Investigation of Bacterial Pathogens Associated with Concentrated Animal Feeding Operations (CAFOs) and their Potential Impacts on a National Wildlife Refuge in Oklahoma: Final Report

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SUMMARY

According to the United States Environmental Protection Agency (US EPA), run off from agricultural lands is one of the greatest contributors of pollutants to surface waters in this country. Major agricultural pollutants include bacterial pathogens and nutrients that originate from animal wastes. Several concentrated animal feeding operations (CAFOs) are located in close proximity to the Salt Plains National Wildlife Refuge in Alfalfa County, Oklahoma; it is our understanding that these facilities are not US EPA registered. The goal of this study was to survey refuge surface water and sediment for known bacterial pathogens of wildlife, especially waterfowl, which may have originated from CAFO wastes. Sampling was conducted during the spring, summer, and fall of 2001 and 2002 at ten sites located on watersheds downstream of agricultural sites. Bacterial indicators of fecal contamination (fecal coliforms and fecal streptococci) were identified and counted in surface water samples. Fecal coliform levels exceeded US EPA human recreational bathing water limits in 22 of 50 samples, and fecal streptococcus numbers exceeded human recreational bathing limits in 47 of 50 samples. Antibiotic resistance profiles were determined for a subset of fecal colifom and fecal streptococcus isolates. The tested isolates were frequently resistant to ceftiofur, enrofloxacin, and tetracycline, antibiotics commonly administered to livestock. In addition, surface water and sediment samples were analyzed for the presence of bacteria (*Clostridium botulinum* type C, toxigenic *Escherichia coli, Erysipelothrix* spp., *Pasteurella multocida, Salmonella* spp., and *Yersinia* spp) with pathogenic potential to wild and domestic animals. Of the organisms surveyed, salmonellae and escherichiae were isolated. The data presented herein will provide a benchmark for future studies investigating the potential long term environmental affects of agricultural practices on the Salt Plains National Wildlife Refuge.

BACKGROUND AND JUSTIFICATION

Human population expansion and concurrent reductions in wildlife habitat have forced wildlife to crowd into smaller areas and to potentially interact more frequently with domestic animals (11). These increased interactions can result in a greater incidence of disease transmission between domestic and wild animals, causing wildlife population declines and economic losses to the livestock industry. Many of these diseases are also transmissible to humans (zoonotic) (11). Further, domestic animal diseases can become endemic in wildlife populations, which can then become reservoirs for reintroduction of disease to domestic animals and humans (9, 11).

The livestock industry has also changed significantly over the past 20 years, as exemplified by the trend towards fewer but larger operations that emphasize intense production and specialization (15). If a livestock facility harbors animals for at least 45 days within any 12-month period and there is no grass or other vegetation in the confinement area during the normal growing season, it is defined by the United States Environmental Protection Agency (US EPA) as an animal feeding operation (AFO) (14). Based upon the number of animals harbored at the facility and upon the potential of the facility to contaminate surface waters, the EPA can further classify an AFO as a concentrated animal feeding operation (CAFO) (14). In the United States, an estimated 15,500 CAFOs annually produce approximately three times more raw excreted waste than is generated by humans (38). Unlike human wastes, which are processed through sanitary treatment facilities, raw animal wastes are typically remediated in open, wildlife-accessible containment lagoons or disposed of through land application. These disposal methods represent a direct threat to wildlife as the causative agents of many diseases are readily excreted through the feces of domestic animals including those that appear clinically healthy (32, 37). In 2000, the agricultural sector was

designated as the leading contributor of pollutants to streams, rivers, lakes, ponds, and reservoirs in the US (16). Thus, CAFOs are designated as potential pollution point sources under section 502 of the EPA Clean Water Act and must develop and implement comprehensive waste management plans in compliance with the National Pollutant Discharge Elimination System program (38).

The Salt Plains National Wildlife Refuge in Alfalfa County, OK, comprises 32,000 acres of salt flats, open reservoirs, woodlands, and agricultural fields. The refuge, designated as one of 18 US sites critical to the survival of shorebirds by the Western Hemisphere Shorebird Reserve Network, provides habitat for approximately 300 migratory bird species, including endangered whooping cranes and interior least terns, and threatened snowy plovers (http://www.greatsaltplains.com).

The presence of several CAFOs (it is our understanding that these facilities are not US EPA registered) as well as the proposed development of additional CAFOs near the refuge (Fig. 1) led to the concern that neighboring agricultural practices may continue to degrade and ultimately destroy critical migratory bird habitat provided by the refuge. For example, in 1998, effluent from a swine CAFO South of the refuge caused a significant fish kill in Spring Creek (D.B. Martin, Personal Communication). In response, whooping cranes and large numbers of migrating waterfowl had to be hazed from contaminated areas. Damage to aquatic invertebrate and amphibian populations was never evaluated.

Salt Plains National Wildlife Refuge personnel asked us to design and complete this study to characterize bacterial and chemical contaminants within the refuge watershed that may have originated from agricultural sources. Bacterial indicators of fecal contamination in refuge surface waters were assessed and water and sediment samples were surveyed for bacteria with pathogenic potential to wild and domestic animals, including *Clostridium botulinum* type C, toxigenic *Escherichia coli*, *Erysipelothrix* spp., *Pasteurella multocida*, *Salmonella* spp., and *Yersinia* spp. The

results of this study provide a summary of the environmental health of surface waters within the Salt Plains National Wildlife Refuge on the days sampling was conducted (six times from May, 2001 to October, 2002) and will serve as a benchmark for refuge personnel to further investigate future impacts of agricultural practices on the refuge watershed.

OBJECTIVES

1) To survey environmental samples for bacterial indicators of fecal contamination and for a subset of known bacterial pathogens of wildlife, especially migratory waterfowl.

2) To analyze antibiotic resistance profiles for representative *E. coli* and fecal streptococcus isolates.

3) To measure chemical parameters in water samples.

METHODS AND PROCEDURES

Sample collection. Water and sediment samples were collected from ten locations within the Oklahoma Salt Plains National Wildlife Refuge (Table 1; Fig. 1) during the months of May, July, and October of 2001 and 2002. With the exception of Sand Creek (no CAFOs immediately upstream), sample sites targeting watersheds downstream of CAFOs or proposed CAFOs were identified with the assistance of refuge personnel. Approximately one liter of surface water was collected in a sterilized plastic bottle at each sample site, placed on ice, and processed within six hours of collection. Additionally, 25 to 50 ml of sediment was collected in a 125 ml sterile plastic container from the top 10 cm of bottom sediment at each sample site. Following collection, sediment samples were mixed using a sterile swab, the sediment-containing swab was immersed in a vial containing 5 ml 10% dimethyl sulfoxide (DMSO) as a cryoprotectant, and the swab was agitated

to remove sediment particles from the swab. DMSO-sediment suspensions and original sediment samples were frozen on dry ice within 6 to 8 h of collection and stored at -70°C until analyzed. Later, DMSO-treated frozen sediment samples were thawed, 2 ml aliquots were each inoculated into 6 ml BHI medium (Becton Dickinson, Cockeysville, MD), and cultures were incubated for 2.5 h at 37°C with shaking. BHI is a non-selective medium used to encourage general bacterial growth within sediment samples prior to selectively culturing for specific organisms. An overview of the bacterial culturing conditions utilized for this study is provided below and is summarized in Table 2. Abbreviations for the bacterial growth media used are defined in Table 3.

Fecal coliforms. The number of fecal coliform bacteria present in water samples was estimated using a membrane filtration procedure (10). Water samples were collected, and 1:10 and 1:100 dilutions were prepared using sterile phosphate buffer. One hundred ml samples were filtered through 47 mm diameter, 0.45 µm mean pore size mixed cellulose acetate/nitrate membranes (Millipore Corporation, Bedford, MA). If for technical reasons less than 100 ml water was filtered, the actual volume filtered was noted and taken into account when calculating colony forming units (CFU)/100 ml. Filters were then placed in membrane petri dishes containing pads saturated with m-Coliblue24® medium (Hach Company, Loveland, CO). Plates were incubated at 44.5°C for 18-24 h in a portable incubator before enumerating colonies. Blue colonies conforming to the criteria for fecal coliforms were counted for each dilution, and colony counts obtained from plates yielding between 10 and 150 colonies were averaged to determine the reported result.

