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ANTIMICROBIAL SUSCEPTIBILITY OF BACTERIA ISOLATED FROM EQUINE URINE

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ABSTRACT

The aim of the present study was to perform a microbiological examination of urine from horses, to isolate and identify bacterial agents and to determine their susceptibility to antimicrobial agents. Samples were taken from 29 horses. Bacterial microflora was isolated from 14 samples. By conventional laboratory methods, 14 strains were identified, of which 7 strains (50%) *Pseudomonas* spp., 5 strains (35.7%) *Klebsiella* spp. and 2 strains (14.3%) *Streptococcus* spp. Gram-negative bacteria showed preserved sensitivity to gentamicin, ceftazidime and fluorinated quinolones and resistance to tetracycline and the combination of trimethoprim/sulfamethoxazole, while streptococci showed sensitivity to amoxicillin, augmentin (amoxicillin/clavulanic acid) and ceftazidime and variable resistance to gentamicin, tetracycline and the combination of trimethoprim/sulfamethoxazole. This is the first study in Bulgaria on the susceptibility to antimicrobial agents of bacterial agents isolated from horse urine. Such studies would be of great importance, both from a scientific and research point of view and of immense practical importance.

Key words*:* horses, urine, bacteria, antibiotic resistance.

Introduction

The global horse industry is developing at an extraordinary pace. This is due to the significant revenues that this sector generates in the global economy (AHC 2017; AHC 2023; Jaqueth *et al.* 2023). Horse breeding is starting to develop more and more intensively in Bulgaria, occupying an important part of the livestock sector. Horses participate in various areas of important economic importance such as sports competitions of various directions, exhibitions, breeding activities, equestrian tourism and various rehabilitation and therapeutic programs with a positive effect on more than 26 pathological conditions in humans and especially in children (White-Lewis 2020). These areas of application determine the close relationships of horses with a wide range of people. At the same time however these contacts create pathways for the transmission and spread of numerous bacteria with zoonotic potential (Gilbert *et al.* 2021), which in most cases are carriers of resistance genes. Many reports present evidence of potential cross-transmission of drug-resistant bacteria from horses to humans (Bramble *et al.* 2011; Van Duijkeren *et al.* 2011; Kaspar *et al.* 2019; Trigo da Roza *et al.* 2019) with severe health consequences.

Information regarding the bacterial causative agents of urinary tract infections in horses, as well as their susceptibility to antimicrobial agents is extremely scarce (Scala *et al.* 2023). According to some authors (Perillo *et al.* 2008; Kabir *et al.* 2024) inflammatory diseases of the urinary tract in horses are uncommon and occur less frequently. Direct causes of their occurrence may be obstruction of urine flow due to formations, urolithiasis, strictures, uroepithelial damage from trauma, some abnormalities in structure and function – vesicoureteral reflux, bladder emptying dysfunction etc.

