AEM Accepts, published online ahead of print on 29 March 2013
Appl. Environ. Microbiol. doi:10.1128/AEM.00246-13

Running Title: *E. coli* O104 in cattle feces

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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Key Words: *E. coli* O104, Shiga toxin-producing *E. coli,* Enteroaggregative *E. coli,* Multiplex PCR, Cattle, Feces

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[†]Contribution no. 13-196-J from the Kansas Agricultural Experiment Station.

A multiplex PCR was designed to detect *E. 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 the O104 serogroup. The isolates of O104 did not carry genes characteristic of the virulent hybrid strain.

In the summer of 2011, a large outbreak of food-borne illness caused by a serotype of Shiga toxin-producing *Escherichia coli* (STEC) 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 gene for Shiga toxin 2 (*stx*2), lacked genes for intimin (*eae*) and enterhemolysin (*ehx*A), and possessed an operon with genes (*aggA, aafA, agg3A,* and *agg4A*) that code for aggregative adherence fimbrial adhesins I, II, and III (AAF/I-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, and whether the strains carry virulence genes characteristic of the hybrid (STEC and EAEC) strain.

In order to detect serogroup O104 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, *ter*D (tellurite-resistance), *eae*, wzx_{O104} (O104 specific O-antigen flippase), $fliC_{H4}$ (H4 specific flagella), *ehx*A and *agg*A (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 the serogroup O104, and then isolate and characterize *E. coli* O104 from PCR-positive fecal samples. Primers for wzx_{O104} (F-GGTTTTATTGTCGCGCAAAG and R-TATGCTCTTTTTCCCCATCG), $fliC_{H4}$ (F-ACGGCTGCTGATGGTACAG and R-

CGGCATCCAGTGCTTTTAAC) and *agg*A (F- CGTTACAAATGATTGTCCTGTTACTAT and R- ACCTGTTCCCCATAACCAGAC) genes were designed with Primer3 software (Version 0.4.0; 17). The primers for *ter*D were according to Bielaszewska et al., (2) and for *stx*1, *stx*2, *eae* and *ehx*A were according to Bai et al. (5). The PCR program was: 94°C for 5 min, 25 cycles for pure culture DNA or 35 cycles for fecal DNA, 94°C for 30 sec, and 65°C annealing for 30 sec and 68°C for 75 sec. The final extension step was a 68°C for 7 min. The specificity of each primer pair for amplifications 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 (ATCC 172801) involved in the outbreak of hemorrhagic colitis in Montana in 1994 (6), a strain (17-2; provided by Dr. Weiping Zhang, South Dakota State University) of EAEC and *E. coli* O157:H7 (ATCC 43894). When tested individually, primers amplified only single bands corresponding to the expected sizes 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 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 amplifications of wzx_{O104}, $fliC_{H4}$ or *agg*A 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 pure culture of O104:H4 and cattle fecal sample 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 x 10^4 CFU/ml. In fecal samples spiked with serially diluted (10-fold) concentrations of O104:H4,

the sensitivity of detection was 1.5 x 10^5 CFU/g (150 CFU per PCR reaction). However, inclusion of an enrichment step (incubation at 40°C for 6 h in *Escherichia coli* broth [EC*;* Oxoid Ltd., Hampshire, UK]) improved the sensitivity to 1.5 x 10^2 CFU/g of feces, which was similar to the sensitivity of detection by mPCR of other STEC (7-10).

The mPCR assay was then used to detect O104 serogroup 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 (2-3 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 (7) from the pre- and post-enrichment samples and subjected to the mPCR assay. Sample-level crude prevalence estimates were calculated based on the overall proportions of samples that tested positive for each gene. Associations between presence of the serogroup O104-specific gene (wzy_{O104}) and *stx*1, *stx*2, *eae* or $fliC_{H4}$ genes 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 for type 1 aggregative adherence fimbriae (AAF/1) required for the phenotypic expression of the aggregative adhesion pattern (11). The *aggA* was chosen because it is more

