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# Fecal Carriage of Extended-Spectrum-β-Lactamase/AmpC-Producing *Escherichia coli* in Horses

**Joost Hordijk,[a]* Evangelia Farmakioti,[a] Lidwien A. M. Smit,[b] Birgitta Duim,[a] Haitske Graveland,[a] Mathijs J. P. Theelen,[a,c] Jaap A. Wagenaar[a,d]**

[a]Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, the Netherlands
[b]Utrecht University, Institute for Risk Assessment Sciences, Utrecht, the Netherlands
[c]Utrecht University, Faculty of Veterinary Medicine, Department of Equine Sciences, Utrecht, the Netherlands
[d]Wageningen Bioveterinary Research, Lelystad, the Netherlands

**ABSTRACT** A nationwide study on the occurrence of extended-spectrum β-lactamase (ESBL)/AmpC in nonhospitalized horses in the Netherlands was performed. Molecular characterization was done, and questionnaires were analyzed to identify factors associated with carriage. In total, 796 horse owners were approached; 281 of these submitted a fecal sample from their horse(s), resulting in 362 samples. All samples were cultured qualitatively in Luria-Bertani (LB) broth and subsequently on MacConkey agar, both supplemented with 1 mg/liter cefotaxime (LB+ and MC+). Positive samples were subsequently cultured quantitatively on MC+. Initial extended-spectrum-β-lactamase (ESBL)/AmpC screening was performed by PCR, followed by whole-genome sequencing on selected strains. Associations between ESBL/AmpC carriage and questionnaire items were analyzed using a univariate generalized estimating equation (GEE) regression analysis, followed by a multiple GEE model for relevant factors. In total, 39 of 362 samples (11%) were determined to be positive for ESBL/AmpC. $bla_{CTX\text{-}M\text{-}1}$-carrying isolates were obtained from 77% of positive samples ($n = 30$). Other ESBL/AmpC genes observed included $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{SHV\text{-}12}$, $bla_{CMY\text{-}2}$, and $bla_{ACT\text{-}10}$. A high association between the presence of $bla_{CTX\text{-}M\text{-}1}$ and IncHI1 plasmids was observed (46% of samples; $n = 18$). Based on core genome analysis ($n = 48$ isolates), six *Escherichia coli* clusters were identified, three of which represented 80% of the isolates. A negative association between ESBL/AmpC carriage and horses being in contact with other horses at a different site was observed. The presence of a dog on the premises and housing in a more densely human-populated region were positively associated.

**IMPORTANCE** Extended-spectrum β-lactamases (ESBLs) are widespread in human and animal populations and in the environment. Many different ESBL variants exist. The dissemination of ESBLs within and between populations and the environment is also largely influenced by genetic mobile elements (e.g., plasmids) that facilitate spread of these ESBLs. In order to identify potential attributable ESBL sources for, e.g., the human population, it is important to identify the different ESBL variants, the bacteria carrying them, and the potential risk factors for ESBL carriage from other potential sources. This nationwide study focuses on ESBL carriage in the open horse population and investigated the molecular characteristics, geographical distribution throughout the Netherlands, and potential risk factors for fecal ESBL carriage in horses. These data can be used for future attribution studies in order to reduce potential transmission of ESBL-producing bacteria between sources.

**KEYWORDS** AmpC, antimicrobial resistance, ESBL, equidae, risk factor, fecal carriage

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Address correspondence to Joost Hordijk, joost.hordijk@rivm.nl.

* Present address: Joost Hordijk, National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

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Extended-spectrum-β-lactamase (ESBL)- or AmpC-type β-lactamase (AmpC)-producing *Escherichia coli* strains are widespread in humans, animals, and the environment (1). Several studies have also reported ESBL/AmpC in horses specifically, mostly from hospitalized horses (2–12), but data on healthy nonhospitalized horses are also available (5, 13–15). In a study from the Czech Republic, similar ESBL-carrying isolates were found in a horse and a human, indicating a zoonotic potential and/or occupational hazard (5). Interestingly, $bla_{CTX\text{-}M\text{-}1}$ from horses has been associated with a specific plasmid, IncHI1 (2, 5, 8, 16). Transfer of IncHI1 plasmids through conjugation is known to be temperature dependent, with an optimum temperature between 22 and 30°C (17, 18), suggesting that dissemination through horizontal transfer mainly plays a role in the environment and not in the horse itself. In addition, horse-associated IncHI1 plasmids have been shown to harbor an FOS operon. The FOS operon harbors genes involved in the metabolism of fructooligosaccharides (19), which naturally occur in several crops and grains. Although not specifically tested in horses, the FOS operon has also been associated with increased *E. coli* intestinal colonization in chickens in an experimental setting (20). Data on risk factors for the carriage of ESBL/AmpC in horses are limited. Multiple people (>5) taking care of the horse and the administration of medication have been reported as potential risk factors in healthy French horses (13).

