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## ANTIMICROBIAL SUSCEPTIBILITY OF *RIEMERELLA ANATIPESTIFER* STRAINS ISOLATED FROM GEESE AND DUCKS IN HUNGARY

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*Riemerella anatipestifer* causes anatipestifer disease in many avian species. A total of 185 *R. anatipestifer* strains isolated in Hungary between 2000 and 2014 from geese and ducks were tested against 13 antibiotics (ampicillin, doxycycline, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, penicillin, spectinomycin, streptomycin, sulphamethoxazole–trimethoprim, sulphonamide compounds, and tetracycline) by the Kirby-Bauer disk diffusion method. The majority of the strains were susceptible to florfenicol (97.9%), ampicillin (95.1%), penicillin (93%), sulphamethoxazole–trimethoprim (92.4%), and spectinomycin (86.5%). The highest resistance rates were observed for flumequine, tetracycline, erythromycin and streptomycin (94%, 91.4%, 75.1% and 71.4% resistance, respectively). The resistance patterns showed some variation depending on the geographical origin of the strains. The average rate of extensive drug resistance was 30.3%, and its proportion tended to increase in the period examined.

**Key words:** *Riemerella anatipestifer*, antimicrobial susceptibility

Anatipestifer disease caused by *Riemerella anatipestifer* infection is prevalent in all countries where intensive goose and duck production is practised. It causes significant economic losses through high mortality, reduced growth rate and the costs of treatment and prevention (Ruiz and Sandhu, 2013). Since its first description in geese by Riemer (1904), the disease has been reported regularly, but the taxonomic position of the aetiological agent remained uncertain for many years (Hendrickson and Hilbert, 1932; Bruner and Fabricant, 1954; Breed et al., 1957). Finally Segers et al. (1993) named the agent *R. anatipestifer* and suggested its transfer to a separate genus (*Riemerella*) based on phenotypic and genotypic characteristics.

*Riemerella anatipestifer* causes anatipestifer disease primarily in young, 2- to 8-week-old goslings and ducklings; however, severe losses have been reported

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in turkey flocks as well (Helfer and Helmboldt, 1977; Smith et al., 1987; Frommer et al., 1990). The disease has also been described in chickens, pheasants, guinea fowl, quails, gulls, budgerigars, and wild waterfowl (Bruner et al., 1970; Karstad et al., 1970; Rosenfeld, 1973; Pascucci et al., 1989; Hinz et al., 1998).

The clinical signs of anatipestifer disease include nasal discharge, coughing, sinusitis, diarrhoea and neural signs such as torticollis, head tremor and lameness (Bisgaard et al., 2008; Fulton and Rimler, 2010). The gross lesions are similar in different avian species and include a slightly enlarged spleen, fibrinous pericarditis, perihepatitis, airsacculitis, catarrhal rhinitis, pneumonia, purulent arthritis, catarrhal enteritis, as well as caseous exudate in the oviducts, skin necrosis and serous-fibrinous meningitis (Dougherty et al., 1955; Smith et al., 1987; Bisgaard et al., 2008). The disease may persist for two weeks without appropriate treatment, and mortality may vary from 10% to 75% (Ruiz and Sandhu, 2013).

In Hungary, anatipestifer disease was first described by Bitay et al. (1979) in ducks, while Ivanics et al. (1996) reported its first occurrence in geese.

Twenty-one serotypes of *R. anatipestifer* are known to date, and no cross-protection has been observed between the different serotypes. In addition, more than one serotype can be present in the same farm (Ruiz and Sandhu, 2013). These facts underline the importance of antibiotics in controlling anatipestifer disease.

The aim of this study was to determine the antibiotic susceptibility patterns of 185 *R. anatipestifer* strains against 13 antibiotics.

## Materials and methods

### *Bacterial strains*

A total of 185 *R. anatipestifer* strains were isolated from 2000 to 2014 from 100 flocks of geese or ducks located at 48 settlements of six counties in Hungary (Fig. 1).

