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# Four Strains of *Escherichia coli* O157:H7 Isolated from Patients during an Outbreak of Disease Associated with Ground Beef: Importance of Evaluating Multiple Colonies from an Outbreak-Associated Product

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**This report describes the investigation of a ground-beef-associated outbreak that involved five genetically distinct patient strains of *Escherichia coli* O157:H7. Human and product isolates were evaluated by pulsed-field gel electrophoresis (PFGE) with two endonucleases. The multiple-strain etiology of this outbreak underscores the importance of isolating and evaluating multiple colonies from outbreak-related products and comparing two endonuclease PFGE patterns of all product and human isolates identified during outbreak periods. This investigation emphasizes the importance of interviewing all confirmed and suspected case patients during the outbreak period, regardless of the PFGE pattern of their isolate, to confirm or rule out an epidemiologic link to the outbreak.**

During November and December 2000, a multistate outbreak of *Escherichia coli* O157:H7 infections was detected by the Minnesota Department of Health (MDH). An MDH case control study identified the association of ground beef consumption with illness. As a result of the case control study, on 4 December 2000 a Green Bay, Wisconsin, plant voluntarily recalled 1.1 million pounds of ground beef that was produced on November 2 and 3 and distributed to 15 states, including Wisconsin. This report describes the findings of an investigation conducted by the Wisconsin Division of Public Health (WDPH) and partners.

The WDPH issued a press release on December 5 describing the outbreak and listing stores in Wisconsin where the recalled ground beef had been distributed. The press release included information on signs and symptoms of *E. coli* O157:H7 infection and the recommendation for symptomatic individuals to seek medical evaluation, including submission of a stool specimen for pathogen isolation. Wisconsin local health departments with stores in their jurisdiction that had received the recalled ground beef made twice-weekly inquiries through December to clinics and hospitals caring for residents in their county. The role of the local health departments was to report possible *E. coli* O157:H7 infections to the WDPH and to forward all *E. coli* O157:H7 isolates to the Wisconsin State Laboratory of Hygiene (WSLH) for pulsed-field gel electrophoresis (PFGE) analysis. WDPH staff conducted telephone interviews of all individuals with confirmed or suspected *E. coli* O157:H7 infection with illness onsets during October through December of 2000. The standard WDPH questionnaire included items regarding demographic information, signs and symptoms of illness, and a food history for the 96-h period prior to illness onset. Questions regarding potential *E. coli* O157:H7 risk exposures that included living on or visiting a dairy farm, consuming unpasteurized milk or milk products, and exposure to another individual with diagnosed *E. coli* O157:H7 infection during the 10 days prior to illness onset were also included. Patients with confirmed or suspect cases were asked about their usual grocery stores, dates and locations of specific ground beef purchases during the month before onset of illness, and whether they had any remaining ground beef available for testing. A case was defined as a Wisconsin resident with laboratory-confirmed *E. coli* O157:H7 infection who had consumed ground beef purchased from a grocery store that received recall-associated product beef and subsequently experienced onset of illness during November or December 2000. Suspect packages of ground beef were defined as those produced by the Green Bay plant during 1 November to 3 November 2000 that were purchased in a retail establishment receiving lots of meat included in U.S. Department of Agriculture Food Safety Inspection Service Class I recall number 74-00. Intact packages of suspect ground beef that were available in homes of Wisconsin laboratory-confirmed case patients were collected by local health department staff and were forwarded to the Wisconsin Department of Agriculture, Trade, and Consumer Protection for isolation of pathogens.