Fecal streptococci. The number of fecal streptococci present in water samples was determined by membrane filtration (10) as described above with the following modifications. Filters were placed in membrane petri dishes containing pads saturated with KF Streptococcal medium (Gelman Sciences, Inc., Ann Arbor, MI). Plates were incubated at 35°C for 46 to 48 h in a portable

incubator. Red colonies conforming to the criteria for fecal streptococci were counted for each dilution, and colony counts obtained from plates yielding between 10 and 150 colonies were averaged.

***Clostridium botulinum* type C.** Efforts were made to identify genetic material from *Clostridium botulinum* type C spores in sediment samples by PCR. Genetic material was extracted from 0.25 to 0.5 g of each sediment sample using the Mo Bio UltraClean Soil DNA Isolation Kit (Solana Beach, CA) according to the manufacturer's instructions followed by a 1% cetyltrimethylammonium bromide (CTAB) extraction. Five µl of each extracted nucleic acid sample were then PCR-amplified using primers targeting the light-chain region of the type C neurotoxin gene as previously described (41).

Toxigenic *Escherichia coli.* The inability of toxigenic *E. coli* strain O157:H7 to ferment sorbitol can be used to screen for this organism in environmental samples. 150 ml water samples were added to 50 ml 4X EcEB (USGS-National Wildlife Health Center, unpublished), and 2 ml aliquots of BHI sediment-enrichment cultures were transferred to 5 ml EcEB. Cultures were incubated 18 to 24 h at 37°C and streaked onto SMAC medium (Becton Dickinson, Cockeysville, MD). SMAC plates were incubated for 18 to 24 h at 37°C and inspected for colorless colonies indicative of non-sorbitol fermenting *E. coli*. Suspected isolates were subcultured onto BAPs (Becton Dickinson) for biochemical characterization using either API-20E or Vitek systems (bioMerieux, St. Louis, MO) and for screening by the 0157:H7 latex agglutination test (Oxoid Limited, Hampshire, England) according to the manufacturer's instructions. Non-sorbitol fermenting *E. coli* strains identified while characterizing the antibiotic resistance profiles (see below) of fecal coliform isolates were included in this analysis as well. Confirmed non-sorbitol fermenting isolates were sent to The Pennsylvania State University Gastroenteric Disease Center for O and H

antigen serotyping and for toxin-gene testing by PCR. Isolates were assayed for the presence of genes encoding heat labile toxin, heat stable toxins a and b, shiga-like toxin types I and II, cytotoxic necrotizing factors 1 and 2, and intimin.

***Erysipelothrix*.** One-hundred fifty ml water samples were added to 50 ml 4X BHIB/S (USGS-National Wildlife Health Center, unpublished) and to 50 ml 4X Packer's medium (3). 2 ml aliquots of BHI sediment-enrichment cultures were each transferred to 5 ml BHIB/S and to 5 ml Packer's medium. Cultures were incubated 18 to 24 h at 37°C. BHIB/S cultures were then streaked onto BHIA/S (USGS-National Wildlife Health Center, unpublished), and Packer's medium cultures were streaked onto BAPs. Plates were incubated 18 to 24 h at 37°C. Following incubation, plates were examined to identify colonies characteristic of *Erysipelothrix* spp. Suspected isolates were subcultured onto BAPs and tested for hydrogen sulfide production using a triple sugar iron agar slant. Hydrogen sulfide producing isolates were further characterized using either bioMerieux API-Coryne or Vitek systems.

***Pasteurella multocida*.** *Pasteurella multocida* isolations were attempted only from BHI-enriched sediment samples. Two ml of each enrichment culture were transferred to 5 ml PmSB (29) and incubated at 37°C for 18 to 24 h in the presence of 5 % CO_2. Each broth culture was streaked onto a BAP and incubated at 37°C for 18 to 24 h. All bacterial colonies were screened based on colony morphology to identify *Pasteurella* spp. Suspected *Pasteurella* isolates were biochemically characterized using the bioMerieux API-20E system.

***Salmonella*.** One-hundred fifty ml water samples were added to 50 ml 4X RV broth (12, 40) (Remel, Lenexa, KA) and to 50 ml 4X DS broth (USGS-National Wildlife Health Center, unpublished). Cultures were incubated at 41.5°C for 18 to 24 h. Two ml aliquots of BHI sediment-enrichment cultures were transferred to 10 ml each of RV and DS media and incubated at 41.5°C for

18 to 24 h. Following incubation, DS and RV cultures were each streaked onto BG agar (23) (Becton Dickinson, Cockeysville, MD) and XLT4 agar (27) (Difco Laboratories, Detroit, MI). Plates were incubated at 37°C for 18 to 24 h. Bacterial colonies were screened based upon morphological and biochemical characteristics to identify potential *Salmonella* spp., and suspected colonies were subcultured on BAPs for biochemical characterization by either the bioMerieux API-20E or Vitek systems. *Salmonella* isolates identified based upon biochemical characteristics were further screened using a polyvalent antisera for *Salmonella* (Becton Dickinson, Cockeysville, MD) before being serotyped at the US Department of Agriculture, National Veterinary Services Laboratory (Ames, IA).

***Yersinia*.** One-hundred fifty ml water samples were added to 50 ml 4X BOS broth (34) and incubated at 23°C for one to five days. Two ml aliquots of BHI sediment-enrichment cultures were transferred to 10 ml BOS medium and incubated at 23°C for one to five days. BOS cultures were streaked onto both CIN (Becton Dickinson, Cockeysville, MD) and DYS (1) plates after one and after five days of incubation. CIN plates were incubated 18 to 24 h at 32°C, and DYS plates were incubated 36 to 48 h at 23°C. Following incubation, plates were examined for colonies characteristic of *Yersinia* spp. Suspected isolates were subcultured onto BAPs and biochemically characterized using either bioMerieux API-20E or Vitek systems.

Antibiotic resistance profiles. Eighteen to 24 h old cultures of bacterial isolates were tested for antibiotic resistance using GNS-207 (gram negative) and GPS-108 (gram positive) test cartridges on the bioMerieux Vitek system according to manufacturer's instructions. Resistance profiles were evaluated for a subset of isolates obtained for the evaluation of fecal coliform and fecal streptococcus levels throughout the refuge. When assaying antimicrobial resistance profiles, species-level identifications of presumptive *E. coli* and fecal streptococcus isolates were determined

using the bioMerieux Vitek system. For both gram negative and gram positive organisms, resistance profiles were measured for at least five isolates, if available, from each sample site. Gram negative isolates were screened for resistance to the antibiotics amikacin, amoxicillin, ampicillin, carbenicillin, ceftazidime, ceftiofur, cephalothin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, nitrofurantoin, piperacillin, tetracycline, tobramycin, and trimethoprim/sulfamethoxazol. Gram positive isolates were screened for resistance to the antibiotics amoxicillin, ampicillin, ceftiofur, cephalothin, chloramphenicol, clindamycin, enrofloxacin, erythromycin, gentamicin, gentamicin-500, oxacillin, penicillin-G, tetracycline, trimethoprim/sulfamethoxazol, and vancomycin.

Chemical analyses. Nitrate, nitrite, iron, and chloride concentrations, as well as total hardness, were measured in water samples using EM Quant Test Strips (EMD Chemical, Inc., Gibbstown, NJ) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Fecal coliforms. Fecal coliforms are a designated subgroup of bacteria closely associated with the gut and intestinal tracts of warm-blooded animals, including humans. The presence of fecal coliforms in water serves as a general indicator of fecal contamination, and detection and quantification of these organisms is based on standardized methodology (28). Fecal coliform counts were conducted as part of this study to provide an assessment of bacterial contamination from fecal sources on the days samples were collected. The US EPA recommends that fecal coliform density not exceed 200 CFU/100 ml of water for human recreational bathing purposes (10). As summarized in Table 4 and Fig. 2, of 50 measurements taken over two years, 28 measurements met human recreational bathing water criteria. Elevated coliform counts were observed in all samples collected

in July, 2002 and may have resulted from a wide-spread rain storm and associated run off that proceeded sampling. Additionally, warm temperatures during the month of July would have been favorable for coliform growth. Elevated coliform counts were also observed in May, 2002 from samples collected at Salt Fork River and Sand Creek. May sampling took place following a smaller rainfall event, and run off in the northern portion of the refuge was evident as elevated amounts of sediment were present in Salt Fork River and Sand Creek. It is possible that a cattle CAFO located upstream of the Salt Fork River sampling sites may have been a contributing factor to the elevated coliform levels observed at these sites.