In horses, however, these inflammations most often occur when the host's immune defense is compromised (WHO 2022). Horses are susceptible to a wide range of infectious diseases, some of which are caused by opportunistic bacteria with high adaptive abilities and resistance mechanisms. The main trigger for the development of such bacterial infections are predisposing factors on the part of the host such as prolonged antibiotic treatment leading to bacterial imbalance, cold, starvation, poor animal hygiene conditions, stress, transport or other pathological conditions leading to a breakdown in the immune system. Many horses with urinary tract infections do not show clinical signs, only the presence of asymptomatic bacteriuria. However, if a bacterial urinary tract infection remains undiagnosed and untreated the consequences can be serious, even life-threatening. The penetration of bacterial pathogens most often occurs ascendingly through the urethra and bladder to the ureters and kidneys. Descending (hematogenous) penetration is a rare phenomenon (Robinson *et al.* 1993). It is believed that bacteria isolated from the excretory system of horses usually originate from the reproductive system, skin or digestive tract, but unlike other microorganisms they must possess abilities and mechanisms that ensure that they overcome the normal defense mechanisms of the excretory tract and successfully colonize the urinary tract. The dynamically changing environment in the excretory system, associated with continuous cycles of production, accumulation and excretion of urine, exposes bacteria to mechanical stress. This requires exceptional resistance and adaptive flexibility of the invading pathogen. Therefore, it is not surprising that *E. coli, Pseudomonas* spp., *Klebsiella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterobacter* spp. are cited in the majority of cases of urinary tract infections. A large number of these bacteria possess virulence factors and a plastic genome that allow them to quickly adapt to stressors of various nature including the presence of antibiotics. A number of reports (Duchesne *et al.* 2019; Isgren *et al.* 2021) reported that an increasing prevalence of multidrug resistance has been recorded in some of the most commonly isolated bacteria from equine urine and its transmission has been demonstrated not only between strains of the same species, but also at the interspecific level. The use of antibiotics in urinary tract infections in horses has been identified as a problem area. On the one hand, due to the very limited number of chemotherapeutic agents that are used in this type of animal and even less in this type of infection (affecting the urinary tract). On the other hand, the incorrect or excessive use of antibiotics generates a strong selective pressure that favors the survival and proliferation of resistant bacteria. Very few studies are conducted on urinary tract infections in horses in Bulgaria and even fewer of them are accompanied by bacteriological examination and determination of the sensitivity of isolates to antimicrobial agents. Such studies would provide valuable information for practice regarding the main microbiological determinants involved in the etiology of urinary tract infections in horses, as well as the best antimicrobial agent against them. In addition, the data would help address the looming global threat of the spread of antimicrobial resistance, which is of paramount importance not only for protecting equine health, but also for public health.

Materials and Methods

For the period July 2024 – March 2025 (9 months), a total of 29 urine samples were taken from horses. The samples were taken from animals with symptoms of the urinary system and from those with risk diseases, that can negatively affect the overall immunity of the body and reflect on the bacterial status of the urinary system. For this reason, the horses were divided into three groups. The first group included 5 horses with clinical symptoms of the urinary system (cystitis, urethritis, urolithiasis). The second group, defined as risk, included 16 horses with diseases of other organs

and systems (colic, rhabdomyolysis, respiratory infections) and the third group – control, which consisted of 8 horses. The samples were taken by catheterization or during spontaneous urination – a middle portion of urine. In two horses an endoscopic examination of the urethra and urinary bladder was performed. The samples were taken in sterile containers following asepsis and antiseptics rules. The microbiological tests were performed in the bacteriological laboratory of the Department of Veterinary Microbiology, Infectious and Parasitic Diseases in FVM at Trakia University. All samples were processed within two hours of obtaining.

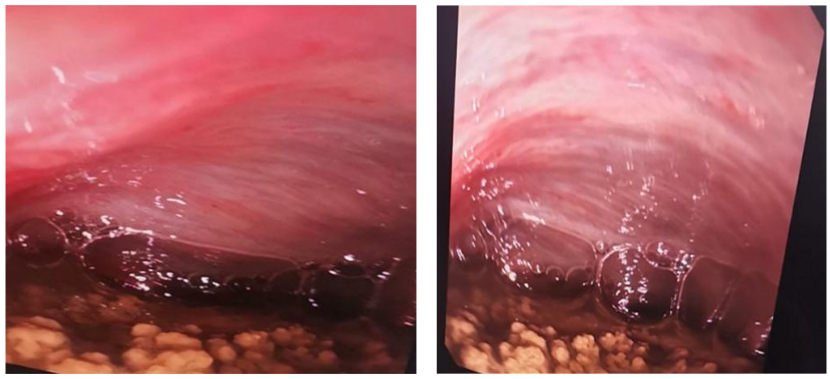

Figure 1: Stones and hyperemia of the mucosa in urinary bladder – endoscopic findings

