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OCCURRENCE OF *Yersinia enterocolitica* SEROTYPE O:9, AND *Citrobacter freundii*, TWO POTENTIAL HUMAN PATHOGENS IN THE THROATS OF TROPICAL PIGS OF GRENADA ORIGIN

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ABSTRACT

A study to determine the occurrence of *Yersinia enterocolitica* and *Citrobacter freundii*, two potential human pathogens in the throats of tropical pigs of Grenada origin and the antimicrobial pattern of the isolates was carried out. During a period of 30 days (15 September 2017 to 15 October 2017), throat swabs from a total of 97 weaned pigs were sampled to isolate and characterize *Y. enterocolitica* by serotyping and resistance testing, and to isolates *C. freundii*. Of the pigs tested, four (4.1%) pigs were positive for *Y. enterocolitica*. The four *Yersinia*-positive pigs included one (2.4%) of 41 male pigs and three (5.4%) of 56 female pigs. There were no significant differences between the proportions of *Yersinia*-positive male and female pigs (p = 0.8437). Of these four *Yersinia*-positive pigs, two were mixed with *C. freundii*, one had slight contamination, and the other was pure. All belonged to serotype 0:9. There were 31 pigs positive for *C. freundii*, and all showed mixed growth. Antimicrobial susceptibility tests against 14 drugs indicated that all isolates of *Y. enterocolitica* were susceptible to third-generation cephalosporins and fluoroquinolones, two classes of antimicrobials recommended for the treatment of *Y. enterocolitica* infection in humans.

Keywords: *Yersinia enterocolitica*, *Citrobacter freundii*, tropical pigs, human pathogens, throats

INTRODUCTION

Enteropathogenic *Y. enterocolitica* is a zoonotic pathogen, causing human disease worldwide with symptoms ranging from gastroenteritis to severe complications of mesenteric lymphadenitis, liver abscesses and postinfectious extraintestinal sequelae (**Batzilla *et al.*, 2011; Valentin-Weigand *et al.*, 2014**). It has also been isolated from cases of diarrhea in dogs in Canada (**Hariharan and Bryenton, 1990**). Recently, an unusual increase of *Y. enterocolitica* in humans occurred in Creuse, central France (**Martin *et al.*, 2015**). Human infections are directly or indirectly derived from animal sources and may be contracted through the ingestion of contaminated food (**Oxoid 2018**). Pigs are considered to be the major reservoir of this pathogen. They may carry this organism in throats, tonsils, tongues, and to a lesser extent in feces (**Hariharan *et al.*, 1995; Schiemann and Fleming, 1981; Singh *et al.*, 2003**). Although the re-emergence and importance of this organism in temperate areas of the world is well documented as indicated by recent publications (**Arrausi-Subiza *et al.*, 2016; Bonardi *et al.*, 2014; Fondrevez *et al.*, 2014; Van Damme *et al.*, 2015; von Altrock *et al.*, 2015**), there is very little information from tropical and subtropical countries. There is no data on the occurrence or properties of this pathogen in animals, including swine raised in the Caribbean. It is known that *C. freundii* an opportunistic human pathogen, may have a colony morphology resembling *Y. enterocolitica* on the selective medium used for isolation of *Y. enterocolitica* (**Oxoid Ltd, 2018**). Published information on *C. freundii*, in pigs is minimal, although it is known that some strains can cause gastroenteritis and hemolytic uremic syndrome (**Tschape *et al.*, 1995**). The objectives of this study were to determine the carriage rates of *Y. enterocolitica* in pigs raised for food, and to characterize the isolates with regard to serotype and antimicrobial susceptibility. We also ascertained the occurrence of *C. freundii* in the throats of the pigs studied.
This study will help determine the possible role of tropical pigs as reservoirs of human pathogenic *Y. enterocolitica* strains and identify drugs which are likely to be effective for treatment, in the event of disease in humans originating from pigs. Slaughter procedures and storage of meat may need modifications to prevent contamination of meat and subsequent multiplication of this psychrophilic organism which can multiply in refrigeration temperatures.

MATERIAL AND METHODS

Study design and sample collection

This study had the approval of the St. George's University Institutional Animal Care and Use Committee (IACUC 16007-R). Young weaned pigs of approximately 16 weeks of age were randomly selected from pig farms in Grenada. For each sampled pig, the gender and age were recorded. All sampling was done in a period of 30 days (15 September 2017 to 15 October 2017). Long guarded body cavity culture swabs (Santa Cruz Animal Health, Dallas, Texas) designed for collection throat samples from animals were used.