Fecal streptococci. Fecal streptococci consist of a number of species from the genera *Streptococcus* and *Enterococcus*. The normal habitat of fecal streptococci is similar to that of fecal coliforms, the gastrointestinal tract of warm-blooded animals, including humans (28). The US EPA recommends that for human recreational bathing purposes, fecal streptococci not exceed a density of 33 CFU/100 ml of fresh water (10). Only 3 of the 50 measurements reported in Table 5 and Fig. 3 met these criteria. Similar to what was observed with the fecal coliforms, elevated fecal streptococcus counts were noted at all sampling sites in July, 2002 and at the Salt Fork River and Sand Creek sampling sites in May, 2002. These elevated counts may have been caused by previously-noted rainfall and resultant run off that occurred prior to sampling. Elevated fecal streptococcus counts were also observed at Clay Creek site two in May 2002 and at the Twin Springs Creek sampling sites in October, 2002. There is a hog CAFO upstream of the Twin Springs Creek sampling sites. It is possible that this CAFO may have contributed to elevated fecal streptococcus counts at these locations.

***Clostridium botulinum* type C.** *C. botulinum* type C, the causative agent of avian botulism, has significant annual impacts on waterfowl populations. Botulism occurs in wildlife following

ingestion of botulinum toxin, and the ability of *C. botulimum* to produce resistant spores allows it to persist in the environment for extended periods of time (36). *C. botulinum* type C genetic material was not detected in sediment samples by PCR. Control experiments indicated that the procedures utilized may not have been fully effective in identifying *C. botulinum* genetic material. Future efforts will be directed towards optimizing both the procedure for extracting genetic material from sediment and the PCR protocol utilized.

Toxigenic *Escherichia coli.* Toxigenic (non-sorbitol fermenting) *E. coli* O157:H7 has been implicated as the causative agent of hemorrhagic colitis in humans (26). The principal reservoir for *E. coli* O157:H7 is cattle (35), and this organism has also been isolated from wild deer (2). *E. coli* O157:H7 can survive up to 21 months outside of an animal host (24). Non-sorbitol fermenting *E. coli* were not recovered in 2001, but 5 isolates were recovered in 2002 (Table 6). None of the non-sorbitol fermenting isolates belonged to the O157:H7 serotype, however 2 of the 5 isolates possessed the gene encoding cytotoxic necrotizing factor 2 (CNF2). CNF2 has been implicated as a virulence factor in *E. coli* strains that cause enteric and extraintestinal infections (30), and necrotoxigenic *E. coli* strains that produce CNF2 have been recovered from the intestinal tracts of domestic animals including cattle (4, 5, 13).

***Erysipelothrix*.** *Erysipelothrix* spp. have been isolated from healthy and diseased mammalian, avian, and amphibian species (32, 33). Major mortality events in eared grebes at the Great Salt Lake in Utah (21) and in brown pelicans off the California coast (USGS-National Wildlife Health Center, unpublished) have been attributed to *Erysipelothrix*. *Erysipelothrix* isolates were not recovered during the course of this study.

***Pasteurella multocida*.** *P. multocida* is the causative agent of avian cholera in wild and domestic fowl (32). In animals, *P. multocida* infections are often acute, resulting in death within 6

to 12 hours (18). *P. multocida* can also be carried latently by birds, causing disease under conditions of stress (32). *P. multocida* has world wide distribution, and in some habitats, avian cholera outbreaks occur among waterfowl on an annual basis.

To evaluate the environmental prevalence of *P. multocida* within the Salt Plains National Wildlife Refuge, attempts were made to cultivate this organism from sediment samples taken at ten sites within and surrounding the refuge. Consistent with the observation that avian cholera has not previously been a problem at the Salt Plains National Wildlife Refuge, *P. multodica* was not cultivated from samples collected during this study.

Salmonella. *Salmonella* spp. are divided into six subgroups encompassing over 2000 different serotypes (31). The natural reservoir for salmonellae is the intestinal tract of both warm- and cold-blooded animals, and the majority of infected animals are subclinically ill excretors. *Salmonella* spp. can survive for up to 7 months outside of an animal host under certain environmental conditions (6). Among wildlife, salmonellosis can cause large-scale mortality events, especially in the young of colony nesting bird species (19).

Twenty-seven *Salmonella* isolates were recovered during the three 2001 sampling events, and 35 isolates were recovered over three sampling events in 2002 (Fig. 4). The greatest number of *Salmonella* serotypes identified in 2001 (59%) were isolated in May from the Twin Springs Creek, Cottonwood Creek, Salt Fork River, and Sand Creek sample sites. This does not correspond to the relatively low fecal coliform and fecal streptococcus counts obtained during this same sampling event (Figs. 2 and 3). The majority of *Salmonella* serotypes identified in 2002 (56%) were isolated in July, during which high fecal coliform and fecal streptococcus counts were also observed throughout the refuge. Thirty nine percent of the *Salmonella* serotypes identified in 2002 were isolated in May. These isolates were obtained from the Cottonwood Creek, Salt Fork River, and

Sand Creek sample sites concurrent with the observation of elevated fecal coliform and fecal streptococcus counts. In two out of three instances, increased frequency of *Salmonella* isolation corresponded with elevated levels of fecal indicator bacteria at refuge sample sites that may have resulted from run off following rain events. Thus, the associations between surface water run off and increased levels of fecal coliforms, fecal streptococci, and salmonellae should be investigated further to evaluate potential adverse impacts to the refuge environment.

The *Salmonella* serotypes most frequently identified in 2001 were 62:G,Z51(Arizona), represented by 6 isolates, Typhimurium, represented by 5 isolates, and Muenchen, represented by 4 isolates. The serotype most frequently identified in 2002 was *Salmonella* Bredeney, isolated 11 times (Fig. 4). Serotype 62:G,Z51(Arizona) tends to be associated with cold-blooded animals (USDA-National Veterinary Services Laboratories, unpublished) and is thus not likely to have originated from CAFO sites. *Salmonella* Typhimurium is commonly isolated from domestic livestock, including cattle, swine, horses, sheep, chickens, and turkeys, exhibiting the highest prevalence in cattle and horses (17). Serotype Typhimurium is also frequently isolated from wild bird tissues analyzed by the USGS-National Wildlife Health Center diagnostic microbiology laboratory (unpublished data). Serotype Muenchen has previously been isolated from various crane species by the USGS-National Wildlife Health Center diagnostic microbiology laboratory (unpublished data), and serotype Bredeney is commonly found in domestic turkey flocks (17). Thus, the *Salmonella* serotypes isolated within the Salt Plains National wildlife refuge are representative of those that colonize both wild and domestic animals.

***Yersinia*.** The genus *Yersinia* contains 11 species that are endemic to a variety of animals, including wild rodents, wild birds, and domestic pigs. *Y. enterocolitica* is found in surface waters and can cause epizootic outbreaks of diarrhea, pneumonia, and spontaneous abortion (22). *Yersinia*

isolates were not recovered from environmental samples collected during this study.

Antibiotic resistance profiles. Antibiotic resistance profiles were determined for a subset of *E. coli* isolates obtained from the fecal coliform bacteria enumerated within the refuge. One-hundred twenty-seven and 131 *E. coli* isolates obtained in 2001 and 2002, respectively, were screened for resistance against a panel of 17 antibiotics (Fig. 5). Of the 2001 isolates, 39 (31%) were resistant to at least one antibiotic, and seven (18%) of the resistant isolates were resistant to two or more antibiotics. Analysis of the 2002 isolates revealed that 29 (22%) were resistant to at least one antibiotic. Ten (34%) of the resistant isolates were resistant to two or more antibiotics. The antibiotic to which the greatest number of *E. coli* isolates exhibited resistance was tetracycline. Twenty nine percent of 2001 isolates and 19% of 2002 isolates were resistant to tetracycline, whereas 5% or fewer of the isolates were resistant to each of the other antibiotics tested.