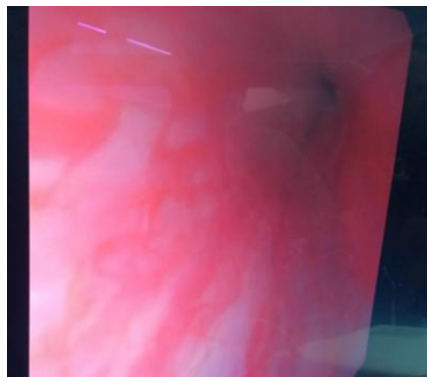

Figure 2: Inflammation of mucosal layer of the urethra – endoscopic examination

Bacteriological studies

Each sample was dilute up to 100 with saline to count colonies and determine clinically significant colonial growth. From the dilution 100µl were inoculated onto blood agar (with 5% defibrinated sheep blood). In parallel, cultures were also made on Mac Conkey agar (Hi Media, India). The cultures were incubated at 37℃ for 24–48 h under aerobic conditions. A clinically significant result was interpreted as a bacterial count in the urine ≥10^3 CFU/ml in horses with urinary system symptoms and levels ≥10^5 CFU/ml in asymptomatic (risk) animals. A morphological characterization of bacterial colonies incl. shape, size, periphery, pigmentation, hemolysis, Gram staining was performed taking into account the size, shape and location of the bacterial cells of the colonial growth. Catalase and oxidase tests were performed. In Gram-negative bacteria, in addition to growth on MacConkey medium and lactose tolerance, cultures were also performed on Kligler's polytropic medium, Simons citrate agar, determination of metabolic type by Hugh Lifeson test,

indole and motility tests and IMViC test. All culture media were produced by Hi Media, India. Additionaly to microscopic examination Gram-positive bacteria were tested for catalase activity and cytochrome oxidase production. Results were indicative for the determination of the isolates as genus Streptococcus spp. Tests for esculin hydrolysis and the presence of CAMP-factor were also performed and group affiliation was determined using a latex agglutination kit (Prolex Streptococcal grouping latex kit, Pro-lab diagnostics, Canada). As a final stage in the phenotypic determination of streptococcal strains was used STREPTO 24 test (Erba Lachema, Czech Republic), which includes 24 substrates. For interpretation of the results was used Erba Expert manual.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing of isolates was performed in vitro, by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Hi Media, India), with 5% defibrinated sheep blood added to the agar for streptococcal isolates. For this purpose, a suspension of a 24-hour culture of each strain tested, corresponding to a density of 0.5 on the McFarland scale, was applied using a sterile swab to the agar surface. Antibiotic discs (Hi Media, India) were used with concentrations as follows: amoxicillin 10 µg, amoxicillin/clavulanic acid – 20/10 µg, amikacin 30 µg, ceftazidime 30 µg, gentamicin 10 µg, enrofloxacin 5 µg, tetracycline 30 µg and trimethoprim/sulfamethoxazole (Co-trimoxazole) 25 µg. After 24 hours of incubation, the zones of growth inhibition were recorded by measuring their diameter. The results were interpreted according to the three-point Bauer–Kirby scale and assessed as susceptible (S), intermediate (I) and resistant (R) according to the current EUCAST and CLSI standards. The intermediates were classified as resistant from a clinical point of view.

Results

Of the 29 horse urine samples tested, bacterial microflora was isolated from 48.3% (n=14) of the samples, with 35.7% (n=5) of the positive samples being from the first group and 64.3% (n=9) from the second group. No microorganisms were isolated from the control group samples. As a result of the microbiological examination, a total of 14 strains were identified, incriminated as possible etiological agents of inflammatory processes in the urinary system. Of these, 7 strains (50%) were identified as *P. aeruginosa*, 5 strains as *K. pneumoniae* (35.7%) and 2 strains (14.3%) as *S. zooepidemicus*. Bacterial microflora was isolated from all samples of the first group. In the second group, 56.3% of the samples were positive. The distribution of bacterial isolates in the two groups is as follows – from the positive samples of the first group of horses, 2 strains (40%) *P. aeruginosa*, 2 strains (40%) *K. pneumoniae* and one strain (20%) *S. zooepidemicus* were isolated, respectively in the second group 5 strains (55.6%) were identified as *P. aeruginosa*, 3 strains (33.3%) *K. pneumoniae* and one strain (11.1%) *S. zooepidemicus* (Table 1).

