Microbiology:

Equine Infectious Disease and Microbial Resistance to Antibiotics

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Introduction

Many infectious diseases previously controlled by antibiotics are reemerging as the bacteria that cause them become resistant to antibiotics. The increasing incidence of these previously controlled infectious diseases is partially due to the overuse and/or misuse of some drugs used to combat infectious diseases. Not every antibiotic is prescribed based on specific laboratory tests. When veterinarians diagnose an infectious disease, they generally administer the antibiotic that has been proven to have the highest success rate in curing that disease. If the animal's symptoms resolve, the job is done. The problem with this strategy, however, is that veterinarians do not always know what bacteria they are killing with the administered drug unless further tests are done. They also cannot be sure whether or not the antibiotic they administer is effective, or alternately if time and an animal's immune response, or competition between the disease-causing pathogen and an animal's normal bacterial flora provided the cure. The short-term results of this strategy are usually adequate: animals are often cured, and if they are not, a different antibiotic can be administered. Research, however, suggests that there may be long-term problems with this approach. In clinical situations it is often inefficient, time-consuming, and too expensive to submit samples for microbiological culture and antibiotic susceptibility testing (1). Practitioners often select antibiotics based on research from university teaching hospitals and veterinary diagnostic laboratories from Canada, the United States and even Europe, since data pertaining directly to their geographic area is often non-existent. Often, veterinarians base their standard of care on data that does not necessarily reflect the actual resistance profiles of the bacteria that cause diseases in his or her region (1).

If the bacteria that veterinarians are trying to kill or the animals' normal flora become resistant to the antibiotics most often used, then there will be no way to combat future diseases caused by these organisms unless new antibiotics are developed. In time, bacteria may become resistant to new drugs as well. Using current strategies of treating diseases without being sure exactly what organisms cause the diseases, we are inadvertently building an army of indestructible organisms that will become more and more resistant with every new drug

that we throw at them. Human medicine has already encountered this problem with resistant strains of Staphylococcus (MRSA- Multidrug Resistant Staph Aureus) (2), Escherichia (food poisoning) (2), and Mycobacteria (tuberculosis) (3).

Many bacteria that animals carry as normal flora are opportunistic: they can cause disease under certain circumstances. For example, an organism that is normally carried in a healthy animal's nose can cause a lung infection if the organism gains access to the lungs, or a wound infection if the organism is introduced into an open wound. It is, therefore, important to determine the antibiotic resistance/sensitivity of organisms that are found as normal flora, as well as those known to cause particular diseases.

Research shows that resistant organisms can spread from one animal to another. One study showed that when one member of a household was treated for ten days with Erythromycin, Trimethoprim-sulfamethoxazole, or Tetracycline for acne, dramatic changes in the normal skin flora of untreated members of the household were observed (2). This finding presents a concern from the human perspective- that uncontrolled diseases in animals could potentially be dangerous to humans (3, 5). Antibiotics are vital to the treatment of bacterial infections
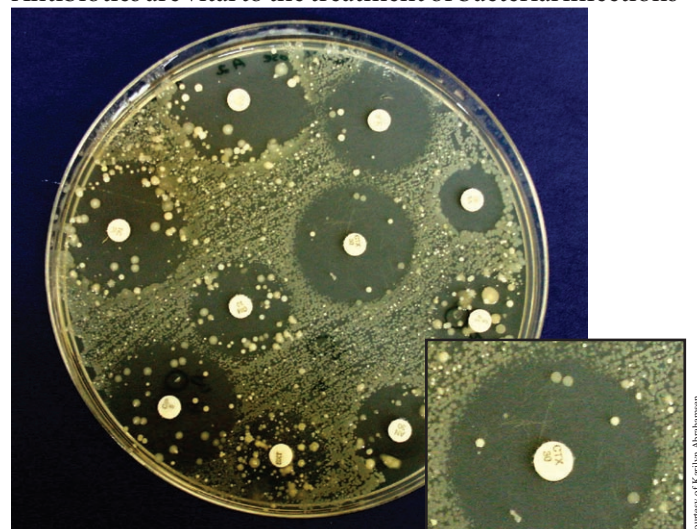

