Viable Antimicrobial Resistant Bacteria are Transported from Cattle Feed Yards via Aerosolized Particulate Matter

**Keywords:** Antimicrobial; Resistance; Cattle; Feed yard; Particulate matter; Dust

**Abstract**

Increased awareness of consequences associated with Antimicrobial Resistant Bacteria (ARB) has given rise to considerable research on how and where resistance to antimicrobial agents occur. A recent investigation that utilized DNA sequencing-based technologies to characterize bacterial communities suggests that ARBs are generated on cattle feed yards and dispersed into the environment via wind-blown Particulate Matter (PM). Despite compelling evidence that bacterial DNA was prominent in fugitive PM, it remained unclear whether the bacterial DNA was derived from viable microorganisms. Thus, the narrow focus of this investigation was to determine whether bacteria associated with airborne PM emanating from cattle feed yards are viable, and if so, whether any cultivable bacteria were resistant to antibiotics. Numerous viable aerobic, microaerophilic, and anaerobic bacteria were successfully cultured from aerosolized, feed-yard-derived PM. Several cultured isolates were resistant to an assortment of antibiotics. This confirms that viable antimicrobial resistant bacteria do indeed travel on airborne PM emanating from cattle feed yards.

**Introduction**

Antimicrobial resistant bacteria and associated illnesses are generally associated with, and attributed to, clinical use (or misuse) of antimicrobial drugs in humans. However, administration of antimicrobials for veterinary applications accounts for roughly 80% of total usage in the United States [1,2]. In 2015 approximately 15.58 million kg of antimicrobials approved for use in food producing animals were sold in the United States, reflecting a 22% increase in sales and distribution since 2009 [3]. Worldwide, antimicrobial use in food-producing animals is expected to increase from 63,000 tons in 2010 to 105,500 tons of antimicrobials in 2030, a 67% increase over 20 years [4]. Extensive use of antimicrobials in agriculture could represent a potentially significant source of ARB which could continue to increase with growing demands for animal protein worldwide [4-9]. Antimicrobials administered to livestock are often incompletely metabolized, resulting in release of both the parent compound and associated metabolites into the environment where they can be dispersed by wind, runoff, and land application [6,7,10-14]. Depending on the antimicrobial entity, 30-90% of the dose can be excreted as the parent compound in urine, and up to 75% may be excreted in manure. Additionally, some reactive metabolites can be converted back to the parent compound once in the environment via bacterial metabolic processes [15]. This is significant because gut bacteria are shed from animals via feces at a rate of approximately 10^11 CFU (colony forming unit) per gram of fecal material [16]. Thus, antimicrobials used in livestock not only exert positive selective pressures in commensal microbial populations, but also in excreta-laden pen floor environments [6,8,17,18].

Recently published data indicate that bacterial communities harboring antimicrobial resistance determinants occur on fugitive PM collected downwind of beef cattle feed yards [12], and by extension, that airborne PM is a dissemination route for ARB to the surrounding environment. Because only a very small proportion of viable inhabitants in any environmental sample can be successfully cultivated in laboratory settings, the study was based on 16S DNA sequence analysis to gain broader understanding of bacterial community structures associated with airborne PM [12]. However, 16S DNA sequence analysis cannot differentiate between DNA derived from viable and non-viable bacteria. Therefore, the narrowly focused objectives of this study were to utilize culture-based methods to determine whether bacteria on fugitive PM collected near cattle feeding operations were viable, and to determine whether any successfully cultured bacteria were resistant to antimicrobials.

**Materials and Methods**

Particulate matter collection

To assess bacterial viability we collected PM samples downwind from eight and upwind from five beef cattle feed yards, located within a 200-mile radius of Lubbock, TX, with holding capacities ranging from 20,000-50,000 head. Each PM sample was collected adjacent to feed yard boundaries in the late afternoon, near dusk, when cattle are most active and during the period of peak PM suspension [19]. A portable high-volume air sampler (Hi-Q Environmental Products; Hi-Q CF-902) was placed on a stable platform facing into the wind, 1-2 m above the ground and approximately 5-10 m from the boundary of the feed yard to collect PM onto a four-inch diameter glass fiber filter (Hi-Q Environmental Products). After sampling, filters were placed in sterile, air-tight containers and transported to the laboratory.