Isolation and identification of *Yersinia enterocolitica* and *Citrobacter freundii*

For the isolation of *Y. enterocolitica* and *C. freundii*, each swab was placed in a tube containing 5 ml of peptone sorbitol bile broth (Sigma-Aldrich, St. Louis, USA). The tube was vortexed, and 1 ml of the suspension was added to 9 ml of Irgasan-Ticarcillin-potassium Chlorate (ITC) broth (Bio-Rad, Marnes La Coquette, France), and incubated for 48 h at 25°C. Then, 10 µl of the culture was streaked on Cefsulodin-Irgasan-Novobiocin (CIN) agar plates (*Yersinia* selective agar base and supplement (Oxoid, Basingstoke, UK), and incubated at 30°C for 24 hours. Suspected *Y. enterocolitica* and *C. freundii* colonies were identified using the API20E (Analytical Profile Index; BioM6rieux, Hazelwood, MO) bacterial identification strips.

Serotyping of *Yersinia enterocolitica*

For the serotyping of the *Y. enterocolitica*, the *Y. enterocolitica* serotyping kit (Denka Seiken Co. Ltd., Tokyo, Japan), which contained one polyvalent antiserum (groups O1 and O2), and 4 types of monovalent antisera for groups O3, O5, O8, and O9) was used.

Antimicrobial susceptibility test

Antimicrobial susceptibility tests was done using the standard disk diffusion method on Mueller Hinton agar (Difco/BD) following recommendation of the Clinical and Laboratory Standard Institute (**CLSI, 2015**). All the Y. enterocolitica were tested for susceptibility to 14 antimicrobials. The antimicrobial disks used were: amoxicillin-clavulanic acid (AmC-30), ampicillin (Am-10), aztreonam (ATM-30), ceftriaxone (CRO 30), ceftazidime (CAZ-30), cefoxitin (FOX-30), cephalothin (CF-30), chloramphenicol (C-30), ciprofloxacin (CIP-5), gentamicin (GM-10), imipenem IPM-10), trimethoprim-sulfamethoxazole (SXT-1.25/23.75), streptomycin (S-10), and tetracycline (TE-30). The inhibition zone sizes were interpreted based on CLSI guidelines. *Escherichia coli* ATCC 25922 was used as quality control strain **(Eguale *et al.*, 2015**).

Statistical analysis

An online data analysis software: http://www.openepi.com/Menu/OE_Menu.htm was used for all the statistical analysis. The OpenEpi-Two by Two table (chi-squared (χ^2) analysis) was used to compare the differences in the proportions of female vs male pigs. The level of statistical significance was set at alpha equal to 0.05 ($\alpha = 0.05$). A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Ninety seven weaned pigs were sampled. By gender, they comprised of 41 (42.3%) male pigs, and the remaining 56 (57.7%) were females. Throat swabs from four (4.1%) pigs were positive for *Y. enterocolitica*. The four *Yersinia*-positive pigs included one (2.4%) of 41 male pigs and three (5.4%) of 56 female pigs. There were no significant differences between the proportions of *Yersinia*-positive male and female pigs ($p = 0.8437$). Of these four *Yersinia*-positive pigs, two were mixed with *C. freundii*, one had slight contamination, and the other was pure. All belonged to serotype 0:9. There were 31 pigs positive for *C. freundii*, and all showed mixed growth. The other isolates included species of no pathogenic significance or poor identifications according to API strips. The United States Centers for Disease Control and Prevention (**CDC, 2016**) estimates *Y. enterocolitica* causes almost 117,000 illnesses, with 35 deaths in the U.S. every year. Raw or undercooked pork contaminated with *Y. enterocolitica* is the most common source of human infection worldwide. *Y. enterocolitica* strains found in pigs and pork are indistinguishable from strains found in humans, further supporting the association between yersiniosis and consumption of pork (**Fredriksson-Ahomaa and Korkeala, 2003**). There are over 70 serotypes of Y. pathogenic serotypes of y. enterocolitica. Most human infections involve serotypes O:3, O:5, O:8, and O:9 (**Schriefer and Petersen, 2011**). Although, O:3 is the leading serotype, human infections due to *Y. enterocolitica*, serotype 09 have been reported from the UK (**Wale *et al.*, 1991**) Germany (**Luedde *et al.*, 2004**), Japan (**Moriki *et al.*, 2010**), and Poland **(Kamińska and Sadkowska-Todys, 2016**). Children are more prone to infection, and serotypes O3 and O:9 predominated in the Netherlands (**Hoogkamp-Korstanje and Stolk-Engelaar, 1995**). In Poland, of 244 cases in 2014, 5.2% were due to O:9 serotype (**Kamińska and Sadkowska-Todys, 2016**).

Although only 4% of the pigs in the present study were positive for *Y. enterocolitica*, the fact that all belonged to the same serotype O:9 is noteworthy. This is in contrast to a study conducted by one of the authors on slaughter hogs in Canada (**Hariharan, *et al.*, 1995**), where majority of the isolates from the tonsils belonged to serotype O:3. The isolation method used in the present study was similar to that used in the Canadian study. The low isolation rates in natural samples may be due to the limited sensitivity of cultural methods (**Fredriksson-Ahomaa and Korkeala, 2003**). On the other hand it may be noted that studies on tropical pigs are lacking, and it is impossible to make comparisons at present.

Citrobacter spp. are opportunistic pathogens that are commensal inhabitants of the intestines on humans and animals. *C. freundii* is a potential foodborne pathogen which has been implicated in human gastroenteritis, hemolytic uremic syndrome, and pigs could be a source (**Bai *et al.*, 2012; Liu *et al.*, 2017; Nimri *et al.*, 2014; Tschape, *et al.*, 1995**).

Table 1. shows the antimicrobial susceptibility (mean zone diameters and interpretation) of four *Y. enterocolitica* serotype O:9 isolates and control strain *Escherichia coli*. Antimicrobial drugs traditionally used to treat human infection, includes cotrimoxazole, doxycycline and chloramphenicol (**Stolk-Engelaar *et al.*, 1995**). Our isolates were susceptible to these drugs, although one isolate showed only intermediate susceptibility to tetracycline. Strains resistant to ampicillin and cephalothin like ours have been found in slaughter pigs in other parts of the world (**Terentjeva and Berzins, 2010**). All four strains in our study were susceptible to third-generation cephalosporins and fluoroquinolones (ciprofloxacin), two classes of drugs recommended for *Y. enterocolitica* infection in humans (**von Altrock, *et al.*, 2015**).

Table 1 Antimicrobial susceptibility of four *Yersinia enterocolitica* serotype O:9 isolates and control strain *Escherichia coli*

Antimicrobial drug and disk potency	Pig # 25	Pig # 31	Pig # 32	Pig # 36	Control strain: *E. coli* ATCC 25922
Amoxicillin/clavulanic acid (AMC-30)	9 (R)	14 (I)	12 (R)	20 (S)	20 (S)
Ampicillin ((AM-10)	0 (R)	10 (R)	0 (R)	12 (R)	21 (S)
Aztreonam (ATM-30)	35 (S)	40 (S)	39 (S)	25 (S)	32 (S)
Ceftriaxone (CRO-30)	37 (S)	39 (S)	39 (S)	27 (S)	33 (S)
Ceftazidime (CAZ-30)	35 (S)	37 (S)	39 (S)	30 (S)	26 (S)
Cefoxitin (FOX-30)	14 (R)	16 (I)	16 (I)	27 (S)	33 (S)
Cephalothin (CF-30)	0 (R)	0 (R)	0 (R)	14 (R)	17 (I)
Chloramphenicol (C-30)	30 (S)	35 (S)	34 (S)	25 (S)	25 (S)
Ciprofloxacin (CIP-5)	39 (S)	39 (S)	43 (S)	31 (S)	33 (S)
Gentamicin (GM-10)	27 (S)	31 (S)	31 (S)	23 (S)	20 (S)
Imipenem (IPM-10)	34 (S)	39 (S)	41 (S)	25 (S)	30 (S)
Trimethoprim-sulfamethoxazole (SXT-1.25/23.75)	31 (S)	34 (S)	36 (S)	24 (S)	22 (S)
Streptomycin (S-10)	25 (S)	27 (S)	25 (S)	17 (S)	17 (S)
Tetracycline (TE-30)	19 (S)	27 (S)	27 (S)	18 (I)	21 (S)

CONCLUSION

Our study showed that presently, tropical pigs of Grenada origin are not major reservoirs for the pathogenic *Y. enterocolitica* serotype O:9, we estimated the occurrence rate in the throats of pigs in Grenada to be 4.1%. This current study also showed that tropical pigs harbor *C. freundii* another potential pathogen that has been associated with human gastroenteritis. Antimicrobial resistance profiles indicated that all isolates of *Y. enterocolitica* were susceptible to third-generation cephalosporins and fluoroquinolones, two classes of antimicrobials recommended for the treatment of *Y. enterocolitica* infection in humans.

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