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Detection of *Escherichia coli* O104 in the Feces of Feedlot Cattle by a Multiplex PCR Assay Designed To Target Major Genetic Traits of the Virulent Hybrid Strain Responsible for the 2011 German Outbreak

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A multiplex PCR was designed to detect *Escherichia coli* O104:H4, a hybrid pathotype of Shiga toxigenic and enteroaggregative *E. coli*, in cattle feces. A total of 248 fecal samples were tested, and 20.6% were positive for serogroup O104. The O104 isolates did not carry genes characteristic of the virulent hybrid strain.

In the summer of 2011, a large outbreak of food-borne illness caused by Shiga toxin-producing *Escherichia coli* (STEC) of serotype O104:H4 was reported in Europe (1). The serotype was unusual in that it was as a hybrid strain of enteroaggregative *E. coli* (EAEC) and STEC (2). The hybrid strain carried the gene for Shiga toxin 2 (stx_2), lacked genes for intimin (*eae*) and enterohemolysin (*ehxA*), and possessed an operon with genes (*aggA*, *aafA*, *agg3A*, and *agg4A*) that code for aggregative adherence fimbrial adhesins I, II, and III (AAF/I to III), typical of EAEC (1, 2). Cattle are a primary reservoir of STEC (3) and shed the organisms in the feces, which can be a source of direct or indirect contamination of food and water leading to human STEC illnesses (4). Therefore, it is of interest to determine whether cattle harbor serogroup O104 strains and whether the strains carry virulence genes characteristic of the hybrid (STEC and EAEC) strain.

In order to detect serogroup O104 isolates with STEC and/or EAEC traits, we designed and validated a multiplex PCR (mPCR) targeting the following 8 genes: stx_1 (Shiga toxin 1), stx_2, *terD* (tellurite resistance), *eae*, wzx_{O104} (O104-specific O-antigen flippase), $fliC_{H4}$ (H4-specific flagellum), *ehxA*, and *aggA* (pilin subunit of aggregative adherence fimbria 1 [AAF/1]; 2). Our objectives were to use the mPCR assay to screen feedlot cattle feces to detect the presence of serogroup O104 and then isolate and characterize *E. coli* O104 from PCR-positive fecal samples. Primers for the wzx_{O104} (F-GGTTTTATTGTCGCGCAAAG and R-TATG CTCTTTTTCCCCATCG), $fliC_{H4}$ (F-ACGGCTGCTGATGGT ACAG and R-CGGCATCCAGTGCTTTTAAC) and *aggA* (F-CG TTACAAATGATTGTCCTGTTACTAT and R-ACCTGTTCCCC ATAACCAGAC) genes were designed with Primer3 software (http://frodo.wi.mit.edu). The primers for *terD* were designed according to Bielaszewska et al. (2), and the primers for stx_1, stx_2, *eae*, and *ehxA* were designed according to Bai et al. (6). The PCR program was 94°C for 5 min, 25 cycles for pure culture DNA or 35 cycles for fecal DNA of 94°C for 30 s, annealing at 65°C for 30 s, and 68°C for 75 s. The final extension step was 68°C for 7 min. The specificity of each primer pair for amplification of the eight genes was validated individually with the DNA of a strain of O104:H4 (ATCC BAA-2326) involved in the 2011 German outbreak, a strain of O104:H21 (BA-178) involved in the outbreak of hemorrhagic colitis in Montana in 1994 (7), a strain (17-2; provided by Weiping Zhang, South Dakota State University) of EAEC, and a strain of *E. coli* O157:H7 (ATCC 43894). When tested individually, primers amplified only single bands corresponding to the expected size of each amplicon: 655 bp for stx_1, 477 bp for stx_2, 434 bp for *terD*, 375 bp for *eae*, 337 bp for wzx_{O104}, 244 bp for $fliC_{H4}$, 199 bp for *ehxA*, and 151 bp for *aggA*. When primers were combined into a single reaction mixture and assay conditions were optimized, 8 distinct bands of the expected amplicons were detected with the pooled DNA of serotypes O104:H4 and O157:H7 (Fig. 1). The EAEC strain was positive for the *aggA* gene only. The mPCR assay did not show amplification of wzx_{O104}, $fliC_{H4}$, or *aggA* genes in any of the 274 strains of STEC, non-STEC, and other related bacteria that were tested (data not shown). All STEC strains used for validation of the assay were positive for at least one of the *stx* genes. The PCR assay sensitivity for detection was determined with a pure culture of O104:H4 and cattle fecal samples spiked with pure culture. The minimum concentration of the pure culture of *E. coli* O104:H4 that amplified the five expected genes (wzx_{O104}, $fliC_{H4}$, stx_2, *terD*, and *aggA*) was 1.5×10^4 CFU/ml. In fecal samples spiked with serially diluted (10-fold) concentrations of O104:H4, the sensitivity of detection was 1.5×10^5 CFU/g (150 CFU per PCR mixture). However, the inclusion of an enrichment step (incubation at 40°C for 6 h in *Escherichia coli* broth [EC broth; Oxoid Ltd., Hampshire, United Kingdom]) improved the sensitivity to 1.5×10^2 CFU/g of feces, which was similar to the sensitivity of detection by mPCR of other STEC (8–11).