Figure 1. Sensitive Antibiotic sensitivity inhibition zones photograph. Zone diameters were measured in mm, recorded, and compared to the standards chart provided by the BBL™ Sensi-Disc™ company to determine resistance or sensitivity. In the photograph, most of the zones around each antibiotic Sensi-disc™ are clearly visible and most zones were sensitive to the antibiotics.
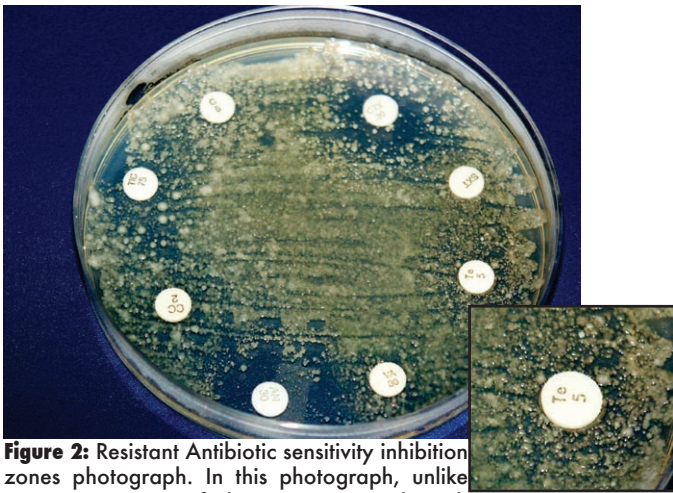
Figure 2: Resistant Antibiotic sensitivity inhibition zones photograph. In this photograph, unlike Figure 1, most of the zones around each antibiotic Sensi-disc™ are not clearly visible and most zones were resistant. Most of the bacteria growing directly next to and on the antibiotic discs were resistant to the antibiotic.

in both humans and animals. However, if animals are treated with antibiotics and develop resistant bacteria, the resistant bacteria can easily be passed on to humans. In one study, researchers placed chickens, previously fed low levels of the antibiotic tetracycline, on a chicken farm and collected fecal samples from the chickens. They also collected fecal samples from farm workers before and after they had worked with the treated chickens. After a period of time, increased numbers of bacteria resistant to multiple antibiotics were found not only in the animals, but also in the farm workers (*2*).

Studies have been done as part of the National Antimicrobial Resistance Monitoring System to explore microbial resistance in certain diseases. The 1998 annual report discussed research investigating bacteria isolates of *Salmonella* and *Campylobacter* obtained from several species of animals. Most of the isolates were resistant to at least one antibiotic tested. Resistance to multiple antimicrobials was also a concern (*6*). Forty percent of the animal *Salmonella* isolates were resistant to 2 or more antimicrobials. This represents an increase from 25% in 1997. In 1998, 18% of *Salmonella* were resistant to 5 or more antimicrobials compared to 11% in 1997 (*6*). This is a disturbing trend. In the two bacteria tested, both became much more resistant to multiple antibiotics within a one year period (*6*).

Veterinarians usually administer antibiotics when an animal shows signs or symptoms of a certain disease, but antibiotics are useless if the bacteria are already resistant. In chronic infections, some vets will culture and test the bacteria for antibiotic sensitivity before choosing which antibiotic
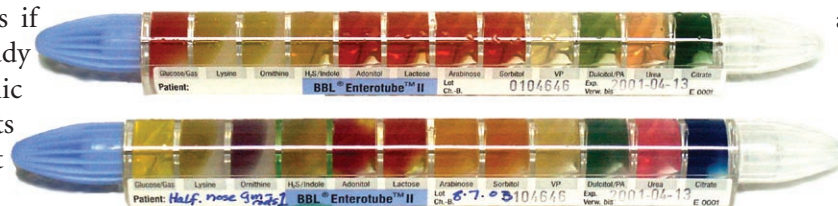
to administer, but in most cases they use the antibiotic that is most commonly recommended to cure the infection without sensitivity testing in order to save time and money. My study was designed to help determine whether that strategy is sound based on a survey of the resistance of cultured bacteria from several equine infectious disease cases and healthy horses' noses.