Resistance profiles against a panel of 15 antibiotics were determined for a subset of the fecal streptococcus isolates enumerated within the refuge (Fig. 6). The isolates tested were identified to the species level and included *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, and *E. gallinarum*. Of the 2001 isolates (110 screened), 107 (97%) were resistant to at least one antibiotic, and 57 (53%) of the resistant isolates were resistant to two or more antibiotics. In 2002, 119 (95%) of the 125 isolates analyzed were resistant to at least one antibiotic, and 63 (53%) of the resistant isolates were resistant to two or more antibiotics. Antibiotics to which the gram positive isolates were most frequently resistant included ceftiofur (≈90% resistant) and enrofloxacin (≈50% resistant). Twelve percent and 7% of the 2001 and 2002 isolates, respectively, were resistant to tetracycline; 2% or fewer of the isolates were resistant to each of the other antibiotics tested.

Antibiotics are used in animal agriculture to promote animal growth and to prevent and treat

disease (8). The use of antibiotics in agriculture for prophylactic and growth-promoting purposes has been implicated in the evolution and widespread dissemination of antibiotic resistant bacteria throughout the environment (7). The development of antibiotic resistance by commensal enteric bacteria, including *E. coli* and various enterococci that colonize the intestinal tracts of both animals and humans provides an avenue for the transmission of antibiotic resistant bacteria from domestic animals into humans (8). As bacterial infections of wild animals are not routinely treated with antibiotics, antibiotic resistant bacteria do not likely pose a direct threat to wildlife health. However, due to the free-ranging nature of wild animals, especially migratory waterfowl that inhabit the Salt Plains National Wildlife Refuge, wildlife may facilitate the transfer of antibiotic resistant bacteria throughout the environment.

In this study, antibiotics to which the bacteria tested most frequently exhibited resistance included tetracycline, ceftiofur, and enrofloxacin. Each of these antibiotics belongs to a different class of pharmaceutical agents and exerts its clinical effects through a unique mechanism of action (8, 20, 39). Thus, the physiological mechanisms that confer bacterial resistance to each antibiotic are also distinct and likely arose independently in response the selective pressure exhibited by each antibiotic.

Tetracycline was the most common antibiotic to which *E. coli* strains exhibited resistance and the third most common antibiotic to which gram positive isolates were resistant. Tetracycline is commonly used in the swine industry, and in the United States, farm animals were treated with 3,488,000 kg of tetracycline per year during the 1990s (7, 8). Among gram positive bacteria analyzed, almost 90% of strains tested were resistant to ceftiofur. Ceftiofur is used worldwide in veterinary medicine to treat respiratory diseases in swine, ruminants, and horses and to treat foot rot

and metritis infections in cattle (20). Also among the gram positive isolates analyzed, approximately 50% were resistant to enrofloxacin, another commonly used veterinary antibiotic (39, 42). Thus, the identification of bacteria resistant to antibiotics commonly used in veterinary medicine in refuge water samples suggests that agricultural practices in areas surrounding the Salt Plains National Wildlife Refuge may have contributed to the dissemination of these organisms within the refuge.

Chemical analyses. Nitrate, nitrite, iron, and chloride concentrations, as well as total water hardness levels, were measured in water samples collected throughout the refuge (Table 7). Of the chemical parameters analyzed, nitrogen levels (measured as nitrate and nitrite) have the greatest potential to impact refuge water quality. Sources that contribute to environmental nitrogen levels include the natural decay of organisms, animal feces, and agricultural fertilizer. EPA limits for nitrogen levels in drinking water are set at 10 ppm for nitrate and 1 ppm for nitrite (10). Nitrate concentrations of 10 ppm or higher were detected in ten water samples obtained from Twin Springs Creek, Cottonwood Creek, and the Salt Fork River (Table 7). Nitrite concentrations of 1 ppm or greater were detected in four samples collected from Twin Springs Creek and the Salt Fork River (Table 7). There are CAFOs located upstream of the Twin Springs Creek and Salt Fork River sampling sites, and it is possible that they may have contributed to elevated nitrogen levels observed at these sites.

CONCLUSIONS

The main goals of this study were to assess levels of bacterial indicators of fecal contamination within Salt Plains National Wildlife Refuge surface water and to identify known bacterial pathogens of wildlife, especially waterfowl, within refuge water and sediment samples. Fecal indicator bacteria frequently exceeded US EPA guidelines for human recreational bathing

waters within the refuge, and elevated bacterial counts observed in May and July, 2002 correlated with rainfall that occurred prior to sampling (Figs. 3 and 4; Tables 4 and 5). High levels of fecal indicator bacteria were observed in Salt Fork River and Twin Springs Creek sampling sites. These sites are downstream of noted cattle and hog CAFOs, respectively (Fig. 1). Counts in excess of US EPA human recreational bathing limits, however, were measured at least once at every sample site. Laboratory efforts to experimentally trace the bacteria identified in this study to agricultural sources were not conducted.

Antibiotic resistance profiles were characterized for representative isolates of fecal indicator bacteria (Figs. 5 and 6). These analyses revealed that high percentages of bacteria of fecal origin isolated from refuge surface water were resistant to antibiotics commonly used in veterinary medicine, including tetracycline, ceftiofur, and enrofloxacin. The threat that antibiotic-resistant bacteria may pose to wildlife has not been characterized, however it is well established that the emergence of resistance in human and veterinary pathogens has exerted significant consequences for the therapeutic use of antibiotics (8).

Erysipelothrix spp., *P. multocida*, and *Yersinia* spp., known pathogens of wildlife, were not identified during the course of this study. This is consistent with the observation that these organisms have not previously been implicated in disease outbreaks among wildlife within the Salt Plains National Wildlife Refuge (USGS–National Wildlife Health Center, unpublished). Continued nutrient loading of the refuge watershed may, however, increase the probability of these pathogens causing future problems to wildlife within the refuge.

Salmonella spp. were isolated from all sample sites within the refuge. Elevated isolation frequencies of salmonellae correlated with rainfall-induced run off, and nutrient inputs from CAFOs or other sources may enhance the growth of these organisms within the refuge watershed. The

presence of salmonellae within refuge surface water poses a potential pathogenic threat both to wildlife utilizing these habitats and to humans visiting the refuge. Necrotoxigenic *E. coli,* similar to strains previously noted to be associated with cattle (4, 5), were also isolated from two sample sites within the refuge (Table 6). The pathogenicity of necrotoxigenic *E. coli* to wildlife has not been characterized, but these organisms can cause infections in humans (30).

RECOMMENDATIONS

While this study demonstrated that antibiotic resistant bacteria of fecal origin, potentially pathogenic *Salmonella* spp., and necrotoxigenic *E. coli* were present in refuge surface waters, the direct threat that these organisms pose to wildlife within the refuge is not known. Sentinel mallard flocks could be utilized to assess the potential of waterfowl to become infected by these organisms and to shed them throughout the environment. Additionally, the role that small mammals inhabiting the refuge may play in disseminating bacteria throughout the environment is not known. Surveys to address the diversity and dispersal patterns of wild mammals within the refuge would provide a starting point for addressing this question. It would also be worthwhile to expand chemical and toxicological monitoring efforts in the refuge to measure nutrient levels in refuge surface waters and to assess possible bioaccumulation of toxic compounds within fish, amphibian, and invertebrate populations. As highlighted by the 1998 fish kill in Spring Creek, potential environmental quality problems within the refuge watershed may first manifest within these groups of sensitive organisms.

