

Antimicrobial resistance profiles and phylogenetic groups of *Escherichia coli* isolated from healthy Thoroughbred racehorses in Japan

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In this study, we investigated the occurrence of antimicrobial resistance in commensal Escherichia coli isolated from healthy Thoroughbred (TB) racehorses in Japan. A total of 212 fecal samples were individually collected from TB racehorses from March 2017 to August 2018 at Japan Racing Association training centers. E. coli was isolated by using selective agar media, deoxycholate-hydrogen sulfide-lactose (DHL) and eosin methylene blue (EMB). A total of 417 E. coli isolates were examined against 10 antimicrobial agents by using the broth microdilution method. The 417 E. coli isolates were phylogenetically grouped using a multiplex polymerase chain reaction. The highest proportion of resistance was observed for streptomycin (30.9%, 129/417) followed by ampicillin (19.4%, 81/417), trimethoprim (15.8%, 66/417), tetracycline (8.4%, 35/417), chloramphenicol (2.6%, 11/417), kanamycin (1.2%, 5/417), nalidixic acid (0.5%, 2/417), cefazolin (0.2%, 1/417), colistin (0.2%, 1/417), and gentamycin (0%). Multidrug-resistant (MDR) E. coli was detected in 7.9% (33/417) of isolates. The proportions of resistance against ampicillin, streptomycin, kanamycin, and chloramphenicol and of multidrug-resistant phenotypes in E. coli belonging to phylogenetic group B2 were significantly higher than those of other groups. This study clarified the distribution of antimicrobial-resistant (AMR) E. coli in Japanese racehorses. A continuous monitoring program for antimicrobial resistance is required to control the spread of AMR bacteria in racehorses.

Key words: antimicrobial resistant, *Escherichia coli*, Thoroughbred

J. Equine Sci.
Vol. 31, No. 4
pp. 85–91, 2020

Antimicrobial resistance is a serious threat to global public health that requires integrated control all over the world [32]. One important factor in the increase of antimicrobial-resistant (AMR) bacteria is the imprudent use of

antimicrobials [3]. The presence of AMR bacteria may lead to treatment failure in infection cases in humans and animals [14, 23, 29]. *Escherichia coli* is a largely commensal bacteria in the gastrointestinal tracts of mammals [20], but it has been reported to be a causal agent in some nosocomial infections in horse clinics [13, 18]. Moreover, it has also been reported to have acquired resistance to multiple antimicrobials [20]. In addition, *E. coli* may act as a reservoir of resistance genes that could be transmitted to other pathogens [25]. Therefore, there have been several investigations of antimicrobial resistance in this microorganism in humans and animals throughout the world [6, 7, 12, 17]. Resistance profiles of bacteria are useful to enhance treatment options

Received: July 7, 2020

Accepted: September 24, 2020

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in cases of AMR bacterial infection [1].

In most countries, information about the volume of antimicrobials used in equine medicine [31] and the availability of those that are pharmaceutically approved is limited [5, 27]. The presence of AMR bacteria affects animal welfare and economically impacts businesses in the horse industry [2], but the occurrence of antimicrobial resistance varies greatly depending on the breeding style in the studied population (e.g., health conditions, breeding area, and feed). Recently, several studies of antimicrobial resistance in horses have been conducted [7, 14, 15, 17, 20, 21]. In Japanese Thoroughbred (TB) racehorses, antimicrobials are still administered in some cases to prevent infection [19], and they are seemingly effective in preventing infection caused by stress during long-distance transportation [11]. However, a comprehensive evaluation of antimicrobial resistance for *E. coli* in horses as an indicator has not been conducted in Japan. In this study, we investigated the occurrence of AMR *E. coli* isolated from healthy TB racehorses in Japan.

Materials and Methods

Sampling, isolation, and identification of E. coli

This study was part of a monitoring study of AMR bacteria isolated from healthy TB racehorse fecal samples in Japan. At the same time, the presence of extended-spectrum β -lactamase (ESBL)- and AmpC β -lactamase-producing bacteria and AMR enterococci was also investigated as per our previous reports [25–27]. Though this study used the same samples as our previous studies, the isolation and identification of *E. coli* were conducted using protocols that were independent from those used in our previous study about ESBL-producing *E. coli* that were isolated using a selective agar medium containing an antibiotic (1 μ g/ml cefotaxime).