All strains were recovered from 5- to 140-day-old birds that had died of anatipestifer disease. Samples taken from the liver, brain, pericardium or lung were cultured on Columbia agar plates (Biolab, Budapest, Hungary) supplemented with 5% defibrinated sheep blood at 37 °C for 24 h in an atmosphere containing 5% CO<sub>2</sub>. A total of 158 strains originated from geese and 27 strains from ducks. The strains were identified by colony morphology, Gram staining and a species-specific polymerase chain reaction (PCR) (Kardos et al., 2007). All isolates were stored at -70 °C until analysed. Table 1 shows the properties of the *R. anatipestifer* strains used in this study.



Fig. 1. Geographic origin of the *Riemerella anatipestifer* strains used in this study

### *Susceptibility testing*

Antibiotic susceptibility was assessed by the Kirby–Bauer disk diffusion method. The isolates were tested for susceptibility to 13 antibiotics: ampicillin (10 µg) [equivalent to the recently used amoxicillin (10 µg)], doxycycline (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), flumequine (30 µg), gentamicin (10 µg), penicillin (10 IU), spectinomycin (100 µg), streptomycin (10 µg), sulphamethoxazole–trimethoprim (23.75 + 1.25 µg), sulphonamide compounds (300 µg), and tetracycline (30 µg) (Biolab, Budapest, Hungary).

The tests were carried out according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013, 2015). Strains were grown on Columbia agar plates supplemented with 5% defibrinated sheep blood at 37 °C in 5% CO<sub>2</sub> atmosphere. Colonies were suspended in 0.85% saline, and the turbidity was adjusted to 0.5 McFarland standard. The disk diffusion analysis was performed on Mueller–Hinton agar (Biolab, Budapest, Hungary) enriched with 5% sheep blood. Inhibitory zone diameters were measured after 24 h of incubation. The resistance breakpoints (Table 2) were interpreted according to the criteria provided by CLSI documents M100-S21 and VET01S (CLSI, 2013, 2015) and the National Food Chain Safety Office, Veterinary Diagnostic Directorate (Budapest, Hungary).

**Table 1**  
Properties of the *Riemerella anatipestifer* strains used in this study

Geographic origin (county)	Host	Year of isolation	Number
Bács-Kiskun	goose	2000	8
		2006	11
		2010	24
		2011	12
		2012	28
		2013	21
		2014	24
	duck	2006	3
		2010	1
		2011	7
		2012	2
		2013	6
		2014	3
Békés	goose	2014	2
	duck	–	–
Csongrád	goose	2010	3
		2011	4
		2012	2
		2013	1
		2014	2
	duck	2013	1
		2014	2
	Hajdú-Bihar	goose	2009
2010			1
2011			1
duck		2014	1
Jász-Nagykun-Szolnok	goose	2004	1
		2010	1
		2013	5
	duck	2013	1
Pest	goose	2012	1
		2013	1
		2014	4
	duck	–	–

**Table 2**  
Resistance breakpoints of the antibiotics used in this study

Antibiotic	Zone diameter (mm)		
	Interpretive criteria		
	Susceptible	Intermediate	Resistant
Penicillin	≥ 24	–	≤ 23
Ampicillin	≥ 24	–	≤ 23
Gentamicin	≥ 15	13–14	≤ 12
Streptomycin	≥ 15	12–14	≤ 11
Spectinomycin	≥ 17	16	≤ 15
Enrofloxacin	≥ 23	17–22	≤ 16
Flumequine	≥ 24	20–23	≤ 19
Tetracycline	≥ 23	–	≤ 22
Doxycycline	≥ 23	–	≤ 22
Erythromycin	≥ 23	14–22	≤ 13
Sulphonamide compounds	≥ 17	13–16	≤ 12
Sulphamethoxazole–trimethoprim	≥ 16	11–15	≤ 10
Florfenicol	≥ 19	15–18	≤ 14

## Results

The majority of the strains were susceptible to florfenicol (97.9%), ampicillin (95.1%), penicillin (93%), sulphamethoxazole–trimethoprim (92.4%) and spectinomycin (86.5%). Enrofloxacin and sulphonamide compounds proved to be less effective (26% and 27% resistance, respectively), although the rate of intermediate susceptibility was unusually high for enrofloxacin (60%). Approximately 50% of the strains were resistant to gentamicin and doxycycline. The highest resistance rates were observed for flumequine, tetracycline, erythromycin and streptomycin (94%, 91.4%, 75.1% and 71.4% resistance, respectively) (Fig. 2).

