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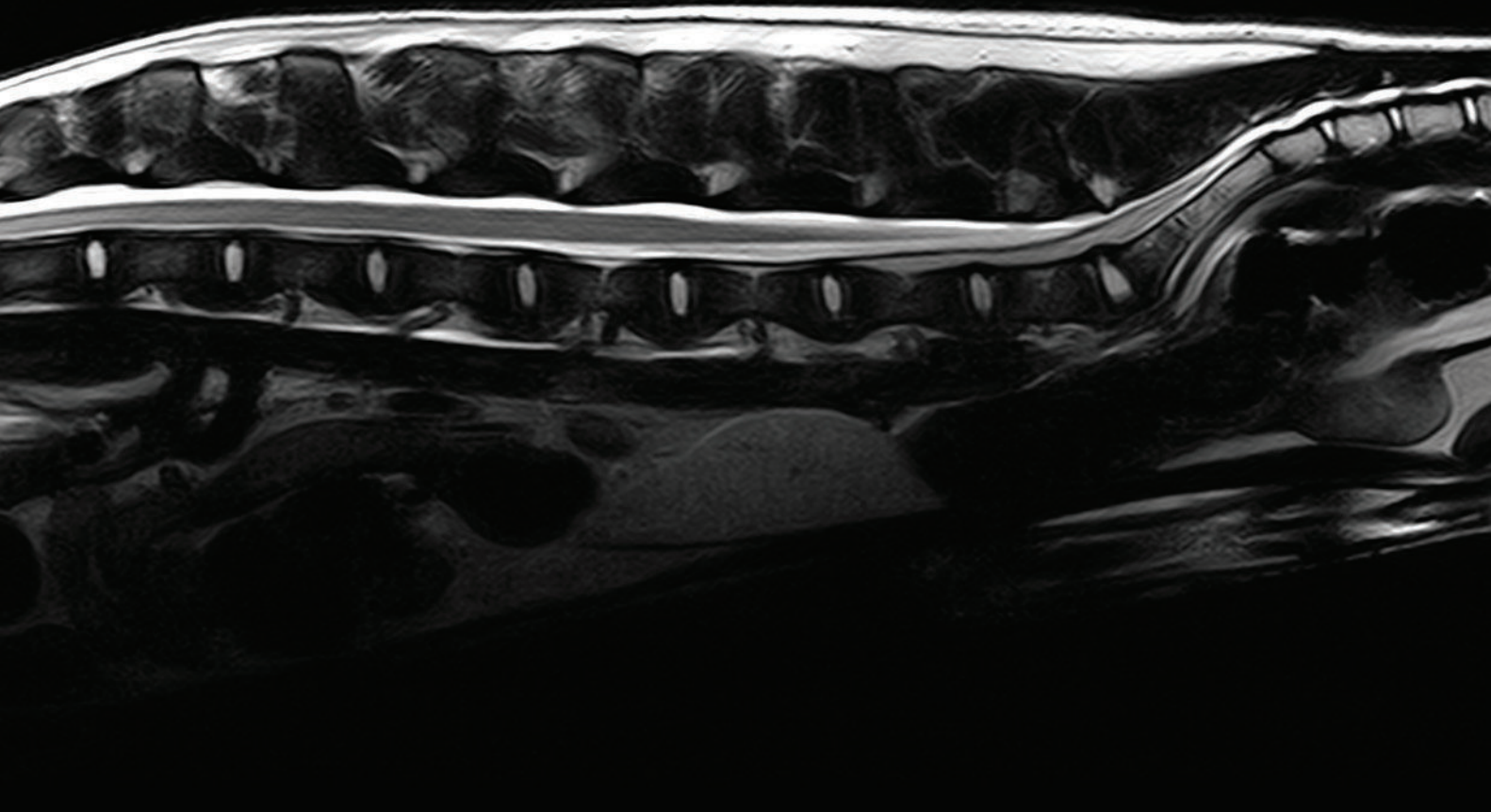
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## STANDARD ARTICLE

# Prevalence, risk factors, and characterization of multidrug resistant and extended spectrum $\beta$ -lactamase/AmpC $\beta$ -lactamase producing *Escherichia coli* in healthy horses in France in 2015

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**Background:** Although antimicrobial resistance is increasingly common in equine medicine, molecular and epidemiological data remains scarce.

**Objectives:** We estimated the prevalence of, and risk factors for, shedding of multidrug resistant (MDR), extended spectrum  $\beta$ -lactamase (ESBL)-producing, and AmpC  $\beta$ -lactamase-producing, or some combination of these in *Escherichia coli* in horses in France. We characterized ESBL/AmpC isolates for antimicrobial susceptibility and the presence of virulence and ESBL/AmpC-associated resistance genes.

**Animals:** Fecal samples from healthy adult horses at 41 premises were collected. A questionnaire was completed by each premises manager. A subset of these samples was tested to build 2 bacterial collections.

**Methods:** Indicator (without enrichment) and specific (enrichment with ceftriaxone) *E. coli* tested for antimicrobial susceptibility. Prevalence of isolates nonsusceptible to antimicrobials was estimated at the horse and the premises level. The ESBL/AmpC and virulence genes were identified by PCR. Multivariable logistic regression was used to investigate risk factors for MDR and ESBL/AmpC isolates at premises.

**Results:** Approximately 44% of horses shed MDR *E. coli*. Resistance most commonly was observed to ampicillin, streptomycin, and amoxicillin/clavulanic acid. Twenty-nine percent of premises housed horses shedding ESBL/AmpC-producing isolates. The ESBL/AmpC gene most commonly identified was *bla*<sub>CTX-M-1</sub>. Virulence gene *iutA* was identified in 1 ESBL/AmpC-producing isolate. Medical treatment, staff numbers, and activity were identified as risk factors for housing horses shedding ESBL/AmpC-producing *E. coli* isolates.

**Conclusions and Clinical Importance:** Prevalence of healthy horses harboring ESBL/AmpC genes and MDR isolates in their intestinal microbiota is substantial. Risk factors could be used to elaborate guidelines to prevent their dissemination.