A total of 212 healthy TB racehorse fecal samples were collected from March 2017 to August 2018 at the Japan Racing Association (JRA) Miho Training Center (103 samples) and the JRA Ritto Training Center (109 samples). Fecal samples were collected from TB racehorses that were diagnosed as clinically healthy by JRA veterinarians. Then fecal samples were immediately transported to our laboratory using cooling boxes. All samples were enriched into trypto-soya broth (TSB; Nissui, Tokyo, Japan) and incubated at 37°C for 24 hr. Then, overnight cultures were spread onto deoxycholate-hydrogen sulfide-lactose (DHL) agar (Nissui) for *E. coli* presumptive isolation and incubated for 24 hr at 37°C. Up to three different pink or red colonies appearing on DHL agar were picked and cultivated on eosin methylene blue (EMB) agar (Nissui) and incubated at 37°C overnight. After that, colonies that appeared as large blue-black colonies with a green metallic sheen were considered

to be *E. coli*, and isolates were streaked onto trypticase soy agar (TSA; Becton, Dickinson and Co., Le Pont-de-Claix, France) and incubated at 37°C for 24 hr. All *E. coli* isolates were stored in TSB with 20% glycerol at –80°C until further work.

Antimicrobial susceptibility test (AST)

Up to two *E. coli* isolates from each sample were selected for AST. Minimum inhibitory concentration (MIC) was determined by using the broth microdilution method as recommended by the Clinical Laboratory Standard Institute (CLSI) guidelines [22]. All isolates were tested against 10 antimicrobial agents with the MIC breakpoints for resistance: ampicillin (ABPC; ≥ 32 μ g/ml), cefazolin (CEZ; ≥ 32 μ g/ml), streptomycin (SM; ≥ 32 μ g/ml), kanamycin (KM; ≥ 64 μ g/ml), gentamycin (GM; ≥ 16 μ g/ml), tetracycline (TC; ≥ 16 μ g/ml), chloramphenicol (CP; ≥ 32 μ g/ml), colistin (CL; ≥ 16 μ g/ml), nalidixic acid (NA; ≥ 32 μ g/ml), and trimethoprim (TMP; ≥ 16 μ g/ml). The breakpoints of each antimicrobial agent were decided based on the CLSI criteria. Those for SM and CL were interpreted based on reports of the Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) system and the European Committee on Antimicrobial Susceptibility Testing, respectively [10, 24]. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 were used as quality control strains. Isolates that phenotypically showed resistance to at least three classes of antimicrobials were considered to be multidrug resistant (MDR) [27].

Phylogenetic group of E. coli

E. coli isolates were assigned to one of the four major phylogenetic groups (A, B1, B2, and D) by using a multiplex polymerase chain reaction according to previous reports [8, 9].

Statistical analysis

The proportions of AST profiles for each antimicrobial were analyzed descriptively by using Excel 2017 (version 15.40; Microsoft, Redmond, WA, U.S.A.). MIC₅₀ and MIC₉₀ are defined as the concentrations required to inhibit 50% and 90% of isolates tested, respectively. The differences in proportions in the AST between *E. coli* isolates from the Miho Training Center and those from the Ritto Training Center were statistically analyzed by the Bonferroni method using RStudio, version 1.2.5033 (RStudio, Boston, MA, U.S.A.), and *P* values <0.05 were considered significant. Comparisons of the proportions in the AST between phylogenetic groups were tested using a two-tailed Fisher's exact test or χ^2 test in js-STAR version 8.9.7j, and *P*<0.05 was considered significant (<http://www.kisnet.or.jp/nappa/software/star/index.htm>).

Table 1. Distribution of minimum inhibitory concentration (MIC) of 417 *Escherichia coli* isolated from Thoroughbred racehorses in Japan

Antibiotic ^{a)}	Resis- tance (%)	MIC ₅₀ (μ g/ ml)	MIC ₉₀ (μ g/ ml)	Distribution of MICs (μ g/ml) ^{b)}													
				≤ 0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	
ABPC	19.4	16	32					2	29	110	195	57				3	21
CEZ	0.2	2	2	1	3	31	161	210	8	2					1		
SM	30.9	8	128				3	25	130	97	33	20	39	29	19	22	
KM	1.2	4	8			4	18	102	193	83	12		1	1		3	
GM	0.0	1	4	5	71	99	114	70	54	4							
TC	8.4	2	8			35	99	113	121	14	1	3	8	11	12		
CL	0.2	0.25	0.5	99	256	45	13	3			1						
CP	2.6	8	16			1	2	9	148	214	32	1	1	1	6	2	
NA	0.5	4	4			3	26	117	215	51	3		1			1	
TMP	15.8	0.5	>256	10	132	158	42	7	2		1		1	3	1	60	

^{a)}ABPC, ampicillin; CEZ, cefazolin; SM, streptomycin; KM, kanamycin; GM, gentamicin; TC, tetracycline; CP, chloramphenicol; CL, colistin; NA, nalidixic acid; TMP, trimethoprim, ^{b)}MIC breakpoints are represented by vertical line borders.