As many of the bacteria isolated during the course of this study possess attributes that suggest they originated from agricultural sites, CAFOs adjacent to the refuge may be sources of these pollutants. The work described herein does not, however, experimentally link bacteria isolated within the refuge to agricultural sources. Short of employing bacterial source tracking techniques

(25) to experimentally trace these organisms to specific point sources, less involved follow-up studies could be done. For example, levels of fecal indicator bacteria could be reassessed both upstream and downstream of the Twin Springs Creek and Salt Fork River CAFOs, sites from which high levels of fecal indicator bacteria were observed. If fecal indicator bacteria originate from these sites, decreasing gradients of indicator organisms should be observed moving downstream from the CAFOs, and lower levels of fecal indicator bacteria should be present upstream of the sites than downstream.

By educating the surrounding community about the health implications of fecal contamination of refuge surface waters and by emphasizing the consequences of widespread environmental dissemination of antibiotic resistant bacteria, perhaps the community will appeal to CAFO operators to voluntarily improve their waste disposal practices. Changes such as ensuring that waste lagoons do not overflow following rainstorms, refraining from applying manure to saturated ground, keeping animals out of streams, and maintaining buffer zones around streams during land application of manure may lead to significant improvements in refuge water quality.

QUALITY ASSURANCE

Planning, data collection, and analysis of information under this contract were completed with the technical care necessary to meet the needs for valid and high quality data. All work done during the contract followed the tenants of Good Laboratory Procedures (GLP) as described in 40 CFR 160 or as institutional policy provided. References and internal quality controls were included when possible, and data were validated with respect to their feasibility and according to the investigators' experience.

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DISCLAIMER

Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Table 1. GPS coordinates of sampling sites (Oklahoma CAFO study – May, 2001 to October, 2002).

Sample Site	Coordinates
Twin Springs Creek – Site 1	N36°40.853’ W098°13.500’
Twin Springs Creek – Site 2	N36°41.045’ W098°13.200’
Clay Creek – Site 1	N36°42.984’ W098°17.048’
Clay Creek – Site 2	N36°43.924’ W098°15.807’
Cottonwood Creek – Site 1	N36°45.222’ W098°18.436’
Cottonwood Creek – Site 2	N36°45.183’ W098°17.265’
Salt Fork River – Site 1	N36°49.201’ W098°21.607’
Salt Fork River – Site 2	N36°47.577’ W098°14.442’
Salt Fork River – Site 3	N36°46.423’ W098°13.287’
Sand Creek	N36°48.662’ W098°12.046’

Table 2. Summary of bacterial culturing methodology (Oklahoma CAFO study – May, 2001 to October, 2002).

Organism	Sample Type	Primary Medium [a]	Primary Incubation	Secondary Medium [a]	Secondary Incubation
fecal coliforms	water	m-Coliblue24®	18-24 h; 44.5°	BAP	18-24 h; 37°C
fecal streptococci	water	KF	46-48 h; 35°C	BAP	18-24 h; 37°C
Escherichia coli	water	EcEB	18-24 h; 37°C	SMAC	18-24 h; 37°C
	sediment	EcEB	18-24 h; 37°C	SMAC	18-24 h; 37°C
Erysipelothrix spp.	water	BHIB/S	18-24 h; 37°C	BHIA/S	18-24 h; 37°C
	water	Packer's Medium	18-24 h; 37°C	BAP	18-24 h; 37°C
	sediment	BHIB/S	18-24 h; 37°C	BHIA/S	18-24 h; 37°C
	sediment	Packer's Medium	18-24 h; 37°C	BAP	18-24 h; 37°C
Pasteurella multocida	sediment	PMSB	18-24 h; 37°C; CO_2	BAP	18-24 h; 37°C
Salmonella spp.	water	DS	18-24 h; 41.5°C	BG and XLT4	18-24 h; 37°C
	water	RV	18-24 h; 41.5°C	BG and XLT4	18-24 h; 37°C
	sediment	DS	18-24 h; 41.5°C	BG and XLT4	18-24 h; 37°C
	sediment	RV	18-24 h; 41.5°C	BG and XLT4	18-24 h; 37°C
Yersinia spp.	water	BOS	1 or 5 d; 23°C	CIN	18-24 h; 32°C
				DYS	36-48 h; 23°C
	sediment	BOS	1 or 5 d; 23°C	CIN	18-24 h; 32°C
				DYS	36-48 h; 23°C

[a] Abbreviations defined in Table 3.

Table 3. Growth medium abbreviations (Oklahoma CAFO study – May, 2001 to October, 2002).

Medium	Abbreviation
Bile Oxalate Sorbose	BOS
5 % Sheep Blood Agar Plate	BAP
Brain Heart Infusion	BHI
Brain Heart Infusion Agar, Selective	BHIA/S
Brain Heart Infusion Broth, Selective	BHIB/S
Brilliant Green	BG
Cefsulodin Irgasan Novobiocin	CIN
Dulcitol-Selenite	DS
Differential *Yersinia* Selective	DYS
Escherichia coli Enrichment Broth	EcEB
KF Streptoccal	KF
MacConkey's Medium with Sorbitol	SMAC
Pasteurella multocida Selective Broth	PmSB
Rappaport-Vassiliadis	RV
Xylose Lysine Tergitol 4	XLT4

Table 4. Fecal coliform counts (Oklahoma CAFO study – May, 2001 to October, 2002).

Sample Site	Fecal Coliform Colony Forming Units Per 100 ml Water[a]					
	May		July		October	
	2001	2002	2001	2002	2001	2002
Twin Springs Creek -Site 1	144	NC	770	2500	NC	380
Twin Springs Creek -Site 2	10	NC	523	2300	NC	19
Clay Creek -Site 1	790	3	NC	3100	NC	100
Clay Creek -Site 2	72	152	NC	2200	NC	10
Cottonwood Creek -Site 1	119	113	177	2200	11	160
Cottonwood Creek -Site 2	100	82	10	2400	3	90
Salt Fork River -Site 1	227	4900	340	9400	22	280
Salt Fork River -Site 2	148	4700	NC	4900	NC	67
Salt Fork River -Site 3	218	3600	98	4400	7	127
Sand Creek	151	5400	144	4600	64	60

[a] "NC" denotes that for technical reasons, counts were not completed.

Table 5. Fecal streptococcus counts (Oklahoma CAFO study – May, 2001 to October, 2002).

Sample Site	Fecal Streptococcus Colony Forming Units Per 100 ml Water[a]					
	May		July		October	
	2001	2002	2001	2002	2001	2002
Twin Springs Creek -Site 1	140	NC	106	10200	NC	3300
Twin Springs Creek -Site 2	30	NC	649	8200	NC	2200
Clay Creek -Site 1	0	84	NC	5300	NC	270
Clay Creek -Site 2	100	2000	NC	2900	NC	750
Cottonwood Creek -Site 1	50	68	81	4600	65	240
Cottonwood Creek -Site 2	20	155	90	8100	118	100
Salt Fork River -Site 1	140	6700	210	7100	105	570
Salt Fork River -Site 2	50	10900	NC	10500	NC	183
Salt Fork River -Site 3	60	8600	69	11200	53	103
Sand Creek	90	8200	154	10500	160	213

[a] "NC" denotes that for technical reasons, counts were not completed.

Table 6. Summary of serotypes and virulence factors identified for non-sorbitol fermenting *E. coli* isolates (Oklahoma CAFO study – May, 2001 to October, 2002).

Sample Site	Date Isolated	Serotype	Virulence Factors
Clay Creek – Site 2	July, 2002	O11:H^{-}	None
Clay Creek – Site 2	October, 2002	O85,167:H28	None
Cottonwood Creek – Site 1	October, 2002	O169:H3 or 16	None
Cottonwood Creek – Site 2	October, 2002	OM:H7 [a]	CNF2 [b]
Salt Fork River – Site 2	July, 2002	O106,166:H24	CNF2 [b]

[a] "M" denotes cross reactivity with multiple O antisera.

[b] Cytotoxic necrotizing factor 2

Table 7. Measurement of chemical parameters in refuge water samples [a] (Oklahoma CAFO study – May, 2001 to October, 2002).

