also often used in daily practice (*Kranz J, 2024*). The involvement of the urinary tract can also result from another inflammatory process and significantly complicate their diagnosis and treatment (*Pietropaolo A*, *2023*).

Therapy for uncomplicated urinary infections is usually empirical—based on a working algorithm from anamnesis data and supporting laboratory results (*Medina M, 2019; Sabih A, 2023*). In many countries, culture testing is not performed for patients with uncomplicated urinary infections, except in cases of pregnancy and recurrences with a high risk of complications (*Lawati H, 2024*).

Most first-line screening methods do not determine the type of uropathogen and its resistance profile. From an epidemiological standpoint, accurate monitoring of the etiological structure and antimicrobial resistance is not feasible. These data are essential for

treating patients with severe urinary infections or at risk of developing sepsis (*Rousham E, 2019*).

Standard urine culture is the "gold standard" in the diagnosis of urinary tract infections. It is based on long-standing knowledge of the etiology and resistance of the most common pathogens and carries the main disadvantage of microbiological testing the need for time to provide an answer. Standard urine culture is indispensable for hospital and complicated urinary tract infections such as those refractory to initial treatment, pyelonephritis, atypical organisms, etc. (*Xu R, 2021).* However, as medical technologies continue to advance, newer diagnostic platforms are increasingly entering routine practice (*Shallcross L, 2020*). The use of automated microbiological urine screening shortens the duration of diagnosis to within hours. Rapid determination of antimicrobial sensitivity is perhaps even more critical for achieving the goal of optimizing appropriate antibiotic therapy (*Roveta S, 2004; Van T, 2017*). New diagnostic platforms, including nucleic acid tests (multiplex PCR, FISH) and mass spectrometry (MALDI-TOF), improve the speed and accuracy of identification to prevent the development of antimicrobial resistance (*Davenport M, 2017; Kaur R, 2020*).

Successful implementation and application of these new technologies have the potential to initiate the era of precision medicine and improve patient care and public health.

2. GOAL AND OBJECTIVES

The goal of this dissertation is to evaluate the automated HB&L Uroquattro system for rapid diagnosis of urinary tract infections.

In this regard, we set the following objectives:

1. To study the etiological spectrum of urinary tract infections in a group of 842 outpatients with symptoms of urinary tract infections.
2. To compare the results of rapid detection of bacterial growth in urine samples obtained with the automated HB&L Uroquattro system and those obtained with the classical culture method, calculating the following indicators: sensitivity, specificity, positive predictive value, negative predictive value, and accuracy.
3. To study the sensitivity to a set of antimicrobial drugs using the Kirby-Bauer disk-diffusion method performed with isolates in pure culture.
4. To study the sensitivity to a set of antimicrobial drugs using the automated HB&L Uroquattro system by working directly with positive urine samples.
5. To study the sensitivity to a set of antimicrobial drugs using the automated VITEK 2 system (bioMérieux, Marcy l'Etoile, France) by working directly with positive urine samples.
6. To determine the "Residual Antibiotic Activity" index in each urine sample and to evaluate the contribution of this test to the correct interpretation of the results obtained from the culture study

3. MATERIALS AND METHODS

3.1. STUDY DESIGN

This study is a prospective research design aiming to evaluate an automated method for rapid diagnosis of UTIs (detection of microbial growth, determination of residual antibiotic activity, testing of sensitivity to antimicrobial drugs) in a group of outpatients over a 7-month period (October 2020 - April 2021) and to compare this method with established microbiological techniques for diagnosing these types of infections. The study included patients with data on UTIs referred by a clinical physician, with symptoms of uroinfection, regardless of their age, gender, and diagnosis. To reflect the data for each patient, a survey was developed, filled out by the patient or their guardian upon sample submission (see appendix 1 of the dissertation). Initial selection included patients with positive findings from the general chemical analysis of urine (nitrites, leukocytes, protein, bacteria in sediment). Laboratory and equipment information systems (HB&L; VITEK 2 Compact) were used to store and analyze the necessary information during the study period.

Criteria for collecting urine samples for the study:

- All urine samples meeting the following conditions were included in the study: first morning urine, collected using the "mid-stream catch" method, after performing hygiene, where patients were instructed to provide the middle portion of urine in a sterile container without touching the inside or the tip of the container. For pediatric patients, pediatric sterile urine collectors were used, with parents instructed to transfer the collector directly into the sterile container without pouring the material. For patients with a urinary catheter, samples were taken from the catheter port after appropriate cleaning (Holroyd S, 2019). For patients with nephrostomy: urine was taken from the connection between the collecting bag and the nephrostomy tube while adhering to antiseptic rules (Yoo M, 2021). All urine samples were transported and processed within 2 hours of sampling.

- Urine samples that could not be delivered to the laboratory within 2 hours of collection were stored in a refrigerator for up to 24 hours or sampled in a container with boric acid preservative.
- Only urine samples that showed bacterial growth > 800 CFU/ml and detected a turbidity level of 0.5 using a McFarland monitor within 4 hours during rapid screening with the automated HB&L Uroquattro system were included in the study.

Criteria for excluding urine samples from the study:

- Urine samples not meeting the above criteria: urine not being first morning, hygiene not performed, sample poured or not mid-stream.
- Improperly stored and transported samples: samples stored at room temperature, spilled samples, samples stored more than 48 hours in an ordinary container without preservative.
- Patients (excluding children and pregnant women) with probable asymptomatic bacteriuria: positive bacteriuria > 10^4 CFU/ml without clinical symptoms and data for uroinfection.
- Turbid samples: urine samples showing initial results greater than 20,000 scattering units at the 30° sensor or 8,000 at the 90° sensor during rapid screening with HB&L, marked as "TURBID" by the device. These samples were excluded from the study according to the manufacturer's recommendation due to the presence of components in the sample that hinder bacterial screening.
- Contaminated samples during rapid screening with HB&L: samples identified as "contaminated" according to the manufacturer's recommendations. Growth curves were monitored in real-time, and all showing atypical growth and not reaching the set positive threshold were marked as contaminated. Atypical growth curves were visualized later and had a different course compared to positive samples, with growth curve recording starting 45 minutes after the tested urines were introduced. Contaminating microflora showed a longer lag phase of 4-5 hours incubation before adapting to new conditions. Pathogenic microorganisms were determined by short replication time, short lag

phase, and rapidly increasing exponential log phase. After determining samples as contaminated, they were excluded from the study.

Studied patients

Prospectively, from October 2020 to April 2021, a total of 1600 urine samples from 842 outpatients with suspected uroinfection were studied. The study included patients with clinical and ultrasound data for acute uroinfection referred by specialists, pregnant women with clinical signs of cystitis and probable bacteriuria, children with congenital urinary system anomalies, dispensary patients with chronic urinary system diseases, patients after kidney transplantation subject to periodic urine control for bacterial growth, patients from nursing homes and hospices, patients with permanent catheters and nephrostomies. All urine samples underwent initial microbial growth screening using the automated HB&L Uroquattro system (screening time of 4 hours) and parallel culture testing for microbial isolation. Persons outside the defined groups or those who refused testing were not included in the study. The study was approved by the Ethics Committee for Scientific Research with protocol No 92/02.04.2020.

Definitions: Confirmed uroinfection: a patient with clinical data for uroinfection, positive rapid screening > 800 CFU/ml, significant bacteriuria according to established criteria, with microbiologically proven uropathogen(s).

3.2. MICROBIOLOGICAL METHODS

3.2.1. SCREENING OF URINE SAMPLES FOR MICROBIAL GROWTH USING HB&L UROQUATTRO DEVICE

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- Microbial identification directly from urine sample determined as positive by the HB&L screening system

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- Determining sensitivity to antimicrobial drugs using the Kirby-Bauer disk-diffusion method
- Phenotypic method for detecting extended-spectrum beta-lactamases (ESBLs)
- Determining sensitivity to antimicrobial drugs using the automated VITEK 2 Compact system
- Determining sensitivity to antimicrobial drugs directly from a positive urine sample after screening using HB&L Uroquattro AST
- Reading and interpretation of results

3.3. DETERMINATION OF RESIDUAL (REMAINING) ANTIBIOTIC ACTIVITY IN URINE SAMPLES USING HB&L DEVICE

3.4. STATISTICAL METHODS

- Correlation analysis
- Predictive analysis

4. RESULTS AND DISCUSSION

4.1. ETIOLOGICAL SPECTRUM OF URINARY TRACT INFECTIONS.

During the studied 2-year period (2020 – 2021), a total of 1600 urine samples obtained from 842 outpatients with symptoms of urinary tract infections (dysuria, polakiuria, fever, presence of leukocytes/nitrites in the general chemical analysis of the urine sample) were examined using rapid HB&L screening and the cultural method. Through the classical culture method, a total of 352 clinically significant, non-repetitive microbial isolates were isolated and identified from 352 patients (41.8%). The main demographic indicators and other characteristics of the studied 352 patients related to urinary tract infections are presented in Table 1.

Table 1. *Characteristics of 352 outpatients with urinary tract infections*

Variables	Total patients n=352 n (%)
Women	239 (67.9)
Men	113 (32.1)
Age 65+	61 (17.3)
Presence of underlying disease/condition	
Kidney transplantation performed	3 (0.8)
Presence of urinary catheter	7 (1.9)
Nephrostomy	4 (1.1)
Pregnancy	43 (12.2)
Antibiotic treatment during the study	113 (32.1)

The average age in the entire group of studied patients (regardless of gender) is 41 years, with 50% of the patients being aged ≤38 years. The highest relative share of patients is in the age range of 20 - 30 years (Figure 1). The average age of female patients is 35 years, with 50% of them being ≤29 years old. The highest relative share of patients is in the age range of 20 - 30 years. The average age of male patients is 55 years, with 50% of them being ≤55 years old. The highest relative share of patients is in the age range of 40 - 50 years.

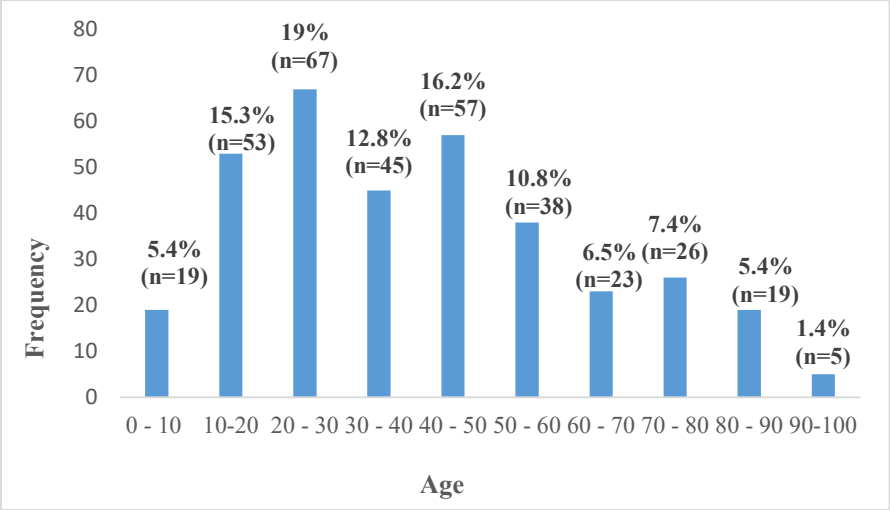

Figure 1. p*resents summarized data on the age distribution of all 352 patients included in the study.*

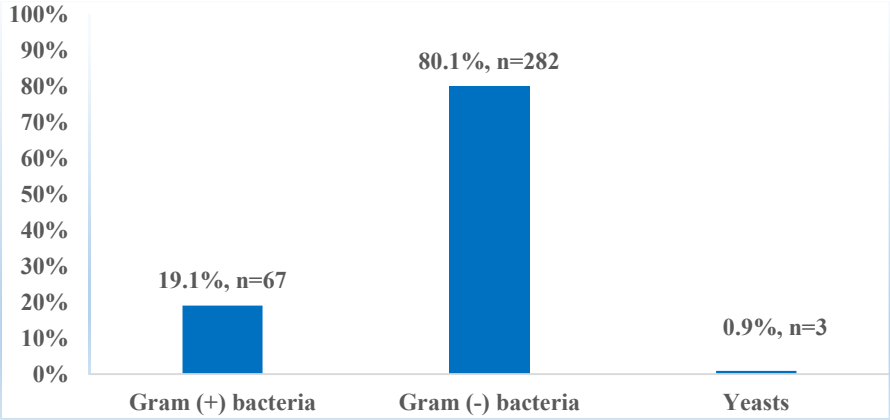

Figure 1. *Age distribution of 352 outpatients with urinary tract infections during the study period.*

In the etiological spectrum of documented urinary tract infections, Gram-negative bacterial species predominate, represented with 80.1% ($p>0.05$). Figure 2 shows summarized data on the causative agents of these infections and their relative share, while the detailed etiological spectrum is presented in Table 2.

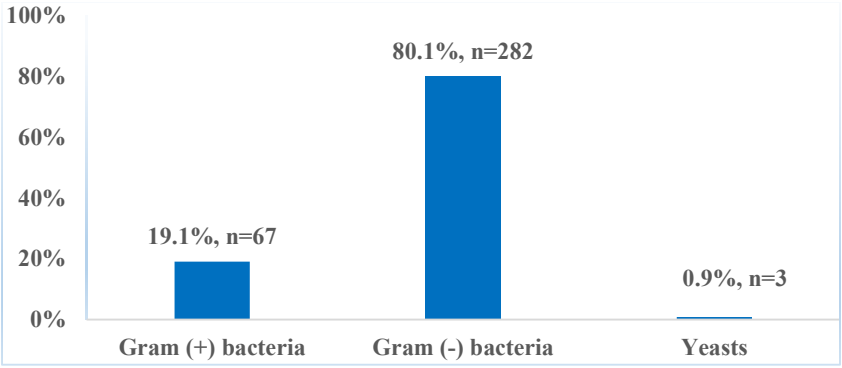

Figure 2. *Spectrum of causative agents of urinary tract infections documented in 352 outpatients in the period 2020-2021 (in number and %).*

Table 2. *Etiological structure of urinary tract infections among 352 outpatients in the period 2020-2021.*

Etiology	Total (female, male) n (%)	Female n (%)	Male n (%)
Общо	352 (100.0)	239 (67.9)	113 (32.1)
Gram negative bacteria	282 (80.1)	186 (66.0)	96 (34.0)
Escherichia coli	200 (56.8)	139 (69.5)	61 (30.5)
Klebsiella pneumoniae	40 (11.4)	25 (62.5)	15 (37.5)
Proteus mirabilis	26 (7.4)	15 (57.7)	11 (42.3)
Citrobacter complex	5 (1.4)	2 (40.0)	3 (60.0)
Enterobacter cloacae complex	5 (1.4)	3 (60.0)	2 (40.0)
Pseudomonas aeruginosa	4 (1.1)	0 (0.0)	4 (100)
Serratia marcescens	1 (0.3)	1 (100)	0 (0.0)
Morganella morganii	1 (0.3)	1 (100)	0 (0.0)
Gram positive bacteria	67 (19.1)	51 (76.1)	16 (23.9)
Enterococcus faecalis	44 (12.5)	30 (68.2)	14 (31.8)
Staphylococcus saprophyticus	20 (5.7)	19 (95.0)	1 (5.0)
Streptococcus agalactiae	2 (0.6)	2 (100)	0 (0.0)
Aerococcus urinae	1 (0.3)	1 (100)	0 (0.0)
Yeasts	3 (0.85)	2 (66.7)	1 (33.3)
Candida spp.	3 (0.85)	2 (66.7)	1 (33.3)

No statistically significant difference was found in the species distribution of uropathogens between the male and female groups (p>0.05). The etiological distribution by gender is presented in Table 3.

