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DETECTION OF PRESUMPTIVE PATHOGENS IN GROUND BEEF FROM SUPERMARKET AND FARMERS' MARKET SOURCES

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ABSTRACT

This study investigates whether specific pathogens are more prevalent in retail meat sold by supermarkets compared to locally sourced markets. Ground beef samples were obtained from conventional 'big box' supermarkets and from local, farmers' markets and examined for the presence of two pathogens, *Escherichia coli* O157,H7 and *Salmonella*. For the detection of *E. coli* O157,H7, homogenized meat samples were enriched overnight in modified EC medium with novobiocin. The enriched cultures were selected onto MacConkey agar with sorbitol, cefixime and tellurite. Presumptive positive colonies were subcultured onto tryptic soy agar with yeast extract and further tested for positive indole and motility, and negative oxidase reactions. For *Salmonella* detection, homogenized meat samples were incubated first in universal pre-enrichment broth, then enriched overnight in Rappaport-Vassiliadis broth, and further plated onto *Salmonella* selective medium. Presumptive *Salmonella* colonies were further incubated on triple sugar iron agar and lysine iron agar to confirm glucose fermentation, sulfide production, and lysine decarboxylase. Oxidase assays were conducted on all presumptive strains. Presumptive colonies of both *E. coli* and *Salmonella* were subjected to rapid identification assays and serological tests to confirm identity. Isolates were then tested for antibiotic sensitivity using the Kirby-Bauer assay. The presence of *E. coli* O157 was observed in one sample of meat sourced from a supermarket, and *Salmonella* was isolated from ground beef purchased from a different retail supermarket. Neither pathogen was detected from ground beef sourced from farmers' markets. Our preliminary results demonstrate a potential difference in the prevalence of both *E. coli* O157 and *Salmonella* species based upon food source.

Keywords: foodborne pathogens, ground beef, *Salmonella*, *E. coli* O157

INTRODUCTION

Escherichia coli naturally resides in the intestinal tract of animals, including humans. Most *E. coli* is harmless. However, there are pathogenic strains such as *E. coli* O157,H7 that can cause abdominal cramps, bloody diarrhea, vomiting, hemolytic uremic syndrome and, in extreme cases, death (Spickler 2009; Blount 2015). *E. coli* O157,H7

produces a Shiga toxin that inhibits protein synthesis and damages the lining of the small intestine in humans (Lowe et al. 2010; Mayer et al. 2012). Cattle do not contain the Shiga toxin receptor, globotriaosylceramide, and therefore are asymptomatic in the presence of *E. coli* O157,H7 (Pruimboom-Brees et al. 2000; Kolenda et al. 2015). Ruminants are thus important reservoir hosts for *E. coli* O157,H7. Some cattle in a herd, termed super-shedders, can remain infected much longer than other cattle and can be responsible for shedding more than 95% of the microorganisms (Spickler 2009). *E.coli* O157,H7 is mainly transmitted to humans through fecal contamination of meat during butchering and packaging (Blount 2015).

Salmonella is another common foodborne pathogen that has been associated with multiple foodborne outbreaks in recent years (Bennett et al. 2015). According to one study, *Salmonella* is a leading cause of hospitalizations and deaths from food borne disease, causing an estimated 1.2 million infections annually in the United States alone (Jackson et al. 2013). Furthermore, it has been shown that major animal-derived food commodities provide the main reservoir for this microorganism, and are also a substantial vehicle for infection (Jackson et al. 2013; Jung et al. 2014).

Beginning in the 1940s, subsidization and the onset of chemical fertilizer paved the way for the incorporation of grains into cattle diets in the finishing stage, to shorten fattening time and increase fat marbling (Sewell 1993; Matos and Wagner 1998; Sumner 2008). Studies have shown that grain diets lower the pH of the cattle rumen from a nearly neutral pH (while eating predominantly grass and forages) to a more acidic pH of 3–4 (Callaway et al. 2009). *E.coli* O157,H7 that evolved to survive in this altered, more acidic environment is therefore more likely to survive in the more acidic stomach of humans (Diez-Gonzalez et al. 1998; Nocek et al. 2002). Furthermore, cattle finished on high grain diets have demonstrated a positive correlation with increased fecal shedding (Callaway et al. 2003) and *E. coli* O157,H7 contamination (de Boer et al. 2009).