A survey was conducted of normal flora in horses' noses, and bacteria cultured from various abscesses, wounds and infections. My goals were to get an idea of the organisms commonly found in healthy horse's noses as well as those found in infected tissue, and to explore whether the organisms were resistant or susceptible to the antibiotics most commonly used in veterinary practice. [Please note that only nasal pharyngeal results are presented here]

It was predicted that many of the cultured organisms would not be resistant to antibiotics as they would likely have been bacteria that the horses picked up from their environment, and so would not necessarily have been previously exposed to antibiotics. It was predicted that a small percentage of the bacteria found in the cultures of various wounds and nasopharyngeal sites would be resistant to some antibiotics, as they could have been previously exposed to antibiotics in the animal tested, or might have been transferred to the tested animal from a stable-mate.

Methods

Bacteria were collected from horses in the Annabessacook Veterinary Clinic in Monmouth, Maine and area stables. Research was conducted at the Bates College Microbiology Laboratory. The main study investigated the normal aerobic equine flora collected from the nasopharyngeal cavities of twenty-seven horses living on several farms in the state of Maine. For each horse, an aerobic bacterial culture was collected using a Fisherbrand® transport swab introduced into one nostril and rubbed gently on the nasal lining. Each swab was then put into its transport tube, which contained Stuart's media. Stuart's media is designed to maintain bacterial viability without supporting growth. Swabs were refrigerated until staining and plating. The bacteria collected on the swabs were Gram stained using standard methods and plated on Mueller Hinton agar, a medium that supports bacterial growth. BBL Sensi-Disc™ antimicrobial susceptibility test discs were placed on the plates to initiate Kirby Bauer antibiotic

Figure 3: Enterotube® photograph. The top enterotube is an uninoculated control tube used for comparison purposes. The bottom tube has been inoculated with bacteria, causing the color changing confirmatory tests.

sensitivity testing. Antibiotics in the discs diffuse out into the agar killing susceptible bacteria. Resistant bacteria however can still grow on the gel unaffected by the antibiotic. Thirteen different antibiotic discs were used. They included 10 µg Gentamicin (GM), 30 µg Amikacin (AN), 5 µg Ciprofloxacin (CIP), 30 µg Ceftriaxone (CRO), 30 µg Cefotaxime (CTX), 2 µg Clindamycin (CC), 10 µg Ampicillin (AM), 75 µg Ticarcillin (TIC), 5 µg Chloramphenicol (C), 25 µg Trimethoprim/Sulfamethoxazole (SXT), 30 µg Tetracycline (Te), 30 µg Vancomycin (Va), and 10 µg Bacitracin (B). The plates were incubated at 37 degrees C for a minimum of 24 hours, allowing the bacterial colonies to grow and the antibiotics in the discs to diffuse into the agar. After bacterial colonies had grown, the zone of inhibition diameters were measured in mm (Fig. 1 and Fig. 2), recorded, and compared to the standards chart provided with the BBL Sensi-Disc™ package to determine resistance or sensitivity to each antibiotic.

Some of the individual bacteria were identified using Enterotubes®. Enterotubes are miniaturized plastic chambers containing 12 different microbiological media that can be easily and quickly inoculated from a bacterial culture (Fig. 3). The Enterotube® method is used for identifying oxidase negative, Gram-negative rod isolates, so after Gram staining, it was necessary to determine whether the bacteria were oxidase negative or oxidase positive. Bacteria can convert carbohydrates to acidic products either aerobically by oxidation or anaerobically by fermentation. An oxidation-fermentation test confirms whether the bacteria are aerobes (oxidase positive) or facultative anaerobes (oxidase negative) (*7*).

