

Temporal rise in beta-lactam and colistin resistance in equine reproductive *Escherichia coli*

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Summary

Antimicrobial resistance in equine-associated bacteria is an increasing concern within the One Health framework, particularly because horses may serve as reservoirs of resistant Gram-negative organisms with potential public health relevance. This study aimed to characterize β -lactam and colistin resistance patterns in *Escherichia coli* isolates recovered from vaginal swabs collected from mares with pneumovagina. A total of 187 vaginal swab specimens were examined by conventional bacteriological methods, and the antimicrobial susceptibility of the *E. coli* isolates recovered was evaluated by the Kirby-Bauer disk diffusion method. A total of 101 *E. coli* isolates were identified, including 83 non-haemolytic and 18 haemolytic isolates. Resistance was observed across multiple antimicrobial classes, and 97 isolates were resistant to between two and twelve antibiotics, whereas only four isolates showed resistance solely to penicillin. Over the study period, resistance to several β -lactams and colistin increased, with statistically supported temporal trends for cefquinome, ceftiofur, amoxicillin, and colistin after false discovery rate correction. Although amoxicillin/clavulanic acid resistance varied significantly across years, its increasing trend did not remain significant after correction. Notably, resistance patterns combining β -lactams and colistin became more frequent in the later years of the study. Taken together, these findings identify the reproductive tract of mares with pneumovagina as an emerging reservoir of multidrug-resistant *E. coli* and support the need for continued surveillance of clinically important β -lactam and colistin resistance in equine reproductive isolates.

Keywords: antimicrobial resistance, β -lactam resistance, colistin resistance, equine reproductive tract, *Escherichia coli*, One Health

Antimicrobial resistance (AMR) is widely recognized as one of the most serious global threats to human and animal health because it compromises the efficacy of antimicrobial agents and limits treatment options across medical disciplines (32). Among resistant Gram-negative bacteria, extended-spectrum β -lactamase (ESBL)- and AmpC-producing *E. coli* have become a major public health concern owing to their rapid international dissemination, clinical relevance, and capacity to acquire multidrug resistance traits (22). In the One Health context, the emergence and spread of resistant bacteria are shaped by interconnected antimicrobial use practices in humans, animals, and the environment, as well as by direct and indirect transmission pathways between these compartments (29). Horses are increasingly considered important

hosts in this interface because of their close contact with humans in sport, breeding, recreation, therapy, and husbandry, which may facilitate the maintenance and interspecies transmission of antimicrobial resistance determinants (9, 20).

β -lactam antibiotics remain central to the treatment of bacterial infections in equine medicine, but their continued utility is threatened by ESBL- and AmpC-producing *Enterobacterales*, which reduce susceptibility to extended-spectrum cephalosporins and related β -lactams. In horses, resistant *E. coli* and other *Enterobacterales* have been reported from fecal, hospital-associated, and reproductive sources, which indicates that equine populations may function not only as recipients of resistant bacteria, but also as reservoirs for their persistence and dissemination (18, 19, 33).

Nevertheless, most available studies have focused on fecal carriage, hospital environments, or broad reproductive microbiology datasets, whereas data specifically addressing resistance profiles of *E. coli* isolated from the mare reproductive tract remain comparatively limited, particularly in relation to resistance to clinically important β -lactams.

Colistin (polymyxin E) is regarded as a critically important last-resort antimicrobial for the treatment of severe infections caused by multidrug-resistant Gram-negative bacteria in human medicine. The identification of plasmid-mediated colistin resistance genes (*mcr*) has intensified concern because these determinants can spread horizontally among bacterial populations and may co-occur with other clinically important resistance genes (1). Although colistin itself is not routinely used in horses, polymyxin B is used in equine practice, and horses may also be exposed to colistin-resistant bacteria through environmental and feed-related pathways, including forage contaminated with resistant microorganisms originating from manure or other waste sources (5, 30, 31). From an epidemiological perspective, the investigation of colistin resistance in equine *E. coli* is therefore justified not only by therapeutic relevance, but also by its potential value as an indicator of mobile resistance dissemination within the broader One Health network.

