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Evaluation of antibiotic resistance in *Proteus* spp: a growing trend that worries Public Health. Results of 10 Years of Analysis

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Running title: Analysis of antibiotic resistance in *Proteus* spp.

SUMMARY

The genus *Proteus* includes several species among which *Proteus mirabilis* is by far the most commonly detected in clinical specimens. In the last twenty years, isolates with multiple acquired resistance genes have been detected, especially in the hospital environment, with a significant impact on the treatment of infections. This research is a ten-year cross-sectional study reporting the detection rates and the antibiotic susceptibility of *Proteus* spp. in clinical specimens from a healthcare setting in Southern Italy. Of all the 1,600 clinical samples sent to the laboratory, 4.4% were positive to *Proteus* spp., with *P. mirabilis* by far the most detected one (83.1%), especially in lower limb ulcers and urines. Moreover, we noted a significant increase of 1200% in the detection rate from 2011 to 2020. Finally, we reported a significant and constantly increasing trend in the detection of antibiotic-resistant strains, ranging from 48.4% in 2011 to 74% in 2020. Our results highlight a clear and significant increase in *Proteus* spp. detection in a typical hospital setting with a parallel increase in the detection of antibiotic-resistant strains. Therefore, *Proteus* spp. can be considered one of the main emerging pathogenic bacteria in the hospital environment

Keywords: *Proteus* spp.; Antibiotic resistance; Hospital-acquired infections.

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INTRODUCTION

Proteus spp. are Gram-negative rod-shaped bacteria belonging to the *Enterobacteriaceae* family, commonly detected in a wide variety of environments, including soil, water, and sewage, but especially as commensals of human and animal gut microbiota (Penner, 2005). First isolated and characterized by Hauser in the late 19th century (Manos *et al.*, 2006), the genus currently includes six species named *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri*, *P. terrae*, and *P. cibarius*, along with the three unspecified genomospecies 4, 5, and 6 (O'Hara *et al*., 2000; Drzewiecka *et al.*, 2016). Among these species, all of the members have been isolated from human clinical specimens except for *P. cibarius* and *P. terrae* (O'Hara *et al*., 2000; Penner, 2005; Manos *et al*., 2006; Hyun *et al*., 2016). *P. vulgaris*, *P. mirabilis*, and *P. penneri* are present in the human gut in various combinations, but in healthy subjects they represent <0.05% (Zilberstein *et al.*, 2007; Yatsunenko *et al*., 2012). Moreover, they are described as opportunistic pathogens with a low level of virulence (Giammanco *et al.*, 2011). Specifically, *P. mirabilis* is by far the most common species detected in clinical specimens, of which 90% from urinary tract infections (UTIs) and catheter-associated urinary tract infections (CAUTIs), and the remaining 10% from respiratory, eye, ear, nose, skin, burn, meningoencephalitis, osteomyelitis and wound infections (Schaffer *et al.*, 2015). UTIs and CAUTIs are common in long-term catheterized patients; they can be complicated by the formation of bladder and kidney stones with permanent renal damage, and by bacteremia with endocarditis and sepsis (Kim *et al.*, 2003; Norsworthy *et al.*, 2017; Ioannou *et al.*, 2020). Indeed, CAUTIs are the most common sources of bacteremia, and bacteremia caused by *P. mirabilis* following UTIs or CAUTIs present a high mortality rate (Hooton *et al.*, 2010; Daniels *et al*., 2014). Although CAUTIs are often polymicrobial, in this context *P. mirabilis* is one of the most commonly detected microorganisms (Nicolle, 2005; Armbruster *et al.*, 2016).