The standard method for isolating *E. coli* O157:H7 from food products was based on the O157VIP/Reveal/Dynabeads/Culture method (8, 13, 14). PFGE was conducted at the WSLH for human and product *E. coli* O157:H7 isolates by using standard Centers for Disease Control and Prevention (CDC) PulseNet methods (7). Briefly, a single colony was subcultured to a blood agar plate and incubated overnight at 37°C. Sufficient growth was then suspended in cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8) and adjusted to an optical density of 1.3 to 1.4 at 610 nm. A 330-μl volume of this suspension was mixed with 15 μl of Proteinase K (20 mg/ml;

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TABLE 1. Demographic characteristics of Wisconsin cases and the PFGE patterns and strain designations of patient and meat *E. coli* O157:H7 isolates, November to December 2000

| Patient or isolate no. | Age (yr) | Gender | Date of illness onset (mo/day/yr) | Hospitalized | HUS | Isolate PFGE pattern determined, by *Xba*I/*Bln*I restrictions | *E. coli* O157:H7 strain designation |
|---|---|---|---|---|---|---|---|
| 1 | 5 | Male | 11/15/2000 | No (seen in emergency room) | No | A/A | 1 |
| 2 | 68 | Female | 11/21/2000 | Yes | No | A/A | 1 |
| 3 | 3 | Male | 11/22/2000 | No | No | A/A | 1 |
| 4 | 58 | Female | 11/28/2000 | Yes | No | A/A | 1 |
| 5 | 25 | Male | 12/7/2000 | Yes | No | A/A | 1 |
| 6 | 47 | Female | 12/10/2000 | Yes | No | A/A | 1 |
| 7 | 57 | Female | 11/19/2000 | Yes | No | A/B | 2 |
| 8 | 84 | Female | 11/23/2000 | Yes | Yes | B/B | 3 |
| 9 | 9 | Male | 11/29/2000 | No | No | B/C | 4 |
| Meat isolate 1 | | | | | | A/A | 1 |
| Meat isolate 2 | | | | | | C/B | 5 |

Sigma) and 300 μl of 1.2% SeaKem Gold Agarose (Bio Whittaker Molecular Applications, Rockland, Maine) in TE buffer (10 mM Tris:1 mM EDTA, pH 8) containing 1% sodium dodecyl sulfate. The agarose was held until use at 55°C in a water bath after being dissolved. This mixture was then pipetted into reusable plug molds (Bio-Rad, Hercules, Calif.). After the plugs solidified, they were transferred to 5 ml of lysis buffer (50 mM Tris:50 mM EDTA, pH 8, and 1% Sarcosine) with 25 μl of Proteinase K. Plugs were lysed at 55°C with shaking at 120 rpm for 2 h. The lysis buffer was then removed, and the plugs were washed two times in 15 ml of distilled water and four more times with TE buffer. All washes were at 55°C with shaking. Separate 2-mm-thick slices of each plug were then restricted with *Xba*I (New England Biolabs, Inc., Beverly, Mass.) and *Bln*I (Roche Molecular Biochemicals, Indianapolis, Ind.) at 35°C for 2 h. Although the standard PulseNet method specifies using 50 U of *Xba*I and 25 U of *Bln*I per 200 μl of reaction buffer, 70 U of *Xba*I and 40 U of *Bln*I per 200 μl were used to avoid the occurrence of partial restriction products (ghost bands). An isolate of *E. coli*, CDC strain G5244, was included as a reference standard on every gel. *E. coli* G5244 was restricted only with *Xba*I and was included in lanes 1, 5, 10, and 15 of every gel for normalization to the software reference standard. Electrophoresis of the samples was performed with a contour-clamped homogeneous electric field mapper (Bio-Rad). The gel was made of 1% SeaKem Gold agarose, and the running buffer was 2.2 liters of standard 0.5× Tris-borate-EDTA. Electrophoresis conditions were as follows: switch times of 2.16 and 54.17 s; angle of 120°; gradient of 6 V/cm; temperature of 14°C; linear ramping factor; and a run time of 20 to 21 h. Gels were then stained for 30 min in 400 ml of distilled water containing 40 μl of a 1% ethidium bromide solution and were destained by being washed three times for 30 min each in 1 liter of distilled water. Gel images were captured with a Gel Doc 2000 gel documentation system (Bio-Rad), and band patterns were analyzed and compared by using BioNumerics software (Applied Maths, Kortrijk, Belgium). In accordance with the CDC PulseNet standard procedure, we used the band-based Dice similarity coefficient and the unweighted pairs geometric matched analysis dendrogram type with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% for band comparison. All test isolates were normalized to the known molecular size bands of the *E. coli* G5244 standard strain. Isolates were considered indistinguishable only if their *Xba*I and *Bln*I band patterns were determined to be indistinguishable by using the BioNumerics software. A strain of *E. coli* O157:H7 was defined as an isolate that yields a specific PFGE band pattern combination when restricted with *Xba*I and *Bln*I.