Table 1: Results of microbiological examination of urine

Microbial Isolate	Positive Samples n=14	Positive Samples from Group I n=5	Positive Samples from Group II n=9
P. aeruginosa	7/50%	2/40%	5/55.6%
K. pneumoniae	5/35.7%	2/40%	3/33.3%
S. zooepidemicus	2/14.3%	1/20%	1/11.1%

Table 2: Antimicrobial susceptibility of bacteria isolated from horse urine

	N of strains		Amx	Amx + Cla	Ak	Caz	G	T	Enr	Cot
P. aeruginosa	7	S	-	-	5/71.4%	7/100%	7/100%	-	7/100%	-
		R	-	7/100%	2/28.6%	-	-	7/100%	-	7/100%
K. pneumoniae	5	S	-	-	-	5/100%	5/100%	-	4/80%	-
		R	5/100%	5/100%	-	-	-	5/100%	1/20%	5/100%
S. zooepidemicus	2	S	2/100%	2/100%	-	2/100%	1/50%	1/50%	-	1/50%
		R	-	-	-	-	1/50%	1/50%	-	1/50%

Legend: S – sensitive; R – resistant; Amx – amoxicillin; Amx+Cla – amoxicillin+clavulanic acid; Ak – amikacin; Caz – ceftazidime; G – gentamicin; T–tetracycline; Enr–enrofloxacin; Cot – trimethoprim/sulfamethoxazole (Co–trimoxazole).

The results of the antimicrobial susceptibility testing of the isolates are shown in Table 2. The data indicate that the *P. aeruginosa* strains exhibit retained susceptibility (100%) to ceftazidime, gentamicin and fluorinated quinolones (enrofloxacin). Due to the limited number of applicable antibiotics in horses, as well as the inherent multidrug resistance of pseudomonads, susceptibility to amikacin was also tested as a second option in the absence of susceptibility to gentamicin. In *P. aeruginosa*, 71.4% of the strains showed susceptibility, and 28.6% were resistant to amikacin. Resistance to tetracycline and the combination of trimethoprim/sulfamethoxazole and amoxicillin/clavulanic acid was confirmed. *K. pneumoniae* strains also showed strong resistance. They were resistant to amoxicillin and the combination of amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole, as well as to tetracycline. Susceptibility to ceftazidime and gentamicin was found (100%), and 80% of the strains also showed sensitivity to enrofloxacin. Streptococci were sensitive to amoxicillin and the combination of amoxicillin/clavulanic acid, as well as to ceftazidime, but showed variable sensitivity to gentamicin, tetracycline and trimethoprim/sulfamethoxazole.

Discussion

The analysis of the results of this study showed a low prevalence of bacterial urinary tract infections in horses. During the study period, only 5 horses with urinary tract inflammation were identified. However our study confirmed the theory of many authors about the role of compromised immune defense of the body in the occurrence and spread of such infections (Perillo *et al.* 2008; Pottier *et al.* 2022; Zheng *et al.* 2024). From the samples of the horses at risk (second group), bacterial microflora was isolated in 56.3% of cases.