P. mirabilis has an impressive number of virulence factors. The enzyme urease is a critical one, but the bacterium also expresses a surprising quantity of other factors, among which several fimbriae and other adhesins. Moreover, a diversity of potent toxins and proteases are produced and, similar to other members of the *Enterobacteriaceae* family, numerous secretion systems including types I, III, IV, V, and VI are present. *P. mirabilis* is able to form biofilm on the catheter surface with a process in which the enzyme urease plays a crucial role in hydrolyzing urea, present in high concentrations in urine, to CO_2 and NH_3. The free ammonia increases the urine pH with the subsequent precipitation of soluble polyvalent anions and cations with the formation of struvite ($MgNH_3PO_4$) or apatite ($CaPO_4$) stones. These crystals can form on the surface and within the lumen of catheters, hindering urine flow with the need to remove and replace the catheter. Through the same mechanism, stones may also form in renal tubules and/or the pelvis, causing inflammation and often

requiring surgical removal (Norsworthy *et al.*, 2017; Armbruster *et al.*, 2018). Finally, the bacterial cells contain an integrative and conjugative element, named ICEPm1, transporting virulence and antibiotic resistance genes, able to self-replicate and self-transfer to other strains and species (Armbruster *et al.*, 2018).

The development and spread of antimicrobial resistance (AMR) have become a crucial public health concern worldwide and involve more and more different settings in addition to hospitals (Venter *et al.*, 2017; Cheng *et al.*, 2019; Facciolà *et al.*, 2021). *P. mirabilis* is naturally susceptible to all b-lactams because the wild-type phenotype does not produce any chromosomally encoded b-lactamase. However, in last twenty years, *P. mirabilis* isolates with multiple acquired resistance genes that complicate the treatment of infections have been reported (Girlich *et al.*, 2020). Specifically, genes encoding narrow spectrum b-lactamases, cephalosporinases and carbapenemases have been described (de Champs *et al.*, 2000; Decré *et al.*, 2002; Naas *et al.*, 2003; Bidet *et al.*, 2005; Schultz *et al.*, 2015; Girlich *et al*., 2015; Nakama *et al.*, 2016). On the other hand, *P. vulgaris* produces a chromosomally encoded inducible class A cefuroximase determining resistance to aminopenicillins and first- and second-generation cephalosporins, with the exception of cefoxitin (Girlich *et al.*, 2020). Some studies have reported a significant increase in ESBL-producing *P. mirabilis* isolates. Tamma *et al.* (2019) carried out a study in a University Hospital in the United States on ceftriaxone non-susceptible *Enterobacterales* isolates over an 8-month period in 2015. The authors reported that *P. mirabilis* accounted for 7.4% of the *Enterobacterales* with identified ESBL or AmpC and that the most common ESBL were CTXM-1 group, CTX-M-9 group and SHV-type, whereas the most common AmpC was DHA-type. The detection of carbapenemases in *P. mirabilis* is still low but has a tendency to increase over time globally. Indeed, Tibbetts *et al.* (2008) first reported KPC-2 carbapenemase in *P. mirabilis* in the United States in 2008, but a few studies from China 2010 (Sheng *et al.*, 2010) and Brazil 2015 (Cabral *et al.*, 2015) reported similar findings. Moreover, the acquisition of metallo-b-lactamase genes such as blaVIM-like genes occurred in Greece in 2006 (Vourli *et al.*, 2006), in Italy in 2008 (Falcone *et al.*, 2010) and more recently in Bulgaria in 2017 (Markovska *et al.*, 2017). Dixon *et al.* (2016) identified IMP-producing *P. mirabilis* in two studies from the United States while the production of NDM in *Proteus* spp. has been reported in a few isolates in France (Girlich *et al*., 2015) and Austria (Valentin *et al.*, 2018). Only two reports of OXA-48 producing *P. mirabilis* isolates have been published so far: one on strains isolated in Palestine in 2012 (Chen *et al.*, 2015) and one reporting data from 2013 to 2014 in Russia (Fursova *et al.*, 2015).

This paper reports the findings of detection rates and antibiotic susceptibility of *Proteus* spp. isolates identified in clinical specimens from a healthcare setting in Southern Italy in the ten-year period 2011-2020.

MATERIALS AND METHODS

Samples

We carried out a cross-sectional study involving clinical specimens from a healthcare setting located in the southern Italian city of Messina, Sicily. This healthcare facility is a multi-specialty clinical institute with a particular focus on Orthopedics and Traumatology, consisting of 9 different wards with 91 hospital beds, in which medical, surgical and rehabilitative activities are performed. The structure is a private clinic belonging to the National Health System network and is an institution accredited with the Joint Commission International system. Since 2011, this healthcare facility has been linked with our Microbiology laboratory, situated in the Messina University Hospital, for the execution of microbiological analyses. We investigated the detection rates of *Proteus* spp. (including *Providencia rettgeri* and *Morganella morgannii*, previously considered belonging to the genus *Proteus*) and the variation of antibiotic susceptibility in strains isolated in the laboratory from various types of biological specimens in the decade 2011-2020.

