Several studies have demonstrated the increased prevalence of resistance to critical antimicrobial drugs among bacterial isolates from canine veterinary patients. Of particular importance are bacteria that produce extended spectrum beta lactamases (ESBL) and carbapenemases, which inhibit extended spectrum cephalosporins and carbapenems, respectively. These drugs are among the most important drugs for the treatment of Gram-negative bacterial infections in human and veterinary medicine. Several studies have shown that eating a raw food diet is a risk factor for canine colonization with ESBL bacteria. Animals colonized with ESBL-producing Enterobacterales and carbapenemase-producing Enterobacterales (CPE) can develop opportunistic infections such as urinary tract infections, cholangiohepatitis, aspiration pneumonia, wound infections, and sepsis caused by these organisms. The clinical outcomes of ESBL-producing bacterial and CPE infection, relative to other pathogens, have not been well described in veterinary patients.

In people, the risk of death associated with ESBL bacterial infection is 2.5 times higher than that of other causes of bacteremia, and 90-day mortality rates are twice as high (36% vs 15%) in...
people colonized with CPE.¹⁵ Though traditionally viewed as agents of hospital-acquired infections, rapidly increasing rates of community (nonhospital-associated)-onset ESBL-producing bacterial and CPE infections have been observed in human populations.²⁰ One study²⁰ of ESBL-producing bacteria of the Enterobacteriales order suggests that the majority of human community-onset infections are due to within household transfer and that food products may account for up to 18% of cases.

Transmission of enteric bacteria has been documented among animals and their owners, including CPE- and ESBL-producing organisms.²¹-²³ ESBL-producing bacteria or CPE can be transmitted from colonized animals that have eaten raw foods to people via the fecal-oral route or directly from the food products to people.²¹ Direct infection from food has occurred in several high-profile human outbreaks of Salmonella linked to unsafe handling of contaminated dry pet foods.²⁴-²⁵ Understanding the potential “silent threat” of antimicrobial resistant bacteria in pet food products is critical in assisting pet owners when they make informed decisions on what to feed their pets.

To establish surveillance and intervention strategies, it is important to understand the diversity of bacterial strains and resistance genes to which animals and people may be exposed via pet food. The aims of this study were to assess the potential contamination of commercial raw pet foods with bacteria that produce extended spectrum beta-lactamase and carbapenemase enzymes and to compare the likelihood of contamination by protein source and preservation method. Additionally, whole-genome sequencing (WGS) was employed to understand the genetic diversity and lineage of isolates.

**Methods**

**Product acquisition**

Products were purchased at either online retailers or brick-and-mortar pet stores within the Philadelphia metropolitan region between February 2021 and June 2021. For online orders, upon receipt at the laboratory, frozen specimens were checked for signs of thaw overnight at 4 °C, which is consistent with feeding directions (if provided) by manufacturers. Thawed products were mixed by sterile spatula and a 25-g sample was weighed into a sterile blender cup. Alternatively, freeze-dried products were shaken in their container prior to removing 25 g aseptically into the blender cup. Then, 225 mL of sterile brain-heart infusion broth was added to the cup and the mixture was blended briefly at 21,500 X g. The homogenate was then incubated at room temperature for 10 minutes with periodic shaking and then allowed to settle by gravity for 10 mins. Broth was decanted into a 2-L specimen bag and 225 mL of double strength tryptone-phosphate broth (2X TP)²⁶ and incubated for 20 hours at 44.0 ± 0.2 °C. Subsequently, a 5-mL aliquot of overnight culture was added to 5 mL of double strength MacConkey broth (2X MAC) supplemented with 5 µg/mL of cefotaxime and was incubated overnight at 35 °C for selective enrichment. Then, 100 µL of the selective enrichment broth was plated to 2 selective chromogenic agars (ChromID Carba Agar, Biomerieux; Spectra ESBL Agar, Remel) and incubated for 16 to 20 hours. Plates were visually examined for colony growth of colors corresponding with presumptive bacteria of the order Enterobacteriales (red/pink or purple/blue colonies for ChromID Carba agar and pink or blue/turquoise on Spectra ESBL agar). A total of 3 representative colonies for each colony morphology and color combination isolated were subcultured to MacConkey agar and frozen at −80°C for future analyses. Oxidase negative nontarget organisms were also included for identification. The first selected colony for each combination was speciated using an automated biochemical card (Vitek 2 GN card; Biomerieux).²⁸ Isolates that were identified as a member of the order Enterobacteriales that grew on the ESBL agar were then confirmed to produce an ESBL by E-test.²⁹ Briefly, presumptive ESBL positive isolates were suspended in sterile saline to a 0.5 McFarland standard and then lawned by sterile swab to a Mueller-Hinton agar plate. Cefotaxime + cefotaxime/clavulanic acid and ceftazidime + ceftazidime/clavulanic acid gradient strips (ESBL E-test strips; Biomerieux) were applied to the inoculated plates and incubated overnight at 35 °C. ESBL results were interpreted as positive, negative, or nondeterminable based on manufacturer’s instructions.²⁹