Because *E. coli* O157,H7 is most often found in fecal matter, fecal shedding caused by grain diets becomes a public health concern. If cattle are secreting more fecal matter with more pathogenic microorganisms, the chances of this fecal matter contaminating meat upon time of slaughter are increased, particularly in commercial feedlots where animals may stand in large amounts of manure. Alternatively, cattle finished on predominately grass and forages retain the normal, neutral pH of the rumen, which prevents the formation of acid resistant strains of *E. coli* O157,H7. This was the basis of our examination to determine the difference in prevalence with varying feed techniques, grain versus grass.

Antibiotics are used in food animals for enhancing growth and for prophylactic treatment of potential diseases (Gustafson and Bowen 1997; Aminov 2010). For this reason, antibiotic resistant strains of foodborne pathogens have become an increasing concern regarding the safety of the commercialized meat industry and its health implications (Hardy 2002). Multidrug resistant isolates of *Salmonella* increased from 39% to 97% between 1979 and 1997 (White et al. 2001).

Studies have shown that in the U.S. there are 9.4 million episodes of foodborne illness and 55,961 hospitalizations each year (Scallan et al. 2011). Other studies report approximately 73,000 estimated annual cases of *E. coli* O157 infections and 1,414,000 estimated total cases of *Salmonella* infections in the United States from 1983 to 1992.

Eighty percent of these cases was estimated to be via foodborne transmission (Mead et al. 1999).

As awareness of meat industry issues rises, movements for alternative methods for obtaining meat are gaining momentum as well. To date, there have been no comparative studies that show the differences in pathogen contamination between meat sourced from conventional feedlot, grain finished cattle, and meat obtained from grazed cattle sold at farmers' markets. This study investigates whether there is a difference in the instance of two common foodborne pathogens, *E. coli* O157,H7 and *Salmonella*, isolated from meat obtained from these alternative methods, and from meat obtained from conventional feedlots.

MATERIALS & METHODS

Bacterial Strains

Reference strains of *Escherichia coli* O157,H7 (ATCC 43888) and *Salmonella enterica* Typhimurium (ATCC 14028) were grown and maintained on brain heart infusion medium (BHI; Becton-Dickinson, Sparks, MD). These strains were used as positive control organisms for all experiments described.

Sample Collection

Three separate samples of nonfrozen ground beef (70% lean, 30% fat ratio) were purchased in approximately 450 g packs from grocery superstores in three different locations in central Georgia on the same day, for a total of nine separate samples. The ratio of 70,30 lean to fat was chosen based on popularity and availability. Similarly, nine total samples of frozen ground beef were purchased from farmers' markets (three samples each from three different markets) located in the same geographical area. Additionally, because local farmers' markets are held on different days of the week in the middle Georgia area, these samples were collected on different days within the same week. Beef from each of the farmers' market vendors was certified to be grass fed to ensure that the difference in feeding strategy was accurate. All ground beef, by law, was processed at USDA inspected facilities. All samples were transported on ice after purchase and frozen. Because all farmers' market samples were frozen at sale, big box samples were also frozen after purchase for the sake of consistency. Samples were refrigerator thawed at 4 °C prior to use.

Enrichment and Isolation of *Escherichia coli*

Thawed ground beef samples were subjected to modified isolation and identification protocols outlined in the FDA Bacteriological Analytical Manual (Feng et al. 2011). Each 25 g sample of thawed ground beef was macerated in a stomacher (Bagmixer, Saint Nom, France) for 120 s with 225 mL of modified EC medium (Becton-Dickinson, Sparks, MD) with 8 mg/L novobiocin (mECMn). Approximately 40 mL of the homogenate was dispensed in sterile baffled culture flasks and incubated, with shaking, for 24 h at 35 °C. Calibrated 1 µl disposable loops were used to streak onto three replicates of cefixime potassium tellurite sorbitol MacConkey (CT-SMAC; Hardy Diagnostics, Santa Maria, CA) plates, giving a total of nine plates from each location. After a 24 h incubation at 35 °C, presumptive *E. coli* O157,H7 colonies were isolated