Microbiological analysis

The clinical specimens were tested with routine microbiological assays to determine the presence of Gram-negative or Gram-positive bacteria causing the infection, and their antibiotic susceptibility. To detect microorganisms, the samples were grown on classic media such as Columbia Blood Agar 5%, CLED Agar, Mannitol-Salt Agar and, depending on the type of sample, culture media such as Hektoen Enteric Agar or SS Agar were added. After incubation at 37°C for 24h, colonies deemed to cause the infection were further investigated, and finally identified by API profiles (bioMerieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

To suggest targeted antibiotic therapy, an evaluation of susceptibility or resistance to antibiotics was performed on these germs using the Kirby-Bauer method. Specifically, the isolates were suspended in sterile water in order to obtain a 0.5 McFarland turbidity standard solution (bioMérieux), corresponding to 1.5×10^8 CFU ml^{-1}, and the suspension was inoculated on plates of Müller-Hinton Agar. Then, antibacterial disks were used to determine the resistance patterns of the isolates against 32 different antibiotics (listed in Table 1), grouped according to four specific categories (cell wall inhibitors, disruptive membrane antibiotics, nucleic acid inhibitors and protein synthesis inhibitors) (Laganà *et al.*, 2018). Products commercially distributed by ThermoFisher (Waltham, Massachusetts, USA) were used to carry out all the culture analyses and the antibiograms. Each bacterial species was classified as resistant (R), intermediately resistant (I), or sensitive (S) according to the breakpoints established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing, 2022) and the Clinical & Laboratory Standards Institute (CLSI) (Clinical & Laboratory Standards Institute, 2022).

RESULTS

From 2011 to 2020, 1600 clinical samples were sent to the laboratory. Figure 1 shows the trend in absolute numbers of the samples sent during the considered period. The figure shows a significant increase in the absolute numbers of samples sent during the considered ten years, with a percentage increase of 198.1% from 2011 to 2020. Specifically, after nine years of constant and progressive rise, with a percentage increase of 348.1% from 2011 to 2019, we observed a significant reduction in 2020, with a decrease of 33.5% compared to 2019. The increase of the sample is absolute, as confirmed by comparing the trend of hospital admissions to that of the samples sent from 2011 to 2020, as shown in Figure 2. The figure shows that despite the reduction in hospital admissions, with a percentage decrease of 40.9% from 2011 to 2020, the number of the samples sent to the laboratory during the same period increased independently over time.

Of all the samples sent, 71 (4.4%), of which 28 (39.4%) and 43 (60.6%) from men and women, respectively, resulted positive for *Proteus* spp. The average age of the patients was 73 ± 13 years (min. 30 - max. 96). Of the total of patients, 52 (73.2%) suffered from co-morbidities and/or risk conditions for infection, of which 46.2% diabetes, 15.4% carriers of urinary catheter, 11.5% heart disease, 11.5% carriers of bone and/or joint prosthesis, 11.5 % bone fracture, and 3.8% obesity. Figure 3 shows the different clinical sample categories in which *Proteus* spp. was detected.

The detected species were as follows: 83.1% *P. mirabilis*, 8.5% *P. rettgeri*, 5.6% *M. morgannii*, and 2.8% *P. vulgaris*.

Table 2 shows the numbers and percentages of *Proteus* spp. detection rates per single year. The table shows an increase of 1200% in the detection rate of *Proteus* spp. from 2011 to 2020.

Finally, we evaluated the susceptibility/resistance of the detected strains to the tested classes of antibiotics active against Gram-negative bacteria, except for colistin, to which all *Proteus* spp. are naturally resistant. Figure 4 shows the percentages of resistance per single antibiotic class. Regarding penicillins, cephalosporins and quinolones, we further stratified the resistance results (Table 3). Furthermore, we evaluated the trend in antibiotic resistance of the detected strains during the considered period (Figure 5).