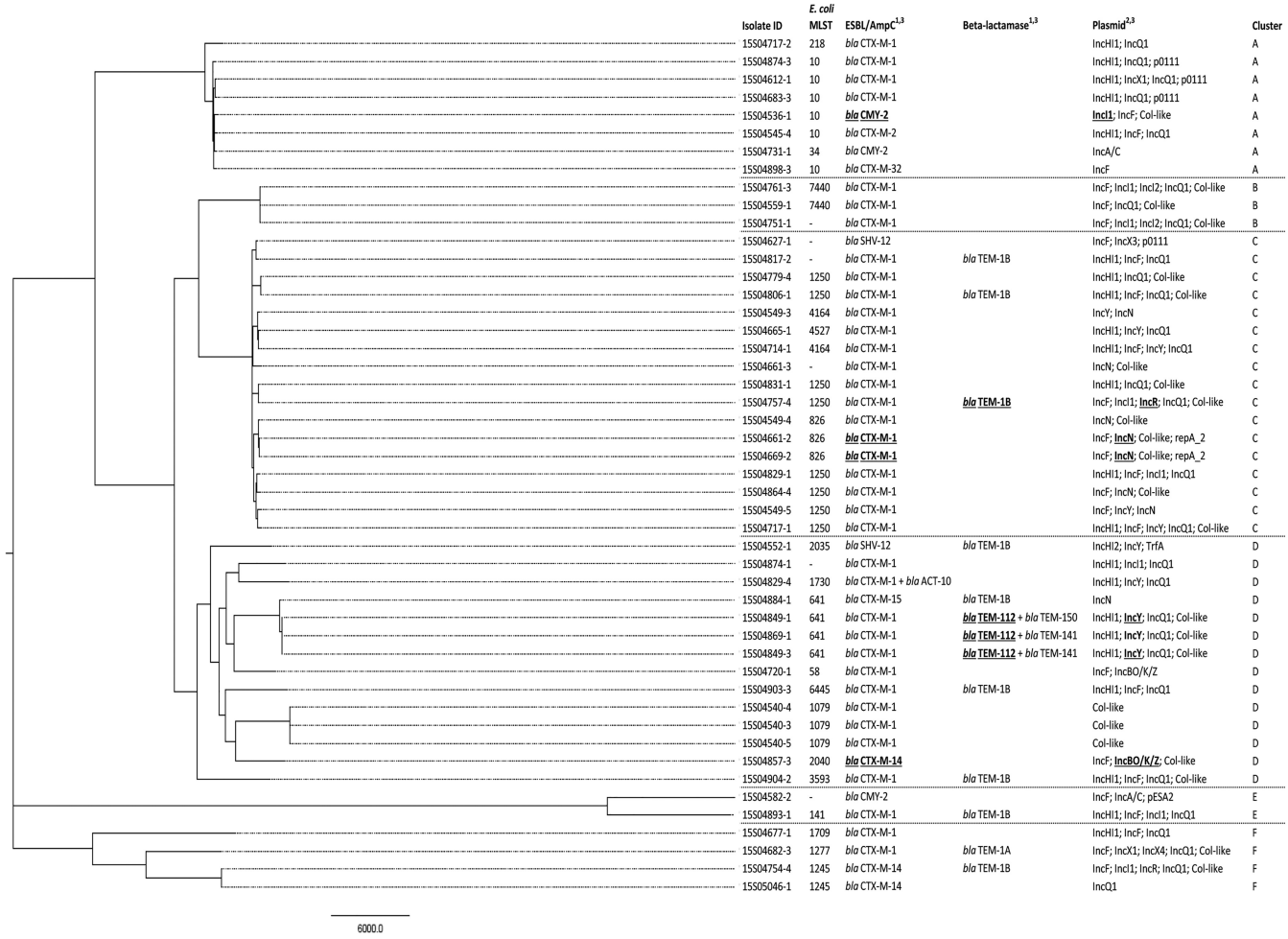
In the present study, we have conducted a nationwide screening for ESBL/AmpC in nonhospitalized horses in the Netherlands to determine the occurrence ESBL/AmpC-carrying bacteria. Molecular analysis was performed to characterize ESBL/AmpC-carrying *E. coli* and to identify the plasmids present. Finally, a questionnaire on animal housing, health, and feed was used to identify determinants associated with carriage.

## RESULTS AND DISCUSSION

**ESBL/AmpC and *E. coli* characterization.** From the 769 registered symposium participants that were approached, 276 submitted a fecal sample of their horses. Several participants that owned more than one horse submitted multiple samples, resulting in 362 samples in total. In total, 39 of 362 samples were positive for ESBL/AmpC-producing *E. coli* (11%, 95% confidence interval [95%CI] = 8 to 14%). From owners that submitted multiple samples, six owners also had multiple positive samples; 13 samples were positive out of 17 submitted samples. Ten owners ($n = 21$ samples) had one positive sample. From the remaining 49 owners ($n = 113$ samples), none of the samples were positive for ESBL/AmpC. From the 39 positive samples in total, 17 samples were positive using the track dilution method, resulting in a mean of $1.5 \times 10^6$ CFU/g of feces (range, $1.06 \times 10^3$ to $2.04 \times 10^7$); 22 samples were positive after enrichment only ($<10^3$ CFU/g of feces). This indicates that the number of CFU/g of feces in general was relatively low.