**Whole-genome sequencing and analysis**

All bacterial isolates identified within the order Enterobacteriales with positive or nondeterminable results for ESBL activity were sequenced. DNA extraction and whole-genome sequencing was performed at a fee-for-service reference laboratory (MiGS Center). Briefly, DNA was extracted from bacteria using a commercial kit (DNeasy Blood and Tissue Kit; Qiagen) with lysozyme pretreatment per manufacturer’s instructions. Libraries were prepared...
(xGen DNA Library Prep kit; Illumina) and paired-end sequencing was performed using a next-generation sequencing platform (NextSeq2000; Illumina) in a 2x151bp format. Raw sequence files were uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and submitted to the pathogen detection pipeline (Bioproject Accession: PRJNA733494) for assembly and antimicrobial resistance gene analysis. Additionally, the pipeline performed single nucleotide variant (SNV) analysis by maximum compatibility algorithm and identifies closely related isolates (< 50 SNVs) within the database. Subsequently assemblies were then downloaded and then typed by multilocus sequence typing (MLST). At the time of publication, genome assemblies were not available for Klebsiella pneumoniae from the pathogen detection pipeline, so raw data files were used to assemble contigs (spADES version 3.12; Center for Algorithmic Biotechnology) and then typed by MLST.

**Statistical analysis**

Overall positivity rates for CPE and ESBL-producing Enterobacterales bacteria were calculated based on identification of a known gene encoding an ESBL enzyme by WGS. Univariable logistic regression was performed to assess the risk of preservation method, supplier, each individual protein source, and total number of protein sources on contamination of raw food products with ESBL-producing Enterobacterales bacteria using a software program (Stata, Version 17; StataCorp). Variables found to be significantly associated (P < .15) with the outcome on univariable analysis were added to a multivariable model in a stepwise manner and retained if they remained statistically significantly associated with the outcome (P < .05 on Wald test).

**Results**

**Product acquisition**

A total of 200 products were purchased from 61 brands/companies; 102 of the products were frozen and 98 were freeze dried. A total of 20 suppliers were used to source products. Twenty-six products contained multiple animal protein sources (26/200, 13.0%) and 174 were single source (174/200, 87.0%). Ninety-one products (91/200, 45.5%) contained poultry (chicken, turkey, quail, or duck), 59 products (59/200, 29.5%) contained beef (including veal or tripe), 20 products (20/200, 10.0%) contained fish (salmon or cod), 19 products (19/200, 9.0%) contained lamb, 15 products (15/200, 7.5%) contained pork, 8 products (8/200, 4.0%) contained venison, 7 products (7/200, 3.5%) contained rabbit, 3 products (3/200, 1.5%) contained goat, and 1 product (1/200, 0.5%) contained alpaca.