## KEYWORDS

antimicrobial resistance, cephalosporinase, equine, microbiota

**Abbreviations:** AMC, amoxicillin-clavulanic acid; AMK, amikacin; AMP, ampicillin; AmpC, AmpC  $\beta$ -lactamase; ATM, aztreonam; BHI, brain heart infusion; bla,  $\beta$ -lactamase; CAZ, cefoperazone; CHL, chloramphenicol; CI, confidence interval; CIP, ciprofloxacin; CIPARS, Canadian Integrated Program of Antimicrobial Resistance Surveillance; CN, cefalexin; CRO, ceftriaxone; Ecl, Reference Laboratory for *Escherichia coli*; ENR, enrofloxacin; ESBL, extended spectrum  $\beta$ -lactamase; ExPEC, extra-intestinal pathogenic *E. coli*; GEN, gentamicin; IFCE, Institut Français du Cheval et de l'Équitation (French Institute of the Horse and Equitation); KAN, kanamycin; MDR, multidrug resistant; NAL, nalidixic acid; PIP, piperacillin; SSS, sulfisoxazole; TET, tetracycline; TIC, ticarcillin; XNL, ceftiofur.

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## 1 | INTRODUCTION

Antimicrobial resistance was declared by the World Health Organization (WHO) in 2014 as the greatest threat for human and veterinary medicine.<sup>1</sup> Equine medicine also is affected, as demonstrated by the recent publication of several case reports.<sup>2,3</sup> Nevertheless, molecular and epidemiological data in this species remains scarce.<sup>4</sup> Since their domestication, horses mainly have been considered working animals or livestock, although more recently some also may be considered companion animals. Therefore, they represent a potential source of contamination by direct contact with their owners or through the food chain. Horses have been overlooked in the global approach to antimicrobial resistance.<sup>5</sup> Nevertheless, they received more antimicrobials per kilogram than did cattle in France in 2013.<sup>6</sup>

*Escherichia coli* is present in the intestinal microbiota of mammals, being mostly commensal although some strains can be pathogenic. Because of its ubiquity, frequent exposure to systemic antimicrobial treatment, and its great genomic plasticity, this bacterium is considered by the European Center of Disease Control as an excellent indicator for antimicrobial surveillance.<sup>7</sup>

An important mechanism of resistance that can be found in *E. coli* is the production of extended spectrum  $\beta$ -lactamases (ESBL), AmpC cephalosporinases (AmpC),<sup>5</sup> or both, resulting in the enzymatic inactivation of  $\beta$ -lactams. The global prevalence of  $\beta$ -lactamase genes (eg, *bla*) has increased considerably over the last 30 years throughout the world, both in humans and in animals. This increase might be due to spatial dispersal of mobile genetic elements or high-risk clones or could arise from antimicrobial pressure. In horses, phenotypic resistance to ceftiofur (XNL) has been documented in foals<sup>8</sup> and in adults.<sup>9</sup> The ESBL gene variant *bla*<sub>CTX-M-1</sub> is most often identified.<sup>10</sup> However, *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-9</sub>,<sup>11</sup> *bla*<sub>CTX-M-15</sub>,<sup>12</sup> and several variants of *bla*<sub>CMY</sub> also have been detected.<sup>10</sup> All these variants also have been found in other animal<sup>10</sup> species and in humans.<sup>13</sup> The gene *bla*<sub>CTX-M-15</sub> is the most prevalent variant in humans because it disseminates through epidemic plasmids and high-risk *E. coli* clones,<sup>14</sup> such as the sequence-type ST131. The  $\beta$ -lactamase genes mostly are carried by plasmids, which also may convey resistance to other antimicrobial classes, thus conferring multidrug resistance (MDR).<sup>15</sup> Moreover, owning a horse is a risk factor for carriage of ESBL in humans,<sup>16</sup> highlighting potential concern for public health. The use of third- and fourth-generation cephalosporins marketed for veterinary medicine is authorized in horses in France, possibly enhancing the dissemination of ESBL/AmpC genes.

Antimicrobial resistance genes may coexist with virulence genes on the same plasmids in pathogenic bacteria.<sup>17</sup> Although *E. coli* is not considered an enteric pathogen in adult horses, extra-intestinal pathogenic *E. coli* (ExPEC) sporadically have been recognized as potential pathogenic agents in horses,<sup>18</sup> and the presence of virulence factors has been reported in *E. coli* isolated from horses.<sup>19</sup> The ExPEC are of public health concern because some isolates may be zoonotic. The presence of highly virulent and antimicrobial-resistant strains in the intestinal microbiota of horses could represent a risk for horse handlers because of the possibility of transmission through close contact.<sup>20</sup>

No data is available on the presence of MDR or ESBL/AmpC-producing isolates and ExPEC in the healthy equine population in France. Our objective was to determine the prevalence of, and risk factors for, shedding MDR or ESBL/AmpC-producing *E. coli* isolates in horses. We characterized potential ESBL/AmpC isolates for antimicrobial susceptibility and the presence of virulence and ESBL/AmpC-associated resistance genes.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling

This work was part of a larger study on resistance to anthelmintics and antimicrobials in horses in France. Sampling details were described elsewhere.<sup>21</sup> Briefly, premises housing more than 40 horses, as mentioned in the French Horse and Riding Institute (IFCE) database, were contacted by telephone and selected on a voluntary basis. For each breeding premises, a riding school within a radius of 50 km was recruited when available. At time of the study, we estimated the number of riding schools in the source population as 1600 and the number of breeding premises as 249 in the study area (only riding schools located in French departments including selected breeding premises were considered). To be included in the study, horses had to be >2 years old and considered healthy by the premises manager. They also had to be present on the premises for >4 weeks before sampling and must not have received anthelmintic treatment within the last 2 months. On the premises, a convenience sample of horses was assembled based on accessibility.

At each premises, between 8 and 36 horses were sampled. Two grams of feces were obtained, either from the rectum or within 5 minutes of defecation and stored at 4°C up to 6 hours. On the collection day, 18 mL of 30% glycerol were added to each sample, and the samples were stored at -20°C before analysis.

A questionnaire was developed based on published risk factors in the horse.<sup>22</sup> It was administered on the farm during an interview with the manager. Questions were directed to the general manager of the premises and were always at the premises level. The questionnaire was written in French and is available on request.