Microbial cultures

Particulate matter was used to inoculate various culture broths and isolation agars to assess microbial viability and antimicrobial resistance on both upwind and downwind samples; however, isolates were only derived and identified from downwind samples because we were not readily able to culture ARB from upwind samples. Isolation of bacteria began with expansion of PM-bound bacteria in generic Tryptic Soy Broth (TSB). Expansion took place in three different
Table 1: Viable aerobic, microaerophilic, and anaerobic bacteria associated with airborne PM downwind of cattle feed yards. Isolates were identified based upon comparative 16S sequence identity to those listed in the greengenes database (Lawrence and Berkley National Lab, 2011), SILVA rRNA database or NCBI bacterial BLAST. Isolates were identified to the closest genus in many cases.

| Sequence | Growth Condition | NCBI | Greengenes | SILVA |
|----------|------------------|------|------------|-------|
|          |                  | Top Matches | Identity | E-value | Top Matches | Identity | Top Matches | E-value |
| Tet resistant #1 | Aerobic | Escherichia coli | 99% | 0 | Escherichia spp. | 100% | Escherichia coli | 0 |
|          |                  |             | Shigella sp. | 100% |           |          |            |        |
| Tet resistant #2 | Aerobic | Escherichia coli | 99% | 0 | Escherichia spp. | 100% | Escherichia coli | 0 |
|          |                  |             | Shigella sp. | 100% |           |          |            |        |
| Tet resistant #3 | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli | 100% | Escherichia coli | 0 |
|          |                  |             | Shigella sp. | 100% |           |          |            |        |
| Tet resistant #4 | Aerobic | Escherichia coli | 99% | 0 | Escherichia spp. | 100% | Escherichia coli | 0 |
|          |                  |             | Shigella sp. | 100% |           |          |            |        |
| Tet resistant #5 | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli str. | 100% | Escherichia coli | 0 |
|          |                  |             | Shigella dysenteriae | 99% | 0 | Shigella sp. | 100% | Escherichia fergusonii | 100% |
| Tet resistant #6 | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli | 100% | Escherichia coli | 0 |
|          |                  |             | Shigella dysenteriae | 99% | 0 | Shigella sp. | 100% | Escherichia coli | 0 |
| 1506-B1-S | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli | 99% | Escherichia coli | 0 |
|          |                  |             | Shigella spp. | 99% | 0 | Shigella flexneri | 99% | Escherichia coli | 0 |
| 1506-B2-S | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli | 99% | Escherichia coli | 0 |
|          |                  |             | Shigella spp. | 99% | 0 | Shigella flexneri | 99% | Escherichia coli | 0 |
| 1506-P4-S | Aerobic | Salmonella enterica | 99% | 0 | Salmonella subsp. enterica | 99% | Salmonella enterica | 0 |
|          |                  |             | Salmonella enterica | 99% | 0 | Salmonella subsp. enterica | 99% | Salmonella enterica | 0 |
| 1479-B1-S | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli | 99% | Escherichia coli | 0 |
|          |                  |             | Shigella flexneri | 99% | 0 | Shigella flexneri | 99% | Escherichia coli | 0 |
| 1479-LB2-S | Aerobic | Escherichia coli | 99% | 0 | Escherichia coli | 99% | Escherichia coli | 0 |
|          |                  |             | Shigella dysenteriae | 99% | 0 | Shigella flexneri | 99% | Escherichia fergusonii | 100% |
| 1479-LP-S | Aerobic | Escherichia coli | 98% | 0 | Escherichia spp. | 99% | Escherichia coli | 0 |
|          |                  |             | Shigella spp. | 98% | 0 | Shigella flexneri | 99% | Escherichia coli | 0 |
| 1478-P2-S | Aerobic | Salmonella enterica | 99% | 0 | Salmonella serovar | 99% | Salmonella enterica | 0 |
|          |                  |             | Salmonella enterica | 99% | 0 | Salmonella enterica | 99% | Salmonella enterica | 0 |
| B-1-1    | Aerobic | Kosakonia cowanii | 99% | 0 | Escherichia coli | 99% | Escherichia hermannii | 0 |
|          |                  |             | Enterobacter cloacae | 99% | 0 | Klebsiella planticola | 0 | Salmonella bongori | 0 |
| A-5-1    | Aerobic | Kosakonia cowanii str. | 888-76 | 99% | 0 | Escherichia coli | 100% | Escherichia hermannii | 0 |
|          |                  |             | Enterobacter subsp. cloacae | 100% | 0 | Klebsiella cf. planticola | 0 | Enterobacter spp. | 0 |
|          |                  |             | Enterobacter spp. | 0 |           |          |            |        |
|          |                  |             | Salmonella bongori | 0 |           |          |            |        |
|          |                  |             | Enterobacter hormaecheii | 0 |           |          |            |        |
**DNA extraction and analysis**