Despite the growing recognition of antimicrobial resistance in equine-associated bacteria, information on concurrent β -lactam and colistin resistance in *E. coli* isolates recovered from vaginal swabs of mares remains limited. Generating such data is important for understanding the potential role of the equine reproductive tract as a niche for resistant bacteria and for informing antimicrobial stewardship in equine practice. Therefore, this study aimed to characterize β -lactam and colistin resistance patterns in *E. coli* isolates recovered from vaginal swabs of mares with pneumovagina.

Material and methods

Ethical approval. According to subparagraph (k) of Article 8 of the Regulation on the Principles and Procedures of Animal Experiment Ethics Committees, published in the Official Gazette dated 15 February 2014 and numbered 28914, samples obtained by swabbing do not require approval from a Local Ethics Committee for Animal Experiments.

Sampling. Between 2020 and March 2023, a total of 187 vaginal swab specimens were collected from 9 to 13-year-old Thoroughbred mares diagnosed with pneumovagina. The mares originated from different horse farms in the Marmara Region. The samples were submitted to the laboratory and processed on the day of collection. The samples were collected under sterile conditions using sterile phosphate-buffered saline or Stuart transport medium (Oxoid; CM0111). Before sampling, the rima vulvae and external vaginal region were cleaned and disinfected repeatedly with paper towels soaked in an alcohol-based antiseptic to minimize external contamination.

Bacteriological examination. Vaginal swabs were examined by conventional culture methods as described by Quinn et al. (23). For bacterial isolation, samples were inoculated onto 5% defibrinated blood agar (Oxoid; CM0271), Gas-sner agar (Merck; 1282), and serum broth. Culture media were incubated aerobically at 37°C for 24 to 48 h. Colonies showing growth characteristics compatible with enteric bacteria were subcultured for purification, and identification was performed using conventional biochemical tests together with API 20E strips (bioMérieux).

Antimicrobial susceptibility testing. Antimicrobial susceptibility of the *E. coli* isolates was evaluated by the Kirby-Bauer disk diffusion method (6). The following antimicrobial disks were used: penicillin (10 U) (P), tetracycline (30 μ g) (TE), erythromycin (15 μ g) (E), florfenicol (30 μ g) (FF), ceftiofur (30 μ g) (EFT), amoxicillin/clavulanic acid (20/10 μ g) (AMC), amoxicillin (10 μ g) (AML), enrofloxacin (5 μ g) (EN), gentamicin (10 μ g) (GM), cefquinome (30 μ g) (CEQ), colistin (25 μ g) (CO), marbofloxacin (5 μ g) (MAR), and sulfamethoxazole/trimethoprim (23.75/1.25 μ g) (SXT). Inhibition zone diameters were interpreted according to CLSI M100 criteria where applicable (11), while colistin results were treated as phenotypic screening data because of the known limitations of disk diffusion for colistin susceptibility testing.

Statistical analysis. Antimicrobial resistance data were summarized as counts and percentages. Because the primary outcomes were categorical resistance classifications and the main objective was to compare annual resistance frequencies and evaluate ordered temporal changes across study years, chi-square tests were selected as the primary statistical approach. Annual differences in resistance frequencies were assessed using chi-square tests across the study years, and linear temporal trends in resistance proportions were evaluated using chi-square tests for trend. Logistic regression or generalized linear models were not used as primary analyses, because the study had limited and uneven yearly sample sizes, including some strata with complete resistance or complete susceptibility, which could lead to sparse-data bias, unstable estimates, or separation. Therefore, the analyses were restricted to proportion-based tests, and the results were interpreted cautiously. Because penicillin resistance was observed in all isolates in every year, P was excluded from statistical testing. To account for multiple testing, Benjamini-Hochberg false discovery rate correction was applied across the 12 testable antibiotics. A focused analysis was performed for CEQ, EFT, AMC, AML, and CO because these antibiotics represented the main resistance targets of the study, namely clinically relevant β -lactam agents and colistin. FDR-adjusted *p* values < 0.05 were considered statistically significant.

Results and discussion

A total of 101 *E. coli* isolates were recovered from the vaginal swabs, including 83 non-haemolytic and 18 haemolytic isolates. Overall resistance rates during the study period were 100% for P, 32.67% for CEQ, 24.75% for MAR, 31.68% for TE, 51.48% for E, 22.77% for FF, 43.56% for EFT, 60.39% for AMC, 52.47% for AML, 27.72% for EN, 53.46% for GM, 24.75% for SXT, and 43.56% for CO.