Table 3. *Etiological structure of urinary tract infections among 352 outpatients in the period 2020-2021, presented by gender.*

Etiology	female n (%)	male n (%)	*p*
Total clinically significant microorganisms isolated from urine cultures	**239 (100)**	**113 (100)**	**0.109**
Gram negative bacteria	**186 (77.8)**	**96 (84.9)**	
Escherichia coli	139 (58.2)	61 (54.0)	
Klebsiella pneumoniae	25 (10.5)	15 (13.3)	
Proteus mirabilis	15 (6.3)	11 (9.7)	
Citrobacter complex	2 (0.8)	3 (2.65)	
Enterobacter cloacae complex	3 (1.2)	2 (1.76)	
Pseudomonas aeruginosa	0 (0.0)	4 (3.5)	
Serratia marcescens	1 (0.4)	0 (0.0)	
Morganella morganii	1 (0.4)	0 (0.0)	
Gram positive bacteria	**51 (21.3)**	**16 (14.1)**	
Enterococcus faecalis	30 (12.5)	14 (12.4)	
Staphylococcus saprophyticus	19 (7.9)	1 (0.8)	
Streptococcus agalactiae	2 (0.8)	0 (0.0)	
Aerococcus urinae	0 (0.0)	1 (0.8)	
Yeasts	**2 (0.8)**	**1 (0.8)**	
Candida spp.	2 (0.8)	1 (0.8)	

The etiological distribution in patients with underlying disease/condition is presented in Table 4.

Table 4. *Etiological structure of urinary tract infections among specific groups of patients (with underlying disease/condition).*

underlying disease/condition	Etiology	n
Patients after kidney transplantation (3)	*K. pneumoniae*	2
	P. aeruginosa	1
Patients with urinary catheter (7)	*E. coli*	3
	E. faecalis	2
	K. pneumoniae	1
	P. aeruginosa	1
Patients with nephrostomy (4)	*E. cloacae*	2
	K. pneumoniae	1
	E. coli	1
Pregnancy (43)	*E. coli*	26
	E. faecalis	8
	S. saprophyticus	5
	K. pneumoniae	2
	S. agalactiae	1
	P. mirabilis	1

Discussion

During the studied two-year period (2020 - 2021), the most frequent and dominant causative agents of urinary tract infections in outpatients were Gram-negative bacteria, proven in 80.1% of the cases. The leading bacterial species were *E. coli* (56.8%), *K. pneumoniae* (11.4%), and *P. mirabilis* (7.4%), with a ratio of Gram-negative to Gram-positive bacteria of 80.1%: 19.9%. Isolates representing the genera *Enterobacter* spp., *Citrobacte*r spp., and *P. aeruginosa* were significantly less frequently proven. Similar results have been reported in numerous other studies conducted in both outpatient and inpatient settings, all confirming the greater relative share of Gram-negative bacteria, primarily representatives of the order Enterobacterales, with the leading uropathogen being *E. coli* (*Davenport M, 2017; Bono M, 2023*). *P. mirabilis, K. pneumoniae*, and *Enterobacter* spp. are among the other relatively frequently proven etiological agents of UTIs, with *K. pneumoniae* being the second most common etiological agent of

community-acquired urinary infections (*Davenport M, 2017; Caneiras C, 2019*), which is also demonstrated in this study. The very high relative share of *E. coli* (58.6%) in the present study confirms its leading role as a uropathogen among the outpatient population. Similar studies in Europe, China, and the USA have found that over 75% of outpatient and hospitalized patients diagnosed with urinary tract infections are associated with *E. coli* (*Flores-Mireles A, 2015; Medina M, 2019; Zhou Y, 2023).*

Among Gram-negative bacteria, a low relative share of *P. aeruginosa* isolates (1.1%) was found, which can be explained by the inclusion of exclusively outpatients in the study, respectively those with community-acquired infections. Zhou's study on the most common uropathogens of community-acquired infections found that this bacterial species causes approximately 1% of uncomplicated and 2% of complicated urinary infections (*Zhou Y, 2023*). It should be noted that *P. aeruginosa* can also be a colonizing agent of the urinary tract, and its isolation from urine should be considered with caution due to the possibility of it being patients with incompletely treated hospital-acquired urinary infections, with catheters and other underlying conditions (*Laupland K, 2007; Gajdács M, 2020*).

Our results show a significantly lower relative share of Gram-positive uropathogens (19.1%) in the etiological spectrum, with *E. faecalis* (12.5%) and *S. saprophyticus* (5.7%) being predominant. A recent study also confirms Gram-positive bacteria in a much lower relative share in the etiological spectrum of urinary infections in outpatients: 16% versus 84% for Gram-negative pathogens in uncomplicated infections and 23% versus 77% in complicated ones (*Zhou*, *Y, 2023*). Similar to our findings, studies conducted in the USA and Canada demonstrate the leading significance of *E. faecalis* in the group of Gram-positive uropathogens (4.1%) (*Sanchez G, 2016; Codelia-Anjum A, 2023*). A study by Seitz et al. reports results very close to ours for the relative share of *E. faecalis* among outpatients with UTIs (10.2%) (*Seitz M, 2017*). Earlier studies conducted by Laupland found representatives of the genus Enterococcus as etiological agents in 5.3% of outpatients with UTIs (*Laupland K,*

2007). Similar data is reported by Zhou et al., who found these microorganisms in 5% of uncomplicated and 11% of complicated urinary infections (*Zhou Y, 2023*). Other authors report differences in the share of *E. faecalis*-associated UTIs related to gender: a higher relative share in men (8.8%) compared to women (1.8%) (*Codelia-Anjum A, 2023*). Conversely, our results show a higher relative share of *Enterococcus* spp. in females compared to males (8.9% vs. 3.9%).

In this study, after enterococci, representatives of the species *S. saprophyticus* are the second most frequently isolated in the group of Gram-positive bacteria (5.7%). Additionally, this microorganism is among the top 5 most frequently proven microbial species, regardless of their Gram classification, and is identified more often in women, which is confirmed by our results—95% of the strains were isolated from women with UTIs. This fact confirms the leading role of *S. saprophyticus* in the etiological spectrum of UTIs in outpatients, especially in women. Some authors identify *S. saprophyticus* as the second most frequent causative agent of community-acquired urinary tract infections (after *E. coli*) (*Ehlers S, 2023; Lala V, 2023*). Ehlers et al. report that in the group of women aged 16-25 years, *S. saprophyticus* causes up to 42% of all urinary infections, while the relative share in men is significantly lower (*Ehlers S, 2023; Lala V, 2023*). *S. saprophyticus* is also associated with polymicrobial urinary tract infections, more often in immunocompromised patients, the very elderly, diabetics, HIV patients and/or those with malignant diseases, and catheterized patients (*Argemi X, 2019*).

In our study, which included predominantly outpatients, no polymicrobial infections were proven. It should also be noted that in patients with nosocomial urinary infections, the elderly, pregnant women, and patients with urinary catheters, *S. saprophyticus* can also be a colonizing agent (*Ehlers S, 2023; Lala V, 2023*). *S. aureus* is a Gram-positive bacterial species that can also cause urinary infections, but mainly through hematogenous routes. In this study, no *S. aureus* isolates were identified as etiological agents of UTIs. This result correlates with many publications reporting a very low relative share of *S. aureus* in the

etiological spectrum of UTIs (0.2-4%), often affecting catheterized patients, those with urological anomalies and/or procedures, males, the elderly, and those with various comorbidities (*Stokes W, 2019; Mason C, 2022*).

The clinical significance of Group B streptococci (*S. agalactiae*) is not always easy to determine, partly due to the difficulties associated with distinguishing infection from colonization. This consideration includes cases of *S. agalactiae* bacteriuria in non-pregnant adults, where the colonization rate of the urethra can exceed 20% (*Mongilardi N, 2022*). According to some authors, *S. agalactiae* is not a common agent of UTIs, causing approximately 1 to 2% of all monomicrobial urinary tract infections (*Goel N, 2020; Mohanty S, 2021*), which aligns with our result of a 0.6% relative share of *S. agalactiae* in the etiological spectrum of outpatient UTIs. In a recent publication by Zhou et al., *S. agalactiae* is isolated in 3% of uncomplicated and 2% of complicated urinary infections (*Zhou Y, 2023*). However, some authors report *S. agalactiae* as an etiological agent in 39% of patients over 70 years old with UTIs living in nursing homes (*Mohanty S, 2021*). *S. agalactiae*-associated UTIs are also frequently reported in pregnant women, diabetics, and immunocompromised individuals, as well as those with urological anomalies, where there is a higher risk of pyelonephritis with the potential for progression to bacteremia and/or urosepsis (*Collin S, 2019*).

In this study, the bacterial species *Aerococcus urinae* was also identified as an etiological agent but represented by a single isolate (0.3%). *A. urinae* is a Gram-positive microorganism associated with cases of urinary infections in both genders, as well as in young children and elderly patients (*Forsvall A, 2019; Hilt E, 2020*). Several authors also report *A. urinae* as a cause of bacteremia, infectious endocarditis and sepsis, infections affecting muscles and bones, as well as necrotizing soft tissue infections (*Senneby E, 2016; Yabes J, 2018; Forsvall A, 2019; Hilt E, 2020*). Other reports link this microbial species with urinary incontinence and overactive bladder (*Price T, 2016; Hilt E, 2020*).

In the etiological spectrum of the UTIs studied among outpatients, fungi of the genus *Candida* were also found in a low relative share

(0.9%). This result correlates with other scientific reports identifying *Candida* spp. in 1% of cases of uncomplicated urinary infections (*Zhou Y, 2023*). Although yeast-like fungi are rarer etiological agents of UTIs, it should be noted that patients with urinary catheters, diabetes, or recent antibiotic therapy are at increased risk of developing mycotic urinary infection (*Kauffman C, 2011; Dias V, 2020*). A study by Dias et al. confirms *Candida* spp. as a cause of UTIs in cases of polymicrobial infections—alongside bacterial pathogens (*E. coli* and *E. faecalis*), as well as mixed infections caused by two different species of *Candida* spp., with the share of these infections being low—4.3% and 8.3% in outpatients and hospitalized patients, respectively (*Dias V, 2020*).

Urinary tract infections are the most common bacterial infection in kidney transplant recipients, affecting approximately one-third of patients and accounting for 50-75% of post-transplant infectious complications (*Cawcutt K, 2019*). In this group of patients, the risk of urosepsis and transplant rejection is very high (*Strohaeker J, 2021*). According to various authors, the most common uropathogens in the follow-up of such patients are *E. coli*, *E. faecalis*, and *K. pneumoniae* (*Strohaeker J, 2021; Khedr L, 2023*). According to Cawcutt et al., the etiology of urinary infections in transplanted patients is determined by whether it is an immediate postoperative, nosocomial, or community-acquired infection. The most common bacterial uropathogens identified by these authors include representatives of the recipient's endogenous microflora (*E. coli, Proteus spp., Klebsiella* spp.), as well as some predominantly nosocomial pathogens such as *P. aeruginosa* (*Cawcutt K, 2019*). During the two-year study period, we identified three patients with UTIs in the post-transplant period, in which *K. pneumoniae* and *P. aeruginosa* were isolated, correlating and confirming the data from publications concerning this high-risk patient group.

Catheter-associated urinary tract infections account for nearly half of hospital-acquired infections. *E. coli* (relative share 65-75%) is followed by other members of the Enterobacteriaceae family, *Enterococcus* spp., coagulase-negative staphylococci (*S. epidermidis*), *P. aeruginosa*, and other non-glucose-fermenting Gram-negative bacteria and *Candida* spp.

(*Rubi H, 2022; Werneburg G, 2022*). According to a study by Rajaramon et al., in cases of short-term catheterization, the most common bacterial species associated with catheter-related UTIs are *E. coli*, *P. mirabilis*, *K. pneumoniae*, *P. aeruginosa*, *S. epidermidis*, *Enterococcus* spp., and *Candida* spp. (*Rajaramon S, 2023*). Our results are for a very small group of catheterized patients (n=7), but nevertheless, the results we obtained fully correlate with those reported in the scientific literature: domination by Gram-negative microflora, with the leading etiological agent being *E. coli*.

Complications related to the placement of a nephrostomy catheter (nephrostomy) include hematuria, urinary tract infections, including pyelonephritis, kidney abscess, and urosepsis. Nosocomial exposure, invasive urological interventions, and catheterization significantly increase the risk of developing UTIs, especially those caused by multidrug-resistant microorganisms (*Scherberich J, 2021*). According to data from Mondal et al., in patients with nephrostomy, *E. coli* also dominates the etiological spectrum, but other microorganisms such as *K. pneumoniae*, *Enterococcus* spp., *Acinetobacter* spp., *P. aeruginosa*, *MRSA*, *MSSA*, and *Candida albicans* are also frequently associated with urinary infections in these patients (*Mondal U, 2022*). In our studied group of outpatients with UTIs, only three had a nephrostomy, with the identified bacterial isolates being related to Gram-negative microflora from the order *Enterobacterales*, without predominance of a specific species.

Another specific group of patients in this study is the group of pregnant women (12.2%). According to various authors, the etiological agents of UTIs in cases of pregnancy are the same as those in non-pregnant patients, with the most frequently isolated microorganism being *E. coli* (60 - 82.5%) (*Balachandran L, 2022; Habak P, 2024*). According to an 18-year retrospective analysis conducted by Habak et al., *S. agalactiae* is often isolated from urine culture during the third trimester of pregnancy and can have a higher relative share than *E. coli* (*Habak P, 2024*). Similar results are reported in a study by Balachandran et al., where *S. agalactiae* is identified in 32%, *E. coli* in 28%, and

Enterococcus spp. and *Klebsiella* spp. in 15% and 14% of cases in advanced pregnancy, respectively (*Balachandran L, 2022*). In our studied group of pregnant patients, Gram-negative microflora is presented in a higher relative share (67.4%), with *E. coli* (89.6%) being the main representative, along with single isolates of other species from the order *Enterobacterales*. In the group of Gram-positive isolates (34.8%), all more frequently associated with UTIs in women uropathogens are confirmed: *S. agalactiae*, *E. faecalis*, and S. *saprophyticus*. The collected data in this study do not allow for detailed analysis of the duration of pregnancy and the specific type of urinary infection (cystitis or pyelonephritis).