In both groups were identified pathogens belonging to three microbial species. These are the Gram-negative *P. aeruginosa, K. pneumoniae* and the Gram- positive *S. zooepidemicus*. This correlates with the studies of other authors (Sprayberry et Robinson 2015; Reed *et al.* 2018-1, 2018-2). However, in contrast to their studies, we did not isolate *E. coli*, which is indicated as the main bacterial cause of urinary tract infections. The most frequently isolated microorganism in our study was *P. aeruginosa* (50%), followed by *K. pneumoniae* (35.7%) and in third place *S. zooepidemicus* (14.3%). In the study of Duchesne *et al.* (2019) was reported a prevalence of 28.9% of *P. aeruginosa*, 43% of *K. pneumoniae* in urogenital swab samples. Isgren *et al.* (2021) found that the most common urogenital pathogens in horses were *E. coli* (31.9%) and β-hemolytic *Streptococcus* spp. (29.5%). Kumas and Maden (2014) reported a case of bladder eversion in a horse as a result of chronic bacterial cystitis caused by *S. zooepidemicus* and *E. coli*. From results obtained by us

can be concluded that following bacteria *P. aeruginosa, K. pneumoniae, S. zooepidemicus* are isolated from the urine of horses. Such can easily provoked inflammation, most often in immunocompromised patients, and the degree of clinical manifestation depends on the host's defenses.

The results of *in vitro* testing using the disk diffusion method showed that few antimicrobials remained active against *P. aeruginosa*. In our study was demonstrated retained susceptibility to gentamicin, ceftazidime and enrofloxacin (100%). Resistance to tetracyclines, sulfonamides and even the combination of amoxicillin/clavulanic acid was confirmed. These results correlate with other authors (Van Spijk *et al.* 2016; Chipangura *et al.* 2017) and confirm its resistance to many classes of antibiotics. In fact, the treatment of *P. aeruginosa* infections is a real challenge not only due to its inherent natural resistance, but also due to its ability to acquire resistance genes, leading to the emergence of new multi-resistant phenotypes. In addition, these bacteria can adhere to tissues and form biofilms, which further limits treatment options and may lead to therapeutic failure. Data on the susceptibility of *K. pneumoniae* to antimicrobial agents in equine medicine are conflicting. However, it is recognized as an organism with inherent drug resistance to many classes of antibiotics. The results of our study confirmed this, as the strains isolated by us were resistant to amoxicillin and its combination with clavulanic acid, tetracycline and trimethoprim/sulfonamide. Susceptibility to gentamicin and ceftazidime was found, and partial sensitivity (80%) to fluorinated quinolones was documented. The current data are in agreement with other studies that have found an increasing prevalence of multidrug-resistant *Klebsiella* spp. (Brisse et Van Duijkeren 2005; Chipangura *et al.* 2017). Although *K. pneumoniae* is usually considered a commensal organism in horses, we believe that this attitude should be reconsidered, both because of the significant risk to equine health and because of the zoonotic potential that can lead to severe infections in humans.

S. zooepidemicus, that causes infections in horses, is generally susceptible to penicillin antibiotics (Malo *et al.* 2016; Awosile *et al.* 2018). However, some studies have reported the development of penicillin resistance (Fonseca *et al.* 2020). A large-scale study in Kentucky in 2022 reported increasing resistance of *S. zooepidemicus* in horses to phenicols, tetracyclines, fluoroquinolones, and potentiated sulfonamides (Lord *et al.* 2022). In our study, the isolated streptococcal strains demonstrated retained susceptibility to amoxicillin, amoxicillin/clavulanic acid, and third-generation cephalosporins (ceftazidime), as well as variable resistance to gentamicin, tetracycline, and potentiated sulfonamide.

Conclusion

From our study can be concluded that the main bacterial agents involved in the etiology of urinary tract infections in horses are bacteria that can be easily provoked, especially in immunocompromised patients, and are relatively resistant to antimicrobial agents. Adequate therapy of inflammatory diseases in horses requires timely identification of the bacterial causative agent and the accurate determination of its sensitivity, respectively resistance to antimicrobial agents. This is a key point for achieving a positive therapeutic effect, especially considering the severely limited treatment options in horses, as well as the possibility of limiting the spread of multidrug-resistant bacteria. Thus, the rule that clinical diagnosis should be accompanied by microbiological studies before antibiotic therapy is once again confirmed.

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Conflict of Interest

The authors declare that they have no conflict of interest regarding authorship and/or the publication of this article.

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