Changes in the antibiotic resistance profiles of the strains over time were also evaluated (Table 3). A tendency of increase was noted in the rate of resistance to erythromycin, florfenicol, spectinomycin and streptomycin from 2000, sulphonamide compounds from 2004, and doxycycline from 2009. The rate of resistance to other antibiotics showed a variable tendency.

We examined the resistance pattern of four strains isolated within three weeks from one outbreak in a farm (Table 4). The strains displayed an expanding resistance pattern, showing resistance to 4, 4, 5 and finally 7 antibiotics out of the 13 antibiotics examined.

Strains isolated from the north-western part of Bács-Kiskun County showed the highest resistance rate to doxycycline, gentamicin, spectinomycin, sulphamethoxazole–trimethoprim, sulphonamide compounds and tetracycline.

Strains collected from the south-western part of Bács-Kiskun County possessed the lowest resistance rate to flumequine, gentamicin, spectinomycin and streptomycin (Table 5).

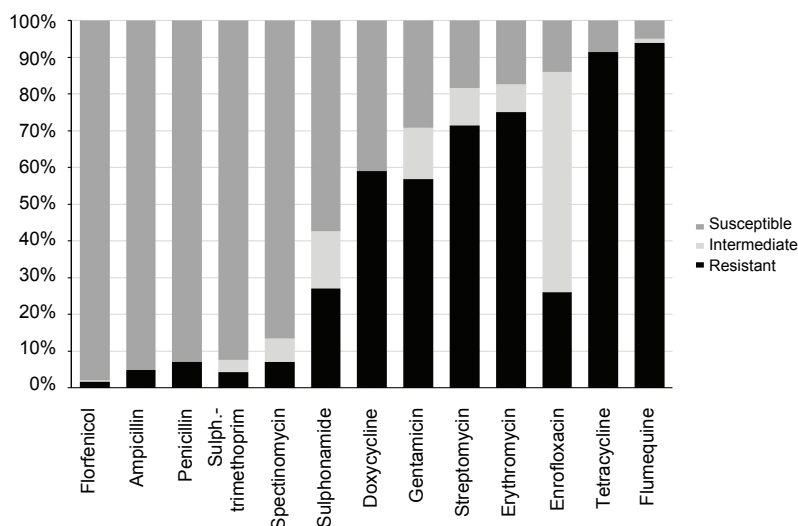


Fig. 2. Antimicrobial susceptibility of 185 *Riemerella anatipestifer* strains tested against 13 different antibiotics

Strains non-susceptible to at least one agent in all but two or fewer antimicrobial categories were defined as extensively drug-resistant (XDR) strains. Non-susceptibility refers to either a resistant, intermediate or non-susceptible result obtained from *in vitro* antimicrobial susceptibility testing (Magiorakos et al., 2012). The average rate of extensive drug resistance was 30.3% for all isolates. No XDR strain was found in 2000. Isolates collected between 2004 and 2006 showed 6.6% extensive drug resistance. The proportion of extensive drug resistance was 38.7% for strains isolated from 2009 to 2010. Isolates collected from 2011, 2012, 2013 and 2014 showed an increasing tendency of extensive drug resistance: 20.8%, 18%, 30.5% and 55.3%, respectively.

The rate of extensive drug resistance showed some variance according to the geographical origin of the strains. The proportion of extensive drug resistance was above average for strains isolated from the north-western part of Bács-Kiskun County and Csongrád County: 36.3% and 40%, respectively. Isolates collected from the north-eastern and south-western part of Bács-Kiskun County showed 31.4% and 31.2% extensive drug resistance, respectively. The proportion of extensive drug resistance among strains isolated from the south-eastern part of Bács-Kiskun County was below average (28.3%).

The proportion of XDR strains isolated from geese and ducks was 31% and 26%, respectively. The rate of resistance to different antibiotics was irrespective of the host species.