National data for Bulgaria regarding the etiological structure and antibiotic resistance of microbial agents associated with urinary infections for an earlier 10-year period (2004-2014) show the leading causative agents as *E. coli* (53%), *Proteus* spp. (10.6%), *Enterococcus* spp. (9.5%), *Klebsiella* spp. (9.5%), and *P. aeruginosa* (4.9%). In a lower relative share (between 1% and 5%), *Enterobacter* spp., *S. saprophyticus*, *S. aureus*, *Acinetobacter baumannii*, *Serratia* spp., and *Candida* spp. are identified. In this 10-year period, a trend for a decrease in the share of *E. coli* isolates is observed from 55.9% in 2007 to 48.2% in 2012, mainly at the expense of *P. aeruginosa* representatives, followed by a new increase in *E. coli* to 54.4% in 2014 (*http://www.bam-bg.net/*).

According to the same surveillance system, for the period 2018-2021, *E. coli* is isolated from the urine of outpatients in a significantly higher relative share in females (71.1%/2018; 69.4%/2019; 71%/2020; 69.1%/2021) compared to males (51.6%; 50.8%; 53.9%; 57.2%). For the same period, an increase in the share of *Klebsiella* spp. isolates is also confirmed in both genders, in both outpatient and hospital settings, with a slightly higher relative share in males in hospital settings—11-16% compared to 10-12% for females. In outpatient settings, in the group of Gram-positive microorganisms, the largest share is of *Enterococcus* spp. (5% in women and 8-11% in men), while in women in outpatient settings, the highest relative share is of *S. saprophyticus* (1.6%) and *S. agalactiae* (2.4%).

Another specific group of patients in this study is the group of pregnant women (12.2%). According to various authors, the etiological agents of UTIs in cases of pregnancy are the same as those in non-pregnant patients, with the most frequently isolated microorganism being *E. coli* (60 - 82.5%) (*Balachandran L, 2022; Habak P, 2024*). According to an 18-year retrospective analysis conducted by Habak et al., *S. agalactiae* is often isolated from urine culture during the third trimester of pregnancy and can have a higher relative share than *E. coli* (*Habak P, 2024*). Similar results are reported in a study by Balachandran et al., where *S. agalactiae* is identified in 32%, *E. coli* in 28%, and *Enterococcus* spp. and *Klebsiella* spp. in 15% and 14% of cases in advanced pregnancy, respectively (*Balachandran L, 2022*).

In our studied group of pregnant patients, Gram-negative microflora is presented in a higher relative share (67.4%), with *E. coli* (89.6%) being the main representative, along with single isolates of other species from the order Enterobacterales. In the group of Gram-positive isolates (34.8%), all more frequently associated with UTIs in women uropathogens are confirmed: *S. agalactiae*, *E. faecalis*, and *S. saprophyticus*. The collected data in this study do not allow for detailed analysis of the duration of pregnancy and the specific type of urinary infection (cystitis or pyelonephritis).

4.2. COMPARATIVE EVALUATION OF THE ACCURACY OF HB&L UROQUATTRO FOR THE PURPOSES OF DIAGNOSING URINARY TRACT INFECTIONS.

In the present study, the performance of the HB&L Uroquattro tool for the purpose of rapid and accurate diagnosis of urinary tract infections was evaluated by comparison with the gold standard in the microbiological diagnosis of these infections, the culture-based method.

Among the total of 1600 urine samples examined, collected prospectively from 842 outpatients over the seven-month study period, the automated HB&L system identified 343 urine samples as positive (21.4%) and 1257 as negative (78.6%). Using the classical culture method, 1248 samples (78%) were determined as negative, and 352

(22%) as positive. Discrepancies were found in nine urine samples (0.6%) obtained from nine patients. These samples were interpreted as negative by the automatic screening but showed bacterial growth of $<10^3$ CFU/ml on agar media (Table 5).

Table 5. *Discrepancies identified between HB&L Uroquattro automated screening of 1600 urine samples and the classical culture method.*

Bacterial species	Number isolates (n)	Culture	HB&L
Candida spp.	3	$<10^3$ CFU/ml	negative
Enterococcus spp.	2	$<10^3$ CFU/ml	negative
Streptococcus agalactiae	2	$<10^3$ CFU/ml	negative
Aerococcus urinae	1	$<10^3$ CFU/ml	negative
Pseudomonas aeruginosa	1	$<10^3$ CFU/ml	negative

Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Accuracy of the HB&L Uroquattro System

In this study, urine samples identified as positive by the HB&L tool and confirmed as positive by the culture method were defined as "true positive." Similarly, urine samples identified as negative by the HB&L tool and confirmed as negative by the culture method were defined as "true negative." During the study, the HB&L Uroquattro system correctly identified 343 samples as positive (97.4%) (true positive) and 1248 samples as negative (100%) (true negative). The present study established a positive predictive value (PPV) for the rapid automatic screening of 100%, a negative predictive value (NPV) of 99.3%, and an overall accuracy of the method of 99.4%.

Discussion:

According to the literature, more than 60% of urine samples sent for microbiological examination due to suspected urinary tract infection remain sterile (*Conkar S, 2018*).

Therefore, performing an initial screening of the samples for the presence of microorganisms using appropriate methods would reduce the time to obtain results by promptly identifying negative samples without missing those positive for microbial growth (*Davenport M, 2017*). Additionally, using screening methods would reduce the costs of laboratory consumables (*Conkar S, 2018*). Screening systems for detecting bacterial growth based on changes in the optical density of the sample have been used since 1980 (*Hale D, 2017*). These systems use a small amount of urine and a laser detector to track changes in the optical density of the sample over a certain period. Improving the integration and automation of this technology has led to the development of systems capable of detecting bacteriuria in less than an hour (*Davenport M, 2017*). One such system is the HB&L Uroquattro apparatus (Alifax, Italy). It performs rapid testing of urine samples within 3 to 5 hours, allowing for direct cultivation of the analyzed sample. Urine samples with high microbial counts are identified as positive within the first hour of examination, while sterile samples are reported as negative by the system up to the fourth hour.

A key element of any acceptable screening system is high sensitivity, which prevents missing infections, and sufficient specificity, which reduces excessive follow-up tests (*Elizabeth D*, *2020*). This study found 97.4% sensitivity and 100% specificity, PPV and NPV of 100% and 99.3% for the automated HB&L screening method performed on 1600 urine samples collected from 842 outpatients with symptoms of UTIs. These results demonstrate the excellent efficiency of the HB&L device for rapid detection of microbial growth and very good correlation with the results obtained with the classical culture method. Our results are consistent with other studies reporting similar sensitivity, ranging between 93% and 99.8%, and specificity - between 90% and 99.7% (*Ilki A, 2010*; *Montgomery S, 2017*; *Harris M, 2021*; *Lee K, 2022*). We did not find any false-positive results. The device also demonstrated excellent efficiency in predicting negative urine samples (99.3% NPV), which greatly eliminates the need for additional microbiological testing

by the culture method. Similarly, Hassan and Montgomery also report very high NPV by the 4th hour - 98.6% and 98.8% respectively (*Brubaker L, 2017; Hassan F*, *2019*). Hassan's study, conducted in a pediatric population with an identical screening method, found 13% positive results, but the threshold criterion was >/= 105 CFU/ml and a duration of 3 hours. The overall diagnostic accuracy reported by the authors is 84%, 92.1% sensitivity, 83% specificity, and identified discrepancies are 1% (n=4) (*Hassan F, 2019*).

Similar to our study, using a larger version of the device (Alfred 60/AST), Lahanas et al. confirm its very good capability to detect negative samples at a threshold of 800 CFU/ml, reporting an NPV of 97.5%, which results in a 72% reduction in the need for subsequent culture testing. In contrast, the authors report poor performance in identifying true positive samples, with a PPV of 47% (*Lahanas S, 2013*). Other authors report relatively low specificity for HB&L (73%) with a sensitivity of 93% (*Ilki A, 2009*). Lower sensitivity than found by us is reported by Cermák et al. (83%), with a specificity of 95%, PPV of 85%, NPV of 93%, and accuracy of 90.7% (*Cermák P*, *2009*). Microbial growth was not proven in about 17% of samples that had a microbial count of ≤ 104 CFU/ml, while 74% of samples with ≥ 105 CFU/ml were identified within three hours (*Cermák P, 2009*). The authors also note that extending the screening time improves the studied indicators (*Cermák P, 2009*).

In our study, nine samples (0.6%) were identified as false negative: they were identified as negative by the automatic screening but demonstrated bacterial growth with a microbial count of < 103 CFU/ml (*Candida* spp., n=3; *Enterococcus* spp., n=2; *Streptococcus agalactiae*, n=2; *Aerococcus urinae*, n=1; and *Pseudomonas aeruginosa*, n=1). This is a result that needs to be considered, as the number of microorganisms detected in UTIs caused by certain bacterial species or slow-growing microorganisms (*Actinotignum schaalii*, *Corynebacterium urealyticum*, *Aerococcus* spp., *Ureaplasma*, *Candida* spp., etc.), as found in this study, is often lower (100 - 100,000) compared to classical UTI pathogens

(*Whiteside S*, *2015*; *Brubaker L*, *2017*). The short working protocol used by the system (3 – 4 hours) may not be sufficient for their detection. Most discrepancies were found for *Candida* spp. isolates, for which HB&L did not yield a positive result by the end of screening. However, standard culture testing isolated *Candida* spp. without the presence of other bacterial species. In a recent study by Athamna, combining automatic screening performed with Alfred 60 (a larger version of HB&L) and MALDI MS, the lowest concordance (84.6%) was shown for *Candida* spp., indicating that candiduria is difficult to adequately identify both by the standard method and by newer identification methods.

In this study, one isolate of *Aerococcus urinae* was identified. During the set time for the automated screening, the sample was indicated as negative (false negative). This is a bacterial species that is usually isolated in culture testing in quantities of $< 10^3$ CFU/ml. The true frequency of UTIs caused by *A. urinae* is likely greatly underestimated, as it can often be misidentified as *Staphylococcus*, *Streptococcus*, or *Enterococcus*. A rare etiological agent of UTIs, *A. urinae* is typically found in elderly patients with multiple comorbidities, with a relative share of 0.15 - 0.8% (*Balouch B*, *2022*). Infections with *A. urinae* are likely to become more common due to advances in laboratory technologies and the aging population.

It should be emphasized that the discrepancies we identified do not pertain to the most common causative agents of urinary infections. The standard media on which urine samples are typically cultured are optimal for isolating microorganisms that most frequently cause UTIs, namely *E. coli*, S. *saprophyticus*, *K. pneumoniae*, and *P. mirabilis*. Most screening methods perform well in diagnosing *E. coli*-associated UTIs (80 - 85% of all urinary tract infections). Hooton et al. demonstrate that the PPV of conventional urine culture is 93% and 99% for *E. coli* with a microbial count of 10^2 CFU/ml and 10^4 CFU/ml, but for *Enterococcus* spp. it is between 10% and 33% and between 8% and 14% for *Streptococcus group B* (*Hooton T, 2013*).

Other potential sources of errors in automated screening are catheter urine samples, which often contain more than one bacterial species, which can be misinterpreted as contamination. Furthermore, in some specific patient groups (children under 3 years old, pregnant women, patients post-kidney transplant, urological surgery, catheterization), the lower number of microorganisms ($< 10^5$) in the urine sample can be clinically significant, and this should be considered to avoid misinterpreting the samples as negative during automated screening (*Roberts A, 2017*). In this study, a cut-off of 10^3 CFU/ml was set due to our chosen maximum screening period of 4 hours using HB&L. A disadvantage of standard culture testing is the differences in microbial counts that different organizations consider significant and clinically relevant (*Lee K*, *2022*). According to CDC recommendations, the diagnosis of UTI includes symptoms of urinary infection and microbial growth > 10^5 CFU/ml. The threshold for UTI in a catheterized sample is set at 103 CFU/ml (*Xu R*, *2021*). Although historically a microbial count of > 10^5 CFU/ml has been accepted for significant bacteriuria, there is increasing doubt that lower values should also be considered (*Werneburg G*, *2023*). Outside of catheter urine, thresholds of 10^3 CFU/ml may fail to detect some sexually transmitted infections (*Tomas M*, *2015*). Other authors report that in 20 - 40% of women with symptomatic urinary tract infections, the microbial count is in the range of 10^2 - 10^4 CFU/ml (*Xu R*, *2021*), which is why a threshold of 10^2 CFU/ml is proposed for patients with dysuric conditions (*Desforges J*, *1993*; *Xu R*, *2021*).

In another comparative study similar to ours, a significantly higher relative share of discordant results - 6.9% (*Ilki A*, *2009*) was proven. To reduce these discrepancies, a preliminary assessment of urine samples for the presence of leukocytes and/or microorganisms is recommended. The authors found that only 0.6% of the 481 urine samples, in which microorganisms and leukocytes were not initially detected, showed positive growth (*Ilki A*, *2009*).

Results similar to ours were reported by Sharma et al. on 1220 urine samples screened simultaneously with HB&L and examined by the

culture method, where 2.1% of the samples showed discrepancies, which the automated system identified as false negatives. According to the same authors, at 102 - 103 CFU/ml, HB&L has a high NPV (96.6%) and sensitivity of 92.7% (*Sharma B*, *2023*).

In the present study, using the culture method as the gold standard in microbiological examination to evaluate the automated screening system, a 22% relative share of positive urine cultures was proven from a total of 1600 urine samples, collected prospectively from 842 outpatients. According to various studies, this percentage varies within relatively wide ranges. For example, a study by Werneburg et al. reported a 41% relative share of positive urine cultures (*Werneburg G*, *2023*). In a similar study from an emergency center in Switzerland, close values (40.2%) were found (*Müller M*, *2018*). A significantly lower percentage of positive urine samples (24.6%) was reported by Ourani in a prospective study in hospital settings. The authors conducted culture testing only after a positive screening method, where urines without additional data for infection such as leukocytes, nitrites were not tested. Exceptions were samples from children under 1 year old, pregnant women, and patients after urological surgical procedures (*Ourani M*, *2020*). In this context, it should be noted that differences in the relative share of positive urine samples are highly dependent on the demographic characteristics of the patients, the diagnostic method used, and the study setting (hospital or outpatient settings). In young age and female gender, culture testing has less clinical value due to the inconveniences associated with the time and cost of the test, while for hospitalized patients with complicated urinary infections (refractory to initial treatment, pyelonephritis, asymptomatic bacteriuria during pregnancy, atypical microorganisms, etc.), culture testing of urine is mandatory (*Xu R*, *2021*). Unfortunately, a disadvantage of this classical method is the time to obtain microbiological results, which is a minimum of 24 to 48 hours. This leads to a delay in etiologically oriented treatment, creating the possibility for the development of unwanted complications (*Xu R*, *2021*).