### 2.2 | Indicator *E. coli* collection: Nonenriched culture, antimicrobial susceptibility testing, ESBL/AmpC gene identification, and descriptive statistics

Because of financial and logistic restrictions, we were not able to test all samples. At first, 3 individual fecal samples per premises were randomly selected, by mixing them up by premises and picking them blinded to horse identification. Because of loss of viability of *E. coli* during transportation observed for the first set of samples (see results), the number of selected samples per premises was increased to 6 on remaining premises where we did not initially obtain samples with viable *E. coli*. One milliliter of the sample suspension in 30% glycerol was inoculated in 4 mL of peptone water. After  $\leq 15$  minutes, 100  $\mu$ L of this suspension was inoculated onto a tryptic soy agar slope

tube which was sent to the Reference Laboratory for *Escherichia coli* (Ecl). On receipt, tubes were incubated for 24 hours at 37°C, scraped and bacterial growth passaged onto MacConkey agar and incubated at 37°C overnight. All colonies, up to a maximum of 3, were selected for each fecal sample. Lactose-positive colonies were selected preferentially, but when not present, lactose-negative colonies were selected and retained for further analysis if indole-positive and citrate-negative.<sup>23</sup> Isolates were confirmed as *E. coli* by PCR detection of the *uidA* gene.<sup>24</sup>

Isolates were tested for susceptibility to the 14 antimicrobial agents examined in the Canadian Integrated Program for Antimicrobial Surveillance (CIPARS 2008) using the disk-diffusion assay as previously described.<sup>25</sup> The following antimicrobial disks were used: azithromycin (15 µg), streptomycin (STR; 10 µg), gentamicin (GEN; 10 µg), trimethoprim-sulfamethoxazole (23.75 µg), sulfisoxazole (SSS; 250 µg), tetracycline (TET; 30 µg), nalidixic acid (NAL; 30 µg), ciprofloxacin (CIP; 5 µg), chloramphenicol (CHL; 30 µg), ampicillin (AMP; 10 µg), amoxicillin/clavulanic acid (AMC; 30 µg), cefoxitin (FOX; 30 µg), ceftriaxone (CRO; 30 µg), and XNL (30 µg). The *E. coli* strain ATCC 25922 was used as a susceptible quality control. Veterinary CLSI 2015 clinical breakpoints were used when available; CLSI 2015 clinical breakpoints were used otherwise.

When isolates were nonsusceptible (intermediate or resistant) to third-generation cephalosporins, we looked for 5 β-lactamase resistance genes (*bla<sub>SHV</sub>*, *bla<sub>TEM</sub>*, *bla<sub>CMY-2</sub>*, *bla<sub>OXA</sub>*, *bla<sub>CTX-M</sub>*) by PCR multiplex. We used the primer sequences described previously.<sup>26</sup> The ESBL/AmpC resistance genes were detected by PCR performed on DNA extracted by heat lysis, as previously described.<sup>27</sup> The protocol was provided by the National Microbiology Laboratory of the Public Health Agency of Canada<sup>28</sup> with control strains ECL3482, PMON38, and CTX-M15. When *bla<sub>CTX-M</sub>* was detected, the CTX-M group was determined by PCR.<sup>29</sup> The amplicon was purified using the QIAquick PCR Purification Kit, according to manufacturer instructions. The variant was identified by Sanger sequencing and compared using the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>).

We estimated the prevalence of horses shedding resistant or nonsusceptible (resistant and intermediate) isolates for each antimicrobial and of premises housing these horses. We then estimated the global prevalence and 95% confidence intervals (CI) of horses shedding isolates nonsusceptible to >1, 3, 5, 7, or 9 classes of antimicrobials and of premises housing these horses. An isolate was considered MDR if nonsusceptible to ≥1 agent of ≥3 antimicrobial classes.<sup>30</sup> Positive MDR status for a horse was defined as detection of ≥1 MDR isolate for that horse. A premise was attributed positive MDR status if it housed ≥1 MDR-positive horse.

At the horse level, prevalence estimates were adjusted for sampling weights to consider the different sampling probabilities of premises (riding school versus breeding) and horses within each premises (ie, the proportion of horses sampled was not constant across all premises). For the estimation of CIs, variances were adjusted for stratification by type of premises (riding school versus breeding) and for clustering to consider potential nonindependence of horses sampled within the same premises. At the premises level, prevalence estimates also were adjusted for sampling weights (at the premises level only)

and stratification. The Surveyfreq procedure of SAS 9.4 was used for estimation, based on the Taylor series method.

### 2.3 | Potential ESBL/AmpC producing *E. coli* collection: Culture, antimicrobial susceptibility testing, ESBL/AmpC and virulence gene identification, and descriptive statistics

Bacteria producing ESBL/AmpC may be shed in small quantities in healthy animals.<sup>31</sup> To improve detection sensitivity and permit more accurate estimation of the proportion of positive premises, 2 different approaches were used for enrichment with CRO. In the first approach, samples used to produce the indicator collection were processed. After receipt at the Ecl and first incubation, plates were scraped and inoculated in 10 mL of MacConkey broth containing 1 mg/L of CRO and incubated overnight at 37°C. When bacterial growth was positive, 100 µL of MacConkey broth was inoculated on MacConkey agar containing 1 mg/L of CRO and incubated at 37°C overnight. Between 1 and 3 lactose-positive isolates per sample were selected.

In the second approach, 8 individual fecal samples per premises were examined individually. When available, these fecal samples were different from those used to produce the indicator collection. In addition, pools of samples from 6 to 10 horses were examined for each premises, and these pooled samples also came from different horses. One milliliter of the initial glycerol/feces mixture was added to 9 mL of Brain Heart Infusion (BHI) broth and incubated 4 hours at 37°C. One hundred microliters of the BHI broth were inoculated on MacConkey agar with CRO, 1 mg/L. When bacterial growth was observed, 1-3 lactose-positive isolates were selected.