**Table 3**  
 Variance of antimicrobial resistance of *Riemerella anatipestifer* strains by the year of isolation

Antibiotic	Resistant, % (number of strains)						
	2000 n = 8	2004–2006 n = 15	2009–2010 n = 31	2011 n = 24	2012 n = 33	2013 n = 36	2014 n = 38
Penicillin	0 (0)	0 (0)	29 (9)	8.3 (2)	6.1 (2)	0 (0)	0 (0)
Ampicillin	0 (0)	0 (0)	22.6 (7)	8.3 (2)	0 (0)	0 (0)	0 (0)
Gentamicin	75 (6)	6.7 (1)	83.9 (26)	83.4 (20)	15.2 (5)	52.8 (19)	73.7 (28)
Streptomycin	0 (0)	6.7 (1)	87.2 (27)	79.2 (19)	90.9 (30)	80.6 (29)	68.4 (26)
Spectinomycin	0 (0)	0 (0)	0 (0)	0 (0)	15.2 (5)	13.9 (5)	7.9 (3)
Enrofloxacin	25 (2)	13.3 (2)	51.6 (16)	37.5 (9)	18.2 (6)	16.7 (6)	18.4 (7)
Flumequine	100 (8)	80 (12)	100 (31)	91.7 (22)	93.9 (31)	94.5 (34)	94.7 (36)
Tetracycline	100 (8)	80 (12)	90.3 (28)	79.2 (19)	100 (33)	91.7 (33)	94.7 (36)
Doxycycline	87.5 (7)	80 (12)	13 (4)	29.2 (7)	69.7 (23)	75 (27)	76.3 (29)
Erythromycin	0 (0)	73.3 (11)	83.9 (26)	62.5 (15)	78.8 (26)	94.4 (34)	71 (27)
Sulphonamide compounds	62.5 (5)	6.7 (1)	25.8 (8)	12.5 (3)	12.1 (4)	22.2 (8)	55.3 (21)
Sulphamethoxazole–trimethoprim	12.5 (1)	13.3 (2)	0 (0)	0 (0)	0 (0)	5.5 (2)	7.9 (3)
Florfenicol	0 (0)	0 (0)	0 (0)	4.1 (1)	3 (1)	2.8 (1)	0 (0)

**Table 4**  
Emergence of the antibiotic resistance of *Riemerella anatipestifer* strains isolated in one outbreak

Isolate ID	Date of isolation	Antibiotic										Resis- tant	Suscep- tible	Inter- medi- ate				
		P	Amp	Gm	S	Spt	Enr	Fim	Te	Do	E				S	Sxt	Ffc	
RA127	05.02.2013	S	S	S	R	S	I	R	R	R	S	R	S	S	S	4	8	1
RA122	12.02.2013	S	S	I	R	S	I	R	R	R	S	R	S	S	S	4	7	2
RA125	25.02.2013	S	S	S	R	S	I	R	R	R	R	R	S	S	S	5	7	1
RA126	28.02.2013	S	S	I	R	R	R	R	R	R	R	R	S	S	S	7	5	1

Abbreviations: P: penicillin, Amp: ampicillin, Gm: gentamicin, S: streptomycin, Spt: spectinomycin, Enr: enrofloxacin, Fim: flumequine, Te: tetracycline, Do: doxycycline, E: erythromycin, S: sulphonomide compounds, Sxt: sulphamethoxazole-trimethoprim, Ffc: florfenicol

**Table 5**

Variance of the antimicrobial resistance of *Riemerella anatipestifer* strains by geographical origin

Antibiotic	Resistant, % (number of strains)					
	North-western Bács-Kiskun n = 22	North-eastern Bács-Kiskun n = 35	South-western Bács-Kiskun n = 32	South-eastern Bács-Kiskun n = 53	Csongrád n = 15	
Penicillin	9 (2)	11.4 (4)	9.4 (3)	5.7 (3)	6.7 (1)	
Ampicillin	4.6 (1)	11.4 (4)	6.2 (2)	3.8 (2)	0 (0)	
Gentamicin	77.2 (17)	57.1 (20)	34.4 (11)	56.6 (30)	66.7 (10)	
Streptomycin	86.3 (19)	88.6 (31)	56.2 (18)	75.5 (40)	73.4 (11)	
Spectinomycin	22.7 (5)	8.6 (3)	3.1 (1)	3.8 (2)	6.7 (1)	
Enrofloxacin	4.6 (1)	22.9 (8)	31.3 (10)	30.2 (16)	46.7 (7)	
Flumequine	95.4 (21)	97.1 (34)	84.4 (27)	94.3 (50)	100 (15)	
Tetracycline	95.4 (21)	94.3 (33)	93.8 (30)	88.7 (47)	86.6 (13)	
Doxycycline	68.2 (15)	42.9 (15)	59.4 (19)	51 (27)	60 (9)	
Erythromycin	68.2 (15)	82.9 (29)	78.2 (25)	81.2 (43)	80 (12)	
Sulphonamide compounds	40.9 (9)	14.3 (5)	25 (8)	22.6 (12)	26.7 (4)	
Sulphamethoxazole-trimethoprim	9 (2)	0 (0)	3.1 (1)	5.7 (3)	0 (0)	
Florfenicol	0 (0)	2.9 (1)	6.2 (2)	0 (0)	0 (0)	