4.3. SENSITIVITY TO ANTIMICROBIAL AGENTS DETERMINED BY THE KIRBY-BAUER DISK DIFFUSION METHOD, PERFORMED WITH BACTERIAL ISOLATES IN PURE CULTURE.

The sensitivity to a range of antimicrobial agents of 282 Gram-negative and 67 Gram-positive bacterial isolates was studied using the Kirby-Bauer disk diffusion method, and the sensitivity to vancomycin in S. saprophyticus isolates was determined using the VITEK 2 automated system.

Gram-negative bacteria

The sensitivity to a range of antimicrobial agents of 278 isolates, representatives of bacterial species from the order *Enterobacterales*, is presented in Table 6.

The observed resistance in this group in descending order is as follows: ampicillin, 66.9% > ciprofloxacin, 38.5% > levofloxacin, 35.6% > trimethoprim/sulfamethoxazole, 35.2% > amoxicillin/clavulanic acid, 33.4% > cefuroxime, 29.1% > ceftriaxone, 15.8% > ceftazidime, 15.1% > fosfomycin, 11.0% > nitrofurantoin, 8.0% > gentamicin, 6.5% > nitroxoline, 2.0%.

The detailed structure of resistance to the studied antibiotics in all isolates, representatives of the order Enterobacterales by bacterial species, is presented in Table 7.

The relative share of ESBL producers among the isolates from bacterial species belonging to the order *Enterobacterale*s is presented in Table 8.

Table 6. *Antibiotic sensitivity of isolates from the order Enterobacterales (n=278), associated with urinary tract infections, determined by the Kirby-Bauer disk diffusion method.*

Antibiotic	Total number tested isolates n (%)	Sensitivity n (%)	Resistance (R+I) n (%)
ampicillin	278 (100.0)	92 (33.1)	186 (66.9)
amoxicillin/clavulanic acid	278 (100.0)	185 (66.6)	93 (33.4)
cefuroxime	278 (100.0)	197 (70.9)	81 (29.1)
ceftriaxone	278 (100.0)	234 (84.2)	44 (15.8)
ceftazidime	278 (100.0)	236 (84.9)	42 (15.1)
meropenem	278 (100.0)	278 (100.0)	0 (0.0)
ciprofloxacin	278 (100.0)	171 (61.5)	107 (38.5)
levofloxacin	278 (100.0)	179 (64.4)	99 (35.6)
trimethoprim/ sulphametoxazole	278 (100.0)	180 (64.8)	98 (35.2)
gentamicin	278 (100.0)	260 (93.5)	18 (6.5)
amikacin	278 (100.0)	278 (100.0)	0 (0.0)
fosfomycin*	200 (100.0)	178 (89.0)	22 (11.0)
nitroxoline*	200 (100.0)	196 (98.0)	4 (2.0)
nitrofurantoin*	200 (100.0)	184 (92.0)	16 (8.0)

*The sensitivity to fosfomycin, nitroxoline, and nitrofurantoin was determined only for *E. coli* isolates.

For *P. aeruginosa* isolates (n=4), aside from the characteristic intrinsic resistance to ampicillin, amoxicillin/clavulanate, cefuroxime, intermediate sensitivity to ciprofloxacin and levofloxacin (sensitivity with increased exposure) was demonstrated - 100%, 50% resistance to ceftazidime, and fully preserved sensitivity to meropenem and amikacin.

Table 7. *Detailed structure of resistance to the studied antibiotics in all Gram-negative bacteria, presented by bacterial species.*

Bacterial species (n)	AM (%)	AMC (%)	CXM (%)	CAZ (%)	CRO (%)	MEM (%)	G (%)	AK (%)	CIP (%)	LEV (%)	TSM (%)	FF (%)	F (%)	NIT (%)
Gram negative bacteria														
E. coli **(200)**	56.5	18.5	19.5	6.5	7.5	0.0	2.5	0.0	32.5	30.5	21.0	11.0	8.0	2.0
K. pneumoniae **(40)**	100.0	77.5	52.5	52.5	52.5	0.0	15.0	0.0	42.5	42.5	80.0	NA	NA	NA
P. mirabilis **(26)**	65.4	50.0	34.6	19.2	19.2	0.0	40.0	0.0	30.7	23.0	57.7	NA	NA	NA
Citrobacter **spp. (5)**	100.0	100.0	100.0	20.0	20.0	0.0	20.0	0.0	40.0	40.0	60.0	NA	NA	NA
E. cloacae **complex (5)**	100	100.0	100.0	0.0	0.0	0.0	80.0	0.0	60.0	60.0	80.0	NA	NA	NA
P. aeruginosa **(4)**	NA	NA	NA	50.0	NA	0.0	NA	0.0	0.0	0.0	NA	NA	NA	NA
M. morganii **(1)**	100	100	100	0.0	0.0	0.0	0.0	0.0	100	100	100	NA	NA	NA
S. marcescens **(1)**	100	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	NA	NA	NA

Abbreviations: A, ampicillin; **AMC**, amoxicillin-clavulanate; **CXM**, cefuroxime; **CAZ**, ceftazidime; **CRO**, ceftriaxone; **MEM**, meropenem; **G,** gentamicin; **CIP,** ciprofloxacin; **АК**, amikacin; **LEV,** levofloxacin; **TSM**, trimethoprim/sulfamethoxazole; **FF**, fosfomycin, **F**, nitrofurantoin; **NIT**, nitroxoline; **NA**, неприложимо.

Table 8. *Relative share of ESBL producers among isolates from the order Enterobacterales (n=278), associated with urinary tract infections, proven by the DDST test.*

Bacterial species (n)	ESBL producers (%)
E. coli **(200)**	6.5
K. pneumoniae **(40)**	52.5
P. mirabilis **(26)**	19.2
Citrobacter **spp. (5)**	20.0

Gram-positive bacteria

The sensitivity to a range of antimicrobial agents of 62 isolates, representatives of bacterial species *E. faecalis* (n=42) and *S. saprophyticus* (n=20) is presented in Table 9 and Table 10.

The observed resistance of *E. faecalis* isolates in descending order is as follows: gentamicin (GHLAR), 19% > ciprofloxacin, 19% > levofloxacin, 16.7% > nitrofurantoin, 7.1%. No isolates resistant to glycopeptides (vancomycin, teicoplanin) and ampicillin were found.

The observed resistance among *S. saprophyticus* isolates, presented in descending order, is as follows: gentamicin, 25% > ciprofloxacin, levofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole, 10% > cefoxitin, 5%. No isolates resistant to glycopeptides (vancomycin, teicoplanin) were found.

Streptococcus agalactiae isolates (n=2) demonstrated complete sensitivity to the tested antimicrobial agents (penicillin, levofloxacin, clindamycin, nitrofurantoin, trimethoprim/sulfamethoxazole, vancomycin, teicoplanin).

The single isolate of *Aerococcus urinae* showed complete sensitivity to all tested antibiotics: ciprofloxacin, levofloxacin, ampicillin, meropenem, nitrofurantoin, vancomycin.

Table 9. *Antibiotic sensitivity of E. faecalis isolates (n=42), associated with urinary tract infections, determined by the Kirby-Bauer disk diffusion method.*

Antibiotic	Sensitivity n (%)	Resistance (R+I) n (%)
ampicillin	42 (100.0)	0 (0.0)
G-HLAR	34 (81.0)	8 (19.0)
ciprofloxacin	34 (81.0)	8 (19.0)
levofloxacin	35 (83.3)	7 (16.7)
vancomycin	42 (100.0)	0 (0.0)
teicoplanin	42 (100.0)	0 (0.0)
nitrofurantoin	39 (92.9)	3 (7.1)

Table 10. *Antibiotic sensitivity of S. saprophyticus isolates (n=20), associated with urinary tract infections, determined by the Kirby-Bauer disk diffusion method.*

Antibiotic	Sensitivity n (%)	Resistance (R+I) n (%)
cefoxitin	19 (95.0)	1 (5.0)
ciprofloxacin	18 (90.0)	2 (10.0)
levofloxacin	18 (90.0)	2 (10.0)
gentamicin	15 (75.0)	5 (25.0)
trimethoprim/sulphametoxazole	18 (90.0)	2 (10.0)
vancomycin	20 (100.0)	0 (0.0)
nitrofurantoin	18 (90.0)	2 (10.0)

Discussion

Gram-negative bacteria

Among the most problematic and monitored resistance in representatives of the order *Enterobacterales* is resistance to beta-lactam antibiotics. This study found significantly reduced activity of aminopenicillins (ampicillin) against Gram-negative bacteria, including E. coli isolates, with resistance reaching 66.9%. Compared to ampicillin, the activity of amoxicillin/clavulanic acid is relatively preserved, but the relative share of resistant Gram-negative isolates exceeds 20% (33.4%). National data show similar results. According to BulStar for the period 2018-2021, *E. coli* isolates demonstrate 26 - 27.1% resistance to amoxicillin/clavulanic acid, and for the second most frequent etiological agents of UTIs, *K. pneumoniae*, the levels are consistently above > 40% for the same period (*Surveillance of antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018. Available at: https:// bam-bg.net//*). In pregnant women, ampicillin and amoxicillin should be avoided as initial therapy due to the relatively high resistance of *E. coli* to these antimicrobial agents (*Habak P, 2024*). In different regions of the world, resistance to amoxicillin/clavulanic acid varies significantly, depending on the population of patients studied and the nature of the urinary infection itself (complicated or uncomplicated) (*Kot B, 2019*). Similar to our results, Martínez-Casanova reported 17.8% in Spain and Gaspari reported 27.5% in a pediatric department in the USA (*Gaspari R, 2005; Martínez-Casanova J, 2021*). A study on the sensitivity of uropathogenic *E. coli* isolates in the USA in 2017 showed 77.9% sensitivity to amoxicillin/clavulanic acid (*Critchley I, 2017*). Overall, it can be said that most published studies indicate that isolates from recent years, related to *Enterobacterales* and causing UTIs, demonstrate reduced sensitivity to the drug. In Europe for the period 2018 - 2019, 74% of the *E. coli* isolates associated with urinary infections were sensitive, and only 42% of *K. pneumoniae* (*Veeraraghavan B, 2020*). Authors from neighboring countries to Bulgaria confirm the increasing levels of resistance to the aminopenicillin group: Romania between 14 and 29.0%, Turkey - 24.3%, Bosnia and Herzegovina - 19.6%, as well as authors from geographically more distant

countries: Mexico - 23.6%, Jordan - 83% (*Kot B, 2019; Petca R, 2020; Şencan İ, 2023*).

Despite the increasing resistance, the use of amoxicillin/clavulanic acid in adults is possible in cases of uncomplicated pyelonephritis, therapy for uncomplicated cystitis during pregnancy, or as perioperative prophylaxis in urological interventions (*Kot B*, *2019*; *Kranz J*, *2024*). In childhood, due to their good safety profile, aminopenicillins remain the treatment and prevention of choice for urinary tract infections, including in newborns (*Buettcher M*, *2021*). In the context of the beta-lactam group of antibiotics, resistance to cephalosporins (especially broad-spectrum) and carbapenem antibiotics is particularly important. The main mechanism of this resistance is associated with the production of various enzymes: TEM, SHV, and CTX-M extended-spectrum β-lactamases; plasmid-encoded AmpC β-lactamases (CMY, ACC, DHA) and carbapenemases of various classes (KPC, VIM, IMP, OXA-48, etc.) (*Bush K*, *2020*; *De Oliveira D*, *2020*; *Markovska R*, *2021*). The cephalosporin group of antibiotics, with their broad spectrum of action, are among the preferred and often used antimicrobial agents for the treatment of both community-acquired and nosocomial urinary tract infections. In this study, we found a relatively high share of isolates of *E. coli* resistant to second-generation cephalosporins (cefuroxime) (> 20%). Marhova et al. reported similar levels of resistance to cefuroxime (25%) in *E. coli* isolates from urine obtained from patients with UTIs in the Plovdiv region (*Marhova M*, *2009*). A large Polish study reported 10% resistance to cefuroxime in E. coli associated with uncomplicated urinary tract infections and 18% in those associated with complicated infections (*Stefaniuk E*, *2016*). A recent study from Romania found 12.6% resistance to this antimicrobial agent in *E. coli* urine isolates (*Duicu C*, *2021*). Authors from other neighboring countries to Bulgaria reported over 35% resistance in 2018 (*Gul A*, *2020*). According to data from Kot et al., resistance to cefuroxime in Belgium, Germany, and Spain is 5.5%, 12.8%, and 16.6%, respectively (*Kot B*, *2019*).

Globally, third-generation cephalosporins are one of the leading groups of antibiotics (along with fluoroquinolones, carbapenems, and other beta-

lactams) associated with increased mortality directly due to antibiotic resistance to them (*Li X*, *2022*). These are strategic antimicrobial agents with a broad spectrum of action, whose use should be limited only to complicated urinary tract infections or hospital-acquired urinary tract infections. A positive result of this study is the low relative share of third-generation cephalosporin-resistant isolates, representatives of the order *Enterobacterales* (15.1%) and specifically *E. coli* (6.5%), a result reflecting the share of ESBL producers among Gram-negative bacteria, confirmed by DDST in all isolates from the study. The share of urine isolates of *E. coli* and *K. pneumoniae* from various studies and countries identified as ESBL producers varies significantly: 4.2% in France (*Raphael E*, *2021*), 10% in the USA (*Gharavi M*, *2021*), and 35.7% in Iran (*Mendelson G*, *2004*). Additionally, it is believed that urinary tract infections are responsible for more than 50% of all bacteremic *E. coli* episodes (*Bonten M*, *2020*). The European Centre for Disease Prevention and Control reports a positive trend in the EU in the time interval between 2016 and 2021 for a statistically significant decrease in the share of E. coli blood isolates resistant to third-generation cephalosporins, with the average share reaching 14.9% and 13.8% in 2020 and 2021 (*ECDC, 2017a*; *ECDC*, *2022*). In 2021, the highest share of countries with resistance below 10% is in the European Union, but unfortunately, Bulgaria has the highest levels among all 28 monitored countries (37.3%). A study by Ermenlieva et al. on urinary infections in children in the Varna region found 6% ESBL producers in outpatient settings (*Ermenlieva NM*, *2016*). These results correlate with our established low relative share of *E. coli* urine isolates resistant to third-generation cephalosporins (6.5%).