All isolates of this collection were confirmed as *E. coli* by the detection of the *uidA* gene using PCR<sup>24</sup> and were tested for susceptibility against 30 antimicrobials by the disk diffusion method. The following disks were used: STR (10 µg), GEN (10 µg), amikacin (AMK; 30 µg), kanamycin (KAN; 30 µg), spectinomycin (100 µg), trimethoprim-sulfonamide (5 µg), SSS (200 µg), TET (30 µg), NAL (30 µg), CIP (5 µg), flumequin (30 µg), enrofloxacin (ENR; 5 µg), CHL (30 µg), florfenicol (30 µg), AMP (10 µg), AMC (30/10 µg), piperacillin (PIP; 100 µg), piperacillin-tazobactam (100/10 µg), ticarcillin (TIC; 75 µg), TIC-clavulanic acid (75/10 µg), cefalexin (CN; 30 µg), FOX (30 µg), cefuroxim (30 µg), CRO (30 µg), ceftazidim (30 µg), XNL (30 µg), cefoperazone (CAZ; 75 µg), cefepime (30 µg), aztreonam (ATM; 30 µg), and imipenem (10 µg). The *E. coli* strain ATCC 25922 was used as a control. Veterinary CLSI 2015 clinical breakpoints were used when available, CLSI 2015 clinical breakpoints otherwise were used when available, and EUCAST clinical breakpoints were used if criteria could not be found in the 2 first sources.

For all isolates in this collection, we looked for 5 β-lactamase resistance genes (*bla<sub>SHV</sub>*, *bla<sub>TEM</sub>*, *bla<sub>CMY-2</sub>*, *bla<sub>OXA</sub>*, *bla<sub>CTX-M</sub>*) by PCR multiplex (PCR and gene identification protocols described above).

The potential pathogenicity of ESBL/AmpC-producing isolates was determined based on the presence of virulence genes which define *E. coli* pathotypes in animals (Enterotoxigenic *E. coli* [*eltB*, *estA*, *estB*], Enteropathogenic *E. coli* [*eae*], Shiga-toxin producing *E. coli* [*stxA* and *stx2A*], and extra-intestinal *E. coli* [ExPEC] [*iutA*]). The PCR

procedures were performed following the protocol of the Ecl available at [http://www.apzec.ca/en/APZEC/Protocols/APZEC\\_PCR\\_e-n.aspx](http://www.apzec.ca/en/APZEC/Protocols/APZEC_PCR_e-n.aspx).

Descriptive statistics were used to present susceptibility profiles per antimicrobial. We also estimated the prevalence and 95% CIs of premises housing horses shedding isolates nonsusceptible to >1, 3, 5, 7, or 9 classes of antimicrobials in this collection, adjusted for stratification and sampling weights as previously described. The ESBL/AmpC status for premises was attributed in the same manner as MDR status.

## 2.4 | Risk factors

Two outcome variables were defined: MDR and ESBL/AmpC status of premises. Putative risk factors from the questionnaire with  $P < .20$  (likelihood ratio test) in univariable logistic regression analysis were selected for inclusion in a multivariable model for each outcome. Pairwise associations between these selected variables were assessed by  $\chi^2$  test. In the presence of significant association ( $P < .05$ ), only 1 of 2 correlated variables was kept based on the biological relevance with the outcome. For the ESBL/AmpC model, the number of horses tested per premises was categorized ( $\leq 10$ , 11-20, and  $\geq 21$ ) and included as a covariate in the full model to consider potential impact on the sensitivity of ESBL/AmpC detection. The final multivariable model then was refined by sequentially omitting variables with  $P > .05$ . However, if the removal of a variable caused a >20% change in the odds ratio of another variable, it was kept in the model as a potential confounder. No interaction was tested. The fit of the final model was evaluated using the Hosmer and Lemeshow goodness-of-fit test. The Genmod procedure of SAS 9.4 software was used.

## 3 | RESULTS

### 3.1 | Indicator collection

Overall, 1061 fecal samples from individual horses were collected from 41 premises, distributed as shown in Figure 1. Because of financial and logistic restrictions, a subset of 195 samples, from 41 premises, was sent to the Ecl. We detected viable *E. coli* in 132 of these 195 samples (68%). A total of 348 *E. coli* isolates were selected from these 132 horses, originating from 38 premises. Prevalence estimates were calculated based on the *E. coli*-positive samples only. Prevalence estimates of horses and premises shedding resistant and nonsusceptible isolates per antimicrobial are shown in Figure 2. Over 40% of the horses and 80% of the premises showed nonsusceptibility to AMP, STR, and AMC.

Nonsusceptibility to third-generation cephalosporins was observed in isolates from 10.6% of horses and 31.7% of premises. None of these isolates demonstrated the presence of *bla* genes by PCR.

Prevalence estimates of nonsusceptibility to 1st-generation quinolone (NAL) was substantial (23.8% of horses and 66.7% of premises) even though nonsusceptibility to fluoroquinolones was lower (3.8% of horses and 10.6% of premises).

Prevalence estimates of horses shedding isolates nonsusceptible to >1, 3, 5, 7, or 9 classes of antimicrobials, and premises housing these horses, are summarized in Table 1. The prevalence of horses

shedding, isolates nonsusceptible to  $\geq 1$  antimicrobial and MDR isolates was high (84.4% and 44.4%, respectively). A total of 79.7% of premises housed horses shedding MDR isolates. In addition, 7.6% of horses shed isolates nonsusceptible to 7 classes of antimicrobials.

### 3.2 | ESBL/AmpC collection

Twenty-nine percent (95% CI, 11.5-46.5) of premises housed horses that shed isolates nonsusceptible to CRO and thus belonging to the ESBL/AmpC collection. In the first approach, 195 samples from individual horses from 41 premises were tested, and we detected positive samples for 5 premises. In the second approach, 744 samples from the 41 premises were tested either in pools or individually. We detected positive samples for 16 premises (including the 5 premises identified using the first approach).

Nonsusceptibility was observed for a wide range of antimicrobial classes among the 50 isolates of this collection originating from the 16 positive premises (Figure 3). Indeed, all isolates were nonsusceptible to AMP, PIP, CN, and CRO, although only 1 isolate was nonsusceptible to AMC and FOX (2nd-generation cephalosporin classified as a cephamycin). In addition, >60% of these isolates were nonsusceptible to aminoglycosides (GEN, KAN, STR), TET, and folate inhibitors (trimethoprim-sulfonamides, sulfizoxazole). The proportion of isolates nonsusceptible to quinolones was lower in this collection than in the indicator collection. Nevertheless, we found 1 isolate that was resistant to fluoroquinolones in this collection. All isolates were susceptible to carbapenems and AMK. For all premises that housed horses shedding ESBL/AmpC isolates (29.0% of total premises), at least isolate was nonsusceptible to 5 classes of antimicrobials, and 2.1% of all premises housed horses that shed isolates nonsusceptible to up to 9 classes of antimicrobials (Table 1).