## Discussion

Due to the lack of a vaccine showing cross-protection among different serotypes of *R. anatipestifer*, the frequent change of serotypes in farms and the common presence of more than one serotype in a farm, antibiotics are still important tools for controlling anatipestifer disease. On the other hand, the threat of emergence of antibiotic resistance makes it extremely important to monitor bacterial pathogens for antibiotic susceptibility.

*Riemerella anatipestifer* strains in this study were generally sensitive to penicillin, with only 7% of the strains proving to be resistant. Earlier studies reported similar findings in Thailand and Taiwan (Pathanasophon et al., 1994; Chang et al., 2003); however, about five years later 58% of the strains showed resistance to penicillin in Taiwan (Yu et al., 2008). During the same period, Chinese authors also observed a high (86.9%) resistance rate to penicillin (Zhong et al., 2009). Ampicillin was also found effective in this study. Interestingly, isolates in China showed remarkable changes in resistance to this antibiotic over time: 58.4% of the strains were resistant in 2009, all strains were sensitive in 2012, and 93% of the strains were resistant in 2015 (Zhong et al., 2009; Sun et al., 2012; Zheng et al., 2015).

Among aminoglycosides and aminocyclitols, spectinomycin was the most effective agent (only 7% resistance), while gentamicin and streptomycin showed higher resistance rates (56.8% and 71.4%, respectively). Aminoglycosides and aminocyclitols are poorly, or not at all, absorbed from the gut, and thus the oral administration of these antibiotics is not recommended in cases of bacterial septicemia (Gálfi et al., 2012). Chinese authors observed a similar tendency of resistance to spectinomycin (11.3%), gentamicin (20.8%), and streptomycin (42.5%) (Zhong et al., 2009). At the same time, strains isolated in Taiwan proved to be highly resistant to all these aminoglycosides and aminocyclitols (Chang et al., 2003). German authors also observed over 90% resistance to gentamicin (Köhler et al., 1995). These findings indicate that there is a variable degree of cross-resistance among aminoglycosides, presumably because of the existence of diverse bacterial enzymes that can inactivate these antibiotics.

Twenty-six percent of our *R. anatipestifer* strains were resistant to enrofloxacin (a Class 2 second-generation fluoroquinolone), while a surprisingly high percent of the isolates (60%) showed intermediate susceptibility to this agent. Flumequine (a Class 1 second-generation fluoroquinolone) proved to be less effective (94% resistance). Authors in Taiwan made a similar observation: 83% of the local strains were susceptible to enrofloxacin, while 81% of them were resistant to flumequine (Yu et al., 2008). In our study, all but 22 out of the 174 flumequine-resistant strains showed decreased sensitivity to enrofloxacin (there were 47 resistant and 105 intermediately susceptible strains). These data support the observation that a fluoroquinolone of advanced generation such as enrofloxacin

could break through the resistance to flumequine, although its sensitivity often decreases after a while (Gálfi et al., 2012).

The rate of resistance to tetracycline was high (91.4%) among the Hungarian strains, while resistance to doxycycline occurred in a lower percentage (59%). Strains from Taiwan showed a similar trend: 69% of the isolates were resistant to tetracycline, whereas only 35% of the strains proved to be resistant to doxycycline (Yu et al., 2008). Chinese *R. anatipestifer* strains were more sensitive: the ratio of resistance was 46.6% to tetracycline and 24.4% to doxycycline (Zhong et al., 2009). This tendency is consistent with the fact that cross-resistance exists among tetracyclines, although some members of this group (like doxycycline or minocycline) can break through this resistance.

