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Identification of macrolide- and rifampicin-resistant *Rhodococcus equi* in environmental samples from equine breeding farms in central Kentucky during 2018

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ABSTRACT

Rhodococcus equi causes severe pneumonia in foals and is most often recognized in people as an opportunistic pathogen. Longitudinal studies examining antimicrobial-resistant *R. equi* from environmental samples are lacking. We hypothesized that antimicrobial-resistant *R. equi* would be detectable in the ground (pasture soil or stall bedding) and air at breeding farms with previous documentation of foals infected with resistant isolates, and that concentrations of resistant isolates would increase over time during the foaling season. In this prospective cohort study, ground and air samples were collected from stalls and paddocks in January, March, May and July of 2018 at 10 horse-breeding farms with history of foal pneumonia attributed to macrolide- or Rifampicin-resistant *R. equi*. Environmental samples were cultured in the presence and absence of macrolides and Rifampicin to select for resistant organisms. Data were analyzed with linear mixed-effects and Hurdle models. Concentrations of total *R. equi* in bedding or air of stalls were significantly ($P < 0.05$) higher in January than other months. The proportion of resistant *R. equi* in soil samples from paddocks was significantly ($P < 0.05$) higher than stall bedding during all months. For each month, air samples from paddocks had a significantly ($P < 0.05$) higher proportion of resistant isolates than those from stalls. Fifty-five percent of resistant soil isolates and 34% of resistant air isolates were considered virulent by identification of the *vapA* gene. Concentrations of resistant *R. equi* isolates did not increase over time during the foaling season. Antimicrobial-resistant *R. equi* can persist in the environment at farms with a history of pneumonia caused by resistant *R. equi* infections, and exposure to resistant isolates in paddocks and stalls appears stable during the foaling season. Resistant isolates in the environment not only pose a risk for disease but also can serve as a repository for dissemination of resistance genes.

1. Introduction

The World Health Organization recognizes antimicrobial resistance of bacteria as one of the greatest threats to human health (World Health Organization, 2014). Human and animal infections with resistant bacteria can be difficult to treat and can decrease survival (Woerther et al., 2010; Giguere et al., 2010). Development of bacterial resistance to antimicrobials seems ineluctable because resistant organisms are frequently observed in clinical environments shortly after the introduction of new antimicrobial molecules (Lewis, 2013). The selective pressure resulting from overuse of antimicrobials in agricultural animal production has contributed to an increase of antimicrobial-resistant organisms (Davies and Davies, 2010; Levy and O'Brien, 2005; Peng et al., 2018). While the presence of resistant bacteria in hospitals is widely recognized (Hwang and Kim, 2018; Lopez-Garcia et al., 2018; Chatedaki et al., 2018), recent studies have also documented antibiotic-resistant bacteria distributed more broadly in the environment, including water supplies and soil (Sarmah et al., 2006; Fernando et al., 2016). Nevertheless, little is known about the ecology or distribution of antimicrobial resistance genes in the natural environment (Nesme and Simonet, 2015).

Rhodococcus equi (R. equi) is a Gram-positive, obligately aerobic

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coccus belonging to the Mycolata taxon, closely related to the tuberculosis pathogen, *Mycobacterium tuberculosis* (Sutcliffe, 1997). It is a facultative intracellular pathogen that causes a life-threatening, pyogranulomatous pneumonia in young horses and, less commonly, in other species, including humans (Giguere et al., 2011; Drancourt et al., 1992)). *R. equi* is also a soil saprophyte with simple growth requirements, and can be isolated from the soil of virtually all horse-breeding farms (Takai and Yamaguchi, 1994; Muscatello et al., 2006a; Cohen et al., 2013). Although *R. equi* is likely present in the environment of all horse farms, the clinical disease is endemic and devastating at some farms, sporadic at others, and unrecognized at many (Giguere et al., 2011).

Over the past 2 decades, control of *R. equi* infections at many farms where the disease is endemic has relied on early detection of subclinical pulmonary disease using thoracic ultrasonography and initiation of therapy before development of clinical disease (Venner et al., 2007). This practice has led to a potentially unnecessary increase in the number of foals treated with antimicrobials at farms because only a fraction of foals that have sonographic lesions progress to develop clinical pneumonia (Venner et al., 2012). High prevalence of macrolide- and rifampicin-resistant *R. equi* at a farm after initiation of mass use of these drugs based on results of ultrasonographic screening has been documented (Burton et al., 2013). Moreover, significantly increased prevalence of *R. equi* resistant to macrolides and Rifampicin was observed when comparing the period between 2007–2017 with 1995–2006 in a retrospective study evaluating records of clinical samples from pneumonic foals submitted to diagnostic laboratories in Kentucky, USA (Huber et al., 2018).