There are no prior data on fecal carriage of ESBL/AmpC in nonhospitalized horses in the Netherlands; therefore, we cannot determine whether there are any changes in occurrence or characteristics of ESBL/AmpC-producing bacteria. However, another study has been performed in an equine hospital in the Netherlands during the same period as the present study (2). That study examined fecal samples of hospitalized horses not suffering from gastrointestinal diseases. In the hospital-based study, 85% of the samples were positive compared to the 11% in the present study. This difference could be due to nosocomial transmission. In other countries (France, Germany, United Kingdom, Poland, and the Czech Republic), the occurrence of ESBL/AmpC-producing bacteria has also been determined in various ways. The occurrence ranged from 4 to 29%. These differences were most likely partially caused by differences in study size and setup, which make the findings difficult to compare (5, 13–15, 21).

A vast majority of 77% of positive samples ($n = 30$) harbored $bla_{CTX\text{-}M\text{-}1}$-carrying isolates. Other ESBL/AmpC genes observed were $bla_{CTX\text{-}M\text{-}2}$ ($n = 1$), $bla_{CTX\text{-}M\text{-}14}$ ($n = 3$), $bla_{CTX\text{-}M\text{-}15}$ ($n = 1$), $bla_{CTX\text{-}M\text{-}32}$ ($n = 1$), $bla_{SHV\text{-}12}$ ($n = 2$), $bla_{CMY\text{-}2}$ ($n = 3$), and $bla_{ACT\text{-}10}$ ($n = 1$) (Fig. 1). The $bla_{CTX\text{-}M\text{-}1}$ gene is a common allele variant in various animal sources (1); in horses in Europe it also is a commonly observed allele variant (2, 5, 8, 9, 16). Horse-associated $bla_{CTX\text{-}M\text{-}1}$ is mostly characterized by its association with the IncHI1




