Serodiagnosis Using Microagglutination Assay during the Food-Poisoning Outbreak in Japan Caused by Consumption of Raw Beef Contaminated with Enterohemorrhagic Escherichia coli O111 and O157

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A microagglutination (MA) assay to identify antibodies to Escherichia coli O111 and O157 was conducted in sera collected from 60 patients during a food-poisoning outbreak affecting 181 patients in Japan which was caused by the consumption of contaminated raw beef. Enterohemorrhagic E. coli (EHEC) O111:H8 and/or O157:H7 was isolated from the stools of some of the patients, but the total rate of positivity for antibodies to O111 (45/60, 75.0%) was significantly higher than that for antibodies to O157 (10/60, 16.7%). The MA titers of antibodies to O111 measured in patients with hemolytic-uremic syndrome and bloody diarrhea were higher than those measured in patients with only diarrhea. In patients from whose stool no isolates of E. coli O111 and O157 were obtained, the positive antibody detection rates were 12/19 (63.2%) for O111 and 2/19 (10.5%) for O157, and the MA titers of antibodies to O111 measured were higher than those to O157. Similarly, the MA titers of antibodies to O111 were significantly higher than those to O157, regardless of the other groups, including groups O111, O111 and O157, and O157. These serodiagnosis results suggest that EHEC O111:H8 stx2 played a primary role in the pathogenesis of this outbreak. Furthermore, our findings suggest that the isolates from the patients’ stool specimens were not always the major causative pathogen in patients with multiple EHEC infections, because the sera from patients from whose stools only O157 was isolated were positive for antibodies to O111. Measuring antibodies to E. coli O antigen is helpful especially in cases with multiple EHEC infections, even with a non-O157 serotype.

Enterohemorrhagic Escherichia coli (EHEC) strains cause a variety of human illnesses, such as uncomplicated diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome (HUS), and related acute encephalopathy. In 1983, Karmali et al. first presented data revealing a possible etiologic role of EHEC in HUS (1). The correlation between HUS and EHEC O157 infection has been widely reported and has supported the conclusion that EHEC is a causative pathogen (2–5). Conversely, HUS has less frequently been reported in outbreaks caused by non-O157 EHEC (i.e., EHEC O104) (6–8). On the other hand, HUS patients without EHEC isolates have frequently been described (3, 4, 9, 10). Detecting EHEC in the stools of patients administered antibiotics long after the onset of diarrhea can be challenging because the organisms are typically isolated only from the diarrhea stool tested soon after onset (9, 11–14). Therefore, alternative laboratory diagnostic methods have been used, such as the detection of Shiga toxin (Stx) protein in the patients’ stools or the detection of antibodies against Stx (15) or lipopolysaccharides (LPSs) of E. coli O antigens in the sera of the patients (16–18). The presence of antibodies to the LPSs of E. coli O antigen in the sera of patients with HUS was first reported by Chart et al. (3) in 1991. Thereafter, antibodies to the LPSs of E. coli O antigen have been measured using methods such as enzyme-linked immunosorbent assay (ELISA) (2, 3, 9, 10), indirect hemagglutination assay (IHA) (4), and passive hemagglutination assay (PHA) (19). In Japan, a direct agglutination test has been used to detect antibodies to E. coli O antigens (20, 21) after mixing a patient’s serum with heat-inactivated whole bacterial cells that were used as the antigen. Therefore, this assay has been called the bacterial agglutination test (21) or the microagglutination (MA) assay using microplates (22). In Japan, this serodiagnostic test is typically performed in research laboratories (23) but not in clinical laboratories because the assay is time-consuming. Nevertheless, the assay is listed under the Infectious Diseases Control Law in Japan as one of the methods for the diagnosis of EHEC infection. In the case of patients with only HUS, their sera are used for detecting antibodies to O antigens of EHEC.

Japan had 2,900 to 4,600 cases of EHEC infection per year from 2001 to 2010 and approximately 100 HUS cases per year from 2006 to 2010 (23). Moreover, outbreaks of food poisoning caused by the consumption of raw beef and the liver of cows contaminated with EHEC are increasing (24). The serogroups of EHEC that have been detected are, from the most to the least frequent, O157, O26, O103, and O111 (23, 24).