During the investigation the WDPH received reports of 74 laboratory-confirmed and 9 suspected cases of *E. coli* O157:H7 infection in Wisconsin residents with onsets during October to December 2000. Following interviews of these patients with laboratory-confirmed or suspect cases, nine laboratory-confirmed cases were identified with a strong epidemiologic link to the outbreak. These patients consumed ground beef purchased from a grocery store that received recall-associated ground beef. The mean patient age was 40 years (range, 3 to 84 years); five were female and six were hospitalized, including one patient who developed hemolytic-uremic syndrome (HUS) (Table 1). The patients were residents of five Wisconsin counties and experienced illness onsets between 15 November and 10 December 2000. One patient had an intact 1-lb package of ground beef that had been purchased from a grocery store that had received recalled ground beef. Wisconsin Department of Agriculture, Trade, and Consumer Protection laboratory staff inoculated 5 g from this intact package of recalled ground beef into enrichment broth. Seven individual colonies were selected from a sorbitol-MacConkey (SMAC) plate streaked with the enrichment broth. Individual colonies were designated A through G, subcultured to new SMAC plates, and forwarded to the WSLH bacteriology laboratory for PFGE analysis. When restricted with *Xba*I and *Bln*I, six of the seven *E. coli* O157:H7 meat isolates were indistinguishable from each other and from the outbreak strain (strain 1, *Xba*I/*Bln*I pattern A/A) initially posted by the MDH on the CDC PulseNet electronic bulletin board. A second *E. coli* O157:H7 meat isolate (strain 5, *Xba*I/*Bln*I pattern C/B) was different from the six strain 1 meat isolates when restricted with *Xba*I and *Bln*I (Table 1).

Four different patient *E. coli* O157:H7 strains were identified during this investigation (Table 1). Six patient isolates were strain 1, the same as for the meat 1 isolate. Of the three remaining outbreak-associated patients, each had a different *E. coli* O157:H7 strain (strains 2 to 4) whose *Xba*I/*Bln*I restriction pattern combinations were distinguishable from each other and from that of *E. coli* O157:H7 strains 1 and 5. The *Xba*I and

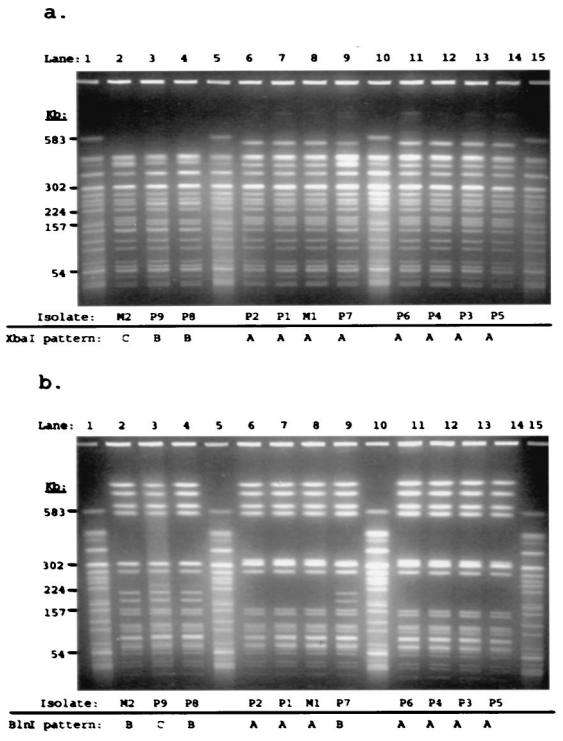


FIG. 1. *Xba*I (a) and *Bln*I (b) restriction profiles of meat 1 (M1), meat 2 (M2), and patient (P1 to P9) *E. coli* O157:H7 isolates. *E. coli* O157:H7 G5244 is the standard strain in lanes 1, 5, 10, and 15. The isolate designation (e.g., M2 in lane 2 of panel a) and the endonuclease pattern designation (A, B, or C) is indicated at the bottom of the gel under each lane.