DISCUSSION

Proteus spp. are resistant, adaptable, and potentially pathogenic components of the human gastrointestinal microbiota. Most commonly, these bacteria can cause UTIs as clinical manifestations, especially in women aged 20-50 years. While in healthy subjects, *Proteus* spp. causes 1-2% of all UTIs (the second cause of UTIs after *E. coli*), in hospitalized patients it accounts for 5%. However, CAUTIs can be caused by *Proteus* spp. in a much higher percentage, ranging from 20% to 45%, especially in catheterized patients. Some risk factors for UTIs by *Proteus* spp. are recognized,

including female sex, long duration of catheterization, inappropriate catheter cleaning or care, sexual activity, unprotected anal sex in men, and immunodeficiency (CD4+ count <200/μL) (Jamil *et al*., 2020).

In this paper, we evaluated the detection rates and the antibiotic susceptibility of *Proteus* spp. strains isolated from clinical specimens collected in a large private healthcare facility. First, we must emphasize the significant increase in the number of samples sent to our laboratory, as confirmed by the percentage increase of 198.1% from 2011 to 2020, despite a parallel decrease in hospital admissions over the same period. This finding suggests a significant improvement in the awareness of healthcare workers concerning the importance of more and closer cooperation with clinics and laboratories in correct patient management, an outcome also derived from a series of informative meetings held over time with healthcare personnel. It is important to highlight that the decrease in the sent samples observed in 2020 is only the result of the COVID pandemic, since most of the facility was converted into a structure for the management of low complexity patients affected by the disease.

Our results highlight a clear and significant increase in *Proteus* spp. Detection, with rates ranging from 1.9% to 8.1% in 2011 and 2020, respectively, and a relative percentage increase of 1200%. As expected, the most-isolated species was *P. mirabilis*. The majority of patients were women in their seventies, often suffering from diabetes and carriers of urinary catheter. Therefore, our results confirmed some of the well-known risk factors for this kind of infection. Probably, the observed significant increase in the *Proteus* spp. detection rate is secondary to an increase in this particular category of patients. At the same time, we evaluated the antibiotic susceptibility of the isolated strains over time. Specifically, we observed in general a very high resistance level to macrolides, tetracyclines, fusidic acid, and mupirocin, while lower resistance levels were found for carbapenems, monobactams, aminoglycosides, and rifampicin. A medium level of resistance was found for all the other tested classes. These results suggest that some important antibiotic resistance concerns, especially regarding carbapenems found in other members belonging to the *Enterobacteriaceae* family, such as *Klebsiella pneumoniae*, have interested *Proteus* spp. relatively little in this studied context. Moreover, concerning penicillins, cephalosporins and quinolones, three of the most used antibiotics, we found a much higher resistance level in the oldest members of these categories. Indeed, tested ureidopenicillins (mezlocillins and piperacillins), III generation cephalosporins (cefotaxime, ceftazidime, and ceftriaxone) and III generation quinolones (ciprofloxacin, levofloxacin, and cinoxacin) showed the lowest resistance levels in their respective class.

However, the antibiotic resistance level found in the detected strains has remarkably increased over time, ranging from 48.4% in 2011 to 74.0% in 2020. This result highlights the ease with which

antibiotic resistance spreads in a health community and how crucial it is to stress the importance of preventing it through the good practice of antibiotic stewardship and, in general, all the measures that can be applied to counteract this critical issue. Moreover, it is important to try to reduce some risk factors, among which are diabetes and catheterization. Previous studies have shown that the presence of a catheter is associated with a 3-10% risk of bacterial infection per day (Nicolle, 2005). Therefore, catheterization should be avoided whenever possible, and when it cannot be avoided, the permanence should be limited and a correct catheter management technique is needed. Specifically, it is recommended that catheters be changed regularly when they cannot be removed (Saint *et al.*, 2016).