Currently, carbapenems (meropenem, imipenem) are considered the first-choice antibiotics for the treatment of infections caused by ESBL-producing Gram-negative bacteria (*Rupp M*, *2003*). In our isolates, no carbapenem-resistant Enterobacterales were identified. This result could be explained by the fact that the studied group of patients in this study are outpatients with community-acquired urinary tract infections. Similar to cephalosporins, fluoroquinolones are broad-spectrum antimicrobial agents,

long used as preferred empirical therapy for urinary infections. To avoid inducing bacterial resistance, these agents are no longer the first-line treatment for uncomplicated cystitis (*Chao Y*, *2019*). According to the recommendations of the European Association of Urology, fluoroquinolones should not be prescribed for uncomplicated urinary infections, even if pathogen sensitivity is proven in a given region (*Kranz J*, *2024*). However, according to the same organization, the significance of fluoroquinolones is maintained as they are an oral alternative for uncomplicated pyelonephritis and in cases of Pseudomonas aeruginosa-associated infections (*Kranz J*, *2024*). Similarly, according to the recommendations of the German Association of Nephrology, fluoroquinolones are also not recommended for the treatment of acute uncomplicated cystitis in otherwise healthy premenopausal women, unless no other alternative exists (*Chao Y*, *2019*). According to guidelines for the treatment of urinary infections published by NICE (National Institute for Health and Care Excellence), in catheter-associated infections, fluoroquinolones are not the drugs of choice for initial therapy, except for intravenous ciprofloxacin, and in cases of recurrent infections (*NICE guideline, 2018).* Authors of the Canadian guideline on obstetrics and gynecology SOGC (The Society of Obstetricians and Gynaecologists of Canada) differ in opinion, as they still recommend quinolones as one of the antibiotics for daily prophylaxis in women with two recurrent UTIs within six months or three within 12 months (*Epp A*, *2017*; *Chao Y*, *2019*).

In our results, high levels of resistance to fluoroquinolones were found in the main uropathogen *E. coli* (over 25%). This result is consistent with national results reported by BULSTAR for urine isolates in the period 2015-2021, which show a trend towards an increase from 16.5% in 2015 to 20.7% in subsequent years (21.4% in 2019; 20.7% in 2020; 19.8% in 2021) (*Surveillance of antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018. Available at: https:// bam-bg.net//).* In many parts of the world, different levels of resistance to fluoroquinolones in *E. coli* isolates, associated with various clinical courses of urinary infections, are observed: over 20% in uncomplicated and community-acquired infections and over

50% in cases of complicated urinary tract infections (*Kot B, 2019*). A 2019 study reported 22% resistance in uropathogenic *E. coli* in Europe, while in hospitalized patients in the USA it was around 31% (*Asadi K, 2019*). A study by Goebel et al. on *E. coli* resistance to ciprofloxacin over a 10-year period (2003–2012) reported an increase from 3.6% in 2003 to 11.8% in 2012 among the entire patient population studied, while in the group of patients over 65 years old, the increase in resistance was even more pronounced: from 11.8% to 29.1% (*Goebel M, 2021*). A very extensive review concerning EU countries also reported increasing resistance to ciprofloxacin in uropathogenic *E. coli* in the period 2006 - 2016: for the UK - from 0.5% to 15.3%, Germany – from 8.7% to 15.1%, and for Spain - from 22.9% to 30.8% (*Kot B*, *2019*). A study conducted in Romania also found reduced levels of sensitivity in *E. coli* and *K. pneumoniae* isolates to levofloxacin, with resistance reaching 29.6% and 15.6%, respectively (*Petca R, 2020*). Fluoroquinolone resistance levels in *E. coli* over 15% are also reported for Turkey when monitoring patients from emergency departments and general practitioners (20.4%) (*Şencan İ, 2023*). It should be noted that significantly higher levels of fluoroquinolone resistance for all pathogens in nosocomial infections are reported in the scientific literature for Europe: 41.9% for cystitis; 44.3% for pyelonephritis; 58.4% for urosepsis, with an average share of fluoroquinolone-resistant uropathogens being 47.1% (*Wagenlehner F*, *2020*). A similar trend for increasing resistance to ciprofloxacin is reported in Asia. For example, in 2008-2014, this resistance increased from 25% to over 40% (*Li X*, *2022*). A study by Prasada et al., covering a 5-year period in India, demonstrated very high levels (over 60%) of fluoroquinolone resistance in uropathogenic E. coli (*Prasada S*, *2019*). Similarly, in North America, although not as significant, resistance increased from 4% in 2008 to 12% in 2017 (*Kot B*, *2019*).

Globally, in almost all 21 regions of the GBD (Global Burden of Diseases), fluoroquinolones, carbapenems, and third-generation cephalosporins are the three leading groups of antibiotics associated with the highest antibiotic resistance (*Li X*, *2022*; *Antimicrobial Resistance Collaborators, 2022*). In many countries, the empirical use of quinolones as

the first choice for the treatment of urinary tract infections is common, which is why it is not surprising that, over the past two decades, along with the excessive use of quinolones, constantly increasing resistance to them is being found (*Şencan İ*, *2023*).

The combined antimicrobial drug trimethoprim/sulfamethoxazole has been one of the recommended first-line agents for uncomplicated urinary tract infections for years, as well as for prophylaxis in the course of chronic infections. It should be noted that trimethoprim/sulfamethoxazole remains a choice for initial therapy in both sexes, but only when data on local resistance of *E. coli* to this agent does not exceed 20% (*Colgan R*, *2011*). In younger ages, the drug can be used in children over 30 days old, as oral therapy for cystitis, as well as for prophylaxis (*Buettcher M*, *2021*). Significant geographical and age differences in resistance levels to the drug are observed, with higher levels reported in male patients, children, and adults over 65 years (*Marchand-Austin A*, *2022*).

Studies on the activity of trimethoprim/sulfamethoxazole against *E. coli* in various European countries (Belgium, Germany, Poland, Switzerland, France, Spain, Bosnia and Herzegovina, and Romania) show resistance ranging between 14.6% and 60% (*Kot B*, *2019*). A multicenter study in the USA, covering 12 emergency centers, reported resistance to the microbial agent of 25.1% (*Wesolek J*, *2022*). In the period 2003 - 2012, resistance levels in *E. coli* to this antibiotic in some states increased from 17% to 22.2% in the 18 - 64 years age group and from 18.5% to 26.7% in the group of patients over 65 years (*Goebel M*, *2021*). Prasada et al. also reported an increasing trend among E. coli isolates in India in the period 2013 - 2017, but in a significantly higher range - 52% - 59.6% (*Prasada*, *2019*). In this study, we found a high level (over 20%) of resistance to trimethoprim/sulfamethoxazole (36%) in the group of isolates from the leading bacterial species E. coli, a result indicating that the drug is not suitable as a choice for empirical therapy of community-acquired urinary tract infections in our region. Slightly lower but also over 20% are the national data (28%) for the same period 2018-2021 (*Surveillance of*

antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018. Available at: https:// bam-bg.net//).

Aminoglycosides gentamicin and amikacin are among the antimicrobial agents of choice for parenteral and combination therapy in cases of more severe or complicated urinary tract infections or infections with limited therapeutic alternatives (*Barlam T*, *2016*). The synergistic effect between aminoglycosides and β-lactams is well documented. A positive result from this study is the low level of resistance to gentamicin in the studied collection of isolates (6.5%) and the fully preserved activity of amikacin. In a multicenter hospital study following female patients in Romania, lower than our results sensitivity to amikacin was reported for *E. coli* (96.0%) and *K. pneumoniae* (88.5%) (*Petca R*, *2020*). Another research group from Romania studying urine isolates but in a pediatric population found significantly higher levels of resistance to gentamicin in *E. coli* (90.3%), *K. pneumoniae* (64.7%), and *P. mirabilis* (92.3%) (*Miron V*, *2021*).

Most researchers report relatively preserved sensitivity to aminoglycosides in *Enterobacterales*, both in hospital and community-acquired infections: in Canada, hospital isolates show 73.6% sensitivity and 85.1% in outpatient cases (*Marchand-Austin A*, *2022*); in Turkey, Şencan also reports low resistance to gentamicin in *E. coli* (10.4%) and *K. pneumoniae* (13.2%) in a study covering outpatient cases of urinary infections (*Şencan İ*, *2023*).

Comparative data from various studies presented by Kot and colleagues for countries outside the European Union show much higher resistance to gentamicin and amikacin among outpatients: Pakistan, 29% and 4%, respectively, Mexico 28.2% and 10% (*Kot B*, *2019*). During the present study, the activity of drugs such as nitrofurantoin, nitroxoline, and fosfomycin against *E. coli* isolates was also tested.

According to the literature, nitrofurantoin is active against the most frequently proven causative agents of urinary tract infections, mainly *E. coli*, and to a lesser extent *Citrobacter* and *Enterococcus*, *Klebsiella* spp. and *Enterobacter* spp. are less sensitive. Bacterial species representative of genera such as *Serratia*, *Acinetobacter*, *Morganella*, *Proteus*, and

Pseudomonas are resistant (*Gardiner B*, *2019*). It should be noted that currently, the European standard for determining sensitivity to antimicrobial agents (EUCAST), used in Bulgaria, does not set breakpoint values for nitrofurantoin testing, except for *E. coli* isolates (http://www.eucast.org/clinical_breakpoints). Due to low resistance levels and a good safety profile, nitrofurantoin is a preferred alternative in pediatric patients as well as in pregnant women (*Habak P*, *2024*).

The European Association of Urology recommends it as a drug of choice for *E. coli*-associated uncomplicated cystitis in women (*Kranz J*, *2024*). The tested isolates showed preserved activity and respectively low resistance levels to the drug in the *E. coli* group (6.5%). National data reported by BulStar for the four-year period 2019-2021 for *E. coli* isolates obtained from hospital and outpatient patients are very similar to our results - 3.9%; 5.1%; 4.9% and 5.7% respectively (Surveillance of antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018. Available at: https:// bam-bg.net//). In several European countries and neighboring countries to Bulgaria (Romania, Poland, France, Turkey), the share of nitrofurantoin-resistant E. coli isolated from outpatient and hospitalized patients is low, ranging from 3% to 3.8%, 4.9% (*Kot B, 2019*; *Petca R, 2020*; *Şencan İ, 2023*). Studies conducted in Australia and the USA among outpatient patients report resistance between 1 and 2% (*Gardiner B*, *2019*; *Squadrito F*, *2024*). In contrast to these data, Gautam et al. found a high relative share of nitrofurantoin-resistant Gram-negative bacteria (20.1%) in India, reporting 44.6% resistance among *Klebsiella* spp. isolates but 8.1% for *E. coli* (*Gautam G*, *2021*). A meta-analysis on resistance to various antimicrobial agents used for the treatment of urinary tract infections, including studies from Europe and South America, found that both the most common uropathogens *E. coli* and *Klebsiella* spp. have very good sensitivity to nitrofurantoin (*Emami A*, *2020*; *Corrales M*, *2022*).

The current data confirm that nitrofurantoin is an adequate choice for initial empirical therapy for uncomplicated community-acquired urinary tract infections or in cases with limited therapeutic alternatives, usually associated with multidrug-resistant isolates.

Fosfomycin is an "old" antibiotic that continues to be recommended as a first choice for the treatment of uncomplicated urinary tract infections in women in many countries. It is most active against *E. coli*, while other uropathogens such as *Klebsiella*, *Proteus*, *Citrobacter*, *Enterobacter*, *Pseudomonas,* and *Enterococcus* show varying levels of sensitivity. *Morganella morganii* and *Acinetobacter* spp. are usually resistant to the drug (*Sorlozano-Puerto A*, *2020*). Fosfomycin remains active, including against isolates that produce extended-spectrum β-lactamases and metallo-β-lactamases (*Kot B*, *2019*). A retrospective cohort study showed that treatment outcomes with fosfomycin are not inferior compared to intravenous ertapenem in cases of outpatient UTIs caused by ESBL producers (*Goebel M*, *2021*).

Compared to nitrofurantoin and nitroxoline, the share of fosfomycin-resistant E. coli isolates in our study is higher (11%) than in other Bulgarian centers (3.2 - 5%) (Surveillance of antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018-2021. Available at: https:// bam-bg.net//). Authors from France found a share of fosfomycin-resistant *E. coli* and *K. pneumoniae* at 2.8% and 86.5%, respectively (*Farfour E*, *2020*). In a study similar to the present one, covering *E. coli* isolates from urine of non-hospitalized women from 20 European centers (Belgium, United Kingdom, Italy, Spain, Russia, etc.), it was proven that the most active (in >90%) were nitrofurantoin (98.5%) and fosfomycin (96.4%). These two drugs remain active against over 90% of cephalosporin-resistant E. coli (*Tutone M*, *2022*). Even after decades of fosfomycin use in European clinics, recent clinical studies show that E. coli urine isolates maintain low resistance levels to the drug (3.6%) (*Bermudez T*, *2023*). Although the use of fosfomycin in the USA remains low compared to trimethoprim/sulfamethoxazole, ciprofloxacin, or nitrofurantoin, it is believed that its use will increase in the next decade (*Bermudez T*, *2023*).

Given the resistance levels to trimethoprim/sulfamethoxazole in our region (>30%), fosfomycin emerges as a good alternative for oral therapy of urinary tract infections in outpatients, including in clinical cases with limited treatment options due to multiple resistance.

Nitroxoline is an antimicrobial agent with a broad spectrum of action against various Gram-negative and Gram-positive bacteria, as well as fungi (*Cherdtrakulkiat R*, *2019*). Recent studies have proven that nitroxoline and its derivatives demonstrate very high activity against all representatives of *Enterobacteriaceae*, including carbapenemase-producing bacteria, particularly *E. coli* and *Klebsiella* spp., isolated from various clinical materials (*Cherdtrakulkiat R*, *2019*; *Fuchs F*, *2021*). Various authors report the broad-spectrum activity of the drug against microbial species such as *A. baumannii*, *E. coli*, *S. aureus*, *S. epidermidis* (*Dobrindt U*, *2022*).

Additionally, nitroxoline has the ability to inhibit biofilm production and is more active against biofilm-producing *A. baumannii* and *E. coli* than against biofilm produced by other bacterial species like *P. aeruginosa* (*Dobrindt U*, *2022*; *Wykowski R*, *2022*). Currently, the drug is not among the first-line medications for the therapy of uncomplicated urinary tract infections but is among the alternatives in cases of complicated, chronic infections, as well as for long-term prophylaxis of recurrent urinary tract infections associated with biofilm production (*Wagenlehner F*, *2017*).