When resistance rates were evaluated by year, P resistance was detected in all isolates throughout the study period. In 2020, resistance was highest for P [45/45 (100%)], followed by AMC [29/45 (64.44%)], E and GM [24/45 (53.33%)], and MAR, TE, FF, EN, and SXT [12/45 (26.66%)]. Resistance to CO was observed in 3/45 isolates (6.66%), whereas no resistance to CEQ, EFT, or AML was detected in 2020. In 2021, resistance remained 100% for P [27/27], followed by AML [24/27 (88.88%)], TE, EFT, and CO [15/27 (55.55%)], CEQ and GM [9/27 (33.33%)], EN [8/27 (29.62%)], AMC [6/27 (22.22%)], E [4/27 (14.81%)], and MAR, FF, and SXT [1/27 (3.70%)].

In 2022, resistance to P, EFT, and AML was detected in all isolates [21/21 (100%)]. AMC and CO showed resistance rates of 18/21 (85.71%), followed by CEQ and E [16/21 (76.19%)], GM [13/21 (61.90%)], SXT [7/21 (33.33%)], and MAR and FF [5/21 (23.80%)]. No resistance to TE or EN was detected in 2022. In 2023, all isolates were resistant to P, CEQ, E, EFT, AMC, AML, EN, GM, and CO [8/8 (100%)]. Resistance rates for MAR, and for TE, FF, and SXT were 7/8 (87.50%) and 5/8 (62.50%), respectively (Tab. 1). Penicillin resistance remained consistently high in all years, whereas resistance to CEQ, EFT, AMC, AML, and CO was more prominent in the later

Tab. 1. Antimicrobial resistance rates of *E. coli* isolates from vaginal swabs of mares (2020-2023)

<i>E. coli</i> isolates (n)	P	CEQ	MAR	TE	E	FF	EFT	AMC	AML	EN	GM	SXT	CO
2020													
NH (12)	R	I	S	S	R	S	S	I	I	S	R	S	S
NH (4)	R	S	S	S	I	S	S	I	I	S	S	S	S
NH (17)	R	S	S	S	S	S	S	R	I	I	S	S	S
H (9)	R	I	R	R	R	R	S	R	S	R	R	R	I
H (3)	R	S	R	R	R	R	S	R	S	R	R	R	R
Total (n) (%)	45 (100)	0 (0.00)	12 (26.66)	12 (26.66)	24 (53.33)	12 (26.66)	0 (0.00)	29 (64.44)	0 (0.00)	12 (26.66)	24 (53.33)	12 (26.66)	3 (6.66)
2021													
NH (9)	R	S	S	R	I	S	S	I	R	S	S	S	I
NH (9)	R	S	S	S	S	S	R	I	R	S	S	S	R
H (1)	R	R	R	R	R	R	R	R	R	I	R	R	R
NH (3)	R	R	S	S	R	S	I	I	I	R	R	S	I
NH (5)	R	R	S	R	I	S	R	R	R	R	R	S	R
Total (n) (%)	27 (100)	9 (33.33)	1 (3.70)	15 (55.55)	4 (14.81)	1 (3.70)	15 (55.55)	6 (22.22)	24 (88.88)	8 (29.62)	9 (33.33)	1 (3.70)	15 (55.55)
2022													
NH (3)	R	I	S	S	I	S	R	I	R	S	S	S	R
NH (11)	R	R	S	S	R	I	R	R	R	S	R	S	R
NH (2)	R	I	S	S	I	S	R	R	R	S	R	R	I
NH (1)	R	R	R	S	R	R	R	R	R	I	I	R	I
NH (4)	R	R	R	S	R	R	R	R	R	I	S	R	R
Total (n) (%)	21 (100)	16 (76.19)	5 (23.80)	0 (0.00)	16 (76.19)	5 (23.80)	21 (100)	18 (85.71)	21 (100)	0 (0.00)	13 (61.90)	7 (33.33)	18 (85.71)
2023													
H (5)	R	R	R	R	R	R	R	R	R	R	R	R	R
NH (2)	R	R	R	S	R	I	R	R	R	R	R	S	R
NH (1)	R	R	S	S	R	S	R	R	R	R	R	S	R
Total (n) (%)	8 (100)	8 (100)	7 (87.50)	5 (62.50)	8 (100)	5 (62.50)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	5 (62.50)	8 (100)
General (n) Total (%)	101 (100)	33 (32.67)	25 (24.75)	32 (31.68)	52 (51.48)	23 (22.77)	44 (43.56)	61 (60.39)	53 (52.47)	28 (27.72)	54 (53.46)	25 (24.75)	44 (43.56)