Sample Site	Sample Date	Nitrate (ppm)	Nitrite (ppm)	Iron (ppm)	Chloride [b] (ppm)	Hardness (mM) [c]
Twin Springs Creek	May, 2001	25	0	0	---	> 4.5
Site 1	July, 2001	0	0	0	---	2.7 to 3.6
	October, 2001	NS	NS	NS	NS	NS
	May, 2002	NS	NS	NS	NS	NS
	July, 2002	0	0	3	0	1.8 to 2.7
	October, 2002	10 to 25	1	2	1500 to 2000	> 4.5
Twin Springs Creek	May, 2001	0	0	0	---	1.8 to 2.7
Site 2	July, 2001	0	0	0	---	1.8 to 2.7
	October, 2001	NS	NS	NS	NS	NS
	May, 2002	NS	NS	NS	NS	NS
	July, 2002	0	0	2	0	1.8 to 2.7
	October, 2002	0 to 10	1	3	3000	> 4.5
Clay Creek	May, 2001	0	0	0	---	3.6 to 4.5
Site 1	July, 2001	NS	NS	NS	---	NS
	October, 2001	NS	NS	NS	NS	NS
	May, 2002	0	0	2	3000	> 4.5
	July, 2002	5	0	3	200	1.8 to 2.7
	October, 2002	0	0	3	3000	> 4.5
Clay Creek	May, 2001	0	0	0	---	> 4.5
Site 2	July, 2001	NS	NS	NS	---	NS
	October, 2001	NS	NS	NS	NS	NS
	May, 2002	0	0	3	1750	> 4.5
	July, 2002	0	0	3	250	1.8 to 2.7
	October, 2002	0	0	0	3000	> 4.5
Cottonwood Creek	May, 2001	25	0	0	---	> 4.5
Site 1	July, 2001	10	0	0	---	> 4.5
	October, 2001	25	0	0	500	> 4.5
	May, 2002	0	0	2	450	> 4.5
	July, 2002	0	0	3	0	0.9 to 1.8
	October, 2002	25	0	2	500	> 4.5
Cottonwood Creek	May, 2001	10	0	0	---	> 4.5
Site 2	July, 2001	0	0	0	---	> 4.5
	October, 2001	10	0	0	2000	> 4.5
	May, 2002	0	0	2	3000	> 4.5
	July, 2002	0	0	3	0	0.9 to 1.8
	October, 2002	0	0	3	3000	> 4.5

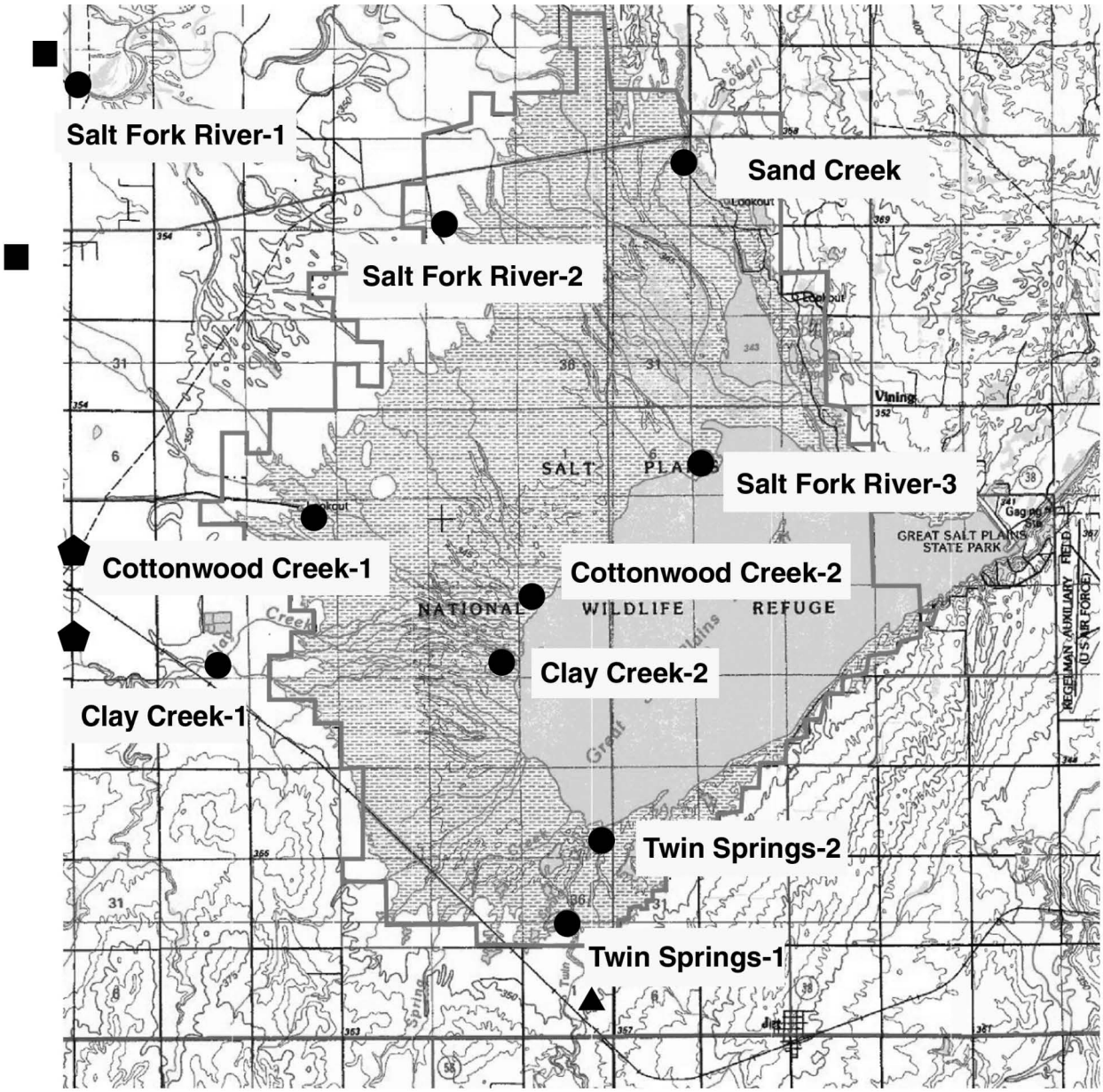
(Continued)

Table 7. Chemical parameters (continued).

Sample Site	Sample Date	Nitrate (ppm)	Nitrite (ppm)	Iron (ppm)	Chloride [b] (ppm)	Hardness (mM) [c]
Salt Fork River	May, 2001	0	0	0	---	> 4.5
Site 1	July, 2001	0	0	0	---	> 4.5
	October, 2001	0	0	0	0	> 4.5
	May, 2002	0	0	3	400	> 4.5
	July, 2002	5	0	3	0	3.6 to 4.5
	October, 2002	0	0	2	0 to 500	> 4.5
Salt Fork River	May, 2001	0	0	0	---	> 4.5
Site 2	July, 2001	NS	NS	NS	---	NS
	October, 2001	NS	NS	NS	NS	NS
	May, 2002	2	0	3	0	3.6 to 4.5
	July, 2002	10	1	5	0	1.8 to 2.7
	October, 2002	0	0	2	500 to 1000	> 4.5
Salt Fork River	May, 2001	0	0	0	---	> 4.5
Site 3	July, 2001	0	0	0	---	> 4.5
	October, 2001	0	0	0	0	> 4.5
	May, 2002	2	0	3	400	> 4.5
	July, 2002	5	2	3	0	1.8 to 2.7
	October, 2002	0	0	3	1500 to 2000	> 4.5
Sand Creek	May, 2001	0	0	0	---	2.7 to 3.6
	July, 2001	0	0	0	---	2.7 to 3.6
	October, 2001	0	0	0	0	3.6 to 4.5
	May, 2002	1	0	2	0	0.9 to 1.8
	July, 2002	5	0	3	0	0.9 to 1.8
	October, 2002	0	0	2	0	2.7 to 3.6

[a] "NS" indicates that due to environmental conditions, no sample was taken.
[b] Measurements for chloride ions were not conducted in May and July of 2001.
[c] Expressed as mmol/l alkaline earth ions.

Fig. 1. Salt Plains National Wildlife Refuge sampling locations (Oklahoma CAFO study - May, 2001 to October, 2002)



● Denotes locations of sample sites.