For the test, several drops of oxidase reagent were applied to a colony of bacteria on an agar plate. A purple color indicated that the bacteria were oxidase positive while no color change indicated that the bacteria were oxidase negative (*7*). Oxidase negative Gram-negative rod colonies of interest were isolated, and Gram stained to confirm isolation. Each Enterotube® was inoculated by touching the end of the inoculating wire to the desired colony and then pulling the wire through each compartment of the tube (*7*). Each tube was then incubated at 37 degrees C for a minimum of 24 hours, allowing the bacterial colonies to grow and cause media color changes in each of the 12 chambers that could be unambiguously scored (Fig. 3). After the tests in each tube were completed, the tests were scored and the

bacteria were identified using the number key provided by the Enterotube® company.

Results

Scientists classify most bacteria into four main groups: Gram-positive rods, Gram-positive cocci, Gram-negative rods and Gram negative cocci. Bacteria from all four groups were found in normal equine flora (Table 2). Gram-positive cocci were the most prevalent and were found in 92.59% of nasal pharyngeal samples. Gram-negative cocci were found in 85.19% of samples, and Gram-negative rods were found in 59.26% of samples. Gram-positive rods, however, were only found in a quarter (25.93%) of the samples.

The results of the twenty-seven horse cultures, and the zone measurements in mm for the thirteen different antibiotics used are shown in Table 1. The numbers of resistant cultures to each antibiotic, expressed as a percent of the total cultures tested for each antibiotic are shown in blue, and are graphed in Figure 4.

Gentamicin (GM) was the most effective antibiotic used in the study. Only 3.70% of the antibiotic sensitivity inhibition zones for GM indicated that the sampled organisms were resistant to this antibiotic. Amikacin (AN) was the second most effective antibiotic with only 11.11% of the samples resistant. Cefotaxime (CTX) was third (15.38%), then Ciprofloxacin (CIP) with 18.52%, Ceftriaxone (CRO) with 22.22%, Clindamycin (CC) with 28.00%, Ticarcillin (TIC) with 33.33%, Ampicillin (AM) with 37.04%, Tetracycline (Te) with 44.44%, Trimethoprim/Sulfamethoxazole (SXT) also with 44.44%, Chloramphenicol (C) with 52.00%, Vancomycin (Va) with 62.96% and lastly, Bacitracin (B) with 68.00%.

Some Horses had interesting bacterial cultures that were isolated and identified. One Vancomycin resistant colony from Bull Run's nose was isolated and inoculated into an Enterotube®. It was identified as an *Escherichia coli*. Most strains of E.coli are harmless and live in the intestinal tract of animals, but the bacteria are opportunistic and can cause a massive infection if given adequate conditions (*8*). One type of bacteria from Buddy's nose which was resistant to Va, Te, Am, SXT, CTX, and CC was isolated and inoculated into an Enterotube®. It was identified as a species of *Shigella*, an opportunistic bacteria that is generally harmless but has been known to cause diarrhea in humans (*8*). One of the organisms isolated from Sassy's nose was identified as *Enterobacter cloacae.* Enterobacter is a species of bacteria that can be found living harmlessly in the intestinal tract, as well as on the skin of organisms, but can also cause opportunistic infections (*8*). One Va resistant large, mucoid, crenellated colony of large Gram-positive cocci was keyed out with a series of tests and identified as *Staphylococcus aureus*. Staphylococcus is another opportunistic bacteria that

	Gram negative rods	Gram negative cocci	Gram positive rods	Gram positive cocci
% Total equine nasal pharyngeal samples containing given bacteria type	59.26%	85.19%	25.93%	92.59%

Table 2: Total equine nasal pharyngeal samples containing given bacteria type. These figures give the calculated percentage of the 27 horse nose samples that contained bacteria of the given type.

can be found living harmlessly on skin surfaces (*8*). Two Halflinger nose colonies were isolated and identified as *Enterobacter cloacae* and *Enterobacter agglomerans*.

Discussion

These results suggest that normal horse nasopharyngeal flora includes a wide variety of organisms. At least one species of *E. coli*, one *Shigella* species, three *Enterobacter* species and one *Staph* were isolated and positively identified from our samples. Gram positive and negative cocci were present in most of the samples (92.59% and 85.19% respectively) and 59.26 % of the samples contained Gram-negative rods. Interestingly only 25.93% of the samples contained Gram positive rods as this type of organism is in high abundance in soil, and grazing horses are likely to inhale these organisms. We expected to find these organisms more often as transient flora, although since soil organisms are adapted to survive at a lower temperature, the finding is not really that surprising.