Results

Antimicrobial susceptibility profile of *E. coli*

E. coli was isolated from 209 of 212 (98.6%) healthy TB racehorse fecal samples. One hundred ninety-nine isolates were obtained from 100 of 103 (97.1%) samples from the Miho Training Center, and 218 isolates were obtained from 109 (100%) samples from the Ritto Training Center. A total of 417 *E. coli* isolates were examined by AST. The distributions of MICs of the 10 antimicrobials for *E. coli* isolates are shown in Table 1. *E. coli* isolates that showed resistance to at least one antimicrobial were isolated from 129 of 212 (60.8%) TB racehorse fecal samples. The highest proportion of resistance was against SM (30.9%, 129/417), followed by ABPC (19.4%, 81/417), TMP (15.8%, 66/417), TC (8.4%, 35/417), CP (2.6%, 11/417), KM (1.2%, 5/417), NA (0.5%, 2/417), CEZ (0.2%, 1/417), CL (0.2%, 1/417), and GM (0%). The proportion of AMR *E. coli* isolates from the Miho Training Center (53.5%, 106/199) was not significantly different from that for AMR *E. coli* isolates from the Ritto Training Center (44.9%, 98/218).

Thirty-three isolates (7.9%) that were resistant to at least three classes of antimicrobials were confirmed to be MDR *E. coli* (Table 2). The most frequent MDR phenotypes were ABPC-SM-TMP (12 isolates), followed by SM-TC-TMP (5 isolates) and ABPC-SM-KM-TC-CP (4 isolates).

Phylogenetic group of *E. coli*

Of the total of 417 *E. coli* isolates in this study, more than half were classified as group B1 (57.3%, 239/417); this was followed by groups A (23.3%, 97/417), B2 (9.8%, 41/417), and D (9.6%, 40/417). The proportion of *E. coli* isolates belonging to group B2 showed the highest resistance (63.4%, 26/41) to at least one antimicrobial, followed by

Table 2. Multidrug resistant of 33 *Escherichia coli* isolated from Thoroughbred racehorses in Japan

Antimicrobial resistance pattern ^{a)}	n (%)
ABPC-SM-KM-TC-CP	4 (12.1)
ABPC-SM-TC-CP-TMP	1 (3.0)
ABPC-CEZ-SM-TMP	1 (3.0)
SM-KM-TC-CP-TMP	1 (3.0)
ABPC-SM-TC-CP	2 (6.1)
SM-TC-CP-TMP	1 (3.0)
ABPC-SM-TMP	12 (36.4)
ABPC-SM-TC	3 (9.1)
ABPC-SM-CP	2 (6.1)
ABPC-SM-NA	1 (3.0)
SM-TC-TMP	5 (15.2)

^{a)}ABPC, ampicillin; SM, streptomycin; KM, kanamycin; TC, tetracycline; CP, chloramphenicol; NA, nalidixic acid; TMP, trimethoprim.

groups B1 (48.1%, 115/239), D (47.5, 19/40), and A (45.4%, 44/97). The proportion of *E. coli* that showed resistance to ABPC, SM, KM, or CP or had an MDR phenotype in group B2 was significantly higher than that in other groups (Fig. 1). The AMR pattern of *E. coli* in each phylogenetic group is presented in Table 3.

Discussion

Commensal bacteria are often exposed to antimicrobials in the course of medical therapy. This can lead to the emergence of antimicrobial resistance in the racehorse community. Moreover, commensals can play a role as both acceptors and donors of antimicrobial resistance [25]. However, few studies have evaluated the occurrence of antimicrobial resistance in *E. coli* as commensal bacteria