DNA was extracted from several bacterial isolates from each oxygen condition using a MoBio Power Soil DNA Isolation Kit according to manufacturer’s directions with modifications including: heating solutions C1 and C6 to 65 °C overnight in a MaxQ 400 incubator (ThermoScientific, Waltham, MA, USA). Bacteria were then plated on Tryptic Soy Agar (TSA) plates for aerobic incubation, Brucella agar for microaerophilic incubation, or anaerobic agar. Aerobic cultures were also propagated on Salmonella selective plates (CHROMagar Salmonella, VWR) for identification of Salmonella. Anaerobic conditions were created using BD GasPak EZ Container Systems with Anaerobe Sachets (VWR). The chamber was evaluated for anaerobic conditions using Anaerobic Indicator Strips (VWR). Microaerophilic conditions used the same chamber as anaerobic conditions but used Campy Sachets (VWR) to create a low oxygen environment. Once mixed cultures of aerobic, anaerobic and microaerophilic bacteria were propagated, plating densities were adjusted to isolate single colonies of bacteria for identification.

| ISDN: 2325-4645 |
|------------------|

C-4-5  | Aerobic  | Staphylococcus arlettae | 98%  | 0  | Staphylococcus spp. | 98%  | Streptococcus spp. | 0  |
B-4-3  | Aerobic  | Bacillus spp. | 99%  | 0  | Bacillus spp. | 99%  | Bacillus spp. | 0  |
B-3-2  | Aerobic  | Bacillus spp. | 99%  | 0  | Bacillus spp. | 99%  | Bacillus spp. | 0  |
B-5-2  | Aerobic  | Staphylococcus hyicus | 98%  | 0  | Staphylococcus sp. | 96%  | Staphylococcus spp. | 0  |
B-5-6  | Aerobic  | Enterobacter cloacae | 99%  | 0  | Enterobacter cloacae | 99%  | Enterobacter spp. | 0  |
|       |         | Klebsiella oxytoca | 99%  | 0  | Klebsiella oxytoca | 99%  | Ledercia spp. | 0  |
|       |         | Pantoea sp. | 0  | | Enterobacter cloacae | 0  | | |
| 1430-4-3-A | Anaerobic  | Clostridium butyricum | 99%  | 0  | Clostridium butyricum | 99%  | Clostridium spp. | 0  |
| 1430-5-4-A | Anaerobic  | Clostridium saccharolyticum | 97%  | 0  | Clostridium xylanolyticum | 99%  | Clostridium spp. | 0  |
| 1439-2-1-A | Anaerobic  | Enterococcus hirae | 99%  | 0  | Enterococcus hirae | 0  | | |
| 1450-4-2-A | Anaerobic  | Clostridium butyricum | 99%  | 0  | Clostridium butyricum | 99%  | Clostridium butyricum | 0  |
| 1493-1-2-A | Anaerobic  | Streptococcus spp. | 99%  | 0  | Streptococcus spp. | 99%  | Streptococcus salivarius subsp. Salivarius | 0  |
| 1493-5-2-A | Anaerobic  | Clostridium spp. | 98%  | 0  | Clostridium butyricum | 0  | | |
| 1493-5-6-A | Anaerobic  | Clostridium spp. | 99%  | 0  | Clostridium butyricum | 99%  | Clostridium butyricum | 0  |
| 1506-2-5-A | Anaerobic  | Escherichia coli | 99%  | 0  | Escherichia coli | 99%  | Escherichia spp. | 0  |
| 1522-3-5-A | Anaerobic  | Enterococcus casseliflavus | 98%  | 0  | Enterococcus spp. | 99%  | Enterococcus casseliflavus | 0  |
| 1493-1-1-C | Microaerophilic  | Bacillus spp. | 99%  | 0  | Bacillus amyloliquefaciens | 99%  | Bacillus spp. | 0  |
| 1506-1-6-C | Microaerophilic  | Klebsiella oxytoca | 97%  | 0  | Enterobacter cloacae | 99%  | Klebsiella oxytoca | 0  |
|       |         | Enterobacter spp. | 98%  | 0  | Klebsiella oxytoca | 99%  | Pantoea sp. | 99%  |
| 1506-3-3-C | Microaerophilic  | Paenibacillus alvei | 94%  | 0  | Paenibacillus spp. | 98%  | Paenibacillus spp. | 0  |
| 1450-2-3-C | Microaerophilic  | Bacillus thuringiensis spp. | 99%  | 0  | Bacillus spp. | 99%  | Bacillus thuringiensis | 0  |
Results and Discussion