**Bacterial culture, identification, and ESBL testing**

ESBL-producing bacteria of the order Enterobacterales were isolated from 20/200 products (10.0%; 95% CI, 7.3% to 16.5%), all of which were frozen. Five of the positive products (5/20, 25.0%) yielded two distinct isolates harboring genes encoding ESBL enzymes and the remainder had a single unique organism. Notably, 75.0% (15/20) of these products came from only 4 manufacturers. Of the positive products, 6 were poultry protein sources (6/20, 30.0% of positive products; 6/72, 7.6% of poultry), which yielded 6 E. coli unique isolates and 1 K. pneumoniae isolate. Five positive products were beef (5/20, 25.0% of positive products; 5/46, 10.9% of beef), which contained 4 E. coli, 2 K.pneumoniae, and 1 Citrobacter braakii isolates. Another 5 positive specimens were pork (5/20, 25.0% of positive products; 5/13, 38.5% of pork) with 5 E. coli and 2 K. pneumoniae isolates. Finally, there were 2 positive “other” single source positive products (2/20, 10.0% of positive products; 2/43, 4.6% of other single source proteins). One positive product contained rabbit (1 E. coli isolate) and 1 contained goat (1 E. coli isolate). The remaining 2 products were blends of beef, poultry, and pork (n = 1) and beef and poultry (1), which represented 10.0% (2/20) of positive products, and 7.7% (2/26) of blend products. Both blend products yielded a single E. coli isolate each. Figure 1 demonstrates these results graphically. No CPE were detected in any raw food products (0/200, 0%).

**Figure 1**—A—Proportion of the 20 positive food products by protein source (inner ring) and of overall proportion of positive samples out of total products sorted by protein source (animal icons and bars). B—Number of positive products by protein source sorted by extended spectrum beta-lactamase (ESBL) genotype.

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The only variable that was significantly associated with the likelihood of having ESBL bacteria was having a pork protein source. Diets with a pork-derived protein source were 8.14 (P = .001; 95% CI, 2.53 to 26.2) times more likely to carry ESBL bacteria than diets with other protein sources. No other protein source, the supplier, or manner of preservation were significantly associated with the likelihood of carrying ESBL bacteria; thus, no multivariable regression modeling was conducted.

Whole-genome sequencing and analysis

A total of 48 distinct isolates were identified for WGS based on unique colony morphology, bacterial species, and E-test results. All identifications made by the Vitek GN card matched identifications made by the pathogen detection pipeline procedure. WGS analysis confirmed the presence of ESBL genes in a total of 25 distinct isolates from 20 products (Table 1). There were 19 *Escherichia coli*, 5 *Klebsiella pneumoniae*, and 1 *Citrobacter braakii*. Phylogenetic comparison of *E. coli* and *K. pneumoniae* can be found in Figure 2. A total of 24 (24/25, 96.0%) of these isolates harbored a bla* _CTX-M-1* gene and 3 *K. pneumoniae* isolates (3/25, 12.0%) harbored known ESBL bla* _SHV* genes. One isolate (RF152A1) harbored 2 _bla_ _CTX-M* genes and another isolate (RF70B1) had 2 _bla_ _SHV* ESBL genes. One group of 2 clonal isolates (RF67A1 and RF69A1) of *K. pneumoniae* had both _bla_ _CTX-M-15* and _bla_ _SHV-28* genes. The most common ESBL enzyme was _CTX-M-27* (n = 8), followed by _CTX-M-55* and _CTX-M-1* (5), and then _CTX-M-15* (including 2 clonal isolates) and _CTX-M-32* (2). One group of 3 clonal isolates (RF81A2, RF82A1, and RF83A1); all harbored a _bla_ _CTX-M-65* gene. Distribution of ESBL enzymes by protein source can be found in Figure 1.

SNV cluster analysis found 7 (7/25, 28.0%) isolates that clustered with previously uploaded NCBI isolates within 50 SNVs. An isolate of *E. coli* (RF80A1) from a frozen goat diet clustered with isolates from bovine feces and beef, which can be viewed using the pathogen detection pipeline isolates browser (https://www.ncbi.nlm.nih.gov/pathogens/) using the accession PDS000085295.3. Three identical isolates of *E. coli* (RF81A2, RF82A1, RF83A1) from frozen beef, pork, and a blend of protein sources from the same manufacturer clustered with both calf fecal and swine intestinal contents isolates (PDS000084106.2). An *E. coli* isolate from a frozen poultry product (RF146A1) was within 50 SNPs of a bovine large intestinal isolate (PDS000095208.1). An *E. coli* (RF152A1) from a frozen beef product clustered into a large tree of 30 isolates including bovine fecal isolates with a difference of 18 SNVs (PDS00012428.9).

The most common sequence types (ST) identified by MLST among the 19 *E. coli* isolates were ST10 (n = 3) and an additional 4 isolates with an ST belonging to Clonal Complex (CC) 10 (ST744 [3], ST1434). Two isolates were ST69 and 1 isolate was ST155, with 1 additional isolate belonging to CC155 (ST58).