Although epidemiological studies of the distribution of virulent *R. equi* in soil and air samples from equine breeding farms have been reported, longitudinal epidemiologic studies of resistant *R. equi* in environmental samples from horse farms are lacking. This information is critical for characterizing acquisition of antimicrobial resistance by *R. equi* and, consequently, for developing strategies to limit the spread of resistant strains that can cause disease in animals and humans. The objective of this prospective study was to determine whether the type of sample (soil/bedding vs air), sampling location (stall vs paddock), and month of sample collection (January, March, May, and July) were associated with the proportion of isolates of *R. equi* resistant to macrolides or rifampicin at horse farms in central Kentucky. We hypothesized that macrolide-resistant isolates of *R. equi* would be found in the environment of farms with history of *R. equi* pneumonia caused by resistant strains, and that there would be a progressive increase in concentrations of antimicrobial-resistant *R. equi* in samples of soil and air over time during the foaling season.

2. Material and methods

2.1. Farm selection

Ten horse-breeding farms located in central Kentucky, USA, were included in this study. These 10 farms had history of recurrent *R. equi* foal pneumonia that included foals diagnosed with pneumonia attributed to isolates of *R. equi* resistant to macrolides, rifampicin, or both. These farms were identified from a previous study from our laboratories (Huber et al., 2018) in which horse-breeding farms were randomly selected for enrollment. Study protocols were reviewed and approved by the Clinical Research Committee of the University of Georgia's College of Veterinary Medicine, and informed client consent was obtained from all participating farms prior to enrollment.

2.2. Sample collection

At each farm, 3 stalls in barns used to house mares and neonatal foals and 3 paddocks used to confine the same group of mares and foals were identified for sampling during the 2018 foaling season. For each sampling site, 1 soil and 1 air sample were collected. Each site at each farm was sampled in January, March, May, and July. All farms were sampled during the last 2 weeks of each month, and all samples were collected between 8 A.M. and noon. Paddocks and stalls from which soil and air samples were collected were being actively used to house mares and foals. Soil samples were collected by scraping the surface soil with a clean metal teaspoon. In the stalls, where soil samples were not available, samples were collected from the surface of the bedding at a location remote from visible evidence of feces. 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.

The air sampling was performed as previously described (Cohen et al., 2013). Briefly, a portable air sampling device was used to collect air samples using culture plates of either modified nalidixicacidnovobiocin-actidione (cycloheximide)-potassium tellurite (NANAT, selective media for *R. equi*), (Grimm et al., 2007; Ladron et al., 2003) 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*). The air sampler was placed on the ground to collect air at approximately 10 cm above the ground. For each sample collection, 500 L of air was aspirated onto the NANAT culture plates at a rate of 100 L/min. Samples were refrigerated at 4 °C until all samples were collected for a given time-point (*i.e.*, a period of approximately 1–3 days), when samples were shipped to the University of Georgia. After receiving the samples, the air collection culture plates were immediately placed in the incubator at 37 °C for 24–36 h. The soil/bedding samples were kept frozen at −80 °C until processed. During the 4 time-points of sample collection, a total of 72 soil samples and 96 air samples were collected, analyzed, and included in this study.

2.3. Sample processing

For each soil/bedding 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*). For both soil and air samples, *R. equi* were identified by colony morphology and counted. Colony forming units (CFU) of *R. equi* were counted in duplicate and the CFU average was calculated. To estimate the number of CFU of total *R. equi* in 1 g of soil, the average CFU was multiplied by 400, to account for the initial dilution in 2000 μL of PBS and for the 10-fold serial dilution ([2000 μL /50 μL] x 10). To estimate the number of CFU of resistant *R. equi* in 1 g of soil sample, the average CFU was multiplied by 40, to account for the initial dilution in 2000 μL of PBS, since no 10-fold serial dilutions were performed for detecting resistant isolates (2000 μL/50 μL). 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 (Clinical and Laboratory Standards Institute, 2013; Giguere et al., 1999).