| Isolate ID | *E. coli* MLST | ESBL/AmpC[1,3] | Beta-lactamase[1,3] | Plasmid[2,3] | Cluster |
|---|---|---|---|---|---|
| 15S04717-2 | 218 | *bla* CTX-M-1 | | IncHI1; IncQ1 | A |
| 15S04874-3 | 10 | *bla* CTX-M-1 | | IncHI1; IncQ1; p0111 | A |
| 15S04612-1 | 10 | *bla* CTX-M-1 | | IncHI1; IncX1; IncQ1; p0111 | A |
| 15S04683-3 | 10 | *bla* CTX-M-1 | | IncHI1; IncQ1; p0111 | A |
| 15S04536-1 | 10 | ***bla* CMY-2** | | **IncI1**; IncF; Col-like | A |
| 15S04545-4 | 10 | *bla* CTX-M-2 | | IncHI1; IncF; IncQ1 | A |
| 15S04731-1 | 34 | *bla* CMY-2 | | IncA/C | A |
| 15S04898-3 | 10 | *bla* CTX-M-32 | | IncF | A |
| 15S04761-3 | 7440 | *bla* CTX-M-1 | | IncF; IncI1; IncI2; IncQ1; Col-like | B |
| 15S04559-1 | 7440 | *bla* CTX-M-1 | | IncF; IncQ1; Col-like | B |
| 15S04751-1 | - | *bla* CTX-M-1 | | IncF; IncI1; IncI2; IncQ1; Col-like | B |
| 15S04627-1 | - | *bla* SHV-12 | | IncF; IncX3; p0111 | C |
| 15S04817-2 | - | *bla* CTX-M-1 | *bla* TEM-1B | IncHI1; IncF; IncQ1 | C |
| 15S04779-4 | 1250 | *bla* CTX-M-1 | | IncHI1; IncQ1; Col-like | C |
| 15S04806-1 | 1250 | *bla* CTX-M-1 | *bla* TEM-1B | IncHI1; IncF; IncQ1; Col-like | C |
| 15S04549-3 | 4164 | *bla* CTX-M-1 | | IncY; IncN | C |
| 15S04665-1 | 4527 | *bla* CTX-M-1 | | IncHI1; IncY; IncQ1 | C |
| 15S04714-1 | 4164 | *bla* CTX-M-1 | | IncHI1; IncF; IncY; IncQ1 | C |
| 15S04661-3 | - | *bla* CTX-M-1 | | IncN; Col-like | C |
| 15S04831-1 | 1250 | *bla* CTX-M-1 | | IncHI1; IncQ1; Col-like | C |
| 15S04757-4 | 1250 | *bla* CTX-M-1 | ***bla* TEM-1B** | IncF; IncI1; **IncR**; IncQ1; Col-like | C |
| 15S04549-4 | 826 | *bla* CTX-M-1 | | IncN; Col-like | C |
| 15S04661-2 | 826 | ***bla* CTX-M-1** | | IncF; **IncN**; Col-like; repA_2 | C |
| 15S04669-2 | 826 | ***bla* CTX-M-1** | | IncF; **IncN**; Col-like; repA_2 | C |
| 15S04829-1 | 1250 | *bla* CTX-M-1 | | IncHI1; IncF; IncI1; IncQ1 | C |
| 15S04864-4 | 1250 | *bla* CTX-M-1 | | IncF; IncN; Col-like | C |
| 15S04549-5 | 1250 | *bla* CTX-M-1 | | IncF; IncY; IncN | C |
| 15S04717-1 | 1250 | *bla* CTX-M-1 | | IncHI1; IncF; IncY; IncQ1; Col-like | C |
| 15S04552-1 | 2035 | *bla* SHV-12 | *bla* TEM-1B | IncHI2; IncY; TrfA | D |
| 15S04874-1 | - | *bla* CTX-M-1 | | IncHI1; IncI1; IncQ1 | D |
| 15S04829-4 | 1730 | *bla* CTX-M-1 + *bla* ACT-10 | | IncHI1; IncY; IncQ1 | D |
| 15S04884-1 | 641 | *bla* CTX-M-15 | *bla* TEM-1B | IncN | D |
| 15S04849-1 | 641 | *bla* CTX-M-1 | ***bla* TEM-112** + *bla* TEM-150 | IncHI1; **IncY**; IncQ1; Col-like | D |
| 15S04869-1 | 641 | *bla* CTX-M-1 | ***bla* TEM-112** + *bla* TEM-141 | IncHI1; **IncY**; IncQ1; Col-like | D |
| 15S04849-3 | 641 | *bla* CTX-M-1 | ***bla* TEM-112** + *bla* TEM-141 | IncHI1; **IncY**; IncQ1; Col-like | D |
| 15S04720-1 | 58 | *bla* CTX-M-1 | | IncF; IncBO/K/Z | D |
| 15S04903-3 | 6445 | *bla* CTX-M-1 | *bla* TEM-1B | IncHI1; IncF; IncQ1 | D |
| 15S04540-4 | 1079 | *bla* CTX-M-1 | | Col-like | D |
| 15S04540-3 | 1079 | *bla* CTX-M-1 | | Col-like | D |
| 15S04540-5 | 1079 | *bla* CTX-M-1 | | Col-like | D |
| 15S04857-3 | 2040 | ***bla* CTX-M-14** | | IncF; **IncBO/K/Z**; Col-like | D |
| 15S04904-2 | 3593 | *bla* CTX-M-1 | *bla* TEM-1B | IncHI1; IncF; IncQ1; Col-like | D |
| 15S04582-2 | - | *bla* CMY-2 | | IncF; IncA/C; pESA2 | E |
| 15S04893-1 | 141 | *bla* CTX-M-1 | *bla* TEM-1B | IncHI1; IncF; IncI1; IncQ1 | E |
| 15S04677-1 | 1709 | *bla* CTX-M-1 | | IncHI1; IncF; IncQ1 | F |
| 15S04682-3 | 1277 | *bla* CTX-M-1 | *bla* TEM-1A | IncF; IncX1; IncX4; IncQ1; Col-like | F |
| 15S04754-4 | 1245 | *bla* CTX-M-14 | *bla* TEM-1B | IncF; IncI1; IncR; IncQ1; Col-like | F |
| 15S05046-1 | 1245 | *bla* CTX-M-14 | | IncQ1 | F |

**FIG 1** Core genome comparison of ESBL/AmpC-positive *E. coli* isolates. Superscript numbers in column headings: 1, allele variants were determined using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/); 2, plasmid incompatibility groups were determined using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/); and 3, alleles and incompatibility reference genes indicated by boldface and underlining are located on the same contig.

plasmid (2, 5, 8, 16), which may carry the FOS operon (19). The total number of isolates obtained was 65, 48 of which were selected for whole-genome sequencing (WGS). *In silico* screening showed that most isolates carried multiple plasmids (Fig. 1). The most abundant plasmid incompatibility groups were IncQ1 (60%), IncHI1 (46%), Col-like plasmids (46%), IncF (31%), IncY (21%), IncI1 (17%), IncN (10%), and several non-frequently occurring incompatibility groups (each <10%). Due to the limitations of the short-read sequences used in this study, we could not directly link $bla_{\text{CTX-M-1}}$ to IncHI1. Nonetheless, the plasmid sequence type (pST) of the IncHI1 plasmids in this study was pST9. This is in line with what has been found previously in the Netherlands, Sweden, and France (2, 8). Only one isolate harbored an ST9-like IncHI1 (one locus had a partial sequence). Also, the *fosK* gene, which is part of the FOS operon, was present in all IncHI1-carrying isolates. Because of their colocalization on the same contig, in four isolates the ESBL/AmpC gene could be directly linked to its carrying plasmid (Fig. 1). In these isolates, $bla_{\text{CTX-M-1}}$ was associated with IncN ($n = 2$), $bla_{\text{CTX-M-14}}$ was associated with IncK/BO/Z ($n = 1$), and $bla_{\text{CMY-2}}$ was associated with IncI1 ($n = 1$). The two $bla_{\text{CTX-M-1}}$-carrying IncN plasmids belonged to pST1, which has also been observed in horses in the Netherlands and the Czech Republic (2, 22). All other ESBL/AmpC genes could not be linked directly to either an incompatibility group or chromosomal location.

