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Prevalence and risk factors associated with emergence of *Rhodococcus equi* resistance to macrolides and rifampicin in horse-breeding farms in Kentucky, USA

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ABSTRACT

The combination of a macrolide and rifampicin has been the mainstay of therapy in foals with *Rhodococcus equi* pneumonia for decades. Recent studies suggest that mass antimicrobial treatment of subclinically affected foals over time has selected for antimicrobial resistance. Our objective was to estimate the prevalence of *R. equi* strains resistant to macrolides and rifampicin at horse breeding farms in Kentucky. A hundred breeding farms in Kentucky were surveyed and *R. equi* were cultured from soil samples. Data were analyzed with logistic regression and generalized linear modeling ($P < 0.05$). Seventy-six percent (76%) of farms yielded resistant *R. equi*, and resistance to macrolides and rifampicin was associated with their use at farms. The present study is the first to report the prevalence and distribution of resistant isolates in the environment of farms in Kentucky, USA. Collectively, previous reports and the data presented herein provide irrefutable evidence of emerging antimicrobial resistance in *R. equi* with alarming prevalence. Widespread dissemination and maintenance of resistance genes in the environment where many other pathogenic bacteria exist is a concern for both animal and human health.

1. Introduction

R. equi is a facultative intracellular pathogen (Hondalus et al., 1993); its ability to persist in, and eventually destroy, alveolar macrophages due to the presence of the virulence gene known as *vapA* (Giguere et al., 1999) is the basis of its pathogenicity. Although *R. equi* may be found in the environment of virtually all horse farms, the clinical disease is endemic and devastating at some farms, sporadic on others, and unrecognized at many (Prescott, 1991). Differences in the prevalence of the disease might reflect variation in environmental conditions (e.g., temperature, dust) and management practices, as well as differences in the number of virulent isolates in the environment (Muscatello et al., 2006; Takai et al., 1991). Soil concentration of *R. equi* at horse breeding farms is usually between10^2 to 10^5 colony forming units (CFU) per gram of soil irrespective of farm history of pneumonia (Takai and Yamaguchi, 1994), and the proportion of virulent (containing pVAPA) *R. equi* may range from < 2% to 23% of all isolates (Cohen et al., 2012).

Thoracic ultrasonographic screening for early detection of pneumonia caused by *R. equi* has become routine practice at some endemic farms, such that the most frequently recognized manifestation of *R. equi* infection at those farms is subclinical pneumonia (Giguere et al., 2011a; Venner et al., 2007). Previous reports have shown that many foals with mild subclinical disease recover spontaneously without therapy (Huber et al., 2018b; Venner et al., 2012). The synergistic combination of a macrolide with rifampicin has been the mainstay of antimicrobial therapy in infected foals since 1980 (Giguere et al., 2012; Nordmann and Ronco, 1992; Prescott and Nicholson, 1984). Prior to 2001, reports of resistance to macrolides and rifampicin were exiguous. Since 2010, however, several reports have documented increasing prevalence of antimicrobial-resistant *R. equi* (Burton et al., 2013; Giguere et al., 2010; Huber et al., 2018a). The increasing resistance of bacteria to macrolides

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and rifampicin extends beyond equine rhodoccocosis because these drugs are widely used in humans to treat tuberculosis (Howard et al., 2018). Moreover, the World Health Organization indicates that macrolides are critically important to human medicine (Cohen and Denning, 2017).

Although previous reports have documented emergence of *R. equi* resistance in clinical disease, the prevalence and spread of resistant isolates of *R. equi* in the horse farm environment is unknown. Epidemiological investigation of the prevalence and factors associated with this emergent problem is crucial for developing strategies to limit the spread of resistant isolates. The objective of this cross-sectional study was to estimate the prevalence of *R. equi* strains resistant to macrolides, rifampicin, or both at horse breeding farms in Kentucky. We hypothesized that we would identify a high prevalence of farms in Kentucky with macrolide- and rifampicin-resistant *R. equi* in environmental samples.