Gentamicin (GM) is the most common antimicrobial used to treat equine musculoskeletal infections because of its high efficacy rate and low cost (*1*). Amikacin is also highly effective but has a higher cost of therapy (*1*). As shown by this study, Gentamicin was the most effective in killing the broadest array of normal equine flora. Only one GM zone was measured as resistant for all twenty seven horses. This is good news for veterinarians, as it confirms that few equine organisms are becoming resistant at this point in time. This study confirmed that Gentamicin is still a highly effective antimicrobial for the equine organisms tested. However, there was one resistant zone, which suggests that organisms are beginning to build a resistance that could someday render Gentamicin ineffective.

Amikacin (AN), Cefotaxime (CTX), Ciprofloxacin (CIP), and Ceftriaxone (CRO), were highly effective, with resistance values under 20% (resistance values reflect the number of horses that had colonies resistant to a given antibiotic compared to the total number of horses sampled.). Clindamycin (CC), Ampicillin (AM), Ticarcillin (TIC), Trimethoprim/Sulfamethoxazole (SXT), and Tetracycline (Te) all had resistance values between 20 and 50%. These numbers are comforting for now, but as resistant organisms become more prevalent, the antibiotics become more and more ineffective, and this can happen at an inexorable rate.

Chloramphenicol (C), Vancomycin (Va), and Bacitracin (B) were relatively ineffective in killing normal equine flora, with resistance values between 50 and 70%. Both Vancomycin and Bacitracin are human antibiotics and are not used much in the veterinary world. Finding resistance to these antibiotics in normal equine flora is surprising since resistance normally develops when bacteria are exposed to the antibiotics. One wonders why the resistance developed. One possibility for bacterial resistance to antibiotics usually used for human disease is the use of some antibiotics in animal feeds.

Age and gender did not significantly affect the numbers of organisms found in these horses (data not shown) although there was a trend towards more resistant organisms with increasing age.

It would be an interesting follow up to this study to re-evaluate some of the younger horses in a few years, to see if and how the resistance of their normal flora changes. It would also be interesting to do this entire study again in a few years using different horses to see if overall resistance to the

Horse Name	AN	Va	Te	SXT	CTX	C	TIC	CC	B	CRO	AM	CIP	GM
Buddy	40	15	20	20+	24	20	26	22	21	20	22	40	30
Halflinger	40	0	22	40	24	8	18	20	5+	40+	20	50+	40
Mariah	25	10+	12	0	20	20	12	20+	0	24	22+	30	24
Molly/Kelsey	20	11	40	28	44	30	48	38	8+	50	0	0	18
Zeus	40	10	8	18	0	26	30	44	0	0	16	36	38
Dolly	32	12	14	14	24	0	0	26	10	22	26	0	24
MC	46	10	34	28	40	50	50+	40	0	30	30	50+	34
Sassy	0	16	16	26	22	22	24	40	28	30	8	40+	22
Blue	28	11	20	34	28	0	44	44	20	24	20	0	30
Maggie	40	18	34	40+	50+	40+	24	30	0	28	0	50+	30+
Derek	26	14	26	15	30	12	0	10	0	14	0	34	16
Molly/Abby	20	10	10	0	X	14	0	12	10	16	18	24	22
Pony	6	0	0	0	24	0	0	0	0	22	12	24	24
Cee	30	20	0	12	32	20	34	32	28	30	34	32	28
Misty	30	10	0	8	40	10	18	10	12	22	25	30	26
Splendor	16/0	0	0	0	0	0	0	0	0	0	0	0	0
Arty	30	0	0	0	40	16	4	0	0	34	0	40	26
Brooke	34	20	30	30	34	16	40	30	20	30	40	36	40
Belle	30	5	0	0	40	30	14	0	0	40	0	40	30
Chloe	30	0	30	0	30	16	0	0	0	30	0	36	30
Winston	34	8	0	24	30	16	24	20	0	24	30	15	32
Crofter	40	20	30	30	34	24	40	40	24	38	40	40	40
Cinder	30	20	20	30	30	20	30	30	10	40	26	34	30
Willy	36	20	26	24	32	18	30	30	19	27	25	34	37
Mae Jac	36	30	30	24	8	20	30	30	30	20	20	40	35
Comet	27	20	30	30	35	X	35	X	X	30	40	40	29
Bull Run	20	18	24	0	36	X	36	X	X	34	38	28	22
Percent resistant	11.11	62.96	44.44	44.44	15.38	52.00	33.33	28.00	68.00	22.22	37.04	18.52	3.70