conserved than the other genes in the cluster, including the master regulator gene *aggR* of the AAF operon, 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 (12). The absence of the *aggA* in cattle feces was not surprising because EAEC pathotype is generally considered as a human diarrheal pathogen (13). However, *E. coli* strains displaying aggregative adherence pattern have been isolated from different animal species, including calves with diarrhea (14, 15). The EAEC strains of animal origin were classified as atypical EAEC because they lacked *aggR* and aggregative adherence fimbrial genes (15). Forty-one fecal samples (16.5%) contained the three genes, wzx_{O104}, $fliC_{H4}$, and *stx*1 or *stx*2 (Table 2). An obvious limitation of a 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 in feces of cattle is based entirely on the detection of the gene that codes for the O antigen of O104. 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, 16-19). Interestingly, the serotype of O104:H4, either Shiga toxigenic or enteroaggregative, has never been reported in animals or food (20). However, strains of O104 with no H antigen (non motile) or different from 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_{104}, *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 cattle, in contrast to the other STEC, 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*$_{104}$, *stx*2, *fliC*$_{H4}$, and *aggR* and reported that 21.7% of cattle tested was positive for *wzx*$_{104}$ 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*$_{104}$, *stx*2, and *fliC*$_{H4}$. In our study, we found 15.3% of fecal samples harbored the combination of *wzx*$_{104}$, *fliC*$_{H4}$ and *stx*2 genes, which does not necessarily mean that *stx*2, and *fliC*$_{H4}$ were carried by O104 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 mPCR were streaked on to 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 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 color of pure culture O104:H4 on the same media; Table 2) were picked and streaked on to

blood agar plates. A single colony from each of the ten isolates of each sample was suspended individually in 1 ml of distilled water and 100 µl suspensions of each of the ten colonies of a sample were pooled together and subjected to the mPCR assay for the 8 genes. If the DNA from the pooled colonies amplified the wzx_{104} gene, then each of the ten colonies was tested individually by the mPCR to identify the pure culture of the serogroup O104. Of the 51 fecal samples, only 10 isolates were positive for the O104 serogroup-specific gene (wzx_{104}). 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, therefore, only seven isolates were considered to be from distinct fecal samples. Five of the 7 isolates possessed *ter*D 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 (5). 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 (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 (n=19) that were in our culture collection and two O9 strains, obtained from the *E. coli* Reference Center, with the primers designed for wzx_{O104} gene, and none of the strains yielded a positive band (337 bp; Fig. 2A). The oligosaccharide unit of the serogroup O104 has the identical structure as the *E. coli* K9 capsular antigen and the gene cluster that codes for O104 has

159 the same genes in the same order as K9 gene cluster (27). The K9 antigen is generally present in
160 strains of *E. coli* serogroups O8, O9, and O9a (28). Published PCR assays designed to detect
161 O104 also were shown to detect the K9 positive O8/O9 *E. coli* (10, 27, 29).

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

176 The research was supported in part by Agriculture and Food Research Initiative Competitive
177 Grant no. 2012-68003-30155 from the USDA National Institute of Food and Agriculture.
178 Authors thank Ashley Smith for collecting fecal samples from the feedlots and Neil Wallace for
179 help in the laboratory.
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TABLE 1. Number (and percentage) of cattle fecal samples positive for genes that encode for *Escherichia coli* O104 serogroup-specific traits before and after enrichment in *Escherichia coli* broth[a]

Genes (encoded protein or function)	No. of samples (n=248) positive	
	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 (97.8)
terD (tellurite resistance)	119 (48.0)	233 (94.0)
aggA (aggregative adherence fimbriae 1)	0	0
wzx_{O104+}*fli*C_{H4}	2 (0.8)	47 (20.0)
wzx_{O104+}*fli*C_{H4} + *stx*1	2 (0.8)	30 (12.1)
wzx_{O104+}*fli*C_{H4} + *stx*2	2 (0.8)	38 (15.3)
wzx_{O104+}*fli*C_{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 *Escherichia coli* broth at 40°C for 6 h.

TABLE 2. Occurrence of serogroup 104 (wzx_{104}), H_4 flagellar antigen ($fliC_{H4}$), Shiga toxins (*stx*1 and *stx*2), intimin (*eae*), enterohemolysin (*ehx*A), tellurite resistance (*ter*D), and enteroaggregative (*agg*A) genes in pooled colonies isolated from cattle fecal samples that were positive for wzx_{104}.

Culture medium for isolation	No. of fecal samples[a]	Colony color of pure culture of *E. coli* O104:H4[b]	Number of pooled colonies positive for:							
			wzx_{O104}	$fliC_{H4}$	*stx*1	*stx*2	*eae*	*ehx*A	*ter*D	*agg*A
MacConkey agar	51	Pink colored	2	13	1	2	2	7	15	0
Rainbow agar	51	Blue-purple centered colonies with purple edges	3	8	2	2	4	10	12	0
Non-O157 STEC Differential agar[c]	42	Dark purple colonies	0	10	1	5	7	8	29	0
CHROMagar™ STEC	42	Light purple colonies	1	19	2	13	11	16	31	0
CHROMagar™ STEC O104	16	Light purple colonies	4	1	3	4	10	11	15	0

[a]Number of fecal samples that were positive for wzx_{104} gene.
[b]Ten colonies from each plate exhibiting the indicated color were pooled together and tested by multiplex PCR for the indicated genes.
[c]Medium described by Possé et al. (24).