2. Material and methods

2.1. Source population and farm selection

A total of 100 horse-breeding farms in central Kentucky, USA with history of recurrent foal pneumonia caused by *R. equi* were randomly selected from a list of all farms that were clients of the Hagyard Equine Medical Institute. The study was approved by the Clinical Research Review Committee of the University of Georgia and client consent for participation was obtained for each farm. A sample size calculation assuming a binomial distribution, alpha of 0.05, precision of ± 5%, and an observed prevalence of farms with resistant isolates of 5% indicated that a minimum of 73 farms would be required. We elected to enroll 100 farms to account for possible loss of follow-up.

2.2. Survey data collection

Each participating farm was asked in 2017 to answer a brief questionnaire gathering information regarding the number of foals born as well as occurrence and treatment of foal pneumonia on that farm for each of the preceding 3 years (*i.e.*, January 2014 to January 2017).

2.3. Soil sample collection

Between June 1st and July 31st 2017, a total of 9 soil samples were collected from each farm. Soil samples were collected by scraping the surface soil with a metal teaspoon. For each location, a volume of 3 teaspoons (approximately 15 g) of soil was collected and placed into an individual sterile plastic bag. All samples from a given farm were placed in a larger sealed plastic bag to avoid cross-contamination. Samples were refrigerated for approximately 5 days, until the end of the sample collection period, when the samples were shipped to the University of Georgia, where they were kept frozen at −80 °C before processing.

2.4. Sample processing

For each soil sample, 1 g was quantitatively cultured by serial 10-fold dilutions on plates of modified NANAT (to select for all *R. equi*), modified NANAT with erythromycin (8 μg/mL; to select for macrolide-resistant *R. equi*), and modified NANAT with rifampicin (50 μg/mL; to select for rifampicin-resistant *R. equi*). *R. equi* were identified by colony morphology and counted; PCR amplification of the *choE* gene of selected colonies from individual plates was performed to confirm validity of morphologic diagnosis (Ladron et al., 2003). Presence of the virulence plasmid was evaluated by PCR-amplification of the *vapA* gene (Giguere et al., 1999).

Colonies growing on NANAT culture plates containing erythromycin were suspected also to carry rifampicin resistance; therefore, all colonies were re-cultured in rifampicin plates to verify possibility of dual resistance. Similarly, colonies growing originally in rifampicin plates were re-cultured on plates with erythromycin. This way, it was possible to identify the colonies that were resistant to only macrolides, only rifampicin, or both. To confirm resistance, MICs of azithromycin, clarithromycin, erythromycin, and rifampicin were determined using E test strips (bioMérieux, Durham, NC, USA) according to the manufacturer's recommendations. Briefly, inocula were prepared from overnight cultures in trypticase soy agar (TSA) by the direct colony suspension method according to the guidelines established by the CLSI, resulting in the recommended inoculum of 1 to 5 × 10^5 CFU (Berghaus et al., 2015; Riesenberg et al., 2014). Concentrations of antimicrobial agents tested represented 2-fold dilutions between 256 and 0.016 mg/L for all macrolides and between 32 and 0.002 mg/L for rifampicin. Control strains tested in parallel and on each test occasion for all methods were *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212.

3. Data analysis

Data were analyzed using descriptive and inferential methods. For descriptive purposes, categorical data were summarized in contingency tables and continuous data were summarized using means and standard deviations, or medians and ranges if data did not appear to follow a normal distribution. The Shapiro-Wilk test for normality was used to assess data distribution. For purposes of inferential analysis, continuous independent variables were recoded as a binary outcome using the median of the population as a cut-point (*e.g.*, 1.45 horses/acre). For inferential analysis, the association of the binary outcome of whether or not macrolide-resistant isolates were recovered from soil samples at the farm with individual dependent variables was analyzed using logistic regression. The association between a variable with isolation of macrolide-and-rifampicin-resistant isolates was expressed as the odds ratio (OR), derived by exponentiation of the estimated co-efficient for the variable in the logistic regression analysis, and 95% confidence intervals calculated using maximum likelihood methods. For the multivariable model, only variables that were associated with the outcome with $P < 0.05$ were retained in the model.

