Clonality and Antibiotic Susceptibility of *Yersinia enterocolitica* Isolated from U.S. Market Weight Hogs

Saumya Bhaduri,[1] Irene Wesley,[2] Harry Richards,[3] Ann Draughon,[3] and Morgan Wallace[4]

Abstract

Pigs are the only known animal reservoir of *Yersinia enterocolitica* strains pathogenic to humans. In this study 106 *ail*-positive pathogenic *Y. enterocolitica* isolates, previously recovered from 2793 swine fecal samples (3.8%) collected during National Animal Health Monitoring System's Swine 2000 study, were examined. The presence of the previously described virulence plasmid, expression of plasmid-associated virulence determinants, and serotype were correlated with genotype, expression of YopE protein, and antibiotic susceptibility. Pulsed-field gel electrophoresis using the enzyme *Xba*I showed that O:3 and O:5 isolates were highly clonal within a serotype regardless of geographic origin. Antimicrobial resistance profiles of 106 isolates of serotypes O:3 and O:5 were evaluated by agar disk diffusion methodology to 16 different antibiotics. All isolates were susceptible to 13 of the 16 tested antimicrobials; resistance was noted to ampicillin, cephalothin, and tetracycline. The presence of the *ail* gene, virulence plasmid, the expression of virulence determinants, and serotypes indicate that these isolates from U.S. swine are potentially capable of causing human foodborne illness.

Introduction

*Y*ERSINIA ENTEROCOLITICA is an enteric commensal bacteria of swine that has been implicated in human gastrointestinal disease (Robins-Browne, 2007). Clinical isolates of all serotypes implicated in human disease harbor a 70-kb plasmid that contributes to virulence of the bacteria and is referred to as the virulence plasmid (pYV) (Bhaduri, 2001, 2002; Skurnik *et al.*, 2002; Bhaduri and Sommers, 2008). Elements encoded by the chromosome are also necessary for virulence (chromosomally encoded virulence factors [CEVF]) (Carniel *et al.*, 2000; Revell and Miller, 2001).

Y. enterocolitica causes an estimated 96,000 cases of human disease annually in the United States (Mead *et al.*, 1999). Case–control studies and DNA-based epidemiological evidence indicate that the majority of outbreaks are due to consumption of uncooked pork as well as contaminated water, milk, and vegetables (Fredriksson-Ahomaa *et al.*, 2006a, 2006b). Swine are acknowledged reservoirs of *Yersinia*, including those species and serotypes that are associated with human illness. In countries where *Y. enterocolitica* is a significant foodborne pathogen, the estimated carrier rate of pathogenic *Y. enterocolitica* ranges from approximately 35% to 70% in swine herds and 4.5% to 100% of individual swine (Davies, 1997; Robins-Browne, 2007). As commensal bacteria of swine, *Y. enterocolitica* is frequently isolated from pig tongues, tonsils, carcasses, pork products, and

[1]Microbial Food Safety Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania.
[2]National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa.
[3]Department of Food Science and Technology, The University of Tennessee, Knoxville, Tennessee.
[4]Dupont Experimental Station, Dupont Qualicon, Wilmington, Delaware.

feces (Bhaduri *et al.*, 1997; Bhaduri and Cottrell, 1997; Jones *et al.*, 2003; Fredriksson-Ahomaa *et al.*, 2006b, 2007). *Y. enterocolitica* is transmitted among swine by the oral–fecal route. Its presence on the surface of freshly slaughtered pig carcasses (Fosse *et al.*, 2008) is likely the result of the fecal contamination or dissemination from the oral cavity during slaughter (Bhaduri, 2001; Bhaduri *et al.*, 2005; Thibodeau *et al.*, 1999).

A broad spectrum of antibiotics has been widely used in agriculture to treat infection and improve growth and feed efficiency in livestock and poultry. The use of approved antibiotics, such as aminoglycosides, β-lactam, cephalosporins, macrolides, sulfonamides, and quinolones in feed in the swine industry is a concern since some of the antibiotics are important in human clinical treatment (Mathew *et al.*, 2007). Aubrey-Damon (2004) reported a higher prevalence of antibacterial resistance in bacteria from feces of healthy pig farmers compared to nonfarmers, possibly due to contact with antibacterial-resistant bacteria from pigs and the farm environment.