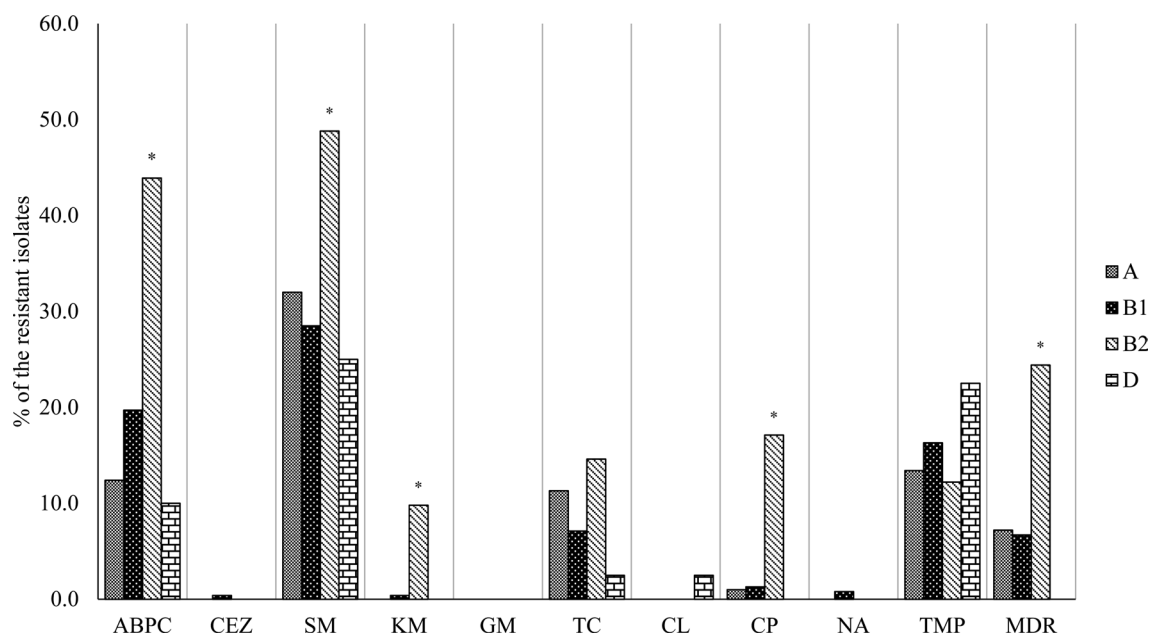


Fig. 1. Antimicrobial resistance in each phylogenetic group (A, B1, B2, and D) of *Escherichia coli* isolates from healthy Thoroughbred racehorse fecal samples in Japan (ABPC, ampicillin; CEZ, cefazolin; SM, streptomycin; KM, kanamycin; GM, gentamycin; TC, tetracycline; CP, chloramphenicol; CL, colistin; NA, nalidixic acid; TMP, trimethoprim; MDR, multidrug resistance). Asterisks indicate statistically significant differences ($P < 0.05$) between the percentages of phylogroup B2 strains compared with other phylogenetic groups for each tested antimicrobial.

in healthy horses [7, 20]. In the present study, nearly half of the total isolates showed resistance to at least one antimicrobial. The positive correlation between the presence of AMR bacteria with previous antimicrobials used has been described previously [16].

A recent study suggested that dihydrostreptomycin is commonly used in Japanese TB racehorses [19]. Our results showed that resistance to SM was the highest (30.9%) among *E. coli* isolated from racehorses. In equine medicine, SM is used as the first-line antimicrobial for the treatment of gram-negative bacterial infections [7]. In contrast to resistance to other aminoglycosides, our results showed that nearly all of the *E. coli* isolates were susceptible to KM and GM (98.8% and 100%, respectively). In Portugal, *E. coli* isolated from healthy Lusitano horse fecal samples showed lower resistance to SM (12.7%; 9/71) than was found in the *E. coli* isolates from the TB horses in our study, and resistance to GM (1.4%, 1/71) was lower than resistance to SM [20]. Resistance to SM among *E. coli* isolates from healthy horse fecal samples in South Korea was also lower than in the *E. coli* isolates from the TB horses in our study [7]. This suggests that the use of SM in equine veterinary practice must be controlled.

Resistance to ABPC was observed in 19.4% of *E. coli* isolates. Susceptibility to CEZ, which is a first-generation cephalosporin, was found in almost all *E. coli* isolates

(99.8%). Intrinsically, *E. coli* is resistant to penicillin, and acquired resistance to β -lactams is mostly mediated by the production of β -lactamases such as TEM-1, TEM-2, SHV-1, and AmpC β -lactamase [18]. Resistance due to ESBL-producing *E. coli* and *Klebsiella pneumoniae* has been reported in TB racehorses in our previous studies [26, 27]. But in South Korea and Portugal, resistance to ABPC in *E. coli* is reportedly lower than that in this study (3.9%, 2/51, and 4.2%, 3/71, respectively) [7, 20].

Combination trimethoprim-sulfamethoxazole (STX) therapy is widely administered orally to treat infections caused by gram-positive and gram-negative bacteria in horses [30]. The highest prevalence (51.0%, 135/264) among tested antimicrobials was observed for TMP-resistant *E. coli* isolated from hospitalized horses in Northwest England [2]. However, in South Korea and Portugal, resistance to STX has been observed at 9.8% (5/51) and 2.8% (2/71), respectively [7, 20]. In our study, 15.8% of *E. coli* were resistant to TMP. The range of MIC values for this antimicrobial was wide, from ≤ 0.125 to > 256 $\mu\text{g/ml}$. This result suggested that the use of TMP in Japanese TB racehorses must be handled more wisely.