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**Table 1**—Characteristics of ESBL-producing bacteria of the order Enterobacterales isolated from canine raw food products.

<table>
<thead>
<tr>
<th>Product ID</th>
<th>Isolate</th>
<th>NCBI accession</th>
<th>Protein</th>
<th>Species</th>
<th>ST</th>
<th>ESBL genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF67B34</td>
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<td>SAMN19414786</td>
<td>Beef</td>
<td><em>K. pneumoniae</em></td>
<td>307</td>
<td><em>bla</em> <em>CTX-M-15, bla</em> _SHV-28*</td>
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<td>SAMN19414787</td>
<td>Poultry</td>
<td><em>K. pneumoniae</em></td>
<td>307</td>
<td><em>bla</em> <em>CTX-M-15, bla</em> _SHV-28*</td>
</tr>
<tr>
<td>RF70B41</td>
<td>A1</td>
<td>SAMN19414788</td>
<td>Pork</td>
<td><em>K. pneumoniae</em></td>
<td>147</td>
<td><em>bla</em> <em>CTX-M-15, bla</em> _SHV-28*</td>
</tr>
<tr>
<td>RF80A35</td>
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<td>SAMN19414790</td>
<td>Goat</td>
<td><em>E. coli</em></td>
<td>69</td>
<td><em>bla</em> _CTX-M-55*</td>
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<td><em>K. pneumoniae</em></td>
<td>Novel</td>
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</tr>
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<td>SAMN19414793</td>
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<td><em>E. coli</em></td>
<td>744</td>
<td><em>bla</em> _CTX-M-65*</td>
</tr>
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<td>RF83A54</td>
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<td>SAMN19414794</td>
<td>Blend</td>
<td><em>E. coli</em></td>
<td>744</td>
<td><em>bla</em> _CTX-M-65*</td>
</tr>
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<td><em>bla</em> _CTX-M-27*</td>
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<td>SAMN19414798</td>
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<td><em>E. coli</em></td>
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<td><em>bla</em> _CTX-M-32*</td>
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<td>SAMN19414799</td>
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<td><em>E. coli</em></td>
<td>58</td>
<td><em>bla</em> _CTX-M-55*</td>
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<td><em>E. coli</em></td>
<td>540</td>
<td><em>bla</em> _CTX-M-27*</td>
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<td>SAMN19414807</td>
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<td><em>E. coli</em></td>
<td>10</td>
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<td>A1</td>
<td>SAMN19414814</td>
<td>Poultry</td>
<td><em>E. coli</em></td>
<td>1434</td>
<td><em>bla</em> _CTX-M-1*</td>
</tr>
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<td>RF147A57</td>
<td>A1</td>
<td>SAMN19414815</td>
<td>Poultry</td>
<td><em>E. coli</em></td>
<td>155</td>
<td><em>bla</em> _CTX-M-1*</td>
</tr>
<tr>
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<td>SAMN19414818</td>
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<td><em>E. coli</em></td>
<td>6775</td>
<td><em>bla</em> _CTX-M-1*</td>
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<td>A1</td>
<td>SAMN19414820</td>
<td>Beef</td>
<td><em>E. coli</em></td>
<td>3900</td>
<td><em>bla</em> <em>CTX-M-27, bla</em> _CTX-M-32*</td>
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<td>A1</td>
<td>SAMN19414821</td>
<td>Beef</td>
<td><em>E. coli</em></td>
<td>10</td>
<td><em>bla</em> _CTX-M-55*</td>
</tr>
<tr>
<td>B1</td>
<td>A1</td>
<td>SAMN19414822</td>
<td>Beef</td>
<td><em>C. braakii</em></td>
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<td><em>bla</em> _CTX-M-27*</td>
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<tr>
<td>B2</td>
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<td>Pork</td>
<td><em>E. coli</em></td>
<td>410</td>
<td><em>bla</em> _CTX-M-55*</td>
</tr>
</tbody>
</table>

*Subscript numbers in the “Product ID” column are coded for brand or manufacturer, and subscript letters are for supplier. RF83 was a blend of beef, poultry and pork, and RF101 was a blend of beef and poultry. ESBL = Extended spectrum beta-lactamase. ST = Sequence type.*
The remaining isolates were all unique (ST154, ST162, ST189, ST398, ST410, ST540, ST2449, ST6775), with none in the same clonal complex. Two K. pneumoniae isolates (RF67A1 and RF69A1) were separated by only 1 SNV and belonged to ST307. The rest of the K. pneumoniae isolates were unique STs (147, 412, and a novel ST with the allelic profile 2, 1, 2, 1, 9, 4, 14). Citrobacter braakii has no established MLST scheme.