In the Varna region, nitroxoline is used in outpatient practice mainly for *E. coli* infections as a drug for combination therapy and step-down therapy. In the present study, we found a very low relative share of nitroxoline-resistant *E. coli* (2%). These results support the use of the drug as an important therapeutic agent in patients prescribed prolonged treatment. According to BulStar data, on a national scale in the period 2018-2021, a low relative share of nitroxoline-resistant *E. coli* (<10%) was also found (6.0%; 9.2%; 6.5%; 9.2%) (Surveillance of antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018-2021. Available at: https:// bam-bg.net//). In an updated German interdisciplinary guideline, nitroxoline is recommended in non-severe cases of uncomplicated urinary tract infections in adult patients, with the authors reporting sensitivity of over 90% among tested Gram-negative, Gram-positive isolates, and yeasts (*Sobke A*, *2018*). Similarly, Dobrindt et al. report resistance of 0.5% to nitroxoline in *E. coli* isolates, including MDR *E. coli*, as well as 5% resistance in *K. pneumoniae* and 32% among MDR *K. pneumoniae* isolates (*Dobrindt U*, *2022*).

Gram-positive bacteria

In the last decade, representatives of *Enterococcus* spp. have been among the leading causative agents of infections (including healthcare-associated infections) - from urinary tract infections to life-threatening infectious endocarditis (*Codelia-Anjum A*, *2023*). Among the most important virulence factors of these microorganisms are their ability to produce biofilm, promoting colonization (including polymicrobial), as well as their antibiotic resistance (*Codelia-Anjum A*, *2023*).

Our results demonstrate completely preserved sensitivity of *E. faecalis* isolates to ampicillin, which remains an important agent in cases of uncomplicated and complicated urinary infections associated with enterococci. Authors from different geographical regions report resistance of enterococci to ampicillin, varying widely: 8.4% in Canada (*Marchand-Austin A*, *2022*), 14.6% for Romania for outpatient isolates (*Petca R*, 2020) to 50% for hospital isolates (*Miron V*, *2021*),

A negative result found in our study is the high relative share of HLAR enterococci (>20%). Aminoglycosides (gentamicin, amikacin) are indicated for severe/complicated urinary infections, usually in combination with ampicillin, and the detection of this type of resistance suggests looking for other synergistic combinations or reliable monotherapy. A high level of resistance to gentamicin (HLAR) has been found among enterococcal isolates in studies in Iran (50%) (*Haghi F*, *2019*), Saudi Arabia (50%) (*Khalil M*, *2022*), and Romania (52%) (*Miron V*, *2021*).

Such isolates are more frequently found in hospital settings, but the significance of this problematic resistance is increasing, as many of the hospitalized patients also require long-term outpatient care (nursing homes, postoperative catheters, permanent catheters). Marchand-Austin and colleagues, following a cohort of patients with urinary infections in outpatient and hospital settings in Canada, reported >90% HLAR (*Marchand-Austin A*, *2022*).

Although less than 20%, our isolates showed reduced activity of representatives of the fluoroquinolone group of drugs (17-19% resistance). According to BulStar data, on a national scale in the period 2018-2021, a high relative share of fluoroquinolone-resistant *E. faecalis* was also found,

with ciprofloxacin resistance in this 4-year period being respectively 30.8%; 30.5%; 30.7% and 29.8%, and levofloxacin - 31.5%; 31.0%; 32.8% and 27.2% (Surveillance of antimicrobial resistance in Bulgaria--a synopsis from BulSTAR 2018-2021. Available at: https:// bam-bg.net//). Probably due to differences in antibiotic policies and approaches, the data related to this type of resistance in the scientific literature vary significantly. For example, Haghi and colleagues report quinolone resistance of around 27% for isolates in Iran (*Haghi F*, *2019*). Even higher levels are reported by Marchand-Austin A and colleagues - 26.7% for *E. faecalis* and 48% in the group of all enterococcal isolates (regardless of the specific species) (*Marchand-Austin A*, *2022*). In a Romanian cohort study among women with urinary infections, 30.2% resistance to levofloxacin was found (*Petca R*, *2020*), and in Turkey among patients of both genders with hospital-acquired infections, ciprofloxacin resistance reached 40.5% (*Şencan İ*, *2023*). According to authors like Khalil and colleagues, quinolones are not a viable therapy for enterococcal infections in Saudi Arabia and Egypt, where resistance is 77% (*Khalil M*, *2022*).

Similar to Gram-negative bacteria, the present study demonstrates preserved sensitivity of *E. faecalis* to nitrofurantoin (93%), making this antimicrobial agent a good empirical choice for the Varna region. Petca and colleagues reported even better activity of the drug, with only 3.3% resistance among tested enterococcal isolates in a Romanian study (*Petca R*, *2020*), while only 1.1% was this type of resistance according to a Canadian study (*Marchand-Austin A*, *2022*). In contrast to these data, results from other geographical regions show resistance to nitrofurantoin among enterococci ranging from 13.3% (Turkey) (*Şencan İ*, *2023*) to 18.8% for Saudi Arabia (*Khalil M*, *2022*).

Among the studied *E. faecalis* isolates, no resistance to the glycopeptide group of antibiotics (vancomycin, teicoplanin) was found. Vancomycin-resistant enterococci (VRE) are more frequently associated with hospital infections, with the most frequently proven species being *E. faecium*, followed by *E. faecalis* (*Levitus M*, *2024*). Due to the limited therapeutic options for VRE, their spread outside hospital settings poses a significant public health risk (*Codelia-Anjum A*, *2023*).

For *S. saprophyticus* isolates, we found very good sensitivity to two of the mainly empirically prescribed oral antimicrobial drugs (*Ehlers S*, *2024*) - nitrofurantoin (95%) and trimethoprim/sulfamethoxazole (90%). Unlike the entire group of coagulase-negative staphylococci, which are characterized by very high levels of methicillin resistance (exceeding 80%), this study found methicillin resistance in only 5%, a result that allows for the use of a wider range of antimicrobial agents for the treatment of *S. saprophyticus* infections. A very close relative share of methicillin-resistant *S. saprophyticus* (5.6%) is reported in a study by Chua and colleagues on an outpatient group of patients with urinary infections. The same authors also report very low levels of resistance to non-beta-lactam antibiotics (2% trimethoprim/sulfamethoxazole, 0% to nitrofurantoin and ciprofloxacin (*Chua K*, *2023*).

According to our results, quinolone resistance (10%) in the group of *S. saprophyticus* isolates is the lowest compared to all other tested uropathogens. Significantly higher quinolone resistance among *S. saprophyticus* isolates is reported in Romania (27.2%) (*Petca R*, *2020*) and Turkey (59.3%) (*Şencan İ*, *2023*).

It should be noted that although not considered as empirical therapy for treating urinary infections in outpatient patients, aminoglycosides (gentamicin) are the drugs for which we found the most compromised activity against *S. saprophyticus* (25% resistance).

In this study, a preserved sensitivity profile was demonstrated for the only *Aerococcus urinae* isolate we identified. Previously considered a contaminant, *A. urinae* is now recognized as a uropathogen in the geriatric population, and in the pediatric population, it is mainly associated with more severe manifestations, primarily against the backdrop of pre-existing urogenital disorders (*Rast D*, *2022*). Sensitivity data for *Aerococcus* spp. are still scarce, with primarily data reported for existing oral antimicrobial agents (fluoroquinolones, nitrofurantoin) (*Carkaci D*, *2017*). The *A. urinae* isolated by us was from a urine sample obtained from an elderly patient, and the isolate showed preserved sensitivity to fluoroquinolones, nitrofurantoin, and ampicillin. A study focusing on *A. urinae*, conducted in nursing homes in the USA, also found very good sensitivity to nitrofurantoin, ampicillin,

penicillin, linezolid, and vancomycin but reduced sensitivity to levofloxacin (63.6%) and ciprofloxacin (44.5%) (*Saad A*, *2023*). Rast and colleagues, studying pediatric patients, reported that appropriate therapy in cases of *A. urinae*-associated urinary infections includes amoxicillin, amoxicillin/clavulanic acid, cefpodoxime, or intravenously administered third-generation cephalosporins (ceftriaxone) (*Rast D*, *2022*).

In the group of *E. faecalis* isolates, the second most common uropathogen, no resistance to ampicillin was found, and resistance to nitrofurantoin was below 10%, making these two groups of drugs suitable for the therapy of *E. faecalis*-associated infections. The activity of fluoroquinolones and aminoglycosides (HLAR) was reduced, with resistance around 20%.

The study did not find any VRE isolates. For *S. saprophyticus*, all antibiotic groups (except aminoglycosides) maintained good activity, with the share of non-sensitive isolates being <=10%. The results of antimicrobial sensitivity testing in this study underscore the importance of periodic monitoring of resistance at the local level to the most commonly used antibiotics in outpatient settings to provide recommendations for empirical treatment.

Good medical practice, however, should always include microbiological examination of urine from patients suspected of having a urinary tract infection to identify the uropathogen and accurately determine its sensitivity to antimicrobial agents.

4.4. SENSITIVITY TO ANTIMICROBIAL AGENTS DETERMINED BY THE AUTOMATED HB&L UROQUATTRO SYSTEM DIRECTLY FROM POSITIVE URINE SAMPLES

From all 122 urine samples identified as positive through screening performed with the HB&L device, a total of 60 Gram-negative and 62 Gram-positive bacterial isolates were isolated and identified to the species level using the automated VITEK 2 system. No discrepancies were found in species identification when conducted using the VITEK 2 device initially directly from the positive urine sample and later with the isolated pure bacterial culture. Table 11 shows the microbial spectrum of the obtained isolates.

Table 11. *Spectrum of bacterial isolates whose sensitivity was determined by the HB&L device.*

Bacterial species	n (%)
Gram negative bacteria	**60 (49.2)**
E. coli	46 (76.6)
Klebsiella pneumoniae	4 (6.6)
Enterobacter cloacae complex	4 (6.6)
Proteus mirabilis	3 (5.0)
Morganella morganii	1 (1.6)
Citrobacter koseri	1 (1.6)
Serratia marcescens	1 (1.6)
Gram positive bacteria	**62 (50.8)**
Enterococcus faecalis	42 (67.7)
Staphylococcus saprophyticus	20 (32.2)
Total bacterial isolates	**122 (100.0)**

Figure 3. *Antibiotic sensitivity of 60 isolates, representatives of the order Enterobacterales, determined by HB&L AST Uroquattro (%).*

Figure 3 shows the sensitivity to six antimicrobial agents determined by HB&L AST Uroquattro for 60 Enterobacterales isolates.

Abbreviation: **CXM**, cefuroxime; **CRO**, ceftriaxone; **MEM**, meropenem; **G**, gentamicin; **AK**, amikacin, **CIP**, ciprofloxacin; **TSM**, trimethoprim/sulfamethoxazole

Figure 4 reflects the sensitivity to five antimicrobial agents determined by HB&L AST Uroquattro for 42 *E. faecalis* isolates.

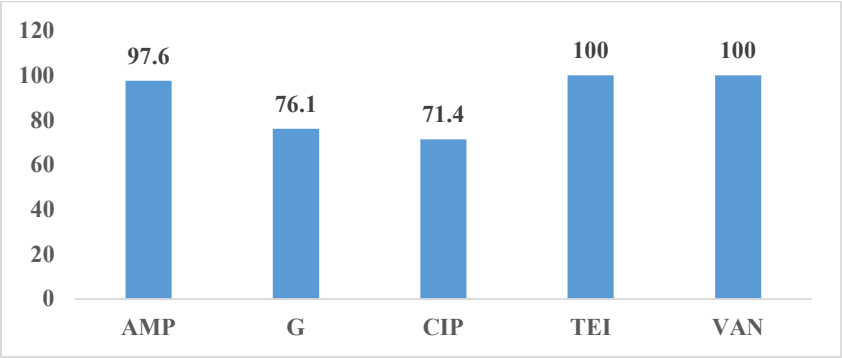

Figure 4. *Antibiotic sensitivity of 42 E. faecalis isolates, determined by HB&L AST Uroquattro (%)*

Abbreviations: AMP, ampicillin; **G**, gentamicin (high level aminoglycoside resistance, HLAR); **CIP,** ciprofloxacin; **VAN**, vancomycin; **TEI**, teicoplanin.

The sensitivity of *S. saprophyticus* isolates (n=20) to three antimicrobial agents, determined by HB&L AST Uroquattro, is presented in Figure 5.

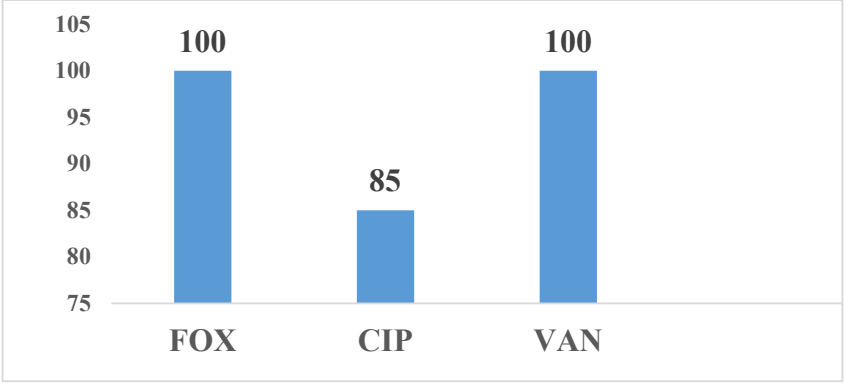

Figure 5. *Antibiotic sensitivity of 20 S. saprophyticus isolates, determined by HB&L AST Uroquattro (%).*

Abbreviations: FOX, cefoxitin, **CIP**, ciprofloxacin; **VAN**, vancomycin

Correlation between antibiotic sensitivity results obtained by HB&L AST and Kirby-Bauer disk diffusion method

In the study, a total of 690 antibiotic sensitivity determinations were performed (HB&L AST directly from positive urine samples versus Kirby-Bauer disk diffusion method from isolates in pure culture).

A categorical agreement of 94.8% compared to the standard method was achieved. A total of 36 errors (5.2%) were found. Of these, 5 were identified as very major errors (VME) (13.9%), 24 as major errors (ME) (66.7%), and 7 (19.4%) as minor errors (mE). Tables 12, 13, and 14 show the correlation results between the two methods for determining sensitivity for different groups of bacteria.

For *Enterobacterales* (n=60), the categorical agreement was 93.3% with 18 major errors, 5 minor errors, and only 1 very major error for trimethoprim/sulfamethoxazole. The lowest correlations in this group were found for gentamicin and ciprofloxacin (91.7%). For all other antimicrobial agents, the correlations were greater than 93%. Full agreement was found for meropenem sensitivity (100%) (Table 12).