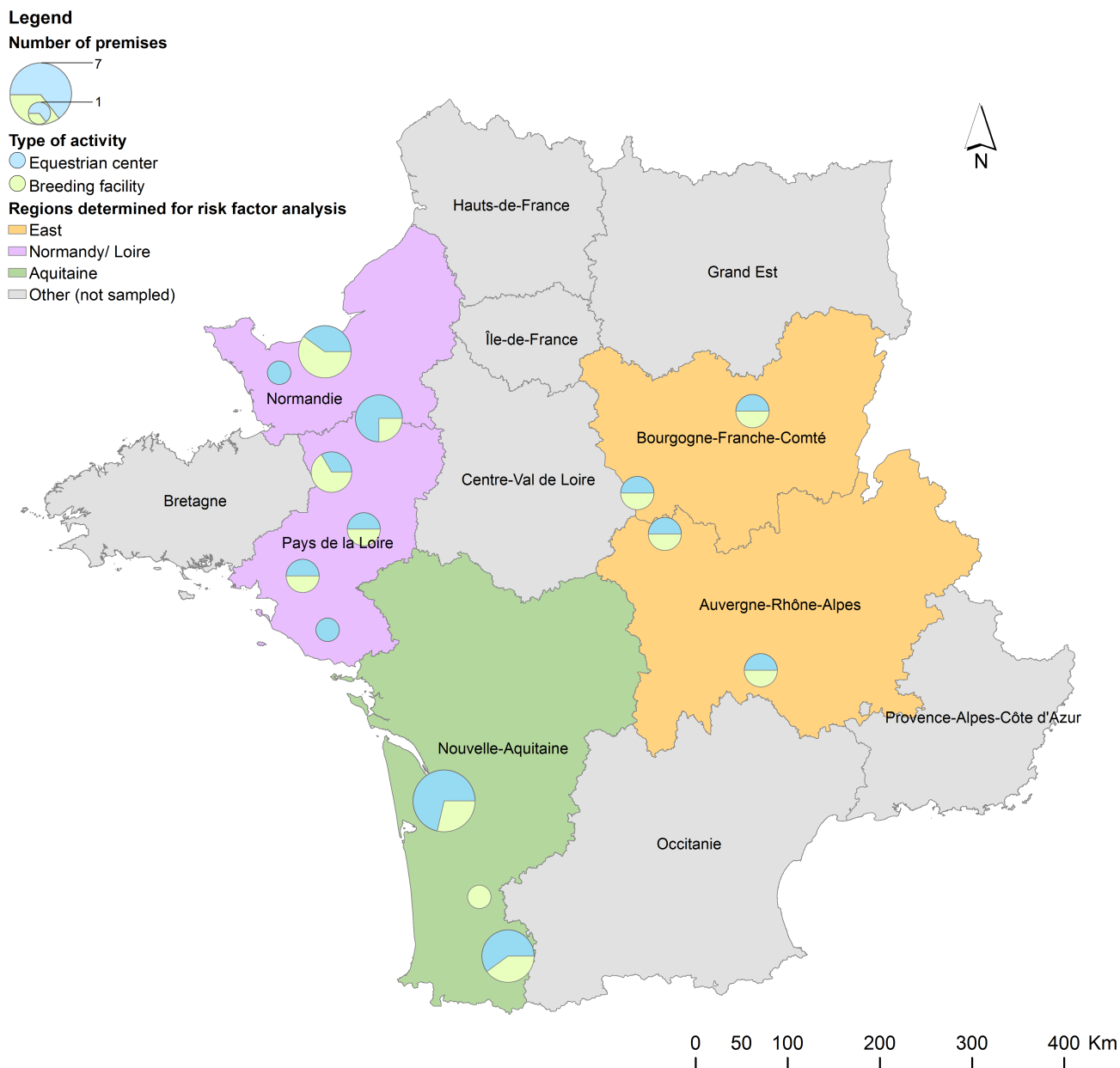
The main ESBL genes identified were *bla*<sub>CTX-M-1</sub> (33/50 tested isolates) and *bla*<sub>CTX-M-2</sub> (8/50), but we also identified isolates that carried the ESBL genes *bla*<sub>CTX-M-14</sub> (2/50) and *bla*<sub>SHV-12</sub> (6/50). Only 1 isolate carried the AmpC gene *bla*<sub>CMY-2</sub>. No isolate with  $\geq 2$  ESBL/AmpC genes was observed.

A high diversity of non- $\beta$ -lactam resistance patterns was observed among isolates that shared the same *bla* gene, but the 1 isolate carrying *bla*<sub>CMY-2</sub> was resistant only to FOX and clavulanic acid. The presence of *bla*<sub>SHV-12</sub> most often was associated with intermediate resistance to CRO and XNL, but with resistance to CAZ (third-generation cephalosporin) and ATM (monobactam). No virulence genes associated with enteric pathogenic *E. coli* were observed. Only 1 isolate carried *iutA*.

### 3.3 | Risk factors

Risk factors were calculated for 38 premises, because 3 questionnaires were not filled out completely. Sixteen potential risk factors were identified from the questionnaire (Table 2). All were dichotomous or categorical.

Five risk factors were considered for multivariate modeling (all  $P < .20$  in univariable analysis) for the ESBL/AmpC outcome, but "transportation" and "contact with wild life" were excluded because they were associated with "the number of persons taking care of the



**FIGURE 1** Geographical distribution of sampled premises over the French territory in a cross-sectional study of 132 healthy adult horses, in 41 premises, performed in 2015. Colored regions were defined for the risk factor calculation. The size of the circle is proportional to the number of premises sampled

horses” and “activity,” respectively. According to the multivariate model, the odds of being an ESBL/AmpC premises were 14.6 times higher ( $P = .03$ ) among the riding schools compared to breeding premises, 9.6 times higher ( $P = .03$ ) if the premises housed a horse that had been medically treated within the last 3 months and 35.7 times higher ( $P = .006$ ) in premises where the staff consisted of >5 persons (Table 3). The Hosmer-Lemeshow goodness-of-fit analysis indicated that our model fitted the data ( $P = .6$ ). Transportation was marginally but not statistically significant ( $P = .06$ ) in the univariate analysis but was excluded from multivariable analysis.