The disinfection of hands (Stilo *et al.*, 2016; Piscitelli *et al.*, 2020), operating theaters (Laganà *et al*., 2015a; Laganà *et al.*, 2017), invasive instruments such as endoscopes (Cristina *et al.*, 2018), and of the general hospital environment (Laganà *et al.*, 2014; Laganà *et al.*, 2015b; Brusaferro *et al.*, 2018) are particularly recommended practices, as it has been well demonstrated how the environment surrounding the patient and healthcare professionals themselves affects the spread of antibiotic-resistant bacterial strains (Facciolà *et al.*, 2019).

Future perspectives are under consideration by various research groups. Many natural compounds with antimicrobial activity, such as honey, citrus essential oils and polyphenols, have been studied to counteract antibiotic resistance (Coniglio *et al.*, 2018; Laganà *et al.*, 2019; Mancuso *et al.*, 2019). Some authors highlight the possibility of using some bacteriophages to hinder the production of biofilm on catheters by *P. mirabilis* in order to prevent CAUTIs (Maszewka *et al.*, 2018; Yazdi *et al.*, 2018; Gomaa *et al.*, 2019). Moreover, various new materials with intrinsic antibiotic activities are being studied for the production of catheters and hospital surfaces (MacPhee *et al.*, 2019; Mancuso *et al.*, 2019; Wang *et al*., 2019; Tailly *et al*., 2020; Laganà *et al*., 2021).

CONCLUSIONS

In conclusion, in recent years the microorganisms belonging to the *Proteus* genus have acquired considerable importance as a cause of HAIs alongside more classic Gram-negative germs such as *Pseudomonas aeruginosa*, *Acinetobacter baumanni*, and *Klebsiella pneumoniae*. In the hospital setting, the antibiotic resistance of these microorganisms is increasing over time, and this could become a serious threat, especially for some categories of patients in critical clinical conditions. Best clinical practices and infection control procedures are the cornerstone for the fight against these kinds of infection.

Conflicts of interest: The authors declare no conflict of interest.

Institutional Revier Board statement: Ethical review and approval were waived for this study because the data were processed on anonymous databases and no author (except the Head of the Laboratory) was aware of sensitive data.

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Table 1: List of antibiotics used to determine the antibiotic resistance patterns of the bacterial isolates

CELL WALL INHIBITING AND DISRUPTING MEMBRANE ANTIBIOTICS	**B-LACTAMS**	**PENICILLINS**	*Natural penicillins*	*penicillin (P, 1 unit,)*
			Aminopenicillins	*amoxicillin (AML, 10 µg)*
				ampicillin (AMP, 10 µg)
			Carboxipenicillins	*carbenicillin (CAR, 100 µg)*
			Ureidopenicillins	*mezlocillin (MEZ, 75 µg)*
				piperacillin (PRL, 100 µg)
			Penicillinase-resistant penicillins	*oxacillin (OX, 1 µg)*
				meticillin (MET, 5µg)
			Combinated penicillins	*amoxicillin + clavulanic acid (AMC, 30 µg)*
		CEPHALOSPORINS	*1st generation*	*cefazolin (KZ, 30 µg)*
			2nd generation	*cefoxitin (FOX, 30 µg)*
				cefuroxime (CXM, 30 µg)
			3rd generation	*cefotaxime (CTX, 30 µg)*
				ceftazidime (CAZ, 30 µg)
				ceftriaxone (CRO, 30 µg)
		CARBAPENEMS	*imipenem* (IMI, 10 µg)	
		MONOBACTAMS	*aztreonam* (AZM, *30 µg)*	
	GLICOPEPTIDES	*vancomycin (VAN, 30 µg)*		
		teicoplanin (TEC), 30 µg)		
NUCLEIC ACIDS INHIBITING ANTIBIOTICS	**INHIBITING DNA TOPOISOMERASIS ANTIBIOTICS**		**QUINOLONES**	*cinoxacin (CIN, 100 µg)*
				nalidixic acid (NA, 30 µg)
				pipemidic acid (PI, 20 µg)
			FLUORQUINOLONES	*ciprofloxacin (CIP, 5 µg)*
				levofloxacin (LEV, 5 µg)
				norfloxacin (NOR, 10 µg)
				ofloxacin (OFX, 5 µg)
	INHIBITING FOLIC ACID SYNTHESIS ANTIBIOTICS		**SULFONAMIDES**	*sulphamethoxazole + trimethoprim* (SXT, 25 µg)
	INHIBITING RNA SYNTHESIS ANTIBIOTICS		**RIFAMYCINS**	*rifampicin* (RD, 30 µg)
	DNA INHIBITORS ANTIBIOTICS		**NITROFURANS**	*nitrofurantoin* (F, 300 µg)
PROTEIN SYNTHESIS INHIBITING ANTIBIOTICS	**30S SUBUNIT INHIBITORS**		**AMINOGLYCOSIDES**	*amikacin* (AK, 30 µg)
				gentamycin (CN, 10 µg)
				netilmicin (NET, 30 µg)
				sisomicin (SIS, 30 µg)
				tobramycin (TOB, 10 µg)
			TETRACYCLINES	*doxycyclin* (DO, 30 µg)
				tetracycline (TE, 30 µg)
				minocycline (MN, 30µg)
			GLYCYLCYCLINES	*tigecycline (TGC, 15 µg)*
	50S SUBUNIT INHIBITORS		**MACROLIDES**	*azithromycin* (AZM, 15 µg)
				eritromycin (E, 15 µg)
			LINCOSAMIDES	*lincomycin* (MY, 2 µg)
				clindamycin (DA,10 µg)
			OXAZOLIDINONES	*linezolid* (LNZ, 10 µg)
			PHENOLIC DERIVATIVES	*chloramphenicol* (C, 30 µg)