In addition, the association between the continuous outcome of the percentage of total CFU that were resistant to macrolides and rifampicin with individual independent factors was analyzed using a generalized linear model with a Gaussian link. The percentage of total CFU that were resistant data were ($\text{logarithm}_{10} + 1$)-transformed to meet distributional assumptions of the modeling; the value of 1 was added for transformation because some percentages were 0.

4. Results

The questionnaires were completed by 72 farms from the 100 farms enrolled in this study initially. Although the statistical analysis was performed only on the farms that completed the questionnaires, the samples collected from the 100 farms were processed. Loss of follow-up was 28% in this study and it was independent of the outcome. Overall, 76% of all 100 farms yielded resistant isolates in at least 1 of the sampling sites. Of the 72 farms that completed the questionnaire, 53% performed thoracic ultrasonography as a screening method for early detection of *R. equi* infection (Table 1), 15% had records of resistant isolates infecting foals in their farms in the last 4 years, 66% reported foals with *R. equi* pneumonia in the last 4 years, 51% reported treating foals with macrolides and 62% with rifampicin, and 24% reported mortality due to *R. equi* infections in foals. Thirty-five percent of farms had < 30 foals born per year. The median horses per acre of the 72 farms was 1.45 (range, 0.08 to 5.86 horses/acre), and the median time that these farms were used for raising horses was 35 years (range, 4 to 222 years). The median total *R. equi* CFU per gram of soil was 5.8 × 10^4, and the mean total resistant *R. equi* CFU was 3.6 × 10^2/g of soil (0.6% of total *R. equi*). The majority (97.7%, 3,550 out of 3,633

Table 1
Continuous (median and range) and categorical (percentage) variables included in the analysis.

Continuous Variables		
Variable	Median (Range)	P value SW test[a]
Number of foals born	30.62 (0.75 to 143.25)	< 0.0001
Number of foals with pneumonia	1.62 (0 to 65.00)	< 0.0001
Number of foals treated with macrolides	1.5 (0 to 65.00)	< 0.0001
Number of foals treated with rifampicin	2.25 (0 to 65.00)	< 0.0001
Number of foals that died from *R. equi*	0 (0 to 2.25)	< 0.0001
Number of horses per acre	1.45 (0.08 to 5.86)	< 0.0001
Number of years raising horses	35 (4 to 222)	< 0.0001
CFU *R. equi*/g soil	51,667 (3,067 to 224,000)	< 0.0001
% Resistant *R. equi*	0.31 (0.00 to 4.03)	< 0.0001
% Resistant and *vapA* +	43% (0 to 100%)	< 0.0001

Categorical Variables		
Variable	Yes (%)	No (%)
Ultrasound screening	53 (74%)	19 (26%)
Confirmed resistant *R. equi*	15 (21%)	57 (79%)
< 30 foals born/year	35 (49%)	37 (51%)
Pneumonia in foals	66 (92%)	6 (8%)
Foals treated with macrolides	51 (71%)	21 (29%)
Foals treated with rifampicin	62 (86%)	10 (14%)
Foals that died from pneumonia	24 (33%)	48 (67%)
> 1.45 horses per acre	35 (49%)	36 (51%)
< 35 years raising horses	31 (44%)	40 (56%)
< 52,000 CFU/g *R. equi* in soil	36 (50%)	36 (50%)

[a] Test for normality with significance indicating non-parametric data.

isolates) of resistant isolates were resistant to both macrolides and rifampicin. The mean rifampicin-only resistant isolates was 6.2 CFU/g (1.7% of total resistant *R. equi*), and macrolide-only resistant isolates was 2.1 CFU/g (0.6% of total resistant *R. equi*). Amplification of *vapA* gene using conventional PCR revealed that 43% of all resistant isolates were virulent (Table 1).

Using logistic regression analysis, the number of foals treated with macrolides, the number of horses per acre, and the concentration of *R. equi* in soil were significantly ($P < 0.05$) associated with increased odds of identifying macrolide-resistant isolates at farms (Table 2). Considering categorical variables, whether macrolides were administered to any foals at the farm and density of > 1.45 horses/acre were significantly ($P < 0.05$) associated with increased odds of identifying macrolide-resistant isolates at farms (Table 2). The best fitting multivariable logistic regression model included terms for the soil concentration of *R. equi*, whether macrolides were used, and density of horses (Table 3). Although the term for horse density/acre was not significant individually, residual deviance of the models indicated that the model including this term was significantly ($P < 0.05$) superior to the nested model excluding it.