Table 1. Antibiotic disc zones recorded in mm for the 27 equine nasal pharyngeal site samples collected. Each horse tested is listed by name in the first column. Black numbers indicate bacterial sensitivity to the antibiotic while red numbers indicate bacterial resistance to the antibiotic. Blue numbers indicate the percent of horses with bacteria resistant to the given antibiotic. An X was used to indicate missing data. See text for antibiotic symbols.

antibiotics increases in the general population.

Conclusions

Antibiotics are routinely used to treat domestic animals and livestock. Antibiotic type, dosage, regimen and degree of use can all affect the development of resistance in a microbial population (*9. 10*). A veterinarian's first priority is to treat his or her patient with the drugs that have proven to be the most effective. However a veterinarian must also make sure the drugs are available and affordable to the animal owner. Many times antibiotics are administered even if an infection is not severe. This tactic ensures that the patient has the safest possible outcome, and it is difficult to argue with this strategy. Veterinarians are doing what is best for their patient at the time. However, developing antimicrobial resistance is a problem that must be considered. What we must do, and what this study attempted to do is gain a better understanding of what bacteria in a veterinarian's area (in this survey, south central Maine) are becoming resistant, and which antibiotics have already been rendered ineffective. In addition, in selecting the optimal antibiotic for each case, vets must also consider other factors besides possible bacteriologic resistance such as the site of infection, adverse side effects, cost of therapy and effects of underlying diseases (*1*).

Animal owners also contribute to the rise in antimicrobial resistance. When caretakers note a decrease in symptoms, they sometimes stop administering the drug to their animals. Many times, if they stop administering the drug before the prescribed time, there is a good chance that the entire pathogen has not yet been eliminated. Circumstances such as this create ideal conditions that select for drug-resistant organisms (*11*). Bacteria that are susceptible to an antimicrobial are killed or put at a competitive disadvantage, while bacteria that have the ability to resist the antimicrobial survive and multiply. Additionally, "bacteria can become resistant when resistance genes are passed from a resistant bacterium to a sensitive one. Therefore, antibiotics may increase the prevalence of resistant bacteria among both target pathogens and normal bacterial flora" (*5*). Money is also cause for concern. Many clients do not have the resources to pay for more expensive antibiotics or follow up treatment and tests to check for resistance that may not ultimately alter the health of their animal.

The issue of increasing resistance needs to be looked into and eventually solved. Veterinarians depend on
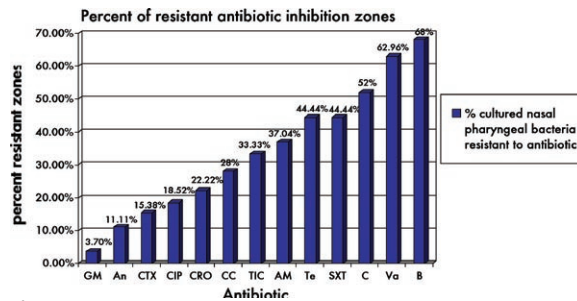

Figure 4. Percent of resistant antibiotic sensitivity inhibition zones. Percents reflect the number of horses out of the total surveyed population that had bacteria resistant to the given antibiotic. Percents were arranged in order of increasing resistance from left to right to show antibiotic efficacy.

antibiotics to treat many of their patients but as more organisms become resistant to more antimicrobials, the problem of treatment becomes more complex (*6*). With no change in our present course of action, treatment will eventually become impossible.

Acknowledgements

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