

***Escherichia coli* pathotype contamination in raw canine diets**

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OBJECTIVE

To investigate the prevalence of *Escherichia coli* contamination and *E coli* virulence gene signatures consistent with known *E coli* pathotypes in commercially available conventional diets and raw-meat-based diets (RMBDs).

SAMPLE

40 diets in total (19 conventionally cooked kibble or canned diets and 21 RMBDs) obtained from retail stores or online distributors.

PROCEDURES

Each diet was cultured for *E coli* contamination in 3 separate container locations using standard microbiological techniques. Further characterization of *E coli* isolates was performed by polymerase chain reaction-based pathotype and virulence gene analysis.

RESULTS

Conventional diets were negative in all culture based testing. In RMBDs, bacterial contamination was similar to previous reports in the veterinary literature, with 66% (14/21) of the RMBDs having positive cultures for *E coli*. Among the 191 confirmed *E coli* isolates from these diets, 31.9% (61/191) were positive for virulence genes. Categorized by pathotype, isolates presumptively belonging to the neonatal meningitis *E coli* pathotype (15.7% [30/191]) were the most common, followed by enterohemorrhagic *E coli* (10.5% [20/191]), enteropathogenic *E coli* (5.8% [11/191]), uropathogenic *E coli* (2.1% [4/191]), and diffusely adherent *E coli* (1.6% [3/191]).

CLINICAL RELEVANCE

The results of this study reaffirmed the bacteriologic risks previously associated with RMBDs. Furthermore, potential zoonotic concerns associated with identified pathotypes in these diets may have significant consequences for owners in the animals' home environment. Potential risk associated with bacterial contamination should be addressed in animals fed RMBDs.

Raw-meat-based diets (RMBDs) have become an increasingly popular dietary choice among pet owners.¹⁻⁴ The benefits of RMBDs have been proposed to be related to both physical and behavioral health.^{2,5} However, scientifically rigorous evaluation of these potential advantages have not been completed and lack supportive evidence.⁶⁻⁸ Of greater concern is that many of these diets do not provide complete and balanced nutrition, and they pose a risk of pathogenic bacterial and other infectious organism transmission to animals and people coming into contact with the diets and feces of animals consuming RMBDs.⁷⁻¹⁸ Specifically, *Escherichia coli* contamination risk is greater in RMBDs than conventional diets, and RMBDs exceed quantities con-

sidered acceptable for human consumption.^{8,10,11,19} Specific *E coli* strains can be pathogenic and therefore may carry a risk to the animal eating the diet.¹⁰ Furthermore, as enteric bacteria, *E coli* strains have also been documented to transfer between animals and owners, and pose a potential zoonotic risk.²⁰

Escherichia coli pathogenicity is strain specific and determined in part by virulence factors inherent to each specific strain. Virulence factors commonly implicated in different *E coli* pathotypes include adhesions, hemolysins, toxins, invasins, cytotoxic necrotic factors, capsules, and effacement factors.^{21,22} Based on virulence factors and clinical presentation, *E coli* strains can be categorized as extraintestinal pathogenic *E coli* (ExPEC) or intestinal

pathogenic *E coli* (InPEC).^{23,24} Subclassifications of InPEC include enteroinvasive *E coli*, enterohemorrhagic *E coli* (EHEC), enterotoxigenic *E coli*, enteropathogenic *E coli* (EPEC), enteroaggregative *E coli*, and diffusely adherent *E coli* (DAEC).^{23,25,26} Likewise, ExPEC can be subdivided into uropathogenic *E coli* (UPEC), neonatal meningitis *E coli* (NMEC), and avian pathogenic *E coli*.^{23,25,26}

Our aim was to investigate the presence of *E coli* contamination in commercially available extruded kibble diets and RMBDs. A secondary aim was to assess for the presence of virulence gene signatures consistent with different pathotypes from all *E coli* isolated from the diets. Our hypothesis was that RMBDs would have an increased frequency of *E coli* contamination, and that those *E coli* would carry virulence genes consistent with *E coli* pathotypes that pose risk to both animal and human health.