■ Denotes locations of cattle CAFOs.

⬟ Denotes locations of proposed cattle CAFOs.

▲ Denotes location of hog CAFO.

Refuge boundary denoted by wide grey line.

Fig. 2. Fecal coliform counts (Oklahoma CAFO study - May, 2001 to October, 2002).

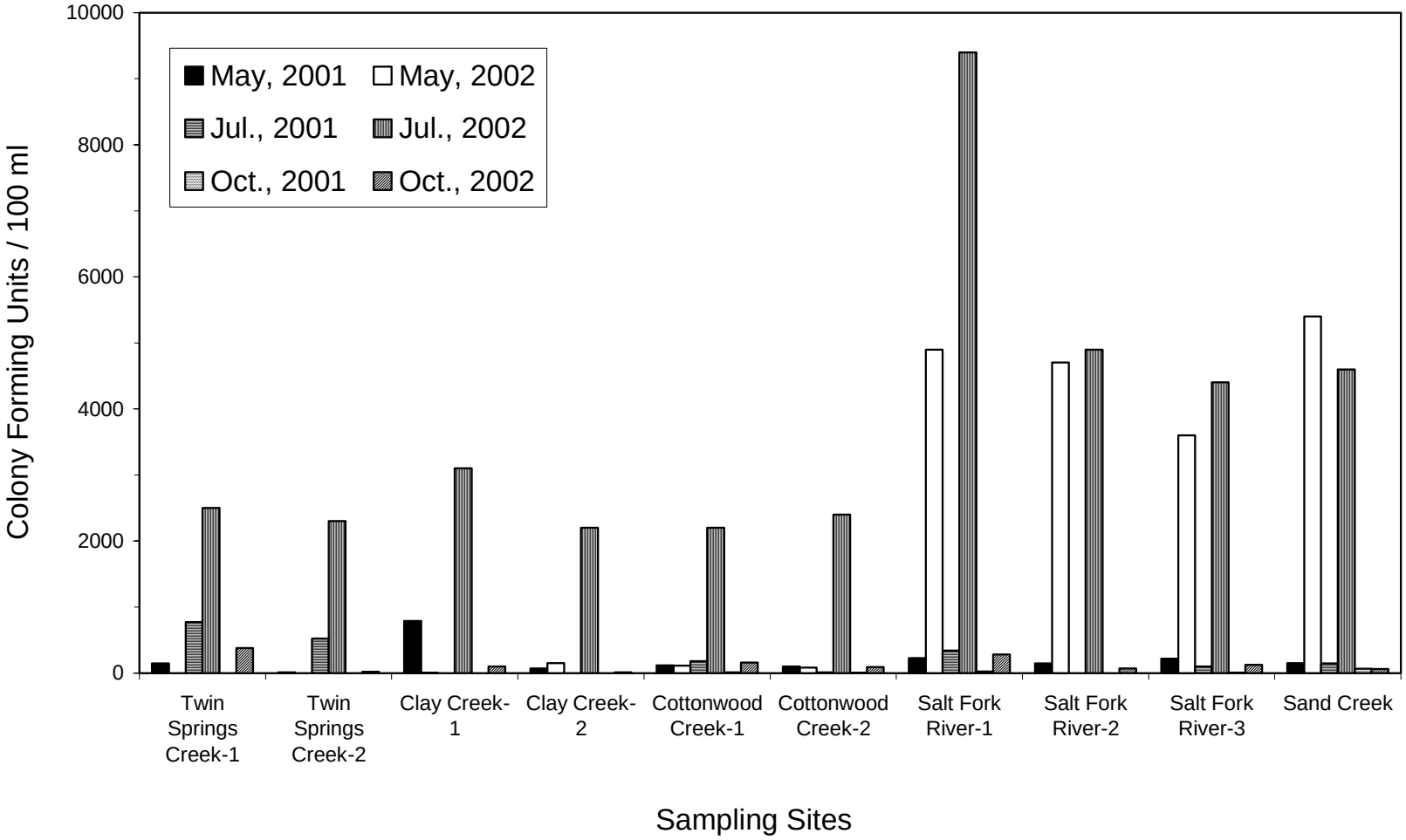


Fig. 3. Fecal streptococcus counts (Oklahoma CAFO study - May, 2001 to October, 2002).

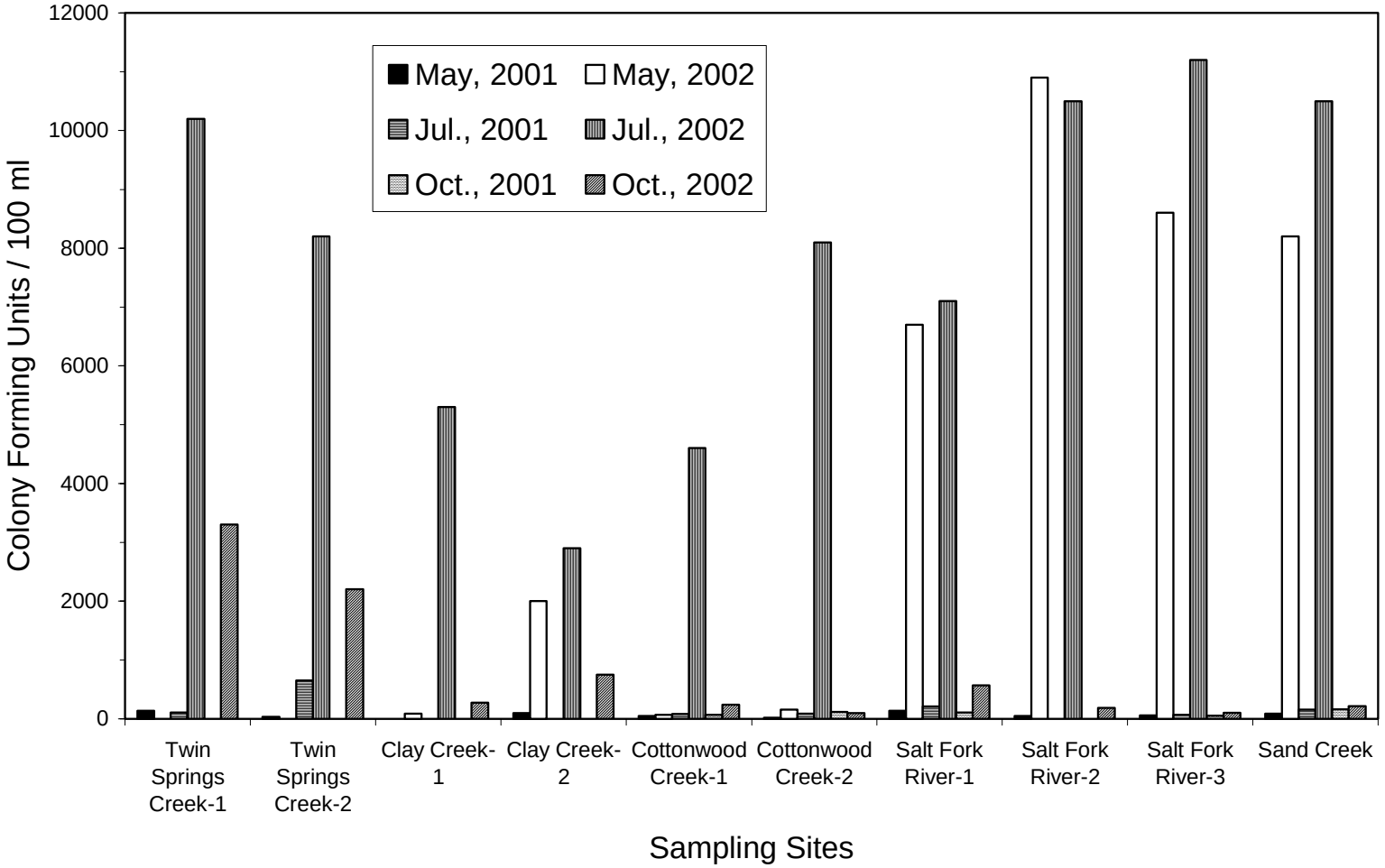


Fig. 4. *Salmonella* serotypes identified during 2001 and 2002 sampling events (Oklahoma CAFO study - May, 2001 to October, 2002).