Using a bivariable generalized linear model analysis with the dependent variable of $\log_{10}$-transformed percentage of virulent *R. equi* isolated from farms, the following variables were significantly associated with the percentage of virulent isolates in soil: number of foals with pneumonia, number of foals treated with macrolides or rifampicin, number of foals that died with *R. equi* pneumonia, and the number of years the farm was used for raising foals (negatively associated) (Table 4). The final multivariable model included the variables $\log_{10}$(number of foals treated with macrolides) and $\log_{10}$(years raising horses) (Table 5). The $\log_{10}$-transformed values of number of foals treated with macrolides was strongly and significantly ($P < 0.0001$ for each) correlated with number of foals with pneumonia, number of foals treated with rifampicin, and the number of foals that died. Years raising horses was not significantly associated with number of horses/acre.

5. Discussion

The present study is the first to report the prevalence and distribution of resistant isolates in the environment of farms in Kentucky, USA. Collectively, previous reports and the data presented herein provide irrefutable evidence of emerging antimicrobial resistance in *R. equi* with alarming prevalence. The combination of a macrolide (such as erythromycin, azithromycin, or clarithromycin) with rifampicin has been the main treatment of *R. equi*-infected foals since 1980 (Giguere et al., 2011b, 2004; Hillidge, 1987), with rare reports of antimicrobial resistance prior to the early 2000s. In 2010, it was reported that the prevalence of macrolide- and rifampicin-resistant *R. equi* was 4% in Texas and Florida between 1997 and 2008, with the majority of resistant isolates identified after 2001 (Prescott and Nicholson, 1984). In 2013, a high prevalence (40%) of clinical isolates of *R. equi* resistant to macrolides and rifampicin was reported from a horse-breeding farm in Kentucky after the farm had implemented screening foals for subclinical pneumonia using thoracic ultrasonography with mass antimicrobial treatment of subclinically-affected foals (Burton et al., 2013). Reports of *R. equi* resistance outside the USA remain rare, although multi-drug-resistant *R. equi* were identified in 2013 from clinical samples of foals that died from *R. equi* at a farm in China (Liu et al., 2014). A recent retrospective report analyzing clinical isolates of foals submitted to diagnostic laboratories showed a significant increase in *R. equi* resistant to macrolides and rifampicin during the period from 2007 to 2017 when compared with the period from 1995 to 2006 (Huber et al., 2018a). The updated World Health Organization ranks macrolides amongst the most critically important antimicrobials (Cohen and Denning, 2017). Therefore, it is of critical importance to understand the causes of emergence of macrolide-resistant strains of pathogenic bacteria, including *R. equi*, and to take steps to limit their spread.

The lack of effective methods for preventing foal pneumonia caused by *R. equi* led to efforts to find alternative methods for controlling morbidity and mortality caused by this pathogen. Around 2001, many farms that suffered from foals with *R. equi* pneumonia on a recurring bases implemented the use of thoracic ultrasonography for earlier detection of disease coupled with treating all foals with subclinical pneumonia with macrolides (with or without rifampicin). This strategy appeared to decrease mortality caused by *R. equi* pneumonia at endemic farms (Venner et al., 2007). Unfortunately, the benefits of this approach came with negative consequences: macrolide- and rifampicin-resistant *R. equi* emerged at a horse-breeding farm after mass antimicrobial use was instituted to treat foals with subclinical pneumonia identified by thoracic ultrasonography (Burton et al., 2013). Evidence exists that many foals with subclinical pneumonia will recover spontaneously without the use of antimicrobials (Huber et al., 2018b; Venner et al., 2012), indicating that mass antimicrobial treatment of subclinical pneumonia may not be warranted. In this study, a significant association between the use of thoracic ultrasonography screening and the use of antimicrobials ($P = 0.019$) was observed. Although while the use of thoracic ultrasonography was not associated directly with increased antimicrobial resistance, the mass antimicrobial use resulting from this screening method was.