Resistance to TC in *E. coli* most commonly occurs in animals, including horses [4]. In South Korea and Portugal (7.8%, 4/51, and 9.8%, 7/71, respectively), the proportions of resistance to TC in *E. coli* isolated from healthy horses

Table 3. Antimicrobial resistant pattern in phylogenetic group of *Escherichia coli* isolated from healthy Thoroughbred racehorses in Japan

Phylogenetic group (n) ^{a)}	Antimicrobial resistance pattern ^{b)}	n
A (44)	SM-TC-CP-TMP	1
	ABPC-SM-TC	1
	ABPC-SM-TMP	2
	SM-TC-TMP	3
	ABPC-SM	1
	SM-TC	3
	SM-TMP	5
	ABPC	8
	SM	15
	TC	3
	TMP	2
B1 (115)	ABPC-SM-TC-CP-TMP	1
	SM-KM-TC-CP-TMP	1
	ABPC-SM-TC-CP	1
	ABPC-CEZ-SM-TMP	1
	ABPC-SM-TC	2
	ABPC-SM-TMP	7
	ABPC-SM-NA	1
	SM-TC-TMP	2
	ABPC-SM	10
	ABPC-TMP	2
	SM-TC	3
	SM-TMP	6
	TC-TMP	2
	ABPC	22
	SM	33
	TC	4
NA	1	
TMP	16	
B2 (26)	ABPC-SM-KM-TC-CP	4
	ABPC-SM-TC-CP	1
	ABPC-SM-TMP	3
	ABPC-SM-CP	2
	ABPC-SM	4
	ABPC-TMP	1
	ABPC	3
	SM	6
	TC	1
	TMP	1
D (19)	ABPC-SM	2
	SM-TMP	3
	TC-TMP	1
	ABPC	2
	SM	5
	CL	1
TMP	5	

^{a)}Total number of AMR *E. coli* isolates in each phylogenetic group.

^{b)}ABPC, ampicillin; CEZ, cefazolin; SM, streptomycin; KM, kanamycin; TC, tetracycline; CP, chloramphenicol; CL, colistin; NA, nalidixic acid; TMP, trimethoprim.

were similar to that in this study [7, 20]. A high prevalence of TC-resistant *E. coli* has also been reported for hospitalized horses in Northwest England [2]. For respiratory tract infections, oxytetracycline is commonly used in combination with a sulfa antimicrobial agent in equine medicine [7]. Even though there was not a high proportion of resistance, the MIC_{50/90} values of *E. coli* for TC and CP were close to the MIC breakpoints for resistance. This information is useful as a warning in equine medicine to prevent the increase in resistance to these antibiotics. Resistance to TC has been reported to be at high prevalence in cattle (18.3–22.5%), pigs (53.8–64.2%), broiler chickens (45.5–61.1%), and layer chickens (22.3–38.5%) in a monitoring study of AMR *E. coli* in livestock from 2011 to 2015 in Japan [28]. TC is the most common antimicrobial used for domestic animals in Japan [28].

Phylogenetic analyses classified *E. coli* isolates into groups B1 (57.3%) and A (23.3%), the major groups, followed by B2 (9.8%) and D (9.6%). It has been reported that most commensal *E. coli* belong to groups A and B1, whereas virulent extraintestinal *E. coli* mainly belong to groups B2 and D [8, 9]. In this study, the proportion of B2 strains of *E. coli* that had resistance to ABPC, SM, KM, or CP or had an MDR phenotype was significantly higher than for other phylogenetic groups. In other studies, the prevalences of MDR *E. coli* were reported to be 3.9% (2/51) and 4.2% (3/71) in healthy horse feces in South Korea and Portugal, respectively [7, 20], and 37.6% in diseased horses in the UK [17]. MDR bacteria, including *E. coli*, have been reported as causative agents of extraintestinal infections in horses [12]. Our results suggest that antimicrobial therapy for MDR extraintestinal *E. coli* infection will be challenging in racehorses.

In conclusion, this is the first study to evaluate commensal AMR *E. coli* as indicator bacteria in healthy TB racehorses in Japan. Our results indicated that racehorses can be a reservoir for AMR bacteria, which might be transmitted to other horses, humans, or the environment through fecal material. One limitation of this study is the lack of information regarding the history of antimicrobial use in horses before entering the JRA training centers. Nevertheless, the information provided is useful for increasing awareness of antimicrobial overuse in equine medicine.

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