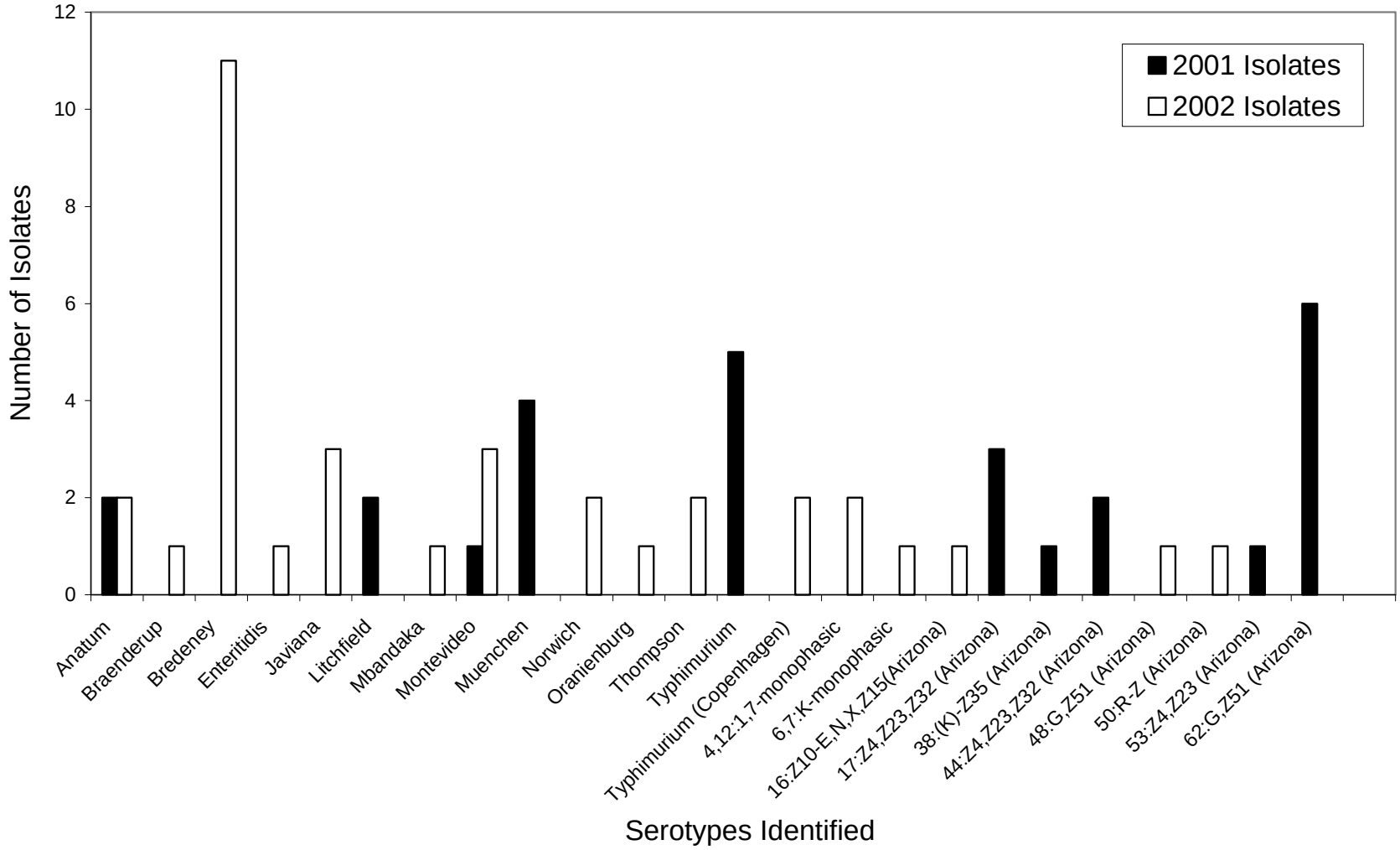


Fig. 5. Antibiotic resistance profiles of *E. coli* isolates[a] from 2001 and 2002 sampling events (Oklahoma CAFO study - May, 2001 to October, 2002).

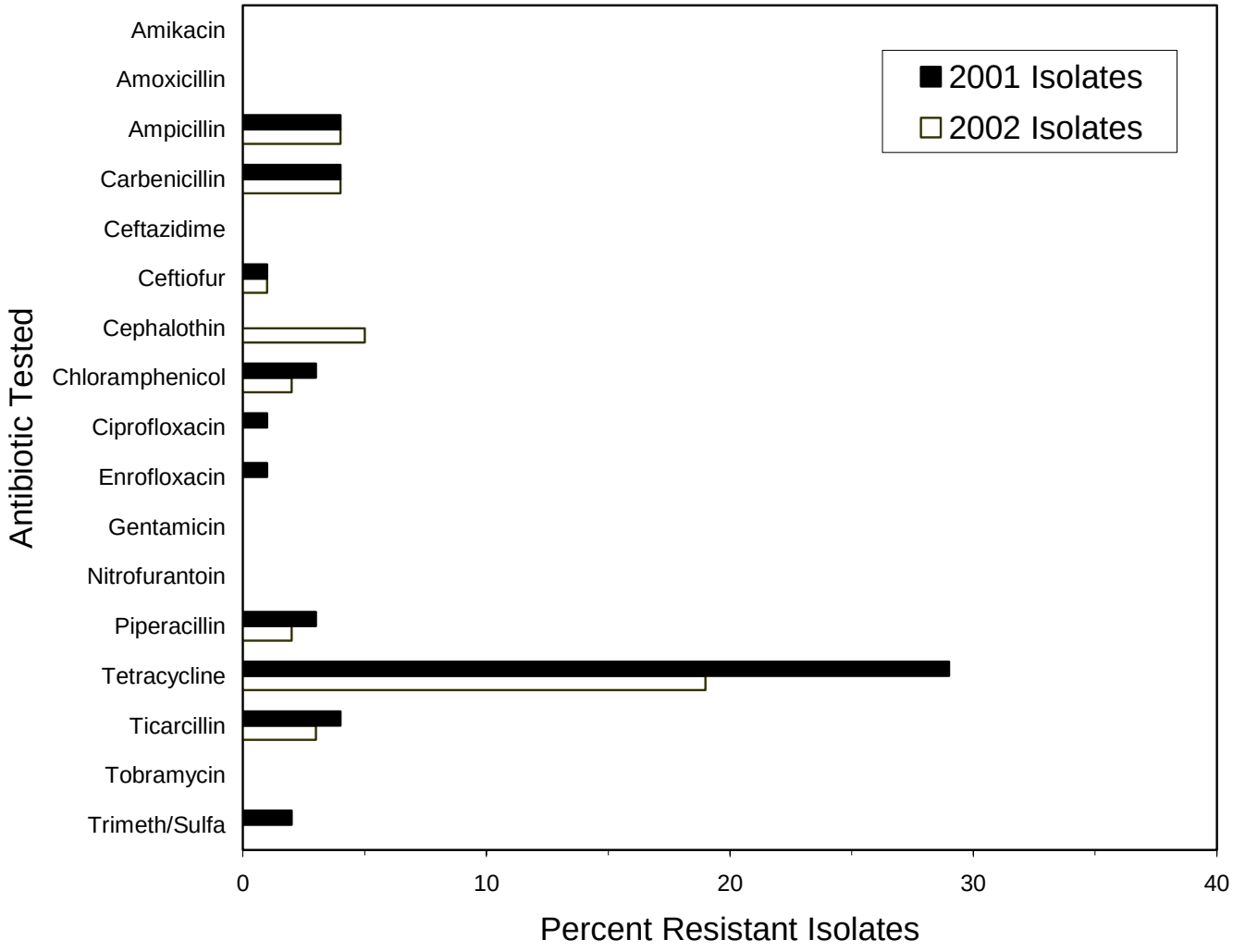


Fig. 6. Antibiotic resistance profiles of gram positive bacterial isolates[a] from 2001 and 2002 sampling events (Oklahoma CAFO study - May, 2001 to October, 2002).