In this study, the majority of *R. equi* isolates resistant to macrolides were also resistant to rifampicin, with less than 3% of resistant isolates showing resistance to either macrolides alone or rifampicin alone. There are no known mechanisms of cross-resistance between macrolides and rifampicin. Thus, it is believed that the combined use of these antimicrobials to treat and prevent *R. equi* infection has contributed to dual resistance by selective pressure to both antimicrobial drugs. Macrolide resistance in *R. equi* is caused by acquisition of the macrolide resistance gene *erm(46)* (Anastasi et al., 2016). Rifampicin resistance results from the substitution of a limited number of highly conserved amino acids in the RNA polymerase β subunit encoded by the *rpoB* gene

Table 2
Odds ratios and 95% confidence intervals (CIs) of derived using logistic regression for the association of a farm yielding positive culture results in soil of macrolide-resistant *R. equi* from a study of 72 farms in central Kentucky. Medians (ranges) are provided for descriptive purposes.

Continuous Variables Variable	No resistant *R. equi* median (Range) n = 18	Resistant *R. equi* median (Range) n = 54	OR (95% CI)	P value
# Born	31.0 (6.5 to 143.2)	30.1 (0.8 to 136.2)	1.0 (0.8 to 1.2)	0.4001
# Pneumonia	1.9 (0 to 13.2)	1.6 (0 to 65.0)	1.0 (0.9 to 1.1)	0.4242
# Macrolides	0 (0 to 15.5)	2.0 (0 to 65.0)	1.1 (< 1.0 to 1.3)	0.1108
# Rifampicin	1.9 (0 to 17.2)	2.4 (0 to 65.0)	1.0 (< 1.0 to 1.1)	0.3323
# Died from *R. equi*	0 (0 to 1)	0 (0. to 2.2)	1.1 (0.2 to 5.6)	0.8834
Horses per acre	1.2 (0.3 to 2.3)	1.6 (0.1 to 5.9)	2.1 (> 1.0 to 4.2)	0.0472[a]
Years raising horses	46 (8 to 222)	35 (4 to 160)	1.0 (< 1.0 to 1.0)	0.2487
Log_{10}(*R. equi*/g soil)	4.52 (3.49 to 4.92)	4.74 (4.26 to 5.35)	33.5 (4.0 to 283.4)	0.0019[a]

Categorical Variables Variable	No resistant *R. equi* (%)	Resistant *R. equi* (%)	OR (95% CI)	P value
< 30 Foals	44% (8/18)	50% (27/54)	1.2(0.4 to 3.6)	0.6842
Pneumonic foals	89% (16/18)	93% (50/54)	1.6 (0.3 to 9.3)	0.6261
Macrolides used	44% (8/18)	80% (43/54)	4.9 (2.7 to 8.7)	0.008[a]
Rifampicin used	89% (16/18)	85% (46/54)	0.7 (0.1 to 3.7)	0.696
Died *R. equi*	33% (6/18)	33% (18/54)	1.0 (0.6 to 1.8)	1
> 1.45 horses/acre	28% (5/18)	57% (30/53)	3.4 (1.1 to 10.9)	0.0437[a]
< 35 years horses	28% (5/18)	49% (26/53)	2.5 (0.8 to 8.0)	0.1266
< 52,000 *R. equi*/g	67% (12/18)	44% (24/54)	0.4 (0.1 to 1.2)	0.1124
Ultrasound	72% (13/18)	74% (40/54)	1.1 (0.3 to 3.6)	0.8777
Confirm resistant	17% (3/18)	22% (12/54)	1.4 (0.4 to 5.8)	0.6179

[a] significant association, P < 0.05.

Table 3
Odds ratios (and 95% CIs) derived by multivariable logistic regression for the association of a farm yielding positive culture results in soil of macrolide-resistant *R. equi* from a study of 72 farms in central Kentucky. The best fitting multivariable logistic regression model included terms for the soil concentration of *R. equi*, whether macrolides were used, and density of horses.

Variable	OR	95% CI	P value
Log_{10}(*R. equi* CFU/g soil)	19.9	1.9 to 206.3	0.0151[a]
Macrolides used	3.7	> 1.0 to 14.0	0.0496[a]
> 1.45 horses/acre	2.3	0.6 to 8.3	0.2191

[a] significant association, P < 0.05.