Colonies growing on NANAT culture plates containing erythromycin were suspected 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 either only macrolides, only rifampicin, or both. To confirm resistance, MICs of azithromycin, clarithromycin, erythromycin, and rifampicin were determined using Etest 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

(Clinical and Laboratory Standards Institute, 2013), resulting in the recommended inoculum of 1 to 5 × 10^5 CFU.[27] 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. (Berghaus et al., 2015)

2.4. Data analysis

The association of the outcome of either CFU or proportion of resistant isolates in soil/bedding with the fixed effects of location (stall vs paddock), month, or their interactions, and random effects of replicate nested within farm, was performed using linear mixed-effects modeling with the nlme package of R statistical software (version 3.4.1 and 3.5.1). To meet distributional assumptions underlying the linear mixed-effects modeling, values of the CFU and proportion of resistant isolates were transformed using the $\log_{10}$ function; because there were 0 values in both CFU and proportion of resistant isolates, a value of 1 was added to each of these variables prior to transformation. Multiple pairwise comparisons among groups were made using the method of Tukey, using the multcomp package in R. To analyze the air data, hurdle modeling was used, since the results from this method of collection were very sparse (Bolker et al., 2009). Hurdle models were fit using R statistical software (version 3.5.1) and the glmmTMB package. For all analyses, significance was considered if $P < 0.05$. The proportion of resistant isolates yielding positive results by PCR for detection of *vapA* were compared between soil and air samples using a chi-squared test.

3. Results

Soil concentrations of total *R. equi* (CFU) were significantly associated with location (paddock vs. stall) in a manner that varied by month (Table 1). Concentrations of total *R. equi* in stall bedding in January were significantly ($P < 0.05$) greater than those in stall bedding for the months of March, May, and July, and also were significantly lower than those in paddocks in all months (Table 1); values in stalls for the months of March, May, and July did not differ significantly ($P > 0.05$). Whereas concentrations of stall bedding decreased significantly after January, there were no significant differences ($P > 0.05$) among months in soil concentrations of *R. equi* in paddocks (Table 1).

There were no significant effects of month or interaction of month with location (paddock vs. stall) on the concentrations of rifampicin- and macrolide-resistant *R. equi* in soil/bedding samples. However, the proportion of resistant isolates was significantly higher in paddock soil than in stall bedding for all months ($P < 0.05$; Table 2).

In air samples, there were significant effects of location that varied by month for the total CFU of *R. equi* (Fig. 1). Specifically, airborne concentrations of *R. equi* in stalls were significantly ($P < 0.05$) greater in January than during March, May, and July, and also were significantly ($P < 0.05$) lower than those in paddocks in all months. Concentrations of airborne *R. equi* in stalls in March, May, and July, did not differ significantly ($P > 0.05$). Concentrations of *R. equi* in paddocks in any month were significantly ($P < 0.05$) higher than those in stalls. Stall concentrations of *R. equi* decreased significantly after January, but there were no significant differences among months in concentrations of *R. equi* in paddocks ($P > 0.05$) (Table 1; Fig. 1). The data for the proportion of resistant *R. equi* in air samples were too sparse to be suitable for fitting multivariable hurdle models. However, when fitting a hurdle model for location only, air samples from paddocks had a significantly ($P < 0.05$) higher probability than stalls of having resistant isolates across all months.

Table 1
Model-based estimated of mean CFU (95% CI) of *R. equi* in soil/bedding samples by month and location from 10 farms in central Kentucky, USA.

Total mean (95% CI) CFU of *R. equi* in soil samples		
Month	Stalls	Paddocks
January	8.57 (6.3, 11.67)[a]	46.02 (33.82, 62.61)[b]
March	2.08 (1.53, 2.83)[c]	41.89 (24.58, 71.38)[b]
May	1.39 (1.02, 1.89)[c]	63.88 (37.48, 108.84)[b]
July	1.34 (0.98, 1.37)[c]	45.06 (26.44, 76.78)[b]

Values with different superscript letters differ significantly ($P < 0.05$).

Table 2
Model-based estimates for proportion (95% CI) of resistant *R. equi* in soil samples by month and location from 10 farms in central Kentucky, USA.

Total mean (95% CI) proportion of resistant *R. equi* in soil samples		
Month	Stalls	Paddocks
January	1.27 (0.97, 1.31)[a]	1.99 (1.82, 2.17)[b]
March	1.06 (0.93, 1.2)[a]	1.86 (1.65, 2.11)[b]
May	1.03 (0.91, 1.16)[a]	1.82 (1.6, 2.04)[b]
July	1.14 (1.01, 1.29)[a]	2.01 (1.78, 2.28)[b]

Values with different superscript letters differ significantly ($P < 0.05$).

Fifty-five percent (1,056/1,920) of the selected resistant isolates recovered from the soil/bedding from the 10 farms were considered virulent by identification of *vapA* gene using conventional PCR. In the air samples, the proportion of resistant isolates positive by PCR for the *vapA* gene was 34% (233/684), which was significantly lower than the proportion of these isolates in the soil (55%; $P < 0.05$).