*Bln*I restriction profiles of the nine patient and the two meat *E. coli* O157:H7 isolates are depicted in Fig. 1a and b, respectively. The Dice similarity index and number of band differences between the three *Xba*I (A to C) and the three *Bln*I (A to C) patterns of the nine patient and two meat isolates is summarized in Table 2. Of the three patient isolates with *Xba*I/*Bln*I combination patterns that varied from meat strain 1 (pattern A/A), only the isolates from patients 7 and 8 had a *Bln*I pattern that was indistinguishable from that of meat strain 2. The isolate from patient 7 had an *Xba*I pattern that matched that of meat strain 1 and a *Bln*I pattern that matched that of meat strain 2. The isolate from patient 8 had the same *Xba*I patterns as patient 9 but had a *Bln*I pattern that matched that of patient 7. The isolate from patient 9 had an *Xba*I pattern that differed from both meat *Xba*I patterns and also had a unique *Bln*I pattern. None of the patient isolates had a *Xba*I/*Bln*I combination pattern that matched that of meat strain 2.

Inclusion of a case patient as part of a food-borne outbreak has traditionally relied on the strength of the epidemiologic association and, when available, confirmatory laboratory documentation. While new molecular techniques allow sophisticated analysis of isolates associated with outbreaks, these technologies should not function as a substitute for epidemiologic information. The goal of molecular analysis should be to provide supporting laboratory evidence that epidemiologically related isolates collected during an outbreak also are genetically related and thus represent the same strain (12). *Xba*I is the PFGE screening endonuclease used by the CDC PulseNet system and the Wisconsin PFGE laboratory to identify clusters of *E. coli* O157:H7 infection. Because the *Xba*I or *Bln*I PFGE pattern of meat strain 2 and Wisconsin case patients 7 to 9 differed by one to three bands from the endonuclease patterns of meat strain 1, they were not initially considered part of the outbreak cluster. However, strong epidemiologic evidence links all nine case patients to the outbreak. Isolation of two distinguishable strains of *E. coli* O157:H7 from a single intact package of recalled ground beef supports the epidemiologic evidence that this was a multistrain *E. coli* O157:H7 outbreak.

The relatedness of *E. coli* O157:H7 isolates with differing PFGE banding patterns derived from analysis of a single enzyme restriction is an area of continuing disagreement. A single mutational genetic event can result in as many as three differences in PFGE banding patterns (12). Some investigators believe that isolates with fewer than three band differences, i.e., strains with one or two band shifts consistent with a single genetic event, should be considered clonally related and subtypes of each other, while strains that differed by three or more bands should be considered unique strains (11, 12). Others maintain that *E. coli* O157:H7 isolates with PFGE patterns that differ from the PFGE pattern of the outbreak strain by more than one band probably are not related (3). Existing guidance for interpreting DNA restriction patterns is based on the assumption that analysis by a single endonuclease is performed (12); in fact, most recently published outbreak investigations have employed a single endonuclease to link product and human isolates as being genetically related (1, 5, 9). In the present outbreak investigation, evaluation of isolates by a second endonuclease revealed interesting homologies between patient and meat isolate patterns that support the occurrence of a multistrain outbreak. The *Xba*I banding patterns of strains 2 to 5 do not fit the pattern expected for a point mutation of strain 1 that would have resulted in the creation or loss of a restriction site, nor do they fit the pattern for the insertion or deletion of DNA into an existing restriction site as described by Tenover and colleagues (12). The endonuclease pattern combination of strains 2 and 3 isolated from patients 7 and 8 are genetically distinct from those of strain 1 and are epidemiologically linked to patient and product outbreak-related *E. coli* O157:H7 isolates. Strain 4, isolated from patient 9, is epidemiologically linked to the outbreak and may represent a minor strain not recoverable from the single intact package of meat that was available for testing.