Explanations: n – number, N – non-haemolytic, H – haemolytic, R – resistant, S – susceptible, I – intermediate. AMC – amoxicillin-clavulanic acid, AML – amoxicillin, CEQ – cefquinome, CO – colistin, E – erythromycin, EFT – ceftiofur, EN – enrofloxacin, FF – florfenicol, GM – gentamicin, MAR – marbofloxacin, P – penicillin, TE – tetracycline, SXT – trimethoprim-sulfamethoxazole.

years, with several antimicrobials showing 100% resistance among the eight isolates recovered in 2023. Temporal trends in resistance to selected β -lactam agents and colistin are presented in Figure 1, which further illustrates increasing resistance to CEQ, EFT, AML, and CO from 2020 to 2023, with AMC showing year-associated variation.

Statistical analysis confirmed the temporal changes observed in the descriptive resistance data. Penicillin was excluded from statistical testing because resistance was detected in all isolates in every study year. After Benjamini-Hochberg false discovery rate correction, significant year-associated variation was detected for all 12 testable antibiotics. Among the focused antibiotics, CEQ, EFT, AMC, AML, and CO showed significant year-associated variation in resistance rates (FDR-adjusted $p < 0.0001$). In the trend analysis, significant FDR-adjusted increasing trends were observed for CEQ, EFT, AML, and CO (FDR-adjusted trend $p < 0.0001$). Although AMC showed significant variation across years, its increasing trend did not remain significant after FDR correction (FDR-adjusted trend $p = 0.0713$). Among the remaining antibiotics, only E showed a significant corrected temporal trend (FDR-adjusted trend $p = 0.0336$), whereas MAR, TE, FF, EN, GM, and SXT did not show significant FDR-adjusted trend effects.

Ninety-seven *E. coli* isolates were resistant to between two and twelve antibiotics, whereas four isolates showed resistance only to P (Tab. 2). The distribution of resistance patterns indicated increasing complexity over time, with later years characterized by broader multidrug resistance combinations. In particular, patterns including resistance to extended-spectrum β -lactam agents together with CO became more frequent from 2021 onwards and were most prominent in 2022 and 2023.

Resistance patterns involving β -lactams (CEQ, EFT, AMC, and AML) together with CO became more prominent over the study period (Tab. 1). With respect to the distribution of β -lactam- and/or colistin-associated resistance patterns over time, the P, P/AMC, and P/AMC/CO patterns were detected in 2020. In 2021, the P/AML, P/CEQ, P/EFT/AML/CO, and P/CEQ/EFT/AMC/AML/CO patterns were observed. The P/EFT/AML/CO and P/CEQ/EFT/AMC/AML/CO patterns, identified in 9 and 6 *E. coli* isolates in 2021, were also detected in 3 isolates in 2022. In 2023, the P/EFT/AMC/AML and P/CEQ/EFT/AMC/AML/CO patterns were identified. The P/CEQ/EFT/AMC/AML/CO pattern, which was observed in 2021 and 2022, was also detected in 8 isolates in 2023. Additional antibiotics co-occurring with β -lactam and colistin resistance patterns are presented in detail in Table 2.

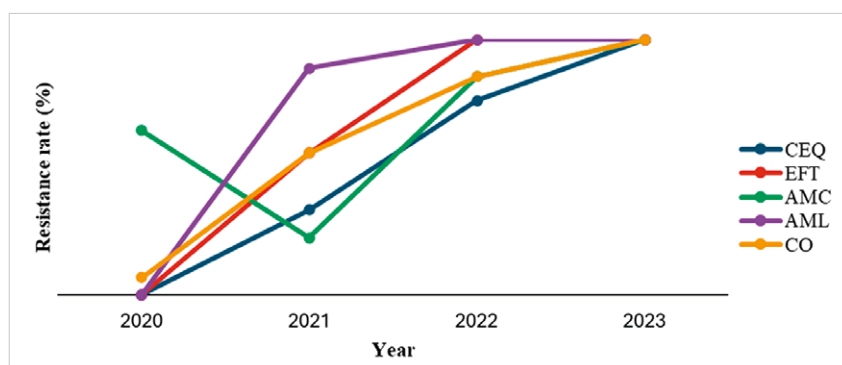


Fig. 1. Temporal trends in resistance rates to selected β -lactam agents and colistin among *E. coli* isolates recovered from vaginal swabs of mares between 2020 and 2023