In the phylogenetic tree that was based on a core genome analysis, six (arbitrarily defined) clusters can be identified. Three clusters are relatively large and represent 80%

of the strains (Fig. 1; clusters A, C, and D). The most abundant STs observed were ST10 (cluster A), ST1250 (cluster C), and ST641 and ST1079 (both cluster D). The sequence types (STs) of clusters A and C show relatively high similarity, only harboring single or double locus variants (SLV/DLV) of ST10 and ST1250, respectively. All STs from cluster A belong to clonal complex (CC) 10. For STs from cluster C, no clonal complex was defined. Cluster D is more diverse, harboring STs that differ by more than two loci. The STs also belonged to multiple clonal complexes (CCs 86, 155, and 23). These findings are in line with the findings of the Dutch hospital-based study (2). In surrounding countries, Germany and France, ST10 and ST1250 or SLV/DLVs were reported (12, 23), suggesting that these are common STs in equine microbiota. In addition to the core genome, analysis of the accessory genome (gene presence or absence) shows a large overlap with the core phylogeny (see Fig. S2 in the supplemental material), suggesting little genetic rearrangement.

**Determinants of ESBL/AmpC carriage.** From the 276 participants, 357 completed questionnaires for individual horses were obtained. Questionnaires regarding five horses were missing; two of these animals were ESBL positive. First, a univariate generalized estimating equation (GEE) regression model was used on variables concerning age, housing, feed, and contact with other animals. The variables "contact with horses at another location" (e.g., during competitions) and "type of feed—other than custom types of horse feed" were negatively associated. In contrast, the "presence of a dog on premises" was positively associated with ESBL/AmpC carriage ($P < 0.1$). These three variables were subsequently analyzed with a multiple GEE regression model, resulting in slightly stronger associations ($P < 0.05$) (Table 1). Both negatively associated determinants need further exploration for conclusions to be drawn. Horses that travel to other locations are mostly horses that participate in competitions. A confounding factor may be housing (e.g., private box and/or field), although housing itself was not a significantly associated factor. Furthermore, none of the custom horse feeds (e.g., oats, grain, hay, and alfalfa) were found to contribute significantly. "Other feed" showed a statistically significant negative association. However, since this category covered a wide range of "miscellaneous" feed or feed additives, no specific components could be determined. Most owners listed grass, carrots, vitamins/additives, or a specific type of feed (e.g., for senior horses). The addition of fructooligosaccharides as a feed additive could not be derived from the questionnaires. A moderate positive association for the presence of dogs on the premises was observed. Another Dutch attribution study also showed that although ESBL diversity in dogs was relatively high, they had little overlap with other sources (note that horses were not included) (24). This means their attribution should be considered with care, and further investigation of dogs present on horse premises should be included in follow up studies. In all, 144 horses were treated with medication (any, including, e.g., pain killers), of which 96 were treated for worms and eight were treated with antimicrobials. Medication use, either "any" or specific antimicrobials, was not identified as a risk factor. Neither was visiting a hospital. It should be noted that eight observations of antimicrobial use is rather low for a risk factor analysis. In contrast, de Lagarde et al. did identify the administration of medication (any) as a risk factor (13). In comparison to our study, de Lagarde et al. identified a significant risk factor that was not included in our study. The number of persons taking care of the horse (0 to 5 or >5) resulted in an increased risk if more than five people were involved (13).

For the geographical analysis, 232 participants (85%) provided a (partial) postal code. The 362 samples that were collected originated from 167 unique postal codes. All observations are plotted in Fig. 2, in which the colors of the circles indicate the proportion of positives (green = 0%, red = 100%). The figure also shows the highly urbanized "Randstad" (red), intermediate zone (yellow), and peripheral zone (blue). The spatial association in ESBL/AmpC carriage showed a higher carriage in more (human) populated regions, with an odds ratio (OR) for the trend of 2.28 (95%CI = 1.08 to 4.82; $P = 0.031$). This association was slightly attenuated after correction for the question-