from the CT-SMAC plates and plated onto tryptic soy agar with 0.6% yeast extract (TSAYE) plates for purity, and incubated at 35 °C for 24 h (Feng et al. 2011). Gram stains as well as oxidase, triple sugar iron (TSI), indole, and motility assays were conducted. Additionally, the API Rapid 20E (Biomereux, Hazelwood, MO) assay for the rapid identification of enterics was conducted on the same samples. Further biochemical assays were conducted on selected samples, including lactose fermentation, sucrose fermentation, glucose fermentation, methyl red (MR), Voges-Proskauer (VP), citrate, urease, nitrate, and catalase. Finally, a polyclonal latex agglutination assay against the *E. coli* O157 antigen (E. coliPRO O157™; Hardy Diagnostics, Santa Maria, CA) was used to confirm the identity of putative O157 samples. These procedures were repeated for each ground beef sample.

Enrichment and Isolation of *Salmonella*

Salmonella species were isolated from the meat samples using a modified protocol based on the FDA Bacteriological Analytical Manual (Andrews et al. 2014) and the USDA Food Safety Inspection Handbook (USDA/FSIS 2014). Each 25 g sample of refrigerator-thawed ground beef was homogenized in a stomacher as previously described with 225 mL of universal pre-enrichment broth (UPB) (Nam et al. 2004). Homogenate samples were incubated at 35 °C for 24 h while shaking. From each tube, 0.1 mL aliquots were removed and placed into a fresh culture tube with 10 mL of Rappaport-Vassiliadis R10 broth (RV broth, Becton-Dickinson, Sparks, MD), and subsequently incubated on a shaker for 24 h at 42 °C (Andrews et al. 2014). After incubation, cultures were streaked for isolation as described earlier onto three replicates of *Salmonella* selective medium (HardyCHROM™; Hardy Diagnostics, Santa Maria, CA). All plates were then incubated at 35 °C for 24 h. Gram stains were conducted on presumptive magenta *Salmonella* colonies as well as various biochemical tests including API Rapid 20E kit assays, oxidase, triple sugar iron (TSI), lysine iron agar (LIA), lactose fermentation, sucrose fermentation, glucose fermentation, methyl red (MR), Voges-Proskauer (VP), citrate, urease, nitrate, and catalase as recommended by the USDA and FDA protocols. Additionally, identification of presumptive *Salmonella* colonies was confirmed with a rapid latex agglutination assay against a wide variety of *Salmonella* antigens (Microgen, Surrey, U.K.).

Antibiotic Sensitivity

Antibiotic sensitivity was tested using the Kirby-Bauer method. Presumptive *E. coli* and *Salmonella* colonies were suspended in sterile saline and turbidity adjusted to a 0.5 McFarland standard. The resultant suspension was used to create bacterial lawns on Mueller-Hinton plates. The antibiotics used were as follows: 30 µg tetracycline, 10 µg streptomycin, 1.25 µg trimethoprim, 23.75 µg sulfamethoxazole, and 30 µg chloramphenicol (Becton-Dickinson, Sparks, MD).

RESULTS

Isolation and Identification of *E. coli* O157,H7

CT-SMAC selective medium was streaked with inoculum enriched in mECMn. Other studies have indicated that UPB is comparable to mECMn for the enrichment of

E. coli 0157 (Nam et al. 2004). However, in our hands this was not the case (data not shown), thus mECMn was used. Several colorless colonies, indicative of presumptive *E. coli* O157, were isolated from all three supermarket locations. Gram stains confirmed that all isolated colonies (n = 3) were Gram-negative bacilli. However, further biochemical tests revealed differing organisms (Table I). All colonies isolated from supermarket locations #1 and #3 were identified as *Enterobacter cloacae*. Three isolates from supermarket location #2 (n = 3) were identified as *E. coli* O157 (Table I, Row 2). Identification was confirmed by latex agglutination using antibodies to the O157 antigen.