Table 2: Detection rates of Proteus spp. in the considered years

Years	Total analysed samples	*Proteus* spp-positive samples (%)
2011	54	1 (1.9)
2012	107	2 (1.9)
2013	143	4 (2.8)
2014	146	3 (2.1)
2015	149	9 (6.0)
2016	201	6 (3.0)
2017	182	8 (4.4)
2018	215	12 (5.6)
2019	242	13 (5.4)
2020	161	13 (8.1)
TOT	1,600	71 (4.4)

Table 3. Percentage of resistance to the main classes of penicillins, cephalosporins and quinolones.

PENICILLINS	Percentage of resistance
Aminopenicillins	77.6
Carbossipenicillins	70.0
Ureidopenicillins	54.9
CEPHALOSPORINS	
I gen. cephalosporins	76.9
II gen. cephalosporins	51.3
III gen. cephalosporins	50.9
QUINOLONES	
I gen. quinolones	76.7
II gen. quinolones	62.2
III gen. quinolones	53.6

Figure 1. Trend of samples sent during the considered period.

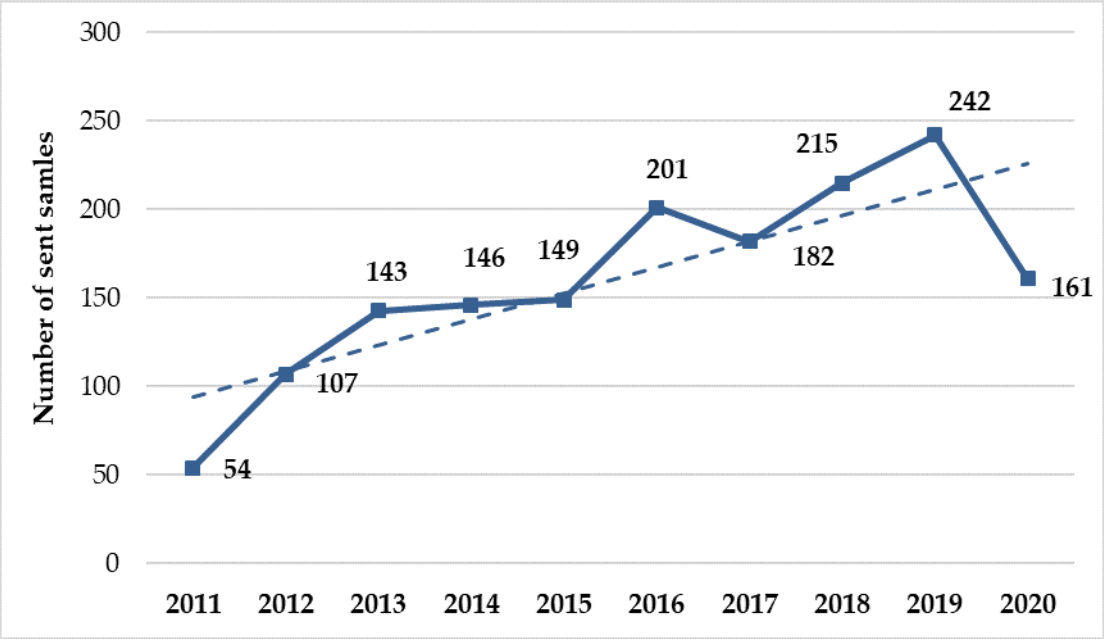

Figure 2. Comparison between trend of hospital admissions and that of sent samples.

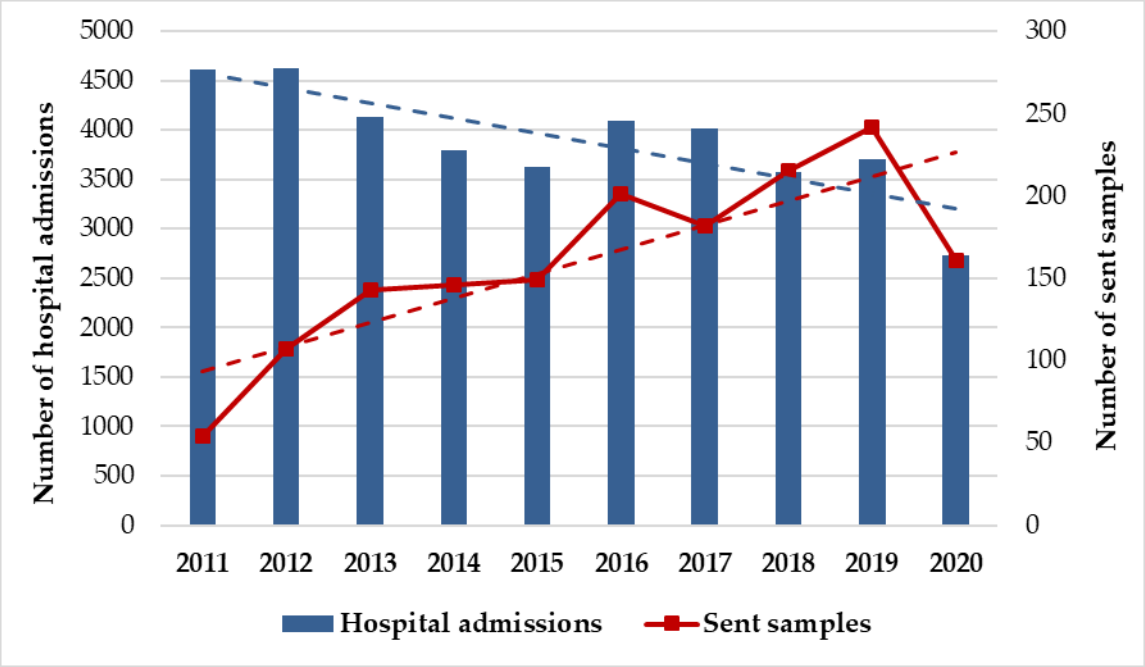

Figure 3. Different clinical sample categories in which *Proteus* spp. was detected.