It is reasonable to expect that more than one strain of *E. coli*

TABLE 2. Dice similarity index and number of band differences between *Xba*I and *Bln*I endonuclease patterns of *E. coli* O157:H7 meat and patient isolates

| Comparison of patterns | % Similarity of patterns (no. of band differences) |
|---|---|
| *Xba*I | |
| A to B | 94 (2) |
| A to C | 97 (1) |
| B to C | 97 (1) |
| *Bln*I | |
| A to B | 91 (2) |
| A to C | 90 (3) |
| B to C | 97 (1) |

O157:H7 might be associated with an outbreak linked to consumption of ground beef. Because production lots of ground beef are comprised of meat from multiple cattle, if present in high enough numbers individual strains contributed by individual cattle could be recoverable from intact packages of ground beef. It has been noted that more than one *E. coli* O157:H7 PFGE subtype may be circulating on a particular farm at any one time (11). In a laboratory investigation of a multistate food-borne outbreak linked to the consumption of undercooked hamburger, Barrett and colleagues reported the recovery of three distinct *E. coli* O157:H7 isolates from three lots of ground beef produced in a plant during a 2-day period (3). The PFGE patterns differed from each other by at least three bands; one was linked to an outbreak in one state, the second isolate was associated with an outbreak in another state, and the third was not linked to a human outbreak. In a prolonged outbreak caused by raw milk, Keene and colleagues observed seven distinguishable PFGE patterns that differed by less than three bands among human and dairy herd *E. coli* O157:H7 isolates (10). Clonal variation in *E. coli* O157:H7 isolates being shed by cattle has also been reported. In a study of Wisconsin dairy cattle, Faith and coworkers observed up to three distinguishable but closely related PFGE patterns among multiple isolates from the same cow (6). Similarly, Akiba and colleagues have noted a shift in the PFGE pattern from a single cow (2). It has been reported that although multiple colonies isolated from a fecal enrichment broth from a single cow tends to produce the same PFGE pattern, multiple PFGE types are sometimes found (4). Louie and colleagues report that human disease may be associated with different subtypes of the same PFGE DNA and that PFGE subtypes of the same *E. coli* O157:H7 strain may be in existence on a farm where *E. coli* O157:H7 is endemic (11). The random nature of our colony selection process in this investigation, the limited number of intact meat specimens available for testing, and the probable loss of low levels of variant genotypes (*Xba*I/*Bln*I pattern types) in the meat specimen tested are three possible reasons why we were unable to detect all four patient specimen genotypes in the meat specimen tested.

Considering the potentially serious outcomes following *E. coli* O157:H7 infection and legal issues associated with *E. coli* O157:H7 outbreak investigations, completeness and accuracy is essential when defining all molecular patterns which may or may not be associated with a food-borne outbreak. During the present outbreak, if the investigators had not conducted a thorough epidemiologic interview of all *E. coli* O157:H7 cases during the outbreak period and had considered only the *Xba*I PFGE pattern initially posted on the PulseNet system they would have missed detecting 33% of Wisconsin's patients with cases related to this outbreak, including one who was hospitalized with HUS. In addition, if multiple colonies from the recalled-meat SMAC plate had not been analyzed, only the originally posted PFGE pattern (strain 1, pattern A/A) would have been identified in association with the outbreak. We strongly recommend that investigators evaluate multiple colonies from outbreak-related product and compare two endonuclease PFGE patterns of all product and human isolates identified during the outbreak period. We further emphasize the importance of interviewing all confirmed and suspected cases during an outbreak period, regardless of the PFGE pattern of the patient isolates, to confirm or rule out an epidemiologic link to the outbreak. Given what is known about genetic variation, clonal turnover, and shedding of multiple strains of *E. coli* O157:H7 by cattle and humans, an evaluation of similar rather than exact PFGE matches in conjunction with epidemiologic data during future outbreak investigations may provide a more comprehensive understanding of *E. coli* O157:H7 strains involved in food-borne outbreaks.

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