Table 4
Odds ratios and 95% confidence intervals (CIs) derived using generalized linear model analysis with the dependent variable of log_{10}-transformed percentage of virulent *R. equi* isolated from a study of 72 farms in central Kentucky.

Continuous Variables Variable	Odds Ratio	95% CI	P value
Log_{10}(# Born)	1	0.9 to 1.1	0.5892
Log_{10}(#Pneumonia)	1.1	> 1.0 to 1.2	0.0166[a]
Log_{10}(#Macrolide)	1.1	> 1.0 to 1.2	0.0033[a]
Log_{10}(#Rifampicin)	1.1	> 1.0 to 1.2	0.0356[a]
Log_{10}(#Died *R. equi*)	1.6	> 1.0 to 2.4	0.0405[a]
Log_{10}(Horses/acre)	1.1	> 1.0 to 1.3	0.0382[a]
Log_{10}(Years raising)	0.9	0.8 to < 1.0	0.0126[a]
Log_{10}(*R. equi*/g soil)	1	0.9 to 1.2	0.5603

Categorical Variables Variable	OR	95% CI	P value
< 30 Foals	1	0.9 to 1.1	0.5909
Pneumonic foals	1.1	0.9 to 1.3	0.2554
Macrolides used	1.1	< 1.0 to 1.2	0.1217
Rifampicin used	1	0.9 to 1.1	0.631
Died *R. equi*	1.1	< 1.0 to 1.2	0.0593
> 1.45 horses/acre	1.1	< 1.0 to 1.2	0.0803
< 35 years horses	1.1	> 1.0 to 1.2	0.0243[a]
< 52,000 *R.equi*/g	1	0.9 to 1.1	0.8546
Ultrasound	1	0.9 to 1.1	0.3728
Confirm resistant	1.1	< 1.0 to 1.2	0.1211

[a] significant association, P < 0.05.

Table 5
Odds ratios (OR) and 95% confidence intervals (95% CIs) derived by multivariable logistic regression for the association of a farm yielding positive culture results in soil of macrolide-resistant *R. equi* from a study of 72 farms in central Kentucky. The final multivariable model included the variables log_{10}(number of foals treated with macrolides) and log_{10}(years raising horses).

Variable	OR	95% CI	P value
Log_{10} (#treated macrolides)	1.1*	> 1.0 to 1.2	0.0076
Log_{10} (#years raising horses)	0.9*	0.8 to < 1.0	0.0260

*significant association, P < 0.05.

(Asoh et al., 2003; Fines et al., 2001). Most macrolide-resistance genes are associated with self-transmissible mobile elements and thus have the capacity to spread among strains, species, and bacterial ecosystems (Giguere et al., 2017). Dissemination and maintenance of resistance genes in the environment where many other pathogenic bacteria are encountered thus presents a large-scale concern for both animal and human health.

This is the first report of prevalence of resistance to macrolides and rifampicin in *R. equi* in environmental samples from horse-breeding farms in Kentucky. While this study has limitations resultant from its cross-sectional design, it reveals alarming evidence of increasing and widespread resistance of *R. equi* to macrolides and rifampicin in the environment at horse farms. The combination of macrolides and rifampicin is the standard treatment of *R. equi* pneumonia in foals, and alternatives are limited (Giguere et al., 2017). The density of horses and foals and the number of animals treated with these antimicrobials were associated with increased proportion of resistant isolates of *R. equi* in the soil. The meaning of the negative relationship between the number of years raising horses and the presence of resistant isolates at farms was unclear. Although it could reflect differences in environmental conditions and management practices (including antimicrobial use) at farms with fewer years of raising horses, it also could be attributed to chance alone or confounding by some other factor. It would be important to replicate this finding before conducting studies to establish a biological basis for this finding. Further studies are needed to further characterize the main risk factors responsible for the current emerging resistance, the molecular characteristics of the resistant isolates, the

persistence of these isolates in the environment, the spread of resistance to other organisms in the soil at breeding farms, and the impact of these resistant isolates on the health of foals.

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