**TABLE 1** Association between questionnaire-reported items and ESBL/AmpC carriage in horses[a]

| Characteristic | No. (%) or mean ± SD | Univariate GEE regression model, OR (95% CI) | Multiple GEE regression model, adjusted OR (95% CI) |
|---|---|---|---|
| Mean age, yr | 12.7 ± 6.7 | 0.99 (0.94–1.05) | |
| In pen, housed individually (reference) | 296 (82.9) | 1 | |
| In pen, housed in group (2 or 3 horses total) | 30 (8.4) | 0.38 (0.06–2.30) | |
| In pen, housed in group (>3 horses total) | 31 (8.7) | 0.79 (0.20–3.14) | |
| In field, housed in group | 280 (79.8) | 0.88 (0.28–2.77) | |
| Medication (any) | 144 (40.7) | 0.68 (0.26–1.77) | |
| Deworming treatment | 96 (27.0) | 0.85 (0.35–2.06) | |
| Antibiotic treatment | 8 (2.2) | 1.65 (0.27–10.06) | |
| Not visited a hospital | 328 (94.0) | 2.10 (0.12–37.86) | |
| Contact horses at other location (e.g., during travel/competition) | 126 (36.2) | 0.41 (0.15–1.14) | 0.37 (0.14–0.95)* |
| Feed, hay | 233 (66.4) | 1.21 (0.43–3.42) | |
| Feed, silage | 168 (47.9) | 0.85 (0.34–2.12) | |
| Feed, kibble | 229 (65.2) | 1.82 (0.65–5.06) | |
| Feed, muesli | 163 (46.4) | 0.71 (0.33–1.55) | |
| Feed, grain | 32 (9.1) | 1.36 (0.53–3.48) | |
| Feed, alfalfa | 104 (29.6) | 0.97 (0.48–1.97) | |
| Feed, oats | 13 (3.7) | NE[c] | |
| Feed, different[b] | 128 (36.5) | 0.30 (0.12–0.75)* | 0.29 (0.12–0.70)* |
| Animals present near housing | 309 (88.3) | 2.39 (0.59–9.69) | |
| Animals present, cattle | 58 (16.6) | 1.30 (0.53–3.17) | |
| Animals present, pig | 18 (5.1) | 0.50 (0.07–3.57) | |
| Animals present, poultry | 105 (30.0) | 0.82 (0.40–1.69) | |
| Animals present, goat | 32 (9.1) | 1.06 (0.32–3.51) | |
| Animals present, dog | 238 (68.0) | 2.39 (0.99–5.75) | 2.67 (1.06–6.74)* |
| Animals present, cat | 229 (65.4) | 1.20 (0.53–2.73) | |
| Animals present, sheep | 69 (19.7) | NE | |
| Animals present, other | 31 (8.9) | 1.09 (0.23–5.16) | |
| No farm animals near housing (reference) | 186 (53.4) | 1 | |
| Farm animals present, professionally | 46 (13.2) | 0.88 (0.31–2.52) | |
| Farm animals present, hobby | 116 (33.3) | 0.59 (0.26–1.35) | |

[a]Results are expressed as odds ratios (OR) for the presence of a characteristic versus the absence (reference), unless indicated otherwise. Asterisks (*) indicate significant values ($P < 0.05$).
[b]"Feed, different" includes all miscellaneous types of feed and feed additives mentioned by the owners. These could include, e.g., vitamins or specific feed for "senior horses."
[c]NE, the OR was not estimable.

naire items included in the multiple GEE regression model, resulting in an OR of 2.11 (95%CI = 0.95 to 4.67; $P = 0.066$). Similar differences were observed in Dutch human population studies, with a higher prevalence of ESBL/AmpC carriage in an urban population (8.6%) (25) than in a rural area (4.5%) (26). Key figures about the current distribution of the equine population in the Netherlands are lacking, but horse population density is traditionally the inverse of the human population. This means the current expected density of the horse population is highest in rural areas. The clusters of ESBL/AmpC-positive *E. coli* shown in Fig. 1 do not correlate with a specific region (data not shown). In combination with the findings of the Dutch hospital-based study, but also the findings from Germany, United Kingdom, Sweden, France, Denmark, and the Czech Republic, in which similar *E. coli* STs and plasmids were observed, the data suggest that the observed ESBL/AmpC carrying isolates are more likely to be horse associated than related to a specific region in Europe. The fact that some owners provided multiple samples may create a sampling bias. Also, it is difficult to establish objectively whether the participating owners are representative sample of Dutch horse owners. Nonetheless, as described above, based on these samples, there does not seem to be a clear clustering of ESBL/AmpC-positive animals at the same location. In addition, since the samples taken were well dispersed over the country, the occurrence of 11% positive samples is most likely a good estimate for the prevalence of ESBL/AmpC-producing *E. coli* among nonhospitalized horses in the Netherlands.

**Conclusions.** In this nationwide study among healthy horses, we show that 11% of the samples were positive for ESBL/AmpC, with the $bla_{CTX\text{-}M\text{-}1}$ type being the most