Table I. Identification of Presumptive *Enterobacteriaceae* Isolated from Supermarket-Sourced Ground Beef, then Selected on CT-MAC and TSAYE Media

Source	*Gram rxn*	*Oxidase*	*TSI*	*Motility*	*Indole*	*API 20E*	*Serology*
Location 1	Neg.	-	Yellow	+	-	*E. cloacae*	-
Location 2	Neg.	-	Yellow	+	+	*E. coli* O157,H7	+
Location 3	Neg.	-	Yellow	+	-	*E. cloacae*	-
Control	Neg.	-	Yellow	+	+	*E. coli* O157,H7	+

Farmers' market samples with presumptive colorless colonies of *E. coli* O157,H7 on CT-SMAC medium were all isolated from location #3 (n = 4). Biochemical testing with API Rapid 20E determined that all four samples were likely presumptive *Enterobacter cloacae* and not *E. coli* O157,H7. Serological assays with anti-O157 antibodies confirmed that none of these farmers' market samples were *E. coli* O157 (Table II).

Table II. Identification of Presumptive *Enterobacteriaceae* Isolated from Farmers' Market-Sourced Ground Beef, then Selected on CT-MAC and TSAYE Media

Source	*Gram rxn*	*Oxidase*	*TSI*	*Motility*	*Indole*	*API 20E*	*Serology*
Location 3	Neg.	-	Yellow	+	-	*E.cloacae*	-
Location 3	Neg.	-	Yellow	+	+	*E. cloacae*	-
Location 3	Neg.	-	Yellow	+	-	*E. cloacae*	-
Location 3	Neg.	-	Yellow	+	-	*E.cloacae*	-
Control	Neg.	-	Yellow	+	+	*E. coli* O157,H7	+

Positive *E. coli* O157 colonies were tested for antibiotic sensitivity as described. While some intermediate resistance was observed, all zones of inhibition were within the margin of error (Table III).

Table III. Antibiotic Sensitivity of Presumptive Pathogens Isolated from Supermarket and Farmers' Market-Sourced Ground Beef

Organism	*TE-30*[a]	*SXT*	*C-30*	*S-10*
E. coli O157,H7 (Location 2)	S[b]	S	S	I
Control *E. coli* O157,H7 ATCC 43888	S	S	S	S
Salmonella spp. (Location 3)	S	S	S	S
Salmonella spp. (Location 3)	S	S	S	I
Salmonella spp. (Location 3)	S	S	S	S
Control *Salmonella typhimurium* ATCC 14028	S	S	S	I

[a]TE-30, tetracycline 30 µg; SXT, trimethoprim 1.25 µg plus sulfamethoxazole 23.75 µg; C-30, chloramphenicol 30 µg; S-10, streptomycin 10µg.
[b]S, susceptible; I, intermediate.

Isolation and Identification of *Salmonella*

All three samples from a single supermarket location showed presumptive colonies of *Salmonella* on *Salmonella* selective medium. API Rapid 20E results indicated that all three samples were *Salmonella*. Standard biochemical tests were conducted to confirm the rapid tests performed with the API Rapid 20E kits. Latex agglutination results confirmed identity as species of *Salmonella* (Table IV).

Table IV. Identification of Presumptive *Salmonella* Selected from Supermarket-Sourced Ground Beef

Source	*Gram rxn.*	*Oxidase*	*TSI*	*LIA*	*API 20E*	*Serology*
Location 3	Negative	-	Yellow	+	*Salmonella*	+
Location 3	Negative	-	Yellow	+	*Salmonella*	+
Location 3	Negative	-	Yellow	+	*Salmonella*	+
Control	Negative	-	Yellow	+	*Salmonella*	+

No presumptive colonies were isolated from the HardyCHROM™ *Salmonella* plates incubated with farmers' market meat homogenates. Therefore, no isolates of *Salmonella* from any farmers' market location were identified.