Materials and Methods

Diet selection

Commercially available conventional and RMBD diets, containing either chicken or beef as a primary protein source, were eligible for inclusion in this descriptive study. Conventional diets were defined as containing no uncooked animal products whereas RMBDs were classified in accordance with the definition by Freeman and Michel⁹ based on the presence of fresh, dehydrated, or frozen uncooked food animal products included in the diet. All diets used in our study were obtained from local retail stores within a 10-mile radius of laboratory or online distributors. The specific diets chosen represented those that were sold consistently in the majority of national retail and online stores based on our experience. This was done in an attempt to provide an analysis of diets presumed to be fed to pets more frequently. All RMBDs were kept at room temperature, refrigerated, or frozen at -20°C from the time of purchase until analysis, according to each individual manufacturer recommendation. For diets shipped to the laboratory, temperature verification was performed at reception of the package to ensure manufacturer-recommended delivery temperatures were maintained. All diets were analyzed within 4 weeks of purchase.

Sample processing

Using standard microbiological techniques, each diet was cultured for the presence of *E coli* in 3 separate sampling locations.^{27,28} For diets in kibble, block, tube, or tray form, the 3 separate samples were taken at 3 different depths (top, middle, bottom) or distances along the container (left, center, or right). For diets in patty or nugget form, the 3 samples were taken from 3 separate pieces at different depths within the container. If the diet was divided into separate packages by the manufacturer, the 1st and 2nd samples were taken from different depths in 1 container and the 3rd sample was taken from a separate container. Sampling and processing of all products was done aseptically with sterile personal

protective equipment, sampling instruments, and collection supplies.

At each sampling site, 1 g of food was homogenized manually and inoculated aseptically to MacConkey broth, and incubated overnight with shaking at 37°C and 200 rpm to select for the growth of Gram-negative bacilli (eg, *Pseudomonas* spp and Enterobacteriaceae). Ten microliters of each broth was streaked for isolation onto MacConkey agar (Difco MacConkey Agar; Becton-Dickinson) and incubated overnight at 37°C in an air incubator. The MacConkey agar allowed differentiation of lactose-fermenting species, including most *E coli* species. Ten single-colony isolates phenotypically characteristic of *E coli* (pink-purple colonies with a pink precipitated halo, indicating lactose fermentation) from the mixed-isolate plates were then streak-plated onto fresh MacConkey agar plates and incubated overnight at 37°C to form pure-isolate plates. If the pure-isolate plate phenotype was consistent with *E coli* as described earlier, the colonies were cryobanked in 75% glycerol (Fisher Chemical) plus 1X PBSS (ie, diluted PBSS 10X; Fisher BioReagents) at -80°C for further testing.

Biochemical confirmation of isolate identity as *E coli*

For biochemical testing, the individual isolates were used to inoculate tryptophan broth (Fisher BioReagents) for indole-positive/-negative status and Luria broth agar (Fisher BioReagents) plates in preparation for oxidase activity testing, and were incubated overnight. Biochemical assays were performed according to standard microbiological techniques: isolates were labeled as consistent with *E coli* when showing both indole positivity, indicating expression of tryptophanase, which catalyzes the production of indole from Kovac's reagent, and oxidase negativity, indicating the isolate's lack of cytochrome c oxidase, which catalyzes the production of indophenols.^{28,29} Each isolate was also screened for the presence of the *uidA* marker gene for molecular confirmation of the isolates as *E coli*, as described later and reported previously.³⁾ In total, each isolate was biochemically identified as *E coli* based on the combination of phenotypic appearance, indole positivity, oxidase negativity, and *uidA* gene presence.

DNA isolation

Genomic DNA was extracted from whole *E coli* cells grown in Luria broth (Fisher BioReagents) cultures from each individual isolate using a commercially available kit (Invitrogen Easy-DNA gDNA Purification Kit; ThermoFisher Scientific) according to manufacturer instructions. Purified genomic DNA was then stored frozen at -20°C until polymerase chain reaction (PCR) analysis.