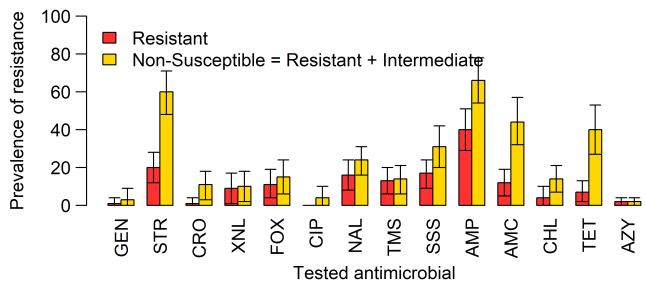
The odds of being an MDR premises were 6.7 (95% CI 1.2–38.4) times higher ( $P = .03$ ) in premises where the staff consisted of >5 persons. This was the only statistically significant variable for this outcome. No regional trends were observed from these analyses.

## 4 | DISCUSSION

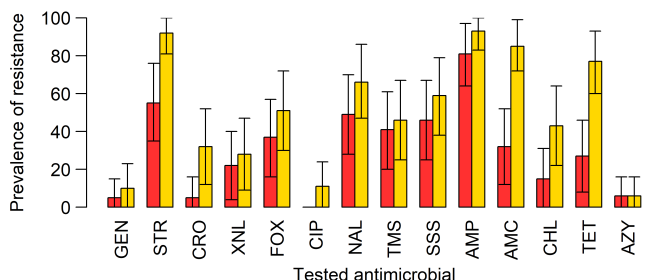
We demonstrated that the fecal microbiota of healthy horses harbors MDR and ESBL/AmpC *E. coli* isolates. The prevalence of premises housing horses shedding ESBL/AmpC *E. coli* isolates (29.0%) is comparable to that found in pig farms in other European countries.<sup>32</sup> This finding is both surprising and worrisome because horses can be considered companion animals.<sup>10</sup> Until now, the focus concerning antimicrobial resistance in animals has been on food-producing animals.<sup>7</sup> These results suggest that companion animals, including horses, also are important in the persistence of antimicrobial resistance genes.

Ceftiofur is a wide-spectrum third-generation cephalosporin approved for use in veterinary medicine that is very well tolerated by horses. Thus, its common use by horse practitioners<sup>33</sup> may promote

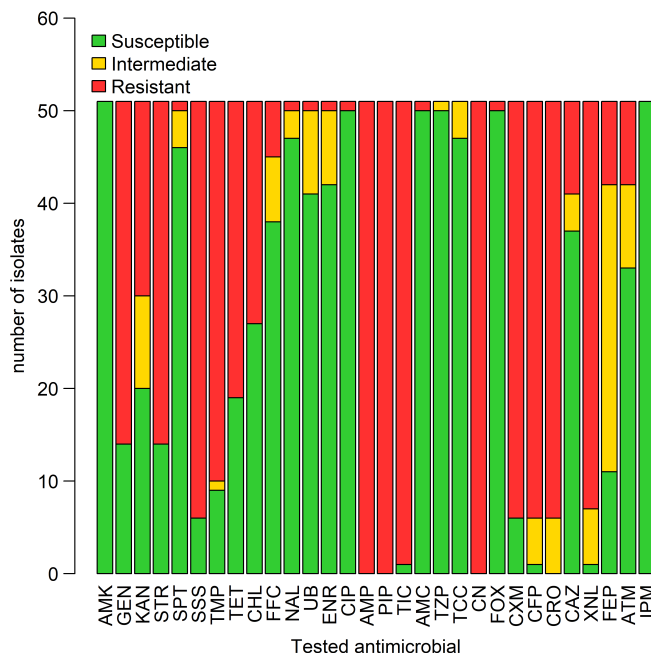
**(A) Prevalence of horses shedding susceptible isolates per antimicrobial (n=132 horses)**



**(B) Prevalence of premises housing horses shedding susceptible isolates per antimicrobial (n=38 premises)**



**Susceptibility pattern of isolates in the ESBL/AmpC collection**



**FIGURE 2** Prevalence estimates of resistance per antimicrobial, at the horse level (A), and at the premises level (B), in a cross-sectional study of 132 healthy adult horses, in 38 premises, performed in 2015, in France. AMC, amoxicillin-Clavulanic acid; AMP, ampicillin; AZY, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; FOX, Cefoxitin; GEN, gentamicin; NA, Nalidixic acid; SSS, Sulfisoxazole; STR, streptomycin; TET, tetracycline; TMS, trimethoprim-sulfamethoxazole; XNL, Ceftiofur. Bars represent 95% confidence intervals. We tested 348 isolates in total

**FIGURE 3** Characterization of susceptibility profiles of *E. coli* isolates in the ESBL/AmpC collection, in a cross-sectional study performed on healthy adult horses, in France, in 2015 (n = 50 isolates). AMC, amoxicillin-Clavulanic acid; AMK, Amikacin; AMP, ampicillin; ATM, Aztreonam; CAZ, Cefoperazone; CFP, Ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CN, Cefalexin; CRO, ceftriaxone; CXM, Cefuroxime; ENR, Enrofloxacin; FEP, Cefepime; FFC, Florfenicol; FOX, Cefoxitin; GEN, gentamicin; IPM, Imipenem; KAN, kanamycin; NAL, Nalidixic acid; PIP, Piperacillin; SPT, Spectinomycin; SSS, Sulfisoxazole; STR, streptomycin; TCC, Ticarcillin-Clavulanic acid; TET, tetracycline; TIC, Ticarcillin; TMP, trimethoprim; TZR, Piperacillin-Tazobactam; UB, Flumequine; XNL, Ceftiofur

ESBL/AmpC gene persistence in this species. Nevertheless, the occurrence of ESBL/AmpC genes in other animal species and in the environment is a global problem affecting both animal and public health<sup>34</sup> and also may contribute to the prevalence of ESBL/AmpC genes in horses. Although most ESBL/AmpC-positive isolates in our study were commensals, ESBL/AmpC genes may be transmissible to potential pathogenic or zoonotic strains.