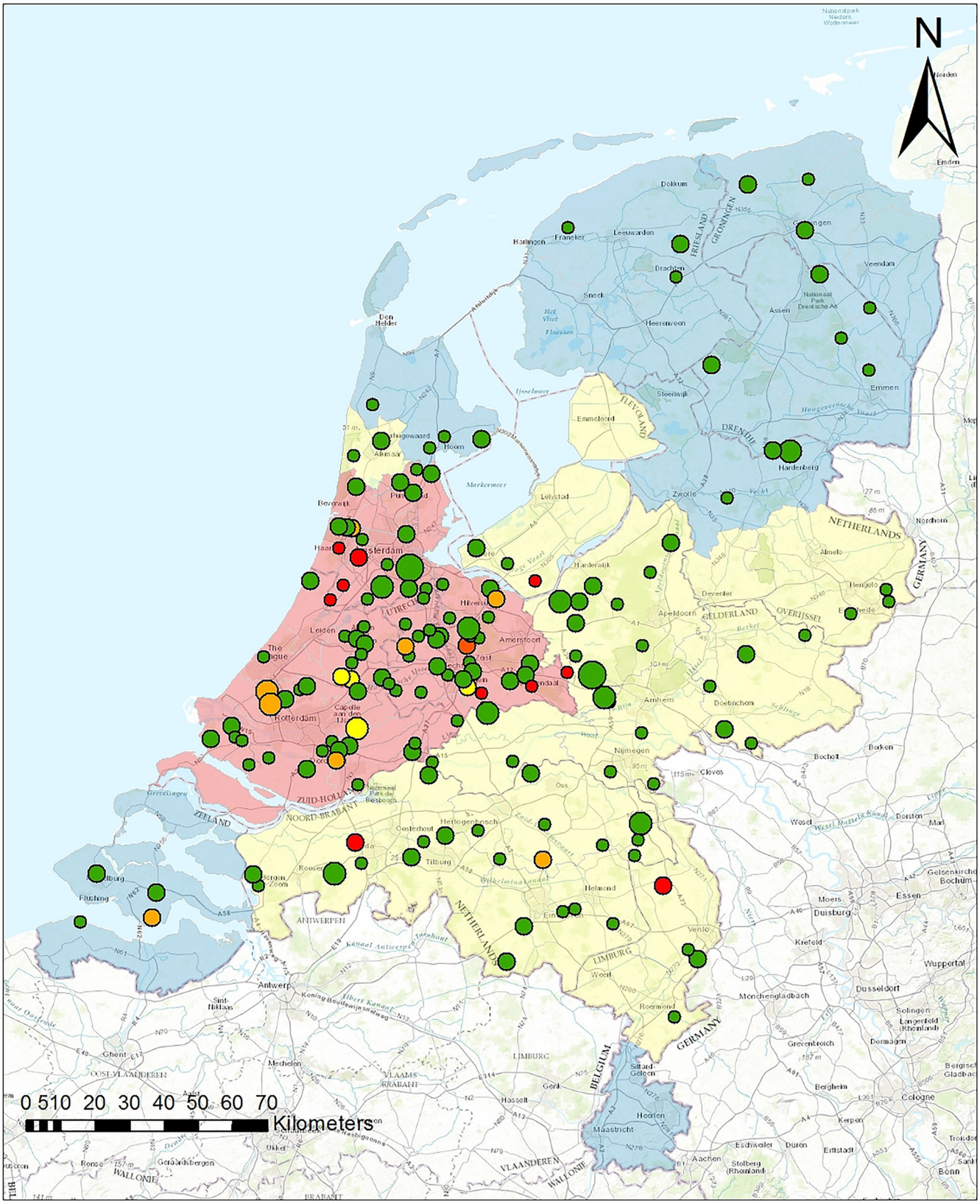



**FIG 2** Spatial distribution of ESBL/AmpC carriage in healthy horses in the Netherlands. A total of 167 circles represent various unique postal codes. The sizes of the circles indicate the number of samples within the postal code area (one to eight samples, not necessarily from the same owner). The circle color indicates the proportion of positives (from green [0%] to red [100%]). Three (human) population density zones were defined: highly urbanized (red), intermediate (yellow), and peripheral (blue). Map created using ArcGis 9.3.1 (Esri, Redlands, CA).

abundant. In general, the level of shedding was relatively low. The associations between equine ESBL/AmpC carriage and human population density, contact with other horses, type of feed, and the presence of a dog require a more in-depth approach to draw strong conclusions. *E. coli* core genome analysis indicated three larger clusters; of these, isolates belonging to ST10-like and ST1250-like strains showed high similarity, harboring single or double locus variants only. Although they could not be directly linked, there is a strong association between the presence of $bla_{CTX\text{-}M\text{-}1}$ and IncHI1. The large overlap in core genome and accessory genome suggests little genetic rearrangement between clusters.

## MATERIALS AND METHODS

**Study design and sampling.** Fecal sample collection took place during a symposium for horse owners at the Equine Hospital of Utrecht University, Utrecht, the Netherlands, in November 2015. Before the meeting, all participants ($n = 769$) that registered were asked to collect a fresh fecal dropping from their horse of the ground and deposit this the same day at the symposium. In addition, participants were asked to fill out a questionnaire including questions about housing (e.g., stable, field, and the number of animals), feed (main components and/or additives), and health (e.g., medication and hospitalization) (see Table S1 in the supplemental material) and to provide a four-digit postal code to identify the region (village/neighborhood level) where the horse was housed. The symposium was advertised directly to the clinic's clientele of the past 2 years (a referral clinic, covering the whole country), through social media, and in horse-related journals. The clientele varied from professionals (e.g., breeders or professional horse trainers) to recreational riders. Each participant depositing a fecal horse sample received a horse-shaped USB-stick as a reward.