Pathotype identification based on virulence genes

Presumptive pathotype identification was performed based on previously published methods that allow for characterization and grouping of isolates based on genetic signatures of virulence factors.³⁰ In brief, single PCRs were performed with a master

mix composed of 1 μL of 20 μM forward primer, 1 μL of 20 μM reverse primer, 1 μL of 10 mM dNTPs (100 dNTP Set; Invitrogen), 5 μL of 10X DreamTaq Buffer (ThermoFisher Scientific), 0.5 μL of DreamTaq DNA Polymerase (ThermoFisher Scientific), and 40.5 μL of ultrapure water. One microliter of each isolate's extracted genomic DNA was added to 49 μL of PCR primer mix, and run through PCR cycling (MiniAmp Plus Thermocycler; Applied Biosystems, ThermoFisher Scientific). The associated pathotype, gene of interest, gene product, primer sequence, amplified fragment size, and PCR run cycle parameters are detailed in **Supplementary Appendix S1**.

Ten microliters of the PCR product was stained with 2 μL of loading dye (6X Loading Dye, ThermoFisher Scientific) and resolved on a 1% agarose (UltraPure Agarose; Invitrogen) Tris-acetate-EDTA (50X TAE Electrophoresis Buffer; ThermoFisher Scientific) gel with ethidium bromide (Invitrogen). Gel electrophoresis was performed at 200 W for 40 minutes, and finished gels were reviewed on a UV visualization system (UVP PhotoDoc-It Imaging System; AnalytikJena). Each gel was evaluated for DNA bands indicative of the presence or absence of target genes.

PCR amplicon sequence confirmation

The PCR amplification products were purified prior to confirmation sequencing using a commercially available kit (QIAquick Purification Kit; Qiagen Sciences). The PCR products were sequenced through a commercial laboratory (EuroFins Genomics), and sequencing results were compared to known *E coli* genome sequences through the National Center for Biotechnology Information BLAST database and RefSeq database for confirmation that the correct gene was sequenced.³¹

Statistical analysis

All data were evaluated using GraphPad Prism version 7.0.0 for Mac. Descriptive statistics are reported for all data collected. Normality was evaluated via the Shapiro-Wilk test and visual inspection. Frequency statistics on categorical data were assessed using Fisher's exact test.

Results

A total of 40 diets (19 conventionally cooked kibble or canned diets and 21 RMBDs) were obtained from retail stores or online through distributors (**Supplementary Appendixes S2 and S3**). The primary protein source in the conventional diets included chicken ($n = 11$) and beef ($n = 8$). The primary protein source in the RMBDs included chicken ($n = 9$), beef ($n = 11$), pork ($n = 1$).

Fisher's exact test revealed that the percentage of culture-positive conventional diets differed significantly from the RMBDs ($P < 0.001$). No conventional diets (0/19) had a positive culture for *E coli* (Supplementary Appendix S2). In contrast, 66% (14/21) of the RMBDs had positive cultures for *E coli*. Among diets that cultured positive, all 3 sampling sites within the product yielded positive cultures in 57%

(8/14) of diets. The remaining diets were positive in the 1st location only in 14.3% (2/14), 2nd location only in 7.1% (1/14), or both the 2nd and 3rd sampling sites simultaneously in 21.4% (3/14).

RMBDs with positive cultures for *E coli* are listed in Supplementary Appendix S). Classified by primary protein source, 8 of the positive-culture diets were beef based and 6 of the culture-positive diets were chicken based.

The tested RMBDs cultured 204 presumptive *E coli* colonies. Of those 204 suspected colonies, 191 were confirmed as *E coli* based on biochemical testing and *uidA* gene presence. Among the 191 confirmed isolates, 31.9% (61/191) were positive for at least 1 virulence gene; 28.3% (54/191) of these isolates were positive for 1 virulence gene only and 3.7% (7/191) were positive for 2 virulence genes. These virulence-gene positives represented isolates from 47.6% (10/21) of the RMBDs. Of these 10 diets culturing virulence gene-positive *E coli*, 7 had virulence gene-positive *E coli* from multiple intradiet sampling sites and 3 had virulence gene-positive *E coli* from only a single sample site. Of those 7 diets with multiple intradiet sampling sites culturing virulence gene-positive *E coli*, the virulence gene signature between samples within the diet was different for 4 diets.