The mPCR assay was then used to detect O104 serogroup isolates in cattle feces. A total of 248 fecal samples were collected based on a convenience sample of eight feedlots located in the Midwest. In 7 feedlots, 24 fresh pen floor fecal samples were collected from 10 different pens (2 to 3 samples per pen). From the eighth feedlot, a total of 80 fecal samples were collected from 18 different pens (4 or 5 samples per pen). One gram of feces was enriched in 9.0 ml of EC broth (Oxoid Ltd.), and DNA was extracted (8) from the pre- and postenrichment samples and subjected to the mPCR assay. Sample-level

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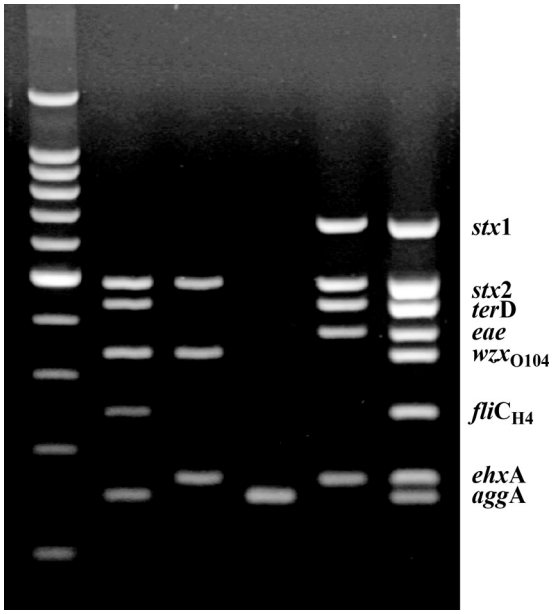

FIG 1 Agarose gel image of amplicons obtained from a multiplex PCR performed with two strains of *Escherichia coli* O104 (German [2nd lane] and Montana [3rd lane] strains), a strain (17-2) of enteroaggregative *E. coli* (4th lane), a strain of *E. coli* O157:H7 (ATCC strain 43894; 5th lane), and pooled DNA of O104 and O157 strains (6th lane). The 1st lane is the molecular size markers (100 bp).

crude prevalence estimates were calculated based on the overall proportions of samples that tested positive for each gene. Associations between the presence of the serogroup O104-specific gene (wzx_{O104}) and the stx_1, stx_2, *eae*, or $fliC_{H4}$ gene within enriched fecal samples were analyzed in generalized linear mixed models specified with a binomial distribution and logit link function. Random effects were used to account for the hierarchical structure of the data (samples within pens and pens within feedlots). Odds ratios and confidence intervals are reported. Due to the small numbers of samples positive by culture-based methods, only descriptive statistics are provided for these data.