The categorical agreement for *E. faecalis* (n=42) was 95.2% with 6 major, 1 minor, and 3 very major errors. The major errors affected the determination of sensitivity to gentamicin (HLAR) (n=2) and ciprofloxacin (n=4), and the very major errors were related to gentamicin (n=1) and the glycopeptide group agents (n=2) (Table 13).

Table 12. *Correlation (%) between antibiotic sensitivity obtained by the HB&L AST system and the reference Kirby-Bauer disk diffusion method for 60 Enterobacterales isolates.*

Enterobacterales **(n=60)**	**CXM**	**CRO**	**MEM**	**G**	**AK**	**CIP**	**TSM**
Number of comparisons	60	60	60	60	60	60	60
Correlation (%)	93.3	93.3	100	91.7	95.0	91.7	95.0

Abbreviations: CXM, cefuroxime; **CRO**, ceftriaxone; **MEM**, meropenem; **G,** gentamicin; **AK**, amikacin, **CIP**, ciprofloxacin; **TSM**, trimethoprim/sulfamethoxazole.

Table 13. *Correlation (%) between antibiotic sensitivity obtained by the HB&L AST system and the reference Kirby-Bauer disk diffusion method for 42 Enterococcus faecalis isolates.*

E. faecalis **(n=42)**	**AMP**	**CIP**	**G**	**VAN**	**TEI**
Number of comparisons	42	42	42	42	42
Correlation (%)	97.6	90.5	92.9	95.2	95.2

Abbreviations: AMP, ampicillin; **CIP**, ciprofloxacin; **G**, gentamicin (high level aminoglycoside resistance, HLAR); **VAN**, vancomycin; **TEI**, teicoplanin.

The categorical agreement for S. saprophyticus was 96.7%. One very major error was found for cefoxitin and one minor error for ciprofloxacin. We demonstrated full agreement for sensitivity to vancomycin (100%) (Table 14).

Table 14. *Correlation (%) between antibiotic sensitivity obtained by the HB&L AST system and the reference Kirby-Bauer disk diffusion method for 20 S. saprophyticus isolates.*

S. saprophyticus **(n=20)**	**FOX**	**CIP**	**VAN***
Number of comparisons	20	20	20
Correlation (%)	95.0	95.0	100

Abbreviations: FOX, cefoxitin**; CIP,** ciprofloxacin**; VAN,** vancomycin**;** * sensitivity determined by the VITEK 2 automated system.

The time to determine sensitivity to antimicrobial agents directly from positive urine samples using the HB&L instrument was between 3 and 5 hours, compared to 48 hours using the standard disk diffusion method from isolated colonies.

4.5. SENSITIVITY TO ANTIMICROBIAL AGENTS DETERMINED BY THE AUTOMATED VITEK 2 SYSTEM DIRECTLY FROM POSITIVE URINE SAMPLES.

For all 122 urine samples identified as positive through HB&L screening, sensitivity to a set of antimicrobial agents was also determined

in parallel using the automated VITEK 2 system, working directly with the positive vials from the HB&L device when reaching a turbidity of 0.5 McFarland. A total of 690 antibiotic sensitivity determinations were performed using the VITEK 2 device directly from positive urine samples, and the results were also evaluated against the results from the Kirby-Bauer disk diffusion method applied to isolates in pure culture.

A categorical agreement of 97% compared to the standard method was achieved, with a total of 21 errors (3%) identified. Of these, 3 were identified as VME (14.3%), 11 as ME (52.4%), and 7 (33.3%) as mE. Tables 15, 16, and 17 show the correlation results between the two methods for determining sensitivity for different groups of bacteria.

For *Enterobacterales* (n=60), the categorical agreement was 97.6% with 4 ME, 5 mE, and only 1 VME for gentamicin. The lowest correlations in this group were found for gentamicin and ciprofloxacin (91.7%). For all other antimicrobial agents, the correlations were greater than 93%. Full agreement was found for meropenem sensitivity (100%) (Table 15).

The categorical agreement *for E. faecalis* (n=42) was 95.2% with 7 ME, 1 mE, and 2 VME. The major errors affected the determination of sensitivity to gentamicin (HLAR) (n=2) and ciprofloxacin (n=4), and the very major errors were related to vancomycin (n=2) (Table 16).

For *S. saprophyticus*, a categorical agreement of 98.3% was determined with one mE for ciprofloxacin. We demonstrated full agreement for sensitivity to vancomycin and cefoxitin (100%) (Table 17).

Table 15. *Correlation (%) between antibiotic sensitivity obtained by the VITEK 2 system directly from positive urine samples and the reference Kirby-Bauer disk diffusion method for 60 Enterobacterales isolates.*

***Enterobacterales* (n=60)**	**CXM**	**CRO**	**MEM**	**G**	**AK**	**CIP**	**TSM**
Number of comparisons	60	60	60	60	60	60	60
Correlation (%)	93.3	93.3	100	91.7	95.0	91.7	95.0

Abbreviations: CXM, cefuroxime; **CRO**, ceftriaxone; **MEM**, meropenem; **G,** gentamicin; **AK**, amikacin, **CIP**, ciprofloxacin; **TSM**, trimethoprim/sulfamethoxazole.

Table 16. *Correlation (%) between antibiotic sensitivity obtained by the VITEK 2 system directly from positive urine samples and the reference Kirby-Bauer disk diffusion method for 42 Enterococcus faecalis isolates*

E. faecalis **(n=42)**	**AMP**	**CIP**	**G**	**VAN**	**TEI**
Number of comparisons	42	42	42	42	42
Correlation (%)	97.6	90.5	95.2	92.8	100

Abbreviations: AMP, ampicillin; **CIP**, ciprofloxacin; **G**, gentamicin (high level aminoglycoside resistance, HLAR); **VAN**, vancomycin; **TEI**, teicoplanin.

Table 17. *Correlation (%) between antibiotic sensitivity obtained by the VITEK 2 system directly from positive urine samples and the reference Kirby-Bauer disk diffusion method for 20 S. saprophyticus isolates.*

S. saprophyticus **(n=20)**	**FOX**	**CIP**	**VAN***
Number of comparisons	20	20	20
Correlation (%)	100	95.0	100

Abbreviations: FOX, cefoxitin**; CIP,** ciprofloxacin**; VAN,** vancomycin**; *** sensitivity determined by the VITEK 2 automated system.

The time to determine antibiotic sensitivity directly from positive urine samples using the VITEK 2 instrument was between 12-18 hours, compared to 48 hours using the standard disk diffusion method from isolated colonies.

Figures 6, 7, 8 show the results of antimicrobial sensitivity testing determined by the automated VITEK 2 system directly from positive urine samples.

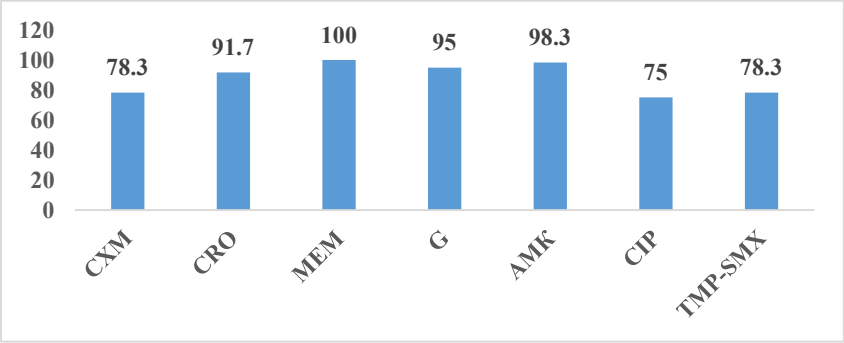

Figure 6. *Antibiotic sensitivity of 60 isolates, representatives of the order Enterobacterales, determined by the VITEK 2 system directly from positive urine samples (%).*

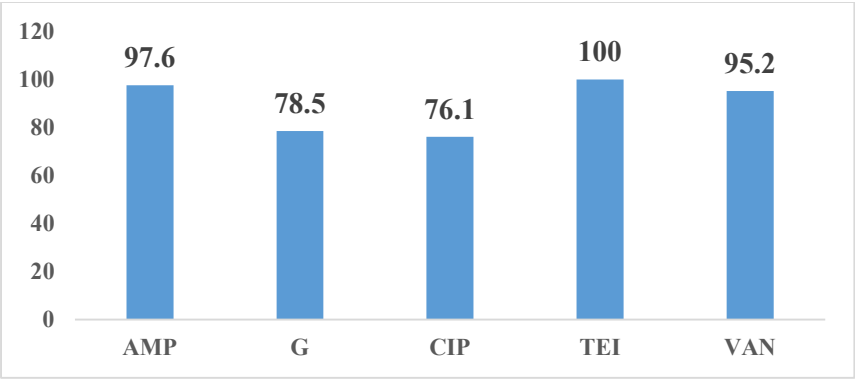

Figure 7. *Antibiotic sensitivity of 42 E. faecalis isolates, determined by the VITEK 2 system directly from positive urine samples (%).*

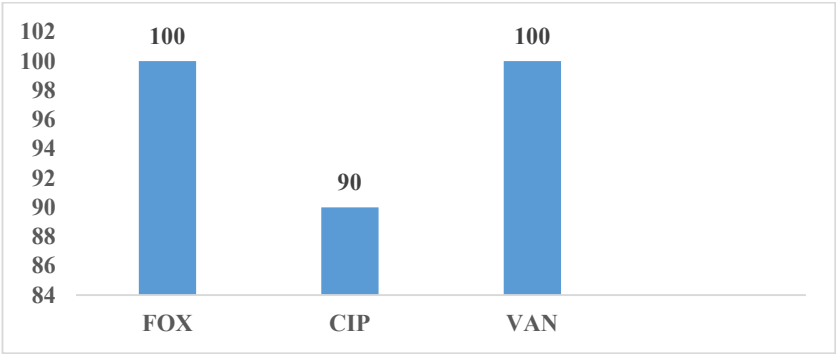

Figure 8. *Antibiotic sensitivity of 20 S. saprophyticus isolates, determined by the VITEK 2 system directly from positive urine samples (%).*

In this study, the results obtained through the automated HB&L screening system are compared with the antibiotic sensitivity results obtained through the classical disk diffusion method. A very good categorical correlation (94.8%) with the reference method was established. This is consistent with studies reporting similar or even higher levels of correlation (93.4%; 94.9%; 95%; 97.1%) (*Anton-Vazquez V*, *2019*; *Sánchez-Carrillo C*, *2019*; *Van den Poel B*, *2020*; *Cupaiolo R*, *2022*). The data obtained indicate that the implementation of the HB&L device for direct antibiotic sensitivity testing in routine

laboratory practice could be highly beneficial, achieving a dramatic reduction in the time to final microbiological result.

In this study, a total of 36 errors were found. The most common were major errors (ME) (66.7%), where the HB&L device categorized the isolate as resistant, while the reference method identified it as sensitive. This is not a critical diagnostic problem, as treatment can be adjusted if necessary, and it does not pose a risk to the patient. However, if the error involves antibiotics considered the first choice for treating urinary tract infections, it could lead to the selection of a drug with a lower efficacy profile, higher recurrence rates, or more side effects.

More concerning are the very major errors (VME), as they can lead to the initiation of incorrect antibiotic therapy and treatment failure, and in cases such as recurrent infections or urosepsis, they can lead to serious complications (*Gajic I*, *2022*). We found 5 very major errors (1 in *Enterobacterale*s, 3 in *Enterococcus* spp., and 1 in *Staphylococcus* spp.). The only very major error among Enterobacterales was for trimethoprime/sulfamethoxazole. This should be taken into account, as trimethoprime/sulfamethoxazole is one of the first-choice agents for treating uncomplicated urinary tract infections in regions where resistance is below 20%.

Among the representatives of the order *Enterobacterales*, the lowest degree of correlation was found for quinolones (ciprofloxacin) and aminoglycosides (gentamicin), but it was mainly associated with minor errors (mE). Among the β-lactam antibiotics tested (cefuroxime, ceftriaxone), a correlation with the cultural method was found in 93.3%, with discrepancies mainly associated with major errors. The β-lactam antibiotic group is a common choice for outpatient treatment of urinary infections in patients of various age groups. The established correlation percentage is relatively high compared to other studies reporting more discrepancies (*Boland L*, *2019*). For example, in a study conducted by Boland and colleagues, an 85.3% correlation rate was reported when testing ceftazidime (*Boland L*, *2019*). Due to subtherapeutic plasma concentrations, oral β-lactams are not recommended as the first choice

in cases of uncomplicated urinary tract infections, except in pediatric age and pregnant women (*Hooton T*, *2012*).

In the group of enterococcal isolates, the lowest categorical agreement was found for ciprofloxacin (90.5%), followed by gentamicin (HLAR) (92.9%) and glycopeptide antibiotics (95.2%). In this group of isolates, 3 very major errors were found: one for gentamicin (HLAR) and 2 for glycopeptides (vancomycin, teicoplanin). Other authors report even lower correlation for aminoglycoside antibiotics (HLAR) (87.5%) but higher correlation for vancomycin (100%) (*Sánchez-Carrillo C*, *2019*). In the study by Anton-Vazquez et al., the agreement for teicoplanin (92%) and vancomycin (90%) was lower than our result (*Anton-Vazquez V*, *2019*). We obtained excellent categorical agreement for ampicillin (97.6%). This result is similar to that reported by Sánchez-Carrillo, who reported full agreement between the two methods used (100%) (*Sanchez-Carrillo C*, *2019*).

Among the staphylococcal isolates studied, we found high correlation for ciprofloxacin (95%) and vancomycin (100%). In contrast, other authors report significant differences when comparing resistance to glycopeptides with different methods, including the detection of very major errors (*Rybak M*, *2013*; *Sánchez-Carrillo C*, *2019*). For cefoxitin, despite the categorical agreement of 95%, we found one very major error, which should be taken into account. In a similar study, the highest agreement was observed for cefoxitin and teicoplanin (92%), and the lowest for vancomycin (80%) (*Anton-Vazquez V*, *2019*). Similar to our result, the authors also report very major errors related to cefoxitin sensitivity (*Anton-Vazquez V*, *2019*). Another study on coagulase-negative staphylococci (including *S. saprophyticus*) found mainly major errors in testing this sensitivity (*Boland L*, *2019*).

It is important to note that no discrepancies in species identification were found in this study when conducted using the VITEK 2 device directly from the positive urine sample on one hand, and with freshly isolated colonies on the other. In this sense, combining the HB&L Uroquattro and VITEK 2 systems can achieve both rapid microbial identification and antibiotic sensitivity through direct inoculation from a

positive urine sample, thus obtaining a result on the day the clinical material is received for testing in the laboratory. Another important advantage of the HB&L system is the possibility of testing sensitivity to a large number of antimicrobial agents, as well as selecting antibiotics depending on the specifics of the isolate and the particular patient (*Sánchez-Carrillo C*, *2019)*.