Up to 75.1% of our strains were resistant to erythromycin. The resistance rate in other countries varied: Chinese authors found 32.7% resistance (Zhong et al., 2009), while authors from Taiwan observed 64% resistance (Yu et al., 2008).

We found only 1.6% resistance to florfenicol, in accordance with data reported from China (Sun et al., 2012).

Twenty-seven percent of our strains were resistant to sulphonamide compounds, while only 4.3% showed resistance to sulphamethoxazole–trimethoprim. Sun et al. (2012) found that Chinese isolates were resistant to sulphonamide, while two thirds of the strains were susceptible to sulphamethoxazole–trimethoprim. These data suggest that the sulphamethoxazole–trimethoprim combination is still effective.

We have also evaluated the changes of antibiotic resistance profiles over time. The rates of resistance to erythromycin, spectinomycin, streptomycin (2000–2014) and sulphonamide compounds (2004–2014) tended to increase. The rate of resistance to other antibiotics showed variable trends. Our results are in accordance with the results of Zhong et al. (2009), who found that the rates of resistance to piperacillin and cefoperazone increased and those to spectinomycin and aztreonam decreased from 1998 to 2005 in China, while no regular patterns were observed for other antibiotics. The authors speculated that the improper use of antibiotics might lead to higher antibiotic resistance rates. Other authors reported similar results, observing a high resistance rate of *R. anatipestifer* isolates from ducks, and explaining it by the overuse and improper application of antibiotics in duck farms (Sun et al., 2012). On the other hand, an increase in susceptibility may occur to antibiotics that had not been used for a while (Zhong et al., 2009).

The average rate of extensive drug resistance was 30.3%. The proportion of extensive drug resistance also tended to increase over time. For strains isolated in 2000, 2004–2006, 2009–2010, 2011, 2012, 2013 and 2014, the rate of extensive drug resistance was 0%, 6.6%, 38.7%, 20.8%, 18%, 30.5% and 55.3%, respectively. These results are in harmony with the findings of Köhler et al. (1995), who observed an increasing proportion of multidrug-resistant isolates over time.

The results obtained for our strains isolated from an outbreak within a three-week period support the assumption that local antibiotic usage may facilitate the emergence of antimicrobial resistance. These strains showed resistance against an increasing number of antibiotics (4 to 7). The history did not provide information about the antibiotics used for therapy; however, resistance emerged to antibiotics commonly used in poultry practice, such as doxycycline, enrofloxacin, gentamicin, and spectinomycin. Although this observation is based on only a few samples, it calls attention to a possible unfavourable side effect of antibiotic usage, the rapid spread of antibiotic resistance. This view is supported by Zhong et al. (2009), who described remarkable differences in resistance patterns that largely depended on antibiotic usage on various farms.

Chang et al. (2003) compared the micro-inhibitory concentrations of kanamycin against strains isolated in Taiwan and in the USA. They found significant differences between the two countries, most likely due to variations in their antibiotic usage. In our study, in conformity with data of the literature, resistance patterns and the proportion of XDR strains showed some correlation with their geographic origin. Again, the most probable reason is the dissimilarity in therapeutic practices.

The World Health Organization has ranked antibiotics according to their relevance in human medicine. The criteria for critically important antibiotics are (1) frequent use in human medicine; (2) an antibiotic that is the only one or one of a few alternative medicines; and (3) evidence of transmission of bacteria or genes to humans from non-human sources. Some agents from these antimicrobial classes belong to critically important antibiotics: aminoglycosides, ansamycins, carbapenems, third and fourth generation cephalosporins, glycopeptides, lipopeptides, macrolides, oxazolidinones, penicillins, quinolones, streptogramins, tetracyclines, and drugs used against mycobacterial diseases. Taking this into consideration when choosing antibiotics for the treatment of food-producing animals may help to maintain the effectiveness of antibiotics relevant in human medicine (Collignon et al., 2009).

The development of antibiotic resistance of pathogenic bacteria and the emergence of multidrug-resistant strains represent a growing threat to animal and human health. The overuse and abuse of antibiotics presumably lead to the selection of resistant bacteria. Our findings as well as data of the literature indicate that the resistance rate of bacterial strains has increased over the last decades. This underlines the significance of reducing the use of antibiotics and the importance of the prudent and correct application of appropriate antibiotics, based on accurate diagnosis and antimicrobial susceptibility testing.

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