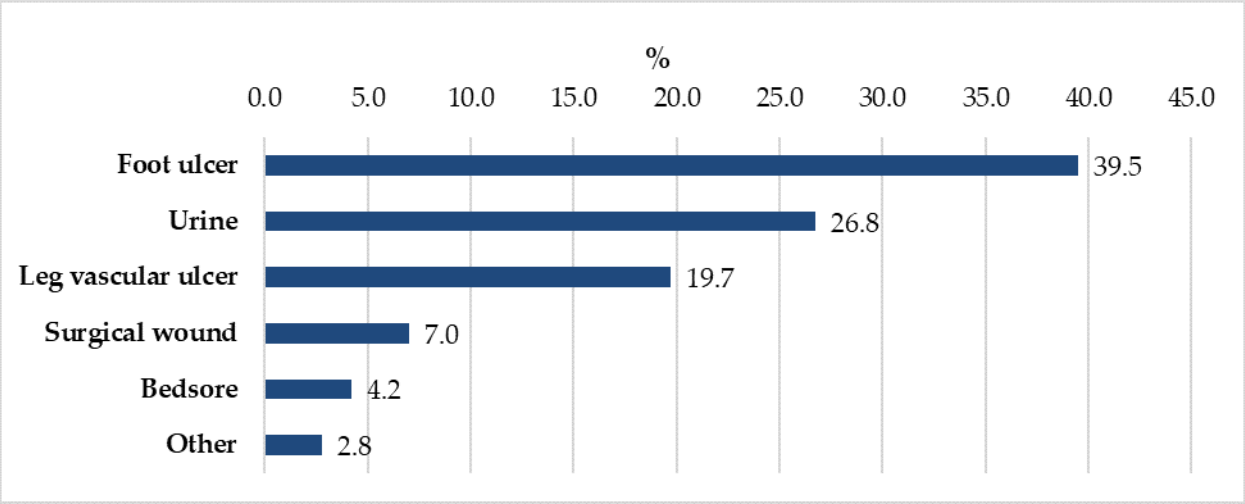

Figure 4. Percentages of resistance showed by detected strains to tested classes of antibiotics.

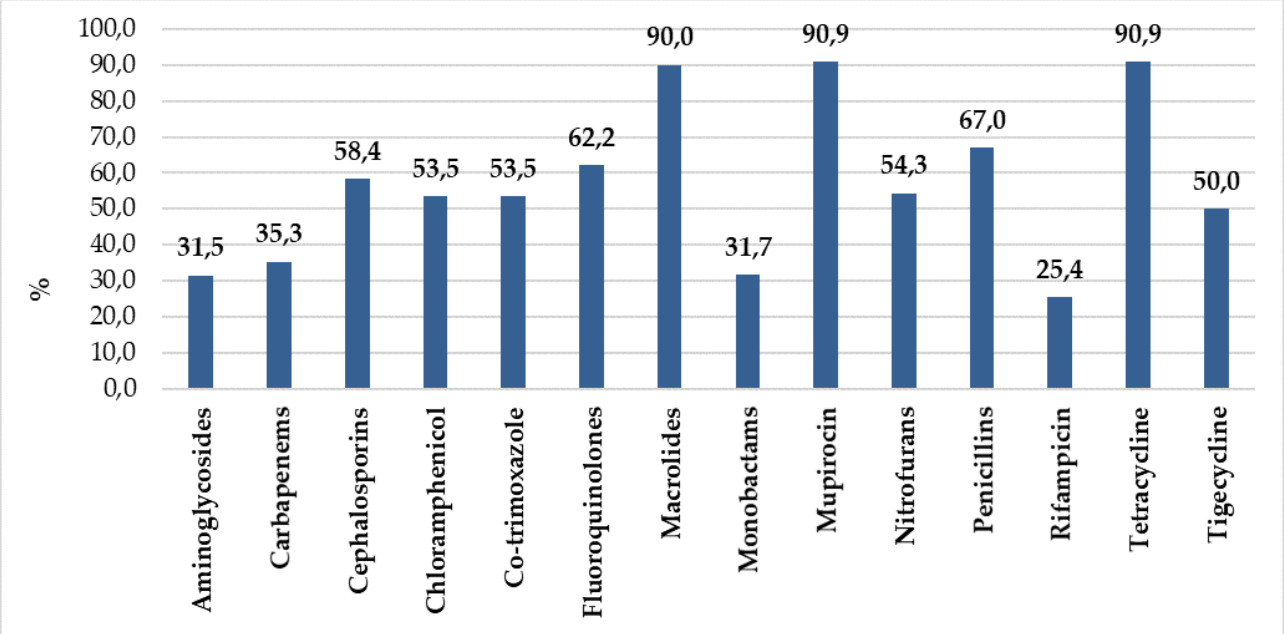

Figure 5. Trend of antibiotic resistance of detected strains during the considered period.