Explanations: CEQ – cefquinome, EFT – ceftiofur, AMC – amoxicillin-clavulanic acid, AML – amoxicillin, CO – colistin. FDR-adjusted significant increasing trends were observed for CEQ, EFT, AML, and CO, whereas AMC showed year-associated variation without a confirmed increasing trend after correction.

Tab. 2. Temporal distribution of multidrug resistance patterns among *E. coli* isolates recovered from vaginal swabs of mares between 2020 and 2023

Year	Number of <i>E. coli</i> isolates	Resistance patterns
2020	12	P/E/GM
	4	P
	17	P/AMC
	9	P/MAR/TE/E/FF/AMC/EN/GM/SXT
	3	P/MAR/TE/E/FF/AMC/EN/GM/SXT/CO
2021	9	P/TE/AML
	9	P/EFT/AML/CO
	1	P/CEQ/MAR/TE/E/FF/EFT/AMC/AML/GM/SXT/CO
	3	P/CEQ/E/EN/GM
	5	P/CEQ/TE/EFT/AMC/AML/EN/GM/CO
2022	3	P/EFT/AML/CO
	11	P/CEQ/E/EFT/AMC/AML/GM/CO
	2	P/EFT/AMC/AML/GM/SXT
	1	P/CEQ/MAR/E/FF/EFT/AMC/AML/SXT
	4	P/CEQ/MAR/E/FF/EFT/AMC/AML/SXT/CO
2023	5	P/CEQ/MAR/TE/E/FF/EFT/AMC/AML/EN/GM/SXT/CO
	2	P/CEQ/MAR/E/EFT/AMC/AML/EN/GM/CO
	1	P/CEQ/E/EFT/AMC/AML/EN/GM/CO

Explanations: AMC – amoxicillin-clavulanic acid, AML – amoxicillin, CEQ – cefquinome, CO – colistin, E – erythromycin, EFT – ceftiofur, EN – enrofloxacin, FF – florfenicol, GM – gentamicin, MAR – marbofloxacin, P – penicillin, TE – tetracycline, SXT – trimethoprim-sulfamethoxazole.

In the present study, vaginal *E. coli* isolates recovered from mares with pneumovagina exhibited resistance to multiple antimicrobial classes, including β -lactams, aminoglycosides, macrolides, tetracyclines, fluoroquinolones, potentiated sulphonamides, and colistin. This overall pattern is consistent with previous reports describing antimicrobial resistance among bacteria isolated from the equine reproductive tract in

different geographic settings (3, 7, 12, 21). Earlier studies from Sweden, India, the United States, Germany, Slovakia, and Italy have documented resistance in reproductive isolates to agents such as penicillin, ampicillin, cephalosporins, gentamicin, polymyxin B, and trimethoprim-sulphonamide combinations, indicating that antimicrobial resistance in equine reproductive bacteria is neither sporadic nor geographically restricted (2, 8, 14, 26). Viewed in this context, our findings add further evidence that the mare reproductive tract may represent a relevant ecological niche for the persistence and circulation of resistant Gram-negative bacteria (27). The novelty of this study lies in its temporally structured assessment of concurrent β -lactam and colistin resistance in vaginal *E. coli* isolates from mares, a niche that remains underrepresented in equine antimicrobial resistance research.

A major finding of the present study is the high frequency of multidrug resistance and the increasing complexity of resistance profiles over the study period. Nearly all isolates were resistant to more than one antimicrobial, and resistance combinations became broader from 2020 to March 2023. This pattern is particularly important because it suggests not only the persistence of resistant strains, but also the possible accumulation or co-selection of resistance determinants under antimicrobial exposure (28). While resistance to penicillin was expected, given its long-standing and widespread use, the year-associated increase in resistance involving cefquinome, ceftiofur, amoxicillin, and colistin is of greater clinical concern, as these agents are of high therapeutic relevance in veterinary and, in some cases, human medicine (15, 17). Resistance to moxycillin/clavulanic acid also showed significant variation across years, but its increasing trend did not remain significant after false discovery rate correction. This distinction is important because it separates descriptive year-to-year variation from statistically supported temporal increases. Accordingly, the temporal shift observed in resistance profiles may reflect selective pressure associated with antimicrobial use in equine practice and highlights the need for caution when empirical treatment is considered (13, 15, 17).