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

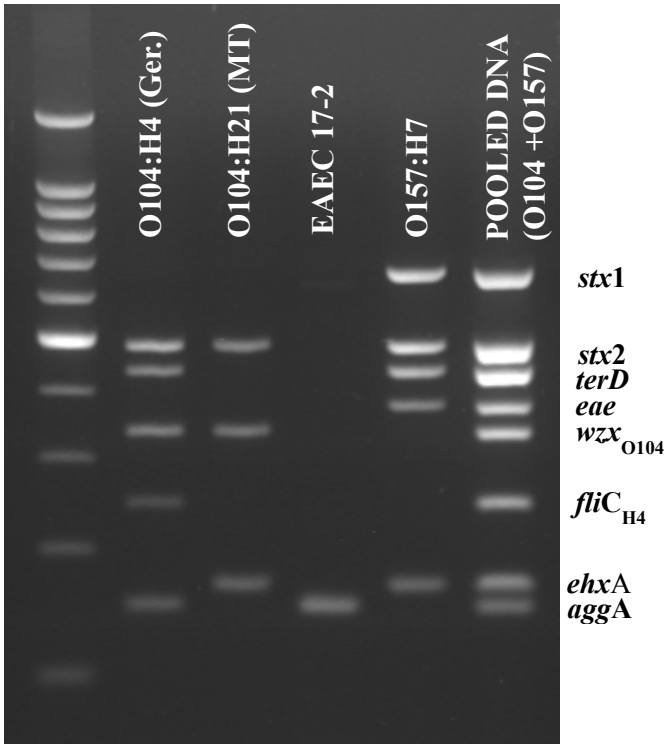
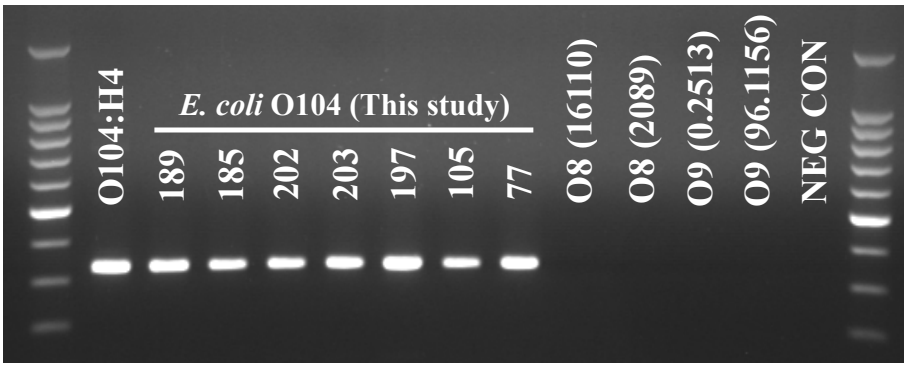


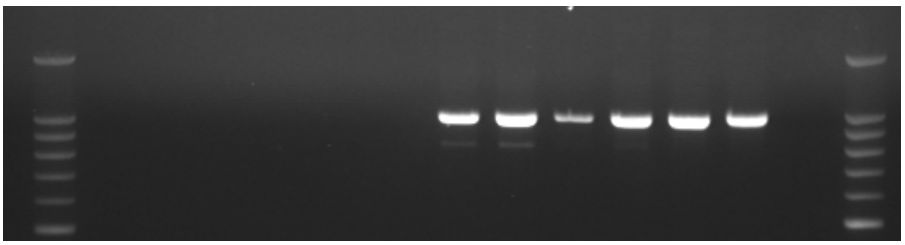
FIG 2. Agarose gel images of amplicons obtained from PCR with primers designed for *Escherichia coli* O104 (A) and O8/O9 (B). Lanes 1 and 15 are molecular size markers (100 bp), lane 2 is O104:H4 (German strain), lanes 3 to 7 are O104 strains isolated in this study, lanes 8 and 9 are strains O104/O8/O9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

A



O104 (337 bp)

B



O8/O9 (1,000 bp)