The comparison we performed between determining antimicrobial sensitivity using the automated HB&L Uroquattro system directly from a positive urine sample and the VITEK 2 system, also directly from a positive sample, showed very close results of categorical agreement of the two approaches compared to the reference method. No major differences were found between the different groups of bacterial isolates: for *Enterobacterales* - 93.3% and 97.6% respectively, for *S. saprophyticus* - 96.7% and 98.3%, and for *E. faecalis* both devices showed the same result (95.2%). The VITEK 2 proved to have a lower number of errors (3% vs. 5.2%). In both methods, the lowest number of VMEs was found: with the HB&L, VMEs were identified in all three categories (1 in *Enterobacterales*, 3 in *Enterococcus* spp., and 1 in *Staphylococcus* spp.), while with the VITEK 2, VMEs were only in the *Enterobacterales* group (1 VME) and the *E. faecalis* group (2 VMEs). Among *Enterobacterales*, VMEs with VITEK 2 related to gentamicin, while with HB&L - to trimethoprime/sulfamethoxazole. In both systems, in the group of enterococcal isolates, VMEs affected glycopeptide antibiotics. Unlike HB&L, no VMEs were found with VITEK 2 when testing sensitivity of staphylococcal isolates.

The undeniable advantage of the HB&L system is the very short period of time from the arrival of the sample in the laboratory to obtaining the result of the antibiotic sensitivity test (on average 5 hours for Gram-negative and 7 hours for Gram-positive isolates). This period includes the screening time (approximately 60-120 minutes to a positive result for bacterial growth, microscopy for Gram identification) and the setting up of the vials for antibiotic sensitivity testing in the HB&L device (3 hours for Gram-negative and 5 hours for Gram-positive). In contrast, with the VITEK 2, the average time to obtaining results was

longer, averaging between 12-18 hours, including the time needed for screening with the HB&L (60-120 minutes), followed by species identification and antibiotic sensitivity determination of the isolate, working directly with the positive urine sample.

4.6. DETERMINATION OF THE "RESIDUAL ANTIBIOTIC ACTIVITY" INDICATOR IN URINE SAMPLES

For the purposes of determining the "residual antibiotic activity" (RAA) indicator, all 1600 urine samples were tested. The overall proportion of RAA-positive samples was 5.7%. Positive RAA tests were confirmed in 91 patients, all with symptoms of urinary tract infections and recent antibiotic therapy and/or intake of supplements with antimicrobial activity. In the entire group of tested patients (n=842), a total of 113 patients (13.4%) reported recent antibiotic treatment and/or intake of supplements with antimicrobial activity (Table 18). The cultural method confirmed microbial growth in 63 patients with a positive RAA result. In 28 patients with a positive RAA urine test, the cultural examination remained negative (Table 18).

Table 18. *Results of the RAA test and classical cultural method applied to urine samples from 113 patients who had recently undergone antibacterial treatment or taken supplements with antimicrobial activity.*

Number patients (n)	Result RAA test	Result culture test	%
n=63	positive	positive	55.8 %
n=28	positive	negative	24.8 %
n=9	negative	positive	8 %
n=13	negative	negative	11.5 %
Total, n=113			100.0

According to the information provided by the patients, among those who demonstrated both a positive RAA test and cultural examination (n=63), a total of twenty-six (41.3%) had been treated with antibacterial agents in the last 10 days prior to the examination due to infections other than urinary tract infections (primarily respiratory), twenty-seven patients (42.9%) had been empirically treated for urinary tract infections with antimicrobial agents prescribed by their general practitioner, and ten

(15.8%) had taken antimicrobial agents without a prescription. Two patients reported that alongside antibiotic treatment, they were also undergoing immunotherapy with preparations containing bacterial lysates. Table 19 shows the distribution of all 113 patients who, during the survey conducted before the microbiological examination, reported recent antibiotic therapy or supplements containing uro-antiseptic ingredients.

Table 19. *Distribution of 113 patients who reported recent antibiotic therapy or supplements containing uro-antiseptic ingredients.*

Ongoing Therapy	n, %
Intake of phytopreparations	48 (42.5)
Antibiotic treatment in the last 10 days for an infection other than a urinary tract infection*	26 (23.0)
Empirically prescribed antibiotic treatment by a general practitioner	27 (23.9)
Self-prescribed antibiotic therapy	10 (8.8)
Total number of patients	113 (100.0)

*Two patients were also undergoing immunotherapy with preparations containing bacterial lysates

Discussion

Determining the "residual antibiotic activity" indicator in urine samples is particularly important for the correct interpretation of microbiological test results in cases where the patient has not reported undergoing antibiotic therapy, to avoid false-negative results or prescribing inappropriate treatment (*Mantzana P*, *2021*). Our study found that 5.7% of the urine samples tested were positive for residual antibiotic activity. Data reported in the scientific literature for this indicator vary and strongly depend on the method applied. For example, using a modified test for determining antibacterial substances in urine, Wilson et al. found a much lower proportion of RAA-positive samples (2.6%) among a total of 14,680 urine samples tested, obtained from outpatients and hospitalized patients in Qatar (*Wilson G*, *2011*). A hospital-based study in Sri Lanka reported a 19.2% proportion of RAA-positive urine samples, proving that about 20% of the samples contained

antibiotics that strongly affected the cultural test results (*Basnayake*, *2020*). Both researchers used manual techniques for measuring RAA. A study conducted by Kussen et al. compared a manual method and the automated screening device Alfred-60 (Alifax, Italy) for detecting RAA in urine samples from hospitalized patients (*Kussen*, *2012*). The authors reported 71.4% sensitivity of the manual method and 92.8% of the automated approach (*Kussen*, *2012*). The lower sensitivity of the manual method is explained by the small sample volume used in the agar diffusion method (*Kussen*, *2012*).

Diagnosing urinary tract infections can be complicated by the presence of antibiotic substances in urine samples subject to cultural testing, which is especially relevant in countries where these medications are purchased without a prescription (*Wilson G*, *2011*). Regarding the RAA test conducted in our study, a very high relative proportion of patients who reported both clinical symptoms of urinary tract infection and recent antibiotic treatment were found to be RAA positive and culture positive. Such a result always requires further assessment of the patient's clinical condition (including additional laboratory tests) and reevaluation of the current antibacterial therapy to determine its adequacy. This is because bacterial growth in the urine sample can be temporarily suppressed by antibiotics, leading to a transient remission of clinical symptoms but may cause complications such as chronic or recurrent asymptomatic infections (*Wilson G*, *2011*).

In this study, we identified an unexpectedly high percentage of culturally negative but RAA-positive urine samples in outpatients with urinary infections. This result is significantly higher than reported by G. Wilson G (0.04%) (*Wilson G*, *2011*), Botão (1%) (*Kussen*, *2012*), Cardozo (7.45%) (*Cardozo*, *2014*), and A. Suresh (15%) (*Suresh A*, *2017*). Significant differences in the relative proportion of RAA (+) / culturally (-) urine samples in the aforementioned studies can be explained by the sample origin (hospitalized or outpatient), the laboratory method used, and differences in some healthcare practices (*Wilson G*, *2011*; *Flores-Mireles A*, *2019*). Analyzing the medical information provided by the patients, our results confirm the findings of

Wilson et al., who found that prior antibiotic intake for infections other than urinary tract infections is among the main reasons for a positive RAA test (*Flores-Mireles A*, *2019*). This fact highlights the importance of the medical history-taking process, especially in outpatients, which can significantly impact the quality of laboratory results, subsequent patient care, and the rational use of antibiotics (*Wilson G*, *2011*). This result also reflects the situation in Bulgaria regarding the consumption of antimicrobial agents in society and the hospital sector in recent years. We should also note that our study was conducted during the COVID-19 pandemic. According to the ECDC (European Centre for Disease Prevention and Control) report for 2020 and 2021, Bulgaria is among the European countries with the highest overall consumption of antimicrobial agents in the region for this period (22.7 – 24.4 DDD/1000 inhabitants per day) (*European Centre for Disease Prevention and Control, 2022*).

5. CONCLUSIONS

After analyzing the results obtained, the following conclusions can be made:

1. The etiological spectrum of urinary tract infections in outpatients during the study period is dominated by Gram-negative bacteria (80.1%). The most commonly identified bacterial species is E. coli (56.8%), followed by E. faecalis (12.5%) and K. pneumoniae (11.4%). Gram-positive bacteria are mainly represented by E. faecalis (65.6%) and S. saprophyticus (29.8%).
2. No statistically significant differences in the distribution of uropathogens in the etiological spectrum of UTIs between the male and female groups were found.
3. Out of a total of 1600 urine samples obtained from 842 outpatients with UTI symptoms, the automated HB&L system detected 21.4% positive samples, while classical microbiological examination detected 22%. The discrepancies between the two methods (0.6%) are mainly associated with rarer pathogens detected in low microbial counts (<103 CFU/ml) and/or slower-growing microorganisms, with the highest number of discordant results determined for Candida spp. isolates.
4. The HB&L automated screening system demonstrated excellent sensitivity (100%), specificity (99.3%), PPV (100%), NPV (99.3%), and accuracy (99.4%), as well as significantly shorter time to result compared to standard testing (4 hours vs. 24 hours).
5. High levels of resistance to ampicillin, trimethoprim/sulfamethoxazole, and fluoroquinolones (over 35%) were found in the Enterobacterales group, but resistance to third-generation cephalosporins was below 20%. Strongly reduced activity of aminopenicillins against E. coli (66.8%) was demonstrated. Carbapenems, aminoglycosides, and nitroxoline have retained activity against E. coli (resistance < 1%, < 6.5%, and < 2% respectively), while resistance to nitrofurantoin and fosfomycin is in the range of 15-20%. Nitroxoline, followed by nitrofurantoin and fosfomycin, can be recommended as first-choice drugs for empirical treatment of uncomplicated, community-acquired urinary tract infections or when other alternatives are lacking due to multiple resistance of the isolates.

6. Among E. faecalis isolates, no resistance to ampicillin was found, and resistance to nitrofurantoin was below 10%, making these two groups of drugs suitable choices for therapy of E. faecalis-associated infections. The activity of fluoroquinolones and aminoglycosides (HLAR) was reduced to around 20%. The study did not identify VRE isolates.
7. Against S. saprophyticus, excluding aminoglycosides, all antibiotic groups retained good activity, with the proportion of non-susceptible isolates ≤10%.
8. The HB&L device demonstrated very good categorical correlation (94.8%) with the standard disk diffusion method for determining sensitivity to antimicrobial agents. Of the 5.2% errors identified, 13.9% were very major errors related to sensitivity determination to trimethoprime/sulfamethoxazole among Enterobacterales (n=1), glycopeptides (n=2), and HLAR among enterococci (n=1), and cefoxitin among staphylococcal isolates (n=1). The highest relative proportion was of major errors (66.7%), with minor errors at 19.4%.
9. The time to determine antibiotic sensitivity directly from positive urine samples using the HB&L AST device is much shorter (between 3 and 5 hours) compared to 48 hours with the standard disk diffusion method from isolated colonies and 12-18 hours when working directly with positive urine samples using the automated VITEK 2 system.
10. The study demonstrates a high relative proportion of urine samples with RAA (5.7%). A high relative proportion of patients in the group undergoing recent antibiotic treatment, who were simultaneously found to have RAA and a positive cultural result (55.8%), with over 40% of these patients having taken antibiotics for infections other than urinary tract infections.

6. SUMMARY OF CONTRIBUTIONS OF THE THESIS WORK

Original Contributions

1. Evaluated the capabilities of the automated HB&L Uroquattro system for rapid diagnosis of urinary tract infections in outpatient settings.
2. Assessed the capabilities of the automated HB&L Uroquattro system for rapid testing of sensitivity to antimicrobial agents.
3. Studied the etiological spectrum of urinary tract infections in a large number of outpatients in the Varna region, as well as local sensitivity to antimicrobial agents (including nitroxoline, nitrofurantoin, and fosfomycin), making recommendations for empirical treatment of uncomplicated community-acquired urinary tract infections.
4. Evaluated the potential of the residual antimicrobial activity (RAA) test for accurate interpretation of microbiological culture results and monitoring therapy effectiveness in cases of urinary tract infections.

Confirmatory Contributions

1. Confirmed the leading role of Gram-negative bacteria, dominated by E. coli, as etiological agents of urinary tract infections in outpatients. In the group of Gram-positive uropathogens, the leading ones are E. faecalis, S. saprophyticus, and S. agalactiae.
2. In line with national data, confirmed high local levels of resistance to aminopenicillins, trimethoprim/sulfamethoxazole, and fluoroquinolones, justifying the restriction of their empirical use in community-acquired urinary tract infections.
3. Automation of the entire microbiological testing process or its individual stages significantly reduces the time to obtain results.

Scientific and Applied Contributions

1. Presented and evaluated a laboratory algorithm including screening of urine samples, followed by microscopic

examination and rapid antibiotic sensitivity testing directly from clinical samples, reducing diagnostic process time by 24-48 hours and fully compatible with other established methodologies.

2. The excellent performance demonstrated by the system highlights its potential for widespread use in routine laboratory practice and its applicability for working with other biological materials obtained from primarily sterile anatomical sites in cases of invasive infections, where rapid microbiological testing is critically important.
3. Introducing the RAA test as part of urine analysis for diagnosing urinary tract infections is an accessible and highly useful additional tool, especially for patients with confirmed bacteriuria undergoing antibiotic therapy (including unreported cases), aiding the interpretation of culture results and preventing false-negative results.

7. SCIENTIFIC PUBLICATIONS AND PRESENTATIONS RELATED TO THE THESIS WORK

Publications in Scientific Journals

1. V. Snegarova-Toneva, D. Niyazi, T. Stoeva. Laboratory Diagnostics of the Urinary Tract. General Medicine, 2023, 25(1), 55-64. (SJR 2023, 0.124, Q4)
2. Snegarova-Toneva V, Niyazi D, Stoeva T. Automated Urine Screening and Residual Antimicrobial Activity Test for Rapid Diagnosis of Urinary Tract Infections in Ambulatory Patients: A Laboratory Evaluation of HB&L Uroquattro Instrument. Acta Medica Bulgarica. 2024;51(s2):29-34. doi:10.2478/amb-2024-0050 (SJR 2023, 0.120, Q4)
3. Snegarova-Toneva V, Niyazi DS, Stoeva T. Evaluation of HB&L Uroquattro System for Rapid Antimicrobial Susceptibility Testing Directly from Positive Urine Samples. Acta Microbiologica Bulgarica. 2024;40(2):199-204. doi:10.59393/amb24400207 (SJR 2024, 0.136, Q4)
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