4. Discussion

In this study, antimicrobial-resistant *R. equi* were recovered from environmental samples of all farms with history of infection of foals with resistant isolates of *R. equi*. A recent study (Huber et al., 2018) demonstrated a statistically significant increase in prevalence of resistance of *R. equi* to macrolides and rifampicin in clinical samples from foals submitted to diagnostic laboratories in central Kentucky between the years 2007 and 2017 relative to the years 1995 to 2006. Collectively, these studies provide alarming evidence of the emergence of resistance in *R. equi* strains to both macrolides and rifampicin in Kentucky, which represent the first-line antimicrobials used to treat *R. equi* infections in foals in that location. Along with *R. equi,* other pathogenic bacteria might be present in soil and water (Peng et al., 2018; Sarmah et al., 2006). Most macrolide-resistance genes are associated with mobile elements and thus have the capacity to spread among strains, species, and bacterial ecosystems (Giguere et al., 2017). Therefore, the maintenance of transferable resistance genes in the environment is of concern not only for animal production but also for human health.

Resistance to macrolides, lincosamides, and streptogramins B in *R. equi* is known to be caused by the presence of the transferable *erm(46)* gene that is transferable to susceptible *R. equi* (Anastasi et al., 2016). The majority of strains isolated in the present study were resistant to both macrolides and rifampicin. There is no known single mechanism for development of co-resistance to these antimicrobials; therefore, this dual resistance is believed to be acquired by selective pressure attributable to concurrent administration of these antimicrobials to treat clinical *R. equi* infections in foals.

The concentration of resistant *R. equi* in soil or air samples did not increase over time in this study as we had hypothesized. There are a number of possible explanations for this finding. First, the study period was relatively short, and most foals are treated for *R. equi* pneumonia during the early summer. Thus, it is possible that we missed the period of maximum antimicrobial pressure or that the effect of maximum pressure required more time to be reflected in environmental samples. Alternatively, it is possible that increasing use of antimicrobials (as

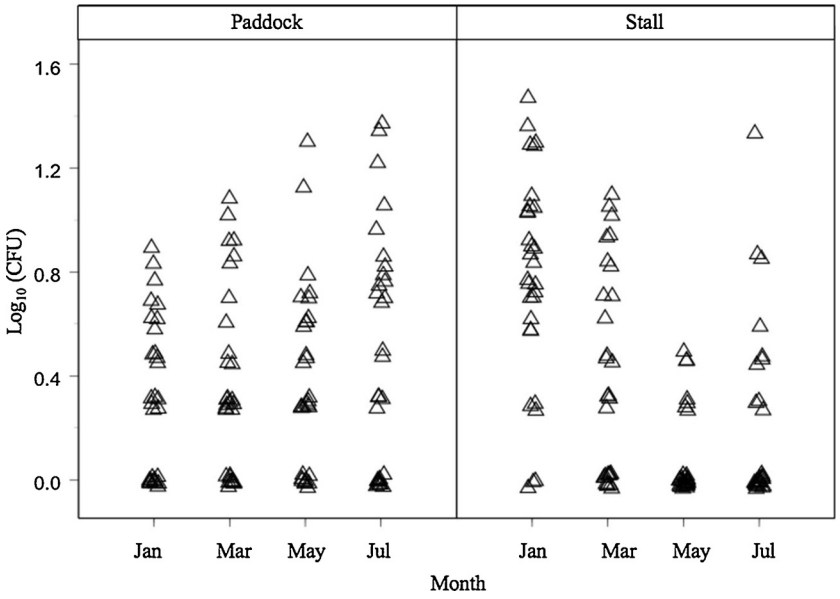

Fig. 1. Total *R. equi* $\log_{10}$CFU in air samples from paddocks and stalls from 10 farms in central Kentucky, USA in January, March, May, and July 2018. Note that there were many 0 values, but that paddock samples tended to increase whereas samples in stalls appeared to decrease, particularly for January relative to other months.

more foals are born and develop pneumonia) indeed resulted in higher rate of spread of resistant organisms in the environment and increased environmental pressure as we hypothesized, but the resistant isolates had reduced fitness such that they did not replicate at low temperatures and thus overall numbers remained static throughout the foaling season. A recent study reported that the possession of rifampicin or macrolide resistance results in decreased *R. equi* fitness in soil (Willingham-Lane et al., 2019). Warmer ambient temperature was associated with an increased concentration of virulent *R. equi* in air samples that were collected from outdoor areas (i.e., paddocks and lanes) in Australia (Muscatello et al., 2006a).