Before enrichment, 3 (1.1%), and after enrichment in EC broth, 51 of the 248 samples (20.6%) were positive for the O104 serogroup-specific gene (Table 1). None of the 248 fecal samples was positive for the *aggA* gene. The *aggA* gene, one of four genes (*aggA* to *aggD*) in a cluster, encodes AAF/1, which is required for the phenotypic expression of the aggregative adhesion pattern (12). *aggA* was chosen because it is more conserved than the other genes in the cluster, including the master regulator gene of the AAF operon, *aggR*, which is typical of EAEC. The AAF adhesins are responsible for the characteristic "stacked brick" aggregative adherence of EAEC demonstrated on Hep-II cells, a human cell line (13). The absence of *aggA* in cattle feces was not surprising, because the EAEC pathotype is generally considered a human diarrheal pathogen (14). However, *E. coli* strains displaying the aggregative adherence pattern have been isolated from different animal species, including calves with diarrhea (15, 16). The EAEC strains of animal origin were classified as atypical EAEC because they lacked *aggR* and aggregative adherence fimbrial genes (16). Forty-one fecal samples (16.5%) contained the three genes wzx_{O104}, $fliC_{H4}$, and stx_1 or stx_2 (Table 1). An obvious limitation of an mPCR that detects serogroup, flagellar type, and virulence genes in a fecal sample is that it does not indicate that the flagellar gene or virulence genes are associated with any particular serogroup. Therefore, our estimation of the crude prevalence of O104 strains in feces of cattle is based entirely on the detection of the gene that codes for the O antigen of O104 strains. *E. coli* of serogroup O104 with H4 flagellar type, which may be an STEC (positive for Shiga toxins and negative for enteroaggregative adhesins and heat-stable enterotoxin) or EAEC (positive for enteroaggregative adhesins and heat-stable enterotoxin and negative for Shiga toxins) has been reported rarely to cause human infections (2, 5, 17–19). Interestingly, the serotype O104:H4, either Shiga toxigenic or enteroaggregative, has never been reported in animals or food (20). However, O104 strains with no H antigen (nonmotile) or an H antigen other than H4 (e.g., H7, H11, H12, H21, etc.) have been reported in cattle feces (20, 21). Wieler et al. (22) tested 2,000 *E. coli* strains isolated from 100 fecal samples from cattle housed in farms located in the outbreak region of Germany with a multiplex PCR designed to detect rfb_{O104}, stx_2, *terD*, and $fliC_{H4}$. None of the strains showed the combination of four genes characteristic of the outbreak strain, which led the authors to conclude that, in contrast to findings for other STEC, cattle were not a reservoir for the O104:H4 serotype. Auvray et al. (23) tested feces from a total of 1,468 French cattle for fecal carriage of O104:H4 by PCR assay targeting wzx_{O104}, stx_2, $fliC_{H4}$, and *aggR* and reported that 21.7% of cattle tested were positive for wzx_{O104} and none of the fecal samples contained the four genes together. Because the full combination of four genes typical of the German outbreak strain was not detected in any cattle feces, the authors concluded that French cattle are not a reservoir of the hybrid pathotype. However, a small proportion (6.1%) of fecal samples contained the three genes wzx_{O104}, stx_2, and $fliC_{H4}$. In our study, we found that 15.3% of fecal samples harbored the combination of wzx_{O104}, $fliC_{H4}$, and stx_2 genes, which does not necessarily mean that stx_2 and $fliC_{H4}$ were carried by O104 strains, as the genes could have been carried separately by distinct serotypes. The presence of wzx_{O104} in enriched fecal samples was positively associated with the presence of $fliC_{H4}$ (odds ratio [OR] of 11.8 with confidence interval [CI] of 1.9 to 71.4; $P < 0.01$) and *eae* (OR of 5.0 with CI of 1.3 to 19.2; $P = 0.02$). However, there was no significant association between the presence of wzx_{O104} and either of the Shiga toxin genes.

Fecal samples ($n = 51$) that were positive for *E. coli* O104 by

TABLE 1 Number of cattle fecal samples positive for genes that encode *Escherichia coli* O104 serogroup-specific traits before and after enrichment[a]