The predominant family of ESBL genes found in the equine microbiota was ESBL *bla*<sub>CTX-M</sub>. This ESBL family has been of public health

concern since the 2000s. In Europe, a dramatic increase in the incidence and diversity of this family has occurred, and over the last 2 decades, a shift from other ESBL enzymes in Enterobacteriaceae such as *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> to *bla*<sub>CTX-M</sub> variants has been observed. The *bla*<sub>CTX-M</sub> encoded ESBL family is characterized by the ability to hydrolyze oxy-imino-cephalosporins (third and fourth generation) and

**TABLE 1** Prevalence estimates of healthy adult horses

	Bacterial collection					
	Indicator		ESBL/AmpC		ESBL/AmpC	
	Horse level (n = 132)		Premise level (n = 38)		Premise level (n = 41)	
Number of resistant antimicrobial classes	Prev (%)	CI (95%)	Prev (%)	CI (95%)	Prev (%)	CI (95%)
≥1	84.4	77.4-91.4	99.2	97.5-100	29.0	11.5-46.5
≥3 (MDR)	44.4	33.1-55.6	79.7	63.5-96.0	29.0	11.5-46.5
≥5	21.9	14.1-29.6	60.2	39.9-80.5	29.0	11.5-46.5
≥7	7.6	1.5-13.7	26.1	7.0-45.2	28.3	10.8-45.8
≥9	0	NA	0	NA	2.1	0.0-4.4

Left of the table: Prevalence estimates of healthy adult horses shedding *E. coli* isolates nonsusceptible to more than 1, 3, 5, 7, or 9 classes of antimicrobials and premises housing these horses based on the indicator collection results in a cross-sectional study of 132 horses, in 38 premises, in France in 2015. Right of the table: Prevalence estimates of premises housing healthy adult horses shedding isolates nonsusceptible to more than 1, 3, 5, 7, or 9 classes of antimicrobial in the ESBL/AmpC collection, based on the ESBL/AmpC collection results in a cross-sectional study on healthy horses, in 38 premises, in France in 2015.

Abbreviations: AmpC, AmpC β-lactamase; CI, confidence interval; ESBL, extended spectrum β-lactamase; MDR, multidrug resistant; Prev, prevalence.



**TABLE 2** Potential risk factors for ESBL/AmpC and multidrug resistant (MDR) status at the premises level, in a cross-sectional study performed on healthy adult horses, in France, in 2015

Risk factor (MDR)	ESBL/AmpC status			MDR status		
	No of stables per category	% of positive stables per category	P (univariate analysis)	No of stables per category	% of positive stables per category	P (univariate analysis)
Number of horses tested			.82	NA		
10 and less	1	0.0				
Between 11 and 20	36	41.7				
21 and more	4	25.0				
Region			.26			.56
Normandy	19	42.1		18	66.7	
Aquitaine	14	50.0		12	75.0	
East	8	12.5		8	87.5	
Transportation			.06			.39
Twice per month or more	27	44.4		24	75.0	
Less than twice per month	11	9.1		10	60.0	
Total number of horses in the stable			.09			.76
Less than 50	13	15.4		9	66.7	
50 and more	25	44.0		25	72.0	
Staff taking care of horses (daily)			.007			.03
5 persons and less	21	14.3		17	52.9	
More than 5 persons	17	58.9		17	88.2	
Decision to administrate antimicrobials without medical advice			.77			.84
Yes	33	33.3		30	70.0	
No	5	40.0		4	75.0	
Contact with wild life			.20			.85
Yes	27	40.7		23	69.6	
No	11	18.2		11	72.7	
Presence of other animals on the farm			.58			.20
Farm animals	4	25.0		4	75.0	
Pets	17	35.3		14	85.7	
Farm animals and pets	12	25.0		11	45.5	
No	5	60.0		5	80.0	
Fertilizer spread on the pasture			.65			.76
Yes	10	40.0		9	66.7	
No	28	32.1		25	72.0	
Stall cleansing frequency			.44			.90
Less than once a week	8	50.0		7	71.4	
Once a week and more	26	34.5		23	73.9	
Activity			.05			.28
Breeding facility	20	55.0		17	82.4	
Riding school	21	23.8		21	66.7	
One person of the staff in contact with human or veterinarian medical environment			.23			.58
Yes	28	28.6		25	68.0	
No	10	50.0		9	77.8	
Vet is specialized in equine medicine			.97			.97
Yes	36	36.1		32	68.8	
No	2	0.0		2	100	
One horse has been hospitalized in the last 3 months			.49			.84
Yes	4	50.0		4	75.0	
No	34	32.4		30	70.0	

(Continues)

TABLE 2 (Continued)

Risk factor (MDR)	ESBL/AmpC status			MDR status		
	No of stables per category	% of positive stables per category	P (univariate analysis)	No of stables per category	% of positive stables per category	P (univariate analysis)
One horse has undergone surgery in the last 3 months			.49			.35
Yes	4	50.0		4	50.0	
No	34	32.4		30	73.3	
One horse has been medically treated in the last 3 months (all treatments considered)			.04			.60
Yes	20	50.0		18	66.7	
No	18	16.7		16	75.0	

A positive MDR status for a horse was defined as the detection of a least 1 MDR isolate for that horse. A premises was attributed a positive MDR status if it housed at least 1 positive MDR horse. *P*-values are derived from the likelihood ratio test in univariate analysis.

monobactams but not cephamycins and carbapenems. These bacteria generally are susceptible to  $\beta$ -lactam inhibitors. The predominance of the *bla*<sub>CTX-M-1</sub> suggests global dissemination of this gene in the equine population. Moreover, it also has been found in all other food-producing animal species in Europe,<sup>13</sup> suggesting dissemination among species.