Discussion

ESBL- and carbapenemase-producing bacteria of the order Enterobacterales bacteria were found. In this study, all ESBL isolates were from frozen food products, and products containing pork were more likely to contain ESBL isolates compared to products without pork. However, the lack of ESBL organisms among freeze-dried products does not rule out the possibility of contamination, as there are several examples of Salmonella recalls linked to freeze-dried products.34,35 ESBL organisms could still be present, and their absence in this study might reflect differences in processing and handling of freeze-dried products (e.g., longer shelf lives could lead to decreased viability of bacteria). ESBL surveys of pork-based raw products for dogs have not been widely performed, but in a recent survey of ground raw pork and beef products intended for human consumption in the United States, ESBL-producing bacteria were more prevalent among pork products.36 It is important to note that, within this study, ESBL organisms were isolated from products with a variety of protein sources including beef and poultry, which suggests that no products are without risks.

This study also characterized the ESBL-producing isolates by WGS. Broadly, the majority of the E. coli (n = 11) isolates belonged to several globally dominant lineages (CC10, ST69, and CC155), which are associated with ESBL carriage and multidrug resistance.37-38 They have all previously been described as agents of extraintestinal infections in companion animals and people and from the feces of food animals or retail meat.39-42 A study43 of raw meat diets in Switzerland also identified E. coli from these lineages that harbored ESBL genes. The presence of these important lineages in raw food products suggests that these may serve as a route of transmission to pets and owners.

Even though no CPE were isolated in this study, bacteria from several high-risk lineages associated with higher-than-normal potential to carry carbapenemase genes were isolated from raw food products. First, an E. coli isolate (RF192A1) from a frozen pork product typed as ST410. This sequence type is considered a high-risk lineage for harboring and acquiring carbapenemase genes, including blaOXA-181 and blaNDM-5.44 Additionally, 3 of the K. pneumoniae isolates were typed within 2 antimicrobial resistant high-risk clonal lineages (ST307 and ST147) that are also associated with several classes of carbapenemases and nosocomial outbreaks.45 Identification of these high-risk organisms suggests that continued surveillance of raw food for CPE is important.

Of the ESBL genes present among study isolates, the most common were those that produce CTX-M type enzymes. CTX-M enzymes have become the dominant ESBL worldwide, with blaCTX-M-15 variant increasing over time to become dominant in most regions.46 This study identified blaCTX-M-15 genes in four isolates of K. pneumoniae from a variety of protein sources. The most commonly identified gene in this study was blaCTX-M-27, found in E. coli and C. braakii from products with a variety of protein sources. Figure 2—Phylogenetic analysis of the E. coli (A) and K. pneumoniae (B) isolates based on multiple sequence alignment of core genes identified by roary (Version 3.11.2). Trees were rooted with American Type Culture Collection (ATCC) strains E. coli 25922 and K. pneumoniae 13883, respectively.

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sources. Recent studies suggest that CTX-M-27 enzymes are rapidly increasing in prevalence across many regions, and reports in food animals have occurred in Asia, Europe, North and South America. It has been suggested that the use of ceftazidime in human hospitals may be responsible for the rapid rise of this variant, given that isolates with CTX-M-27 have a higher minimum inhibitory concentration for the drug compared to the closely related CTX-M-14. Studies investigating the role of certain veterinary drugs in the emergence of particular ESBL enzymes are lacking. Ceftazidime use is prohibited in food production animals, but the gene encoding CTX-M-27 has been reported in E. coli isolated from swine, beef cattle, and poultry. In this study, food containing all of these major proteins also had E. coli with a blaCTX-M-27 gene present. It has been suggested that a more diverse set of plasmids replicons are associated with blaCTX-M-27 food animal isolates than isolates from humans. A complex population structure likely implies that more work is needed to elucidate the role of food, food animals, and companion animals in the emergence of the CTX-M-27 genotype within the Enterobacteriales.