Overall, 15.7% (30/19) of isolates were positive for *ibeA*, 10.5% (20/191) of isolates were positive for *stx2*, 5.8% (11/191) of isolates were positive for *eaeEae*, 2.1% (4/191) of isolates were positive for *papC*, and 1.6% (3/191) of isolates were positive for *daaE*. Categorized by pathotype, these virulence genes made isolates presumptively belonging to the NMEC pathotype (15.7% [30/191]) the most commonly detected, followed by EHEC (10.5% [20/191]), EPEC (5.8% [11/191]), UPEC (2.1% [4/191]), and—the lowest—was DAEC (1.6% [3/191]) (Supplementary Appendix S3 and **Figure 1**).

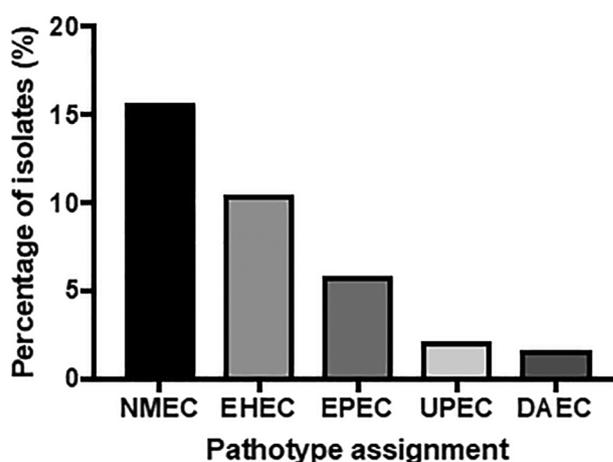


Figure 1—The prevalence of individual pathotypes among the total isolates cultured from all diets. The data displayed include all isolates with positive results cultured from the 14 raw-meat-based diets. DAEC = diffusely adherent *E coli*; EHEC = enterohemorrhagic *E coli*; EPEC = enteropathogenic *E coli*; NMEC = neonatal meningitis *E coli* pathotype; UPEC = uropathogenic *E coli*.

Discussion

This study investigated the frequency of *E coli* contamination among conventionally cooked diets and RMBDs. The *E coli* strains cultured from the diets were evaluated further to determine whether they were consistent with known pathotypes of concern to humans and animals. *Escherichia coli* with known virulence genes were detected in nearly one third of *E coli* isolated from the RMBDs. No *E coli* was isolated from either kibble or canned conventional diets. As such, the bacteriologic risks associated with *E coli* contamination were only associated with RMBDs in our study. The virulence genes detected in the *E coli* isolates suggest the presence of potentially pathogenic *E coli* strains in these diets, including both ExPEC and InPEC types.

The primary InPEC pathotypes identified included EHEC and EPEC, with a smaller proportion of DAEC. Both EHEC and EPEC pathotypes have been associated with gastrointestinal disease in humans following exposure to contaminated environmental sources.^{21,32} Key virulence factors in these pathotypes include intimin (*eae* gene), Shiga toxin (*stx2*), and plasma-encoded bundle forming pilus (*bfp* gene). In EHEC isolates, it has been shown previously that the occurrence of multiple virulence genes associated with EHEC is associated with more severe disease symptomatology, including worse gastrointestinal symptoms and hemolytic uremic syndrome.^{33,34} In this cohort of *E coli* isolates, there were none that contained multiple virulence genes from the EHEC pathotype. Regardless, this single virulence-gene pathotype has been associated with disease outbreaks and mortality in human populations, and thus still poses a potential risk to both animals and people in the local environment.^{35,36} Similarly, traditional EPEC strains contain the locus of enterocyte effacement pathogenicity island containing both the *eae* and *bfp* genes. In our study, the *bfp* gene was not identified in these isolates. Although consistent with the EPEC pathotype, these may or may not be pathogenic, and our study did not test for isolate pathogenicity directly. EPEC without the *bfp* gene has been noted to play equivocal disease pathophysiology roles in previous studies.^{23,25,37} However, specific strains of EPEC containing only the *eae* gene have been shown to be a cause of gastrointestinal disease in people.³⁸