Gene(s) (protein or function)	No. (%) of samples ($n = 248$) positive for gene(s):	
	Before enrichment	After enrichment
wzx_{O104} (O104 antigen flippase)	3 (1.2)	51 (20.6)
$fliC_{H4}$ (H4 flagellar antigen)	103 (41.5)	214 (86.3)
stx_1 (Shiga toxin 1)	37 (14.9)	144 (58.1)
stx_2 (Shiga toxin 2)	100 (40.3)	188 (75.8)
eae (intimin)	92 (37.1)	204 (82.3)
*ehx*A (enterohemolysin)	210 (84.5)	243 (98.0)
terD (tellurite resistance)	119 (48.0)	233 (94.0)
aggA (aggregative adherence fimbria 1)	0	0
wzx_{O104} + $fliC_{H4}$	2 (0.8)	47 (19.0)
wzx_{O104} + $fliC_{H4}$ + stx_1	2 (0.8)	30 (12.1)
wzx_{O104} + $fliC_{H4}$ + stx_2	2 (0.8)	38 (15.3)
wzx_{O104} + $fliC_{H4}$ + stx_1 or stx_2	2 (0.8)	41 (16.5)

[a] Fecal samples were enriched by incubating 1 g of feces in 9 ml of EC broth (Oxoid) at 40°C for 6 h.

TABLE 2 Occurrence of *E. coli* O104 serogroup-specific genes[a] in pooled colonies isolated from cattle fecal samples that were positive for wzx_{O104}

Culture medium for isolation	No. of O104-positive fecal samples[b]	Colony color of pure culture of *E. coli* O104:H4	No. of pooled colonies[c] positive for:							
			wzx_{O104}	$fliC_{H4}$	stx_1	stx_2	*eae*	*ehxA*	*terD*	*aggA*
MacConkey agar	51	Pink	2	13	1	2	2	7	15	0
Rainbow agar	51	Blue-purple centers with purple edges	3	8	2	2	4	10	12	0
Non-O157 STEC differential agar[d]	42	Dark purple	0	10	1	5	7	8	29	0
CHROMagar STEC	42	Light purple	1	19	2	13	11	16	31	0
CHROMagar STEC O104	16	Light purple	4	1	3	4	10	11	15	0

[a] Pooled colonies were tested for serogroup O104 (wzx_{O104}), H_4 flagellar antigen ($fliC_{H4}$), Shiga toxin (stx_1 and stx_2), intimin (*eae*), enterohemolysin (*ehxA*), tellurite resistance (*terD*), and enteroaggregative (*aggA*) genes.
[b] Number of fecal samples that were positive for the wzx_{O104} gene.
[c] Ten colonies from each plate exhibiting the indicated color were pooled and tested by multiplex PCR for the indicated genes.
[d] Medium described by Possé et al. (24).

mPCR were streaked onto MacConkey agar (BD, Sparks, MD), Rainbow agar (Biolog, Inc., Hayward, CA), non-O157 STEC differential agar (24), CHROMagar STEC (CHROMagar Microbiology, Paris, France, distributed by DRG International, Mountainside, NJ), and CHROMagar STEC with O104 supplement (CHROMagar Microbiology) plates. The proprietary supplement, probably containing cephalosporin, was designed specifically to allow the growth of organisms with the extended-spectrum beta-lactamase phenotype, a characteristic feature of the German outbreak strain. All plates were incubated at 37°C for 24 h, and 10 presumptive colonies per plate (based on colony appearance and the color of pure culture O104:H4 on the same medium; Table 2) were picked and streaked on to blood agar plates. A single colony from each of the 10 isolates of each sample was suspended individually in 1 ml of distilled water, and 100-μl suspensions of each of the 10 colonies of a sample were pooled and subjected to the mPCR assay for the 8 genes. If the DNA from the pooled colonies amplified the wzx_{O104} gene, then each of the 10 colonies was tested individually by the mPCR to identify the pure culture of serogroup O104. Of the 51 fecal samples, only 10 isolates were positive for the O104 serogroup-specific gene (wzx_{O104}). None of the 10 isolates was positive for Shiga toxin genes, *eae*, or *aggA*. Of the 10 isolates, two isolates on three occasions were from the same fecal sample on two different agars, and therefore, only seven isolates were considered to be from distinct fecal samples. Five of the 7 isolates possessed *terD* and *ehxA*, while the other two isolates did not possess any of the other 4 genes. The seven isolates were submitted to the *E. coli* Reference Laboratory at Pennsylvania State University for serogroup confirmation. Of the seven isolates, five were confirmed as O104 with H7 flagellar type and two were identified as O8:H11 and O8:H21 by the *E. coli* Reference Center. The H7 flagellar type of the five O104 isolates was confirmed with the primers designed to identify $fliC_{H7}$ of *E. coli* O157 (6). The flagellar types (H11 and H21) of the two O104/O8 isolates were confirmed by PCR assays (25, 26). A PCR described by Wang et al. (27), designed for O8/O9 strains (F-GGCATCGGTCGGTATTCC and R-TGCGCTAATCGCGTCTAC), was performed on the seven isolates. The two isolates identified as O8 by the *E. coli* Reference Center yielded positive bands (1,000 bp), and the other 5 isolates were negative (Fig. 2B). We then retested *E. coli* O8 isolates (n = 19) that were in our culture collection and two O9 strains, obtained from the *E. coli* Reference Center, with the primers designed for the wzx_{O104} gene, and none of the strains yielded a positive band (337 bp) (Fig. 2A). The oligosaccharide unit of the O antigen of serogroup O104 has a structure identical to that of the *E. coli* K9 capsular antigen, and the gene cluster that codes for