The second most common ESBL genes in this study were blaCTX-M-55 and blaCTX-M-1, all of which were found in E. coli isolates. Bacteria harboring both variants have been reported in food animals and people, with studies that suggest potential transmission via the food chain. Identification of isolates producing these variants in raw pet food products suggests a potential role in transmission to pets. This could also represent a risk for transmission to people if pets were to become colonized or if improper handling of the product occurs. Previous studies have shown that owners and pets can share fecal microbiota, including ESBL bacteria, and that pet owners may be at higher risk of ESBL carriage than nonpet owners. A review of UK raw pet food manufacturer and supplier websites showed that a significant number (61%) lacked resources to educate owners in proper handling or risks of the products. The same study also showed that even owners who were aware of the risks of foodborne illness associated with raw diets often lacked knowledge on safe and proper handling. In another study of pet owner feeding habits, approximately half of owners who fed raw food diets reported washing their hands after feeding their pets, which was slightly less than the 58% of owners that reported this habit regardless of diet type.

SNV analysis revealed close relationship (< 50 SNVs) of several raw food isolates to previously characterized fecal isolates from food-producing animals. In multiple cases (RF80A1, RF146A1), the raw protein source and production-animal did not match. This may suggest that clones move across production systems, or alternatively, that cross-contamination with fecal bacteria may occur at food manufacturers processing several species of animals. This is also supported by two instances in this study of multiple products of different protein source from the same manufacturer containing genetically identical bacteria (RF67A1/RF69A1 and RF81A2/RF82A1/RF83A1), as well as the fact that almost three-quarters of the ESBL isolates came from only 4 manufacturing companies. Additionally, a study of raw companion animal diets detected undeclared DNA of additional protein sources in several commercial raw products. This may suggest that the manufacturer may play a more significant role in likelihood of contamination than protein source alone. Pet food production can be quite complex. Small companies often depend on the same manufacturers, but unfortunately, because of limited labeling, it can be difficult to identify the manufacturer. In conventional pet foods, multiple brands or products can be contaminated with foodborne pathogens during common processes (e.g., coating of food items with fats after extrusion) because of shared equipment and ingredients. Cross-contamination of allergens has also been posited in companion animal food production made by comanufacturers.

The lack of CPE in this study may, in part, reflect the fact that carbapenems are not licensed or are outright banned for use in food-production worldwide. Approximately 1% of sales of third-generation cephalosporins are used in food animals in the United States, and their use is limited to labelled therapeutic purposes in major species. Use of third-generation cephalosporins may drive selection of ESBL-producing bacteria in food animals and their environment. CPE- and ESBL-producing bacteria often harbor resistance to many additional classes of drugs more commonly used in food production (e.g., tetracyclines, aminoglycosides); therefore, use of these classes may contribute to reports of isolation of CPE from retail meat and from the feces of food-producing animals. Lack of identification of CPE in this study does not exclude the possibility of contamination of food products. As CPE become more prevalent globally, it will be important to continue surveillance for potential sources of spread. One limitation of this study was that the media used for isolation of carbapenemase-producing bacteria are not specifically designed for OXA-48-producing organisms; therefore, this study may not have adequately assessed products for bacteria with this class of enzyme.

This study focused on raw dog food products given the previously published links between consumption and colonization; however, this study was not designed to evaluate other commercial pet food product or treats types. Moreover, this study did not evaluate the likelihood of transmission of these bacteria from the food products to pets or owners, and it is currently unclear how often exposure to a positive product would lead to animal or owner colonization. The relative abundance of the resistant isolates to other flora within the product was also not evaluated, and it cannot be determined what role this factor may play in overall risk. Finally, we only sought to identify ESBL bacteria in this study, even though other mechanisms of resistance to third-generation cephalosporins such as hyperexpression of ampC beta lactamases (especially if plasmid-mediated) may also be of clinical concern.
Future studies could include other important mechanisms of antimicrobial resistance.

Based on the results of this study, we recommend that owners who choose to feed raw food to their pets should take extra care when handling frozen raw food products and raw food products containing pork. Pet owners, regardless of what type of food they feed their pets, should handle all products with adequate hygiene to prevent their own exposure to pathogens.

Acknowledgments

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