Viable bacteria

Using these methods we were able to culture and identify viable bacteria that reside on airborne PM emerging from beef cattle feed yards. Of the top ten phyla of bacteria sequenced in [12], we were able, with limited culture effort, to propagate strains from Firmicutes and Proteobacteria. The limited number of cultured genera was not unexpected given that only about 1% of environmental bacterial are culturable [27]. Furthermore, only three distinct culture agars were used in this study; thus, it is probable that additional genera would have been cultured if additional growth media formulations were utilized. Genera of easily cultured aerobic bacteria from downwind fugitive PM included Escherichia, Shigella, Enterobacter, Staphylococcus, Bacillus and Klebsiella (Table 1). Additionally, Salmonella was cultured from PM collected downwind of two of the eight feed yards. Microaerophilic and anaerobic cultures contained bacteria from the genera of Clostridium, Enterococcus, Streptococcus, Pantoea and Paenibacillus (Table 1). Though it has been suggested that failure to culture gram negative bacteria from airborne particulates is a result of desiccation and/or irradiation [28], culture efforts in this study resulted in the propagation of a diverse array of gram negative taxa from airborne PM samples downwind of cattle feed yards. Many of the identified isolates from aerosolized PM were common bovine gut microbiome inhabitants.

Antimicrobial resistance

Antimicrobial resistance was assessed in mixed cultures of aerobic, anaerobic and microaerophilic cultures by evaluating zones of inhibition in classic disc diffusion tests (Kirby-Bauer) [29], where we evaluated resistance to the following broad-spectrum antimicrobials; tetracycline, streptomycin, chloramphenicol, erythromycin, and novobiocin. This suggests that a spectrum of antibiotic resistant bacteria inhabit PM collected downwind of cattle feed yards, and further implicates feed yard-derived airborne PM as a transport mechanism facilitating environmental dissemination of ARB.

Conclusion

Since several strains of viable antimicrobial resistant bacteria reside within feed yard pen material, aerosolization of these moieties could result in respiratory exposure of the herd, feed yard workers and surrounding communities to potentially pathogenic and antibiotic resistant bacteria [19,30]. In light of recent evidence that free-living bacteria retain antibiotic resistance cassettes without the presence of a selective pressure and that this retention comes at no physiological cost to the bacterium [31], the potential for resistance determinants to be spread laterally to other free-living bacteria is probable. This increases risk of localized antibiotic resistant bacterial infections and adds to the spread of ARB, especially in environments similar to West Texas where long-range transport of aerosolized particles is exacerbated by frequent, powerful wind events [19,31].

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