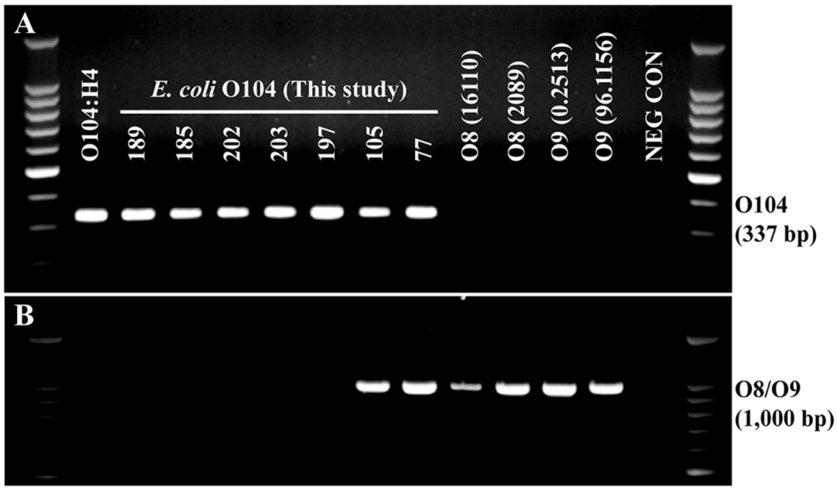

FIG 2 Agarose gel images of amplicons obtained from PCR with primers designed for *Escherichia coli* O104 (A) and O8/O9 (B). The 1st and 15th lanes are molecular size markers (100 bp), the 2nd lane is O104:H4 (German strain), the 3rd to 7th lanes are O104 strains isolated in this study, the 8th and 9th lanes are O104/O8/O9 strains isolated in this study, the 10th and 11th lanes are O8 strains, the 12th and 13th lanes are O9 strains, and the 14th lane is the negative control. Numbers in parentheses indicate the size of the bands.

O104 has the same genes in the same order as the K9 gene cluster (27). The K9 antigen is generally present in strains of *E. coli* serogroups O8, O9, and O9a (28). Published PCR assays designed to detect O104 also were shown to detect K9-positive O8/O9 *E. coli* (11, 27, 29).

In conclusion, the 8-gene PCR assay will be useful to confirm putative isolates of serogroup O104 and determine the presence of major traits that are characteristic of STEC and EAEC pathotypes. Additionally, the assay could be used to screen fecal samples for the prevalence of serogroup O104 before subjecting the samples to culture-based detection and isolation. Although mPCR detected 21% of fecal samples as positive for serogroup O104, isolates were only recovered by culture-based procedure from a few (7/51; 13.7%) of the PCR-positive samples. Culture-based detection may have been limited by lack of selectivity in the isolation procedure, and further research to refine culture methods is needed. Because none of the fecal samples contained *aggA*, the enteroaggregative gene, and none of the strains isolated carried $fliC_{H4}$, *aggA*, *or stx* genes, cattle do not appear to be a likely reservoir for *E. coli* O104:H4 with characteristics of STEC and EAEC. Further research is needed to determine the predominant pathotype (STEC, EAEC, or nonpathogenic) of serogroup O104 strains that are shed in cattle feces.

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