In April and May 2011, an outbreak of food poisoning caused by EHEC O111:H8 and O157:H7 occurred in Toyama, Fukui, Ishikawa, and Kanagawa Prefectures in Japan. The Toyama pre-
government finally reported that 181 patients, including 21 patients with acute encephalopathy and 5 deaths among 34 patients with HUS, were affected by the outbreak that occurred at 6 out of 20 restaurants of a barbecue restaurant chain. The causative food was epidemiologically determined to be a raw beef dish called *yukhoe*. In this outbreak, EHEC O111:H8 strains with stx1 (O111 stx1, stx2) and O157:H7 strains with stx1, stx2, or stx1 and stx2 (O157 stx1 and stx2) were isolated from the stool specimens of the patients. Furthermore, *E. coli* O111:H8 strains without stx (O111 stx-negative strains) were also isolated from 52 patients. This O111 stx-negative strain is a non-Stx-producing *E. coli* strain that has a genetic background identical to that of the O111 stx1 strain, as determined by using pulsed-field gel electrophoresis (PFGE) analyses and multilocus variable-number tandem-repeat analysis (MLVA) (M. Watahiki, J. Isobe, K. Kimata, T. Shima, J. Kanatani, A. Nagata, K. Kawakami, M. Yamada, H. Izumiya, S. Iyoda, T. Morita-Ishihara, J. Mitobe, J. Terajima, M. Ohnishi, and T. Sata, unpublished data). Various combinations of strains exhibiting serotypes or toxin types of the EHEC and O111 stx-negative strains were isolated from stool specimens from the patients. However, none of the *E. coli* strains was isolated from 102 patients, including 14 HUS patients, and, consequently, the local public health center delayed starting a precise survey and declaring that a food-poisoning outbreak had occurred in order to prevent the infection from spreading further.

In this study, we measured the antibodies to the O antigens of *E. coli* O111 and O157 in the sera of patients with and without any isolates of EHEC or *E. coli* by using the MA assay, and we observed the antibody response to O111 and/or O157 in the patients. We studied the relationships of these results to the symptoms and isolates of the patients, and we also evaluated the usefulness of serodiagnosis in examining the antibody response of the host and in determining the major pathogen in cases with multiple EHEC infections in this outbreak.

**MATERIALS AND METHODS**

**Food-poisoning outbreak in Toyama Prefecture.** In the food-poisoning outbreak in 2011, 175 of 181 (96.7%) patients were from Toyama Prefecture, including 31 of 34 HUS patients (91.2%) and 4 of the 5 deaths that occurred in this prefecture. The 175 patients in Toyama corresponded to the following case definition for outbreak-related illness: the patients had consumed *yukhoe* at the implicated restaurants from 17 April through 25 April 2011 and developed one or more gastrointestinal tract symptoms and signs or HUS or had culture-confirmed infection with isolates of EHEC O111 stx1 and/or O157 stx1 and stx2. The age distribution of the 175 patients was 1 to 70 years (mean, 25.5 years; 90 men and 85 women), and 156 patients (89.1%) had consumed *yukhoe*. E. coli and acute encephalopathy accounted for the 4 deaths. The 31 HUS patients were aged 1 to 63 years (mean, 20.5 years; 11 men and 20 women). The EHEC strains isolated from the stools of the patients included 89 O111 strains consisting of 37 O111 stx1, and 52 O111 stx-negative strains and 57 O157 strains consisting of 9 O157 stx1, 24 O157 stx2, and 24 O157 stx1 and stx2 strains.

**Serum specimens.** The 280 serum specimens used in this study were collected from 60 of the 175 patients and were obtained from 13 hospitals. These 60 patients were aged 0 to 15 years (13 patients), 16 to 60 years (45 patients), and >61 years (2 patients). Among the samples from these patients, 17 serum specimens were collected as single specimens from 17 patients, whereas the other serum specimens were collected as multiple specimens from 43 patients (2 to 20 serum specimens per patient); specifically, 2 to 19 serum samples were obtained from HUS patients. Among the 60 patients, 28 developed HUS (HUS patients), 13 did not develop HUS but had bloody diarrhea (bloody diarrhea patients), and 19 had only diarrhea (diarrhea patients). Furthermore, the 60 patients were divided into the following groups according to the serogroup profiles of the E. coli strains isolated from their stool specimens (Table 1): 19 patients with neither EHEC nor stx-negative *E. coli* O111 (group A), 21 patients with only O111 stx1 and O111 stx-negative strains (group B), 15 patients with O111 stx1, O111 stx-negative, and O157 stx1 and stx2 strains (group C), and 5 patients with only O157 strains (group D). The patients with O111 stx2 and/or O111 stx-negative strains were included in the same group because, as mentioned in the introduction, the O111 stx-negative strains had a genetic background identical to that of the O111 stx1 strains according to PFGE and MLVA analysis (data not shown). We examined 49 serum samples stocked in our laboratory as negative controls; these serum samples were comparable to the serum samples from the patients involved in this outbreak. As positive controls, we used immune rabbit serum with antibodies specific to each of the O antigens of the *E. coli* strains (O1, O18, O26, O111, and O157; Denka Seiken, Tokyo, Japan).