**Strain isolation.** All fecal samples were stored at 4°C until they were processed in the following days. Of each fecal sample, 0.5 g was enriched in 4.5 ml of Luria-Bertani (LB) broth with 1 $\mu$g/ml cefotaxime (LB+) (Oxoid-Tritium, the Netherlands) and incubated overnight at 37°C. Enriched broth was subsequently inoculated ontoa MacConkey agar with 1 $\mu$g/ml cefotaxime (MC+; Oxoid-Tritium) overnight at 37°C. Samples showing growth on MC+ were analyzed quantitatively using the track dilution method (detection limit, $10^3$ CFU/g of feces). In brief, 0.5 g of fecal sample was suspended in 4.5 ml of saline. Subsequently, 100 $\mu$l of suspension was mixed 1:1 with saline in a 96-well microtiter plate and further diluted 1:10 five more times. Then, 20 $\mu$l of each dilution was spotted onto a 12-$cm^2$ MC+ plate, which was tilted at an ~45° angle until the droplets almost reached the bottom of the plate. The plates were incubated overnight at 37°C. The numbers of CFU (i.e., CFU in the first dilution with separately growing colonies [~<10 CFU]) were used to calculate the concentration CFU/g of feces.

For further typing, three colonies were selected from track dilution plates. Only one colony was selected if the sample was only positive after selective enrichment and subsequent inoculation on MC+. Species of selected isolates was confirmed by using a matrix-assisted laser desorption ionization–time of flight method.

**DNA extraction.** For PCR screening, a boiled lysate was made from one colony in Tris-EDTA. DNA isolation for WGS was performed using the UltraClean microbial DNA isolation kit (Qiagen) according to the manufacturer's protocol. All DNA was stored at 4°C until further analysis.

**ESBL/AmpC and *E. coli* characterization.** To identify the most common ESBL/AmpC genes ($bla_{CTX\text{-}M}$, $bla_{TEM}$, $bla_{SHV}$, $bla_{CMY}$, and $bla_{OXA}$), all lysates were screened by PCR as described earlier (27). In addition, multilocus sequence types (MLST) were determined by PCR according to the *E. coli* MLST scheme curated by Enterobase (https://enterobase.warwick.ac.uk). PCR products were purified with ExoSAP-IT (Affymetrix) and subsequently sent for sequencing (BaseClear, the Netherlands). ESBL/AmpC annotations as reported on www.lahey.org/studies were used as a reference.

At least one isolate of each positive sample was selected for WGS on a NextSeq platform (Illumina). If multiple isolates were available within a sample, each isolate with a different combination of ESBL/AmpC allele variant and *E. coli* ST was selected ($n = 48$). Genome sequences were assembled using SPAdes (28). Core genome alignments were determined using Parsnp v1.2 (29), corrected for recombination regions using Gubbins, and visualized using FigTree (https://github.com/rambaut/figtree) (30). The accessory genome was determined by using Prokka for annotation and subsequently running Roary to determine gene presence or absence (31, 32). Tanglegrams of the pangenome (core genome + gene presence/absence) were constructed using Dendroscope, as described by Huson (https://github.com/husonlab/dendroscope3).

Resistance genes and plasmid incompatibility groups were determined with ResFinder (33) and PlasmidFinder (34) by using the ABRicate tool (35). *In silico* MLST typing was performed, using the scheme defined by Jolley and Maiden, as described by T. Seemann (36, 37).

**Statistical analysis.** A complete case analysis was conducted in SAS v9.4 (SAS Institute). Observations with missing/unknown values (around 2% missing values for most variables) were excluded. First, univariate associations between ESBL/AmpC carriage and questionnaire items were studied by GEE regression analysis with the logit link function using PROC GENMOD. The GEE approach is used to model dependence in response data, since observations of horses belonging to the same owner may be positively correlated. The GEE model was fit with an exchangeable covariance structure, in which the observations within a cluster (horses belonging to the same owner) are assumed to be equally correlated. Next, a multiple GEE model was conducted, mutually adjusting for variables with $P < 0.1$ in the univariate analysis.

Geographical analysis was done based on the centroid of four-digit postal code areas. The association between ESBL/AmpC carriage and three Dutch regions (the highly urbanized "Randstad," intermediate zone and peripheral zone [38]) was analyzed using GEE. The univariate spatial association was subsequently adjusted for questionnaire items associated with ESBL/AmpC carriage. Results were expressed as odds ratios (OR) with 95% confidence intervals (95%CI).

**Data availability.** All DNA sequences were deposited in the European Nucleotide Archive (ENA) under project number PRJEB34847.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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J.H., E.F., J.A.W., H.G., and B.D. designed the study. E.F., J.H., M.J.P.T., and H.G. conducted sample and data collection. J.H., E.F., and L.A.M.S. performed the microbiological, molecular, and computational analysis. J.H. prepared the manuscript. L.A.M.S., B.D., H.G., M.J.P.T., and J.A.W. discussed, read, contributed to, and approved the final manuscript.

Fecal samples were collected after natural defecation. No animals were directly subjected to sampling, and therefore all sampling was performed within the guidelines of the Dutch Animals Act (STB-2011-345) (39) and the Animal Welfare Body Utrecht (https://www.ivd-utrecht.nl/en/), meaning no additional license was required.

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