Like *E. coli* O157, the *Salmonella* isolated from supermarket sourced ground beef did not demonstrate a significant pattern of resistance to the antibiotics tested (Table III).

DISCUSSION

Isolation and Identification of *E. coli* O157

These preliminary results suggest a prevalence of *E. coli* O157 in supermarket-sourced ground beef as compared to meat from farmers' markets. With nine samples from supermarket stores, a single isolate (11%) of *E. coli* O157 was found. Other studies, using similar USDA and FDA methodologies, reported 3 to 4% of ground beef samples as positive for the isolation of *E. coli* O157 (Gomez-Aldapa et al. 2013; Mansouri-Najand et al. 2015). Farmers' markets samples tested negative for the presence of *E. coli* O157. Interestingly, a study in South Africa reported that the prevalence of *E. coli* O157 was higher in feces from commercial (14–20%) than communal (noncommercial) cattle (5%) (Ateba et al. 2008). Despite the encouraging data of this current study, a larger sample size is needed to draw any meaningful conclusions.

It should be noted that the API Rapid 20E used for the identification of presumptive colonies, in accordance with the manufacturer's protocol, yielded results for *E. coli* O157,H7 that were sometimes inconsistent. Commercial identification kits like the API system have been used for decades and have been shown to be effective and accurate, particularly when supplemented with additional testing. However, a recent study reported that identification of *E. coli* isolates by rapid commercial systems like the API should be interpreted with care (Abulreesh 2014). More efficient, labor-saving and sensitive alternatives to the API system exist, but cognizance must be taken of cost and accessibility to the technology (Law et al. 2014; Zhao et al. 2014). Therefore, in this current study, standard biochemical tests were also run to confirm the results obtained with the rapid API identification kits.

Isolation and Identification of *Salmonella*

Recent studies have reported that the prevalence of *Salmonella* in ground beef is still a significant health concern (Griese et al. 2013; CDC 2015). Guo and colleagues estimated that ground beef contributes to 28% of human salmonellosis in the United States (Guo et al. 2011). In our study, *Salmonella* was isolated from 100% of the ground beef samples purchased from a single supermarket location. Overall, one third of our samples from conventional supermarket sources tested positive for *Salmonella*. No instances of *Salmonella* from farmers' market samples were identified. Previous studies have reported the prevalence of *Salmonella* in ground beef at rates of 20% (White et al. 2001), although these rates are affected by processing locations and seasons (Barkocy-Gallagher et al. 2004). Studies have indicated that a major source of *Salmonella* in ground beef may be lymph nodes and even contamination from carcass hides (Koohmaraie et al. 2012). Lymph nodes are routinely included in beef trimmings intended for further manufacturing, such as grinding, and may thus "be an important source of *Salmonella* in ground beef" (Gragg et al. 2013). It is feasible that the processing of beef in commercial slaughterhouses does not lend itself to the removal of these lymph nodes, as opposed to the individual, small nonassembly line processing found in farmers' market sources.

Antibiotic Sensitivity Testing

There was no discernible resistance to any of the antibiotics tested on either *E. coli* O157 or *Salmonella* isolates. Other studies have shown significant resistance to antibiotics such as sulfonamides, tetracycline, and chloramphenicol from *E. coli* (Tadesse et al. 2012) and *E. coli* O157 (Nizza et al. 2010) isolated from multiple sources including food animals. Similarly, studies have reported antibiotic-resistant strains of *Salmonella* isolated from food animals (DiMarzio et al. 2013). Since the disk-diffusion assay used in this current study has been shown to be comparable to other methods of testing antibiotic sensitivity (Nayak et al. 2007), the contradictory result is most likely due to the small sample size of isolates tested.

This preliminary study confirmed the presence of both *E. coli* O157 and *Salmonella* species from supermarket-sourced ground meat, and none in ground beef farmers' market sources. Although of small sample size, this study is a good foundation for comparative studies on the prevalence and isolation of *E. coli* O157 and *Salmonella* from animal food products sourced and processed from conventional feedlots and meat obtained from grazed cattle and sold at farmers' markets.

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