**Bacterial strains used for antigen preparation.** Antigens were prepared from the original *E. coli* isolates obtained during this outbreak: O111:H8 stx2 (isolate E165) and O157:H7 stx1 and stx2 (isolate E045-1). In addition, we used other isolates stocked in our laboratory: *E. coli* O26:H11 (isolate EC3273), *E. coli* O1:H6 (isolate EC3061), and *E. coli* O18:H7 (isolate EC2922). *E. coli* O1, O18, and O26 strains were also used as controls because *E. coli* O1 and O18 strains are frequently isolated from healthy Japanese people and *E. coli* O26 is the second major serogroup of EHEC identified in Japan (23).

**MA assays were performed as described by Vuddhakul et al.** (20) with minor modifications. The bacterial strains were grown on Trypticase soy
agaru (Becton, Dickinson and Company, Le Pont de Clai, France) at 35°C for 18 h. The cells were suspended in saline solution and autoclaved at 121°C for 1 h. The cells were then centrifuged at 2,000 × g for 15 min, the supernatants were discarded, and the cell pellets were resuspended in 5 ml of saline. After washing twice with saline, the cells were centrifuged at 200 × g for 5 min, and the supernatants were mixed with equal volumes of 2% formalin–saline and then incubated at 35°C for 1 h. The suspensions were again washed using saline and finally adjusted with saline to a 3 to 4 McFarland standard by using a turbidity meter (Densimat; Sysmex-biometerieux, Marcy l’Etoile, France). The suspensions were subsequently used as E. coli O antigens.

Determination of titers of antibodies to O antigen of E. coli. The test sera were diluted 1:10 using sterile saline, inactivated by heating at 56°C for 30 min, and then centrifuged at 10,000 g for 1 min. Next, 25-μl aliquots of the test sera were diluted 2-fold by mixing with 25 μl of sterile saline in 96-well V-shaped microtiter trays (BM Equipment, Tokyo, Japan). The highest dilution giving a clear agglutination pattern was considered the endpoint. The MA antibody titers were recorded as the reciprocal of the endpoint dilution of the test sera, yielding final serum dilutions ranging from 1:20 to 1:40,960. Lastly, to confirm the immunoglobulin class of the antibodies identified using the MA assay, the positive sera from the patients were treated with 2-mercaptoethanol (2ME; Wako Pure Chemical Industries, Tokyo, Japan) as described previously (19, 25).

Statistical analysis. We investigated the relationship between the antibodies to E. coli O antigens in the sera and the serogroup types of the E. coli strains isolated from patients’ stools by using the HALBAU program (version 7.0). The level of significance was set at 0.05. The rates of positivity for serum antibodies to O111 and O157 strains were compared by using Fisher’s exact test. The serum antibody titers were common logarithmic transformations, and titers of less than 10 were defined as 2, the minimal detectable value. Statistical significance was determined by using U tests (for 2 groups) or Kruskal-Wallis tests (among 3 groups or more) and then applying the post hoc Bonferroni method.

Ethical considerations. This study was approved by the Ethical Review Board at the Toyama Institute of Health (approval no. 2 in 2012).

RESULTS

Specificity of MA assay. The rabbit immune sera with antibodies to the E. coli serogroups (O1, O18, O26, O111, or O157) reacted specifically with each of the E. coli O antigens prepared from original and laboratory isolates. No cross-reaction was observed (data not shown).

The MA titers of antibodies to E. coli serogroups O111, O157, and O26 for the 49 negative-control serum specimens are shown in Fig. 1a (Controls). The highest MA antibody titers in the negative-control sera were ≤1:80 for E. coli O111 and O157 and ≤1:40 for E. coli O26. The sera with MA antibody titers of <1:20 included 45 serum specimens (91.8%) with E. coli O111, 27 serum specimens (55.1%) with E. coli O157, and 40 serum specimens (81.6%) with E. coli O26. The MA titers of sera with antibodies to E. coli O1 and O18 were under 1:20 (data not shown). The sera with MA antibody titers of 1:20 to 1:40 were suggested to reflect potential EHEC infections in Japan (21). Collectively, MA antibody titers below 1:80 were considered