The occurrence of resistant bacteria in the soil were higher in paddocks than in stalls in all months. Direct comparison of soil concentrations in paddocks and stalls, however, is misleading because whereas soil was collected from paddocks, bedding was collected from stalls because most stalls were bedded with straw over either concrete or macadam. It is also possible that, because participating farms had prior history of foals with *R. equi* pneumonia that management changes were implemented to reduce *R. equi* concentrations in stalls, such as more frequent removal of feces from stalls. Concentrations of total *R. equi* collected over time, however, did vary between location. While in paddocks there was no significant change in soil proportions over the study period, occurrences in stall bedding were highest in January. Explanations for these results include reduced ventilation during winter months or that more horses were stalled or stalled longer during the colder months (and therefore defecated more in bedding).

Concentrations of total *R. equi* in air samples were significantly higher in stalls during January than in later months. In paddocks, airborne concentrations were significantly lower than stalls in January, but tended to rise in paddocks over time, although the rise was not statistically significant (Fig. 1). The extent to which these findings holds true at other farms and the implications for foal health of higher airborne concentrations in stalls during earlier months in the foaling season remain to be determined. The airborne data for the resistant isolates in air were too sparse to permit multivariable modeling, but bivariate hurdle modeling indicated that resistant isolates were significantly more likely in air of paddocks than stalls. These results conflict with previous studies of airborne concentrations of total *R. equi* and virulent *R. equi* indicating that concentrations were higher in stalls than paddocks (Muscatello et al., 2006b). However, in a study conducted at 2 horse-breeding farms in central Kentucky that had historical *R. equi* pneumonia in foals, the concentration of virulent *R. equi* in air samples collected from stalls did not differ significantly from that in air samples collected from paddocks, and the airborne concentration of virulent *R. equi* did not vary significantly by month (Kuskie et al., 2011). The reason for the discrepancy among this study and ours is unknown. It is possible that the resistant organisms were able to better persist in soil than in bedding because bedding lacked organic material and was presumably removed frequently, and thus there was greater availability of resistant organisms in paddocks to become airborne. In this study, no information about climate conditions were collected at each time-point, such that we are unable to determine how factors such as ambient temperature, humidity, precipitation, and wind could have influenced our results, which might explain the difference between our results and previous studies. Nevertheless, collectively, these results indicate that the exposure to macrolide-resistant isolates in air, soil or bedding is relatively constant over a single breeding season.

Of the resistant isolates found in soil, the *vapA* gene was detected in 55% (1,056/1,920), whereas 34% (233/684) of the resistant isolates recovered from the air were positive for *vapA*. Identifying resistant isolates of *R. equi* in the environment does not necessarily indicate that these organisms can cause disease, because presence of the virulence gene *vapA* is necessary to allow *R. equi* to replicate inside macrophages and cause disease (Giguere et al., 1999). The presence of virulent and resistant *R. equi* in air samples might be clinically more relevant, since the respiratory tract is believed to be the main route of infection in foals. Irrespective of their capacity for virulence, these resistant isolates do serve as a reservoir for the resistance gene(s) that can be transferred to virulent strains or to other environmental and pathogenic organisms.

Additional epidemiological studies are needed to understand the determinants and risks associated with environmental exposure to antimicrobial-resistant *R. equi* at breeding farms. This report provides important new information regarding the distribution of macrolide- and Rifampicin-resistant isolates in the environment at horse-breeding farms with prior history of rifampicin- and macrolide-resistant infection of foals. Our results indicate that exposure to resistant *R. equi* on these farms is widespread and consistent across the foaling season. Many of the resistant isolates were also virulent, but irrespective of virulence for foals, these resistant isolates are also a repository for potential dissemination of the resistance gene(s) to other organisms, including important human pathogens. There is thus an urgent need to better understand and control the occurrence of macrolide- and Rifampicin-

resistant *R. equi* at horse-breeding farms.

5. Conclusions

Macrolide- and rifampicin-resistant isolates of *R. equi* can persist in the environment of farms at which foals were diagnosed with pneumonia attributed to *R. equi* resistant to these antimicrobials. Expanded knowledge about the epidemiology of antimicrobial-resistant strains provides information to develop strategies to limit the spread of resistant isolates. More studies are needed to characterize the risk factors at horse-breeding farms that contribute to emergence and increased concentrations of resistant *R. equi* strains.

Declaration of interest

The authors of this study have no conflict of interest to be declared.

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