The statistically supported temporal increase in resistance to cefquinome, ceftiofur, amoxicillin, and colistin deserves particular attention. From an antimicrobial stewardship perspective, this pattern is concerning because third- and fourth-generation cephalosporins are critically important in equine practice (15, 17), whereas colistin resistance has disproportionate public health relevance because of its association with mobile resistance mechanisms (1, 4). ESBL- and AmpC-producing *Enterobacterales* are known to compromise the efficacy of extended-spectrum cephalosporins, and the parallel increase observed in the present study may be consistent with the circulation of such mechanisms among the isolates examined (10, 16). Moreover, the simultaneous

increase in phenotypic colistin resistance, despite the absence of routine colistin use in horses, raises concern regarding indirect selection, environmental exposure, or horizontal transfer of resistance determinants, such as *mcr*-associated elements. Because molecular testing was not performed, the present findings should be interpreted as phenotypic evidence of resistance rather than confirmation of specific ESBL, AmpC, or *mcr*-mediated mechanisms. Nevertheless, the convergence of resistance to β -lactams and colistin supports the need for molecular investigation in future studies.

These findings also have practical implications for antimicrobial stewardship in horses. Empirical treatment remains common in equine medicine, particularly when rapid clinical decisions are required. The present results, however, illustrate the risk of relying on historical expectations of susceptibility in the absence of culture and antimicrobial susceptibility testing (13). The continued or repeated use of broad-spectrum and critically important antimicrobials may favor the maintenance of multidrug-resistant organisms within equine populations and increase the likelihood of co-selection for resistance to unrelated antimicrobial classes. In this regard, the prudent use of ceftiofur, cefquinome, fluoroquinolones, and polymyxin-related compounds is especially important, and treatment decisions should be supported as far as possible by microbiological evidence and stewardship principles (15, 17). The potential co-occurrence of phenotypic colistin resistance with β -lactam resistance further reinforces the need for more restrictive and evidence-based prescribing practices in equine clinical settings (4, 28).

This study has several limitations. Resistance was assessed phenotypically, and molecular confirmation of resistance determinants was outside the scope of this study. Colistin susceptibility was evaluated by disk diffusion, which is not a gold-standard method. Therefore, the absence of broth microdilution limits the definitive interpretation of the colistin results. The isolates were obtained exclusively from mares with pneumovagina. Detailed clinical histories, including previous antimicrobial treatments, were unavailable, and healthy controls were not included. Accordingly, the findings should not be generalized beyond the sampled clinical population. In addition, the small number of isolates recovered in 2023 ($n = 8$) warrants cautious interpretation of the reported percentages. Despite these limitations, the temporal increase in resistance to selected β -lactams and colistin warrants continued surveillance. Future studies should include healthy mares, detailed clinical data, broth microdilution, and molecular characterization of ESBL-, AmpC-, and *mcr*-associated determinants.

This study suggests that the reproductive tract of mares with pneumovagina may represent a previously underexplored reservoir of multidrug-resistant *E. coli*. The findings provide phenotypic evidence of widespread multidrug resistance among vaginal iso-

lates from mares with pneumovagina, with statistically supported temporal increases in resistance to cefquinome, ceftiofur, amoxicillin, and colistin. Although amoxicillin/clavulanic acid showed significant year-associated variation without a confirmed increasing trend after correction, the overall expansion of β -lactam and colistin resistance patterns is of clear clinical and One Health concern. These results emphasize the need to move beyond empirical antimicrobial use in equine reproductive practice and to prioritize routine culture-based diagnosis, antimicrobial susceptibility testing, prudent prescribing, and longitudinal surveillance. Molecular characterization of ESBL, AmpC, and *mcr*-associated determinants is warranted to define the mechanisms underlying these phenotypic resistance patterns and to assess their potential dissemination within equine populations and across the animal–human–environment interface.

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