The 2 ExPEC pathotypes that were identified included NMEC and UPEC.³⁹ Food animals and pets have both been considered as potential reservoirs for ExPEC as well as sources of transmission between hosts, presumably through fecal oral contamination.^{40,41} Furthermore, meat for consumption has been identified frequently as a source of the ExPEC contaminants.⁴² Specifically, ExPEC strains have been cited to be more likely contaminants in poultry meat.⁴³⁻⁴⁵ However, the number of positive isolates and diets in our study precluded evaluation of this potential association statistically. Further studies investigating the type of RMBD ingredients and their association with ExPEC contamination is an area for future research.

Previous work has shown that the microbiome and metabolome of dogs fed an RMBD differs from those fed a conventional diet.⁴⁶⁻⁴⁹ It is unknown to what degree dietary bacteriologic invasion may be driving these changes. The exact cause of the reported microbiome differences is unknown but may be multifactorial, including macronutrient content, dietary preparation, and bacteriologic content. Interestingly, previous work has shown that dogs eating RMBDs had a greater fecal abundance of Enterobacteriaceae, and specifically *E coli*.⁴⁶ This research has shown that dietary protein concentration may affect *E coli* abundance in dogs and rat models.^{46,50,51} In addition, it has long been established that food-borne contamination with *E coli* can affect the microbial ecosystem in the species ingesting the diet.⁵² Further work is necessary to investigate the individual effects of diet contamination and diet composition, such as protein concentration, driving microbiome changes in dogs fed RMBDs. Complete nutritional profiles were not provided by many of the RMBD companies, which precluded an exact comparison of protein concentration in these diets and further analysis.

Last, the presence of a specific virulence genes associated with pathotypes does not imply definitively that the microbe is pathogenic.²¹ Additional in vitro and in vivo testing beyond the scope of this study is needed to assess further the true pathogenicity of the *E coli* strains isolated from RMBDs. The presence of an individual virulence gene or genes still needs to be matched appropriately to host susceptibility to cause disease.³⁵ However, pathotype identification has been shown to be useful in identifying strains with pathogenic potential.²¹ The documentation of *E coli* contaminants with genes present for vital virulence factors in our study has important animal and human health implications. Expansion of this work looking at engraftment of dietary contaminant *E coli* on the intestinal ecosystem, host health, and public health are important areas for future research.

This study had limitations that are important to consider when interpreting results. It did not assess the magnitude of *E coli* contamination in each diet and whether the degree of *E coli* would clinically pose a risk to the animal ingesting the diet or the human exposed. Furthermore, the method of diet selection was semiobjective and based on the frequency of availability of the diets listed at major retailers. This method encompassed readily available brands but does not predict directly the frequency of use of the specific diets selected. This study done with different diets may yield different results. Similarly, each diet was sampled from a single order from a single batch from the manufacturer. If serial analyses of these diets were to be performed, the results could change because contamination may or may not be consistent and predictable.

In conclusion, these results reaffirmed previous reports regarding the bacteriologic risks associated with RMBDs. Based on the potential zoonotic concerns, these bacteriologic risks might have severe

and potentially life-threatening consequences. These risks and contamination issues should be addressed in animals fed RMBDs, and owners interested in feeding their pets raw diets should be educated on public health concerns, especially in the absence of a documented RMBD health benefit. Based on the finding of specific pathotypes of concern among these diets, future research on raw diets should include tracking specific *E coli* pathotypes identified in RMBDs and their implications on the host animal's microbiome, health, and environmental impact.

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Supplementary Materials

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