In addition to CTX-M ESBLs, the AmpC resistance gene *bla*<sub>CMY-2</sub> is frequently found in food production animals in North America,<sup>35</sup> but it also has been found in Europe.<sup>36</sup> To the best of our knowledge, this gene has not previously been identified in healthy horses, and this finding reinforces the idea that AmpC genes may spread through the healthy animal population.

The ESBL/AmpC genes often are carried by MDR plasmids, which play a key role in their dissemination.<sup>15</sup> The high prevalence of ESBL/AmpC and MDR *E. coli* isolates observed in our study may be explained by the combination of the presence of such plasmids and antimicrobial pressure. Nevertheless, their mobile genetic elements, such as transposons and conjugative plasmids, and their ability to disseminate, should be investigated further.<sup>13</sup>

Our approach of examining an indicator *E. coli* collection identified extensive drug resistance in *E. coli* in the intestinal microbiome of healthy horses in France, as previously observed in the United Kingdom.<sup>4</sup> The large amount of nonsusceptibility to AMP in the indicator collection could be explained by the frequent use of penicillin in equine medicine.

TABLE 3 Parameter estimates and odds ratio from a multivariable logistic regression modeling ESBL/AmpC-positive status at the premises level, based on the results of a cross-sectional study performed on 38 premises housing healthy adult horses, sampled in France in 2015

Risk factor	Odds ratios		
	Estimate	95% CI	P
Riding school versus breeding facility	14.6	1.3-164.6	.03
One horse has been medically treated in the last 3 months (all treatments considered) versus no horse treated	9.6	1.2-76.9	.03
More than 5 persons taking care of horses daily versus 5 persons or less	35.7	2.9-500.0	.006

Abbreviations: AmpC, AmpC  $\beta$ -lactamase; CI, confidence interval; ESBL, extended spectrum  $\beta$ -lactamase.

Acquired resistance to quinolones and fluoroquinolones is a result of the appearance of chromosomal mutations. Generally, the mutations appear in sequence and involve the genes *gyrA* (coding for the gyrase) and *parC* (coding for a subunit of the topoisomerase). The first mutation confers resistance to quinolones and the combination of the 2 mutations confers resistance to fluoroquinolones. Hence, nonsusceptibility to NAL generally is considered a predictor for fluoroquinolone treatment failure.<sup>37</sup> The finding in the indicator collection that >55% of premises featured this type of resistance suggests that ENR should be used with caution in horses.

Although we detected nonsusceptibility to third-generation cephalosporins in the indicator collection, none of the isolates carried tested ESBL/AmpC genes, suggesting that other ESBL/AmpC genes may be present in the population, (eg, *bla*<sub>OXA</sub>, *bla*<sub>PER</sub>, *bla*<sub>GES-1</sub>, and *bla*<sub>VER-1</sub>) or other mechanisms of resistant to cephalosporins may be present (eg, an efflux pump). These other mechanisms would less likely be transmissible by mobile elements but also could account for treatment failure when using cephalosporins and therefore could still have an impact on the health of horses.

In the indicator collection, we detected viable *E. coli* in only 68% of the fecal samples. This viability rate is low compared to other published studies in healthy horses,<sup>4</sup> and compared to internal data available in our laboratory. Some *E. coli* isolates may have been lost during shipping. This issue questions the validity of the indicator collection results, but if it had any effect on the results it would have underestimated resistance. Indeed, antimicrobial resistance has a fitness cost,<sup>38</sup> and resistant isolates should have died first. Nevertheless, the level of resistance we found in the indicator collection, based only on *E. coli*-positive samples, is comparable to that of other studies.<sup>4</sup> Therefore, we do not believe shipping had considerable influence on the results of the indicator collection.

Our results showed that medication represented a risk factor for ESBL/AmpC-positive premises. Antimicrobial treatment already has been identified as a risk factor at the individual level in other studies.<sup>22</sup> Although our results pertain to the premises level, we could hypothesize that medically treated horses are more likely to shed ESBL/AmpC isolates. Based on this information, we could suggest isolating horses that are medically treated or at least implementing appropriate biosecurity measures. As an example, limiting contact between treated and healthy horses and handling healthy horses

before treated horses might be beneficial to limit antimicrobial gene dissemination. More longitudinal studies are needed to establish the duration of shedding in nonhospitalized horses and to develop more accurate recommendations. Nevertheless, based on other studies,<sup>39,40</sup> the isolation of the horses should be for at least 2 weeks.

The finding that the number of persons taking care of horses influences the presence of ESBL/AmpC genes or the presence of MDR isolates has not been documented previously, to our knowledge. Although more studies are needed to confirm such an association, considering that this information is easy to obtain, it could be helpful for elaborating guidelines to improve the health of horses. For example, it could help horse practitioners to define "at-risk" populations and justify the performance of antimicrobial susceptibility testing more frequently.

Riding schools also seem to be more at risk, as compared to breeding premises, for the presence of ESBL/AmpC genes. This risk factor is relevant because riding schools are an interface with the general population. This finding could provide the impetus to set up prevention measures to limit dissemination of ESBL/AmpC genes and MDR isolates. Appropriate recommendations could include washing hands after touching horses or limiting contact between horses and infants at this type of premises, although further studies are needed to quantify the risk of transmission from horses to humans, and reciprocally.

Our study has some limitations. First, horses within a premises were not selected randomly. However, we have no reason to believe that this selection process would have biased our results, considering that the people in charge of field sampling were blinded to the outcome status and drug use history of the horses. In addition, CIs for odd ratios were wide because of the small sample size of premises. Based on these results, it is not possible to compare the strength of association among the identified risk factors, and this low precision should be considered in interpreting the impact of these risk factors in the horse population. Small sample size also limited the statistical power of our study, and thus true associations with risk factors might have been missed. Finally, because many risk factors were tested, some risk factors may have been statistically significant based on chance alone, and thus more studies should be conducted to confirm these associations.

In conclusion, we found a high prevalence of ESBL/AmpC genes and MDR isolates in the microbiota of healthy horses. Surveillance of ESBL/AmpC gene dissemination and quantification of MDR isolates would be beneficial to characterize the nature and extent of the risk they represent, with the aim of limiting their transmission among horses, but also to other species including humans and to the environment.

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## CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

## OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

## HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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