We reported previously that a total of 106 *ail*-positive *Y. enterocolitica* clones from 2793 fecal samples (3.8%) were isolated during the 2000 National Animal Health Monitoring System's Swine 2000 survey. These isolates were initially characterized by the presence of virulence plasmid, expression of plasmid-associated virulence determinants, expression of YopE protein, and serotype (Bhaduri *et al.*, 2005; Bhaduri and Wesley, 2006) (Table 1). The primary goal of

the present study was to further characterize these *ail*-positive swine NAHMS isolates by pulsed-field gel electrophoresis (PFGE) profiles. In addition, resistance profiles to 16 National Antimicrobial Resistance Monitoring System (NARMS) antibiotics, including amikacin, amoxicillin, ampicillin, cephalosporins (cefoxitin, ceftiofur, ceftriaxone, cephalothin), chloramphenicol, ciprofloxacin, aminoglycosides (gentamicin, kanamycin, streptomycin) naldixic acid, sulfamethoazole, tetracycline, and trimethoprim, were examined. Among the antimicrobials evaluated, gentamicin is used with a swine label for baby pigs 1 day of age. Some of the antibiotics serve as surrogates for the actual drug used in swine medicine and are relevant to current hog production. For example, ciprofloxacin is not used in veterinary medicine, but enrofloxacin (another fluoroquinolone) is licensed. Resistance to nalidixic acid measures a change that is intermediate in the development of ciprofloxacin resistance. Cefoxitin and ceftriaxone are not veterinary drugs, but serve as markers for cephalosporins since not all ceftiofur-resistant bacteria are resistant to cefoxitin. Ceftiofur is the only cephalosporin licensed for use in swine. Chloramphenicol is prohibited from use in veterinary medicine, but it may serve as a marker for fluorfenicol resistance. Trimethoprim is not licensed for use in swine, but is included in the standard NARMS panel. Since plasmid genes may encode resistance to antibiotics, it is important to know whether resistance is plasmid and/or chromosomally

Table 1. Presence of pYV, Evaluation of pYV-Associated Virulence Characteristics, and Serotype of *ail*-Positive *Y. enterocolitica* Isolated from Swine Fecal Samples

Isolate group state	Total no. of isolates	Presence of pYV[a]	pYV associated virulence phenotypes[b]	Serotype O:3	Serotype O:5
Illinois	4	4 (+)ve	4 (+)ve	0	4
Indiana	25	24 (+)ve	24 (+)ve	25	0
Iowa	21	21 (+)ve	21 (+)ve	12	9
Minnesota	18	18 (+)ve	18 (+)ve	6	12
Nebraska	29	28 (+)ve	28 (+)ve	28	1
Ohio	1	1 (+)ve	1 (+)ve	0	1
South Dakota	8	8 (+)ve	8 (+)ve	8	0
Total	106	104 (+)ve	104 (+)ve	79	27

[a]75-kb pYV was detected by multiplex polymerase chain reaction assay using primers targeting the chromosomal *ail* gene and the plasmid *virF* gene.
[b]pYV associated virulence phenotypes colony morphology: YEP+ cells appeared as small colonies on BHA; CV binding: YEP+ cells showing dark violet colonies on BHA; Lcr: YEP+ cells appeared as pinpoint colonies on BHO; CR uptake: YEP+ cells showing red dark pinpoint colonies on CR-BHO; AA: agglutination of YEP+ cells; HP: YEP+ cells agglutinates forming clumps showing hydrophobicity.

encoded. These findings would then be correlated with our previous descriptions of the virulence plasmid, cytotoxicity factor, and serotypes (Bhaduri and Wesley, 2006).

Materials and Methods

Ail-*positive* Y. enterocolitica *isolates*

A total of 106 *ail*-positive (CEVF: <u>a</u>ttachment <u>i</u>nvasion <u>l</u>ocus) *Y. enterocolitica* isolated from 2793 fecal samples were stored at −70°C with 10% glycerol in cryogenic vials as previously described (Bhaduri *et al.*, 2005). These 106 *ail*-positive *Y. enterocolitica* isolates were examined in the present study as described below.

Preparation of pYV-less strains from YEP+ isolates

The pYV-less (YEP−) strains were obtained from large, flat white colonies that emerged spontaneously from swine fecal and GERO:3 YEP+ cultures grown at 37°C on Congo red–brain heart infusion agarose as described (Bhaduri and Sommers, 2008). The absence of pYV in these YEP− strains was confirmed by a polymerase chain reaction (PCR) assay targeting *virF* gene as described (Bhaduri, 2003). These YEP− strains were used as controls for the following experiments.

PFGE

PFGE was performed using the standard Food Safety Inspection Service (FSIS) protocol for *Salmonella* with minor modifications (Cook *et al.*, 1998). Briefly, *Y. enterocolitica* isolates were grown on BHA plates (18 hours at 37°C), suspended in buffer (100 mM Tris, 100 mM EDTA) to an OD_{610} of 1.0 ± 0.1. An equal volume of 1.4% Low Melt agarose, 1% SDS containing 1 mg/mL proteinase K (Roche, Indianapolis, IN) was added. Aliquots were dispensed into disposable 0.1-mL plug molds (Bio-Rad, Hercules, CA) and allowed to solidify for 20 minutes. Plugs were transferred to 50-mL conical bottom tubes containing 5 mL of lysis buffer (50 mM Tris, 50 mM EDTA, 1% sarcosine, 0.1 mg/mL proteinase K) and incubated (54°C for 2–4 hours in a shaking water bath). Plugs were washed twice in H_2O and twice in 0.5 M TBE (650 mL each wash) in a PVC plug washer for 30 minutes

per wash step. Plugs were sectioned into thirds and digested with 50 U *Xba*I (New England Biolabs, Beverly, MA) overnight at 37°C. Plugs were incorporated into 1% Pulsed Field Grade Agarose gels (Bio-Rad) and pulsed-field electrophoresis was performed using a CHEF mapper XA system (Bio-Rad) in 0.5×TBE at 14°C, 200 V with pulses ramping from 4 to 40 seconds over 19 hours. Gels were stained with ethidium bromide, photographed (Chemi Doc; Bio-Rad), and images saved as TIFF files.

Cluster analysis

TIFF files were analyzed using BioNumerics software (Applied Maths, Austin, TX). Cluster analysis using the Dice correlation for band matching with a 1% position tolerance and hierarchic UPGMA was used to generate a dendrogram describing the relationship of *Y. enterocolitica* isolates.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the microdilution method according to National Committee for Clinical Laboratory Standards (NCCLS, 2001) guidelines using the Sensititre plate protocol (Trek Diagnostics System, Inc., Cleveland, OH). The plates were incubated (16 hours at 35°C) and resistance was scored via visual examination. The antibiotics tested consisted of a standard panel of 16 NARMS antibiotics, including amikacin, amoxicillin, ampicillin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, naldixic acid, streptomycin, sulfamethoazole, tetracycline, and trimethoprim. Resistance to an antibiotic was confirmed using standard disk diffusion methodology (NCCLS, 2001). Breakpoints to establish resistance were selected based on NCCLS recommendations for Enterobacteriaceae.

Results and Discussion

One *ail*-positive isolate from each positive fecal sample was evaluated for clonality by PFGE and antimicrobial susceptibility patterns, and the results were correlated with our previous initial report of virulence profiles (Bhaduri *et al.*, 2005; Bhaduri and Wesley, 2006).
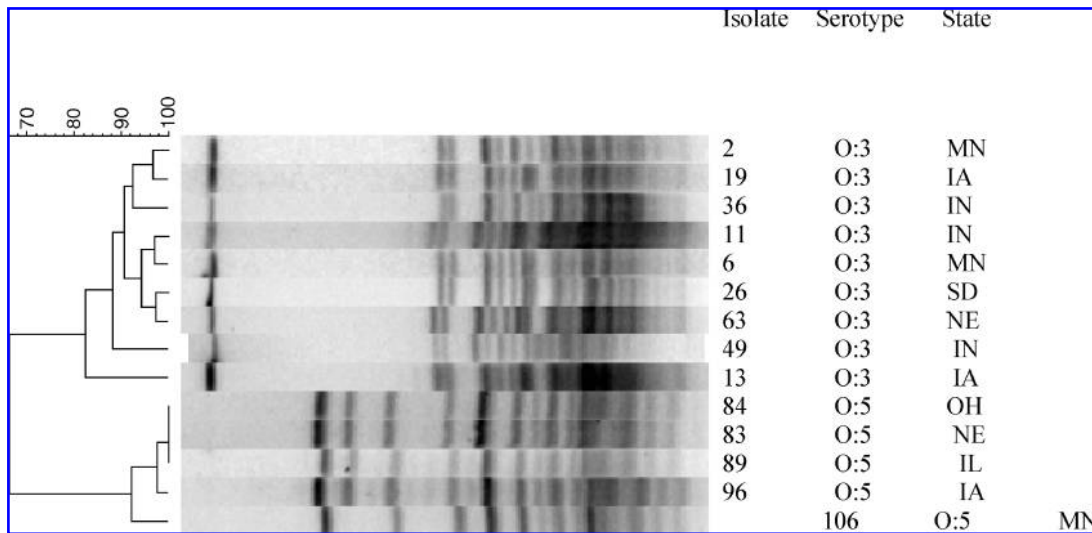
FIG. 1. Representative portion (a subset of total 106 fecal YEP$^+$ isolates including serotypes O:3 and O:5) of a dendrogram showing relatedness between and within O:3 and O:5 serotypes, following digestion with *Xba*I. Levels of similarity were calculated with Dice coefficient and cluster analysis was performed by UPGMA.

Genomic analysis

*Xba*I was the sole enzyme used in this study since it yields the most discriminating macro-restriction fragments for *Y. enterocolitica* (Fredriksson-Ahomaa *et al.*, 1999). By PFGE (Fig. 1) O:3 and O:5 *ail*-positive isolates could be distinguished. However, isolates were highly clonal within a serotype and exhibited minor variations that could not be correlated with geographic origin. Thus isolates from different farms within the same state or from different states displayed nearly indistinguishable PFGE profiles. That O:3 and O:5 pulsotypes exhibit only minor variations within a serotype, regardless of geographic origin, indicates high clonality and that the genome of *Y. enterocolitica* is stable, an observation that concurs with others (Fredriksson-Ahomaa *et al.*, 1999).

Antibiotic susceptibility in ail-positive isolates

Antibiotic resistance of 106 YEP$^+$ swine fecal isolates was studied to obtain basic data of resistance patterns. A high degree of antibiotic susceptibility was observed in the sampled population of *ail*-positive *Y. enterocolitica* from swine feces. All of the strains ($n = 106$) were susceptible to amikacin, amoxicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, naldixic acid,

streptomycin, and trimethoprim. Similar patterns of susceptibility were observed among strains isolated from pig tonsils in Switzerland, southern Germany as well as in human strains (Bucher *et al.*, 2008; Fredriksson-Ahomaa *et al.*, 2007). Resistance to ampicillin was shown in all of the 106 isolates. Ampicillin resistance due to production of β-lactamases is well described in the literature (Bucher *et al.*, 2008). Of the 106 isolates, 87.7% were resistant to cephalothin, and 27.4% were resistant to tetracycline (Table 2). All of the isolates resistant to tetracycline were also resistant to cephalothin. Higher percentage of resistance (72–100%) to cephalothin was found among four states (Table 2); moderate resistance (13–69%) to tetracycline was distributed among three states and O:3 and O:5 while no isolate from Nebraska was resistant (Table 2). Likewise, Funk *et al.* (2000) in screening *ail*-bearing isolates of serotype O:5 from hog tonsils in the Midwest concluded that the majority of isolates were resistant to ampicillin, penicillin, and cephalothin. However, Funk *et al.* (2000) could not correlate the presence of the *ail* gene with antimicrobial resistance. Since it is not known if antibiotic resistance is associated with pYV or chromosomal gene(s), we tested all 16 antibiotics to determine the resistance pattern of isogenic YEP$^-$ strains generated from YEP$^+$ isolates. The

Table 2. Number and Percentage of Strains and Serotypes of YEP[+] Isolates Resistant to Cephalothin and Tetracycline

	Resistant (%)[a]	
	Cephalothin	Tetracycline
Geographic origin (*n* = no. of isolates)		
Indiana (25)	72.0	40.0
Iowa (21)	76.2	42.0
Nebraska (28)	100.0	0.0
South Dakota/Minnesota (26)	100.0	15.4
Serotype distribution		
O:3 serotype (80)	85.0	13.8
O:5 serotype (26)	100.0	69.2
Total isolates (106)[b]	88.7	27.4

[a]Number of resistant strains divided by total number of isolates (*n* = 106).

[b]The total isolates (*n* = 106) includes one strain from Ohio, one strain from Colorado, and four strains from Illinois. Each of these six strains was resistant to both antibiotics.

presence or absence of the pYV did not have a significant effect on the resistance profile. These overall susceptibility/resistance results are consistent with what others have reported in the literature (Fredriksson-Ahomaa *et al.*, 2007; Bucher *et al.*, 2008).

Conclusions

Porcine isolates of *Y. enterocolitica*, which retained the chromosomal *ail* gene, virulence plasmid, pYV, and pYV-associated virulence phenotypic characteristics including cytotoxicity factor, YopE, and serotype, were further analyzed by PFGE and antimicrobial profiles. Macrorestriction patterns demonstrated a high degree of clonality among isolates of the same serotype, regardless of geographic origin indicating stability of the genome. This pathogen was sensitive to 13 of 16 antimicrobials. The YEP[+] isolates (104/106) were resistant to only ampicillin (100%), cephanlothin (87.7%), and tetracycline (27.4%). Thus, these swine isolates, which may enter the food chain by fecal contamination of the carcass during the slaughter, are potential pathogens capable of causing human diseases.

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Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Saumya Bhaduri, Ph.D.
Microbial Food Safety Research Unit
Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
600 East Mermaid Lane
Wyndmoor, PA 19038

E-mail: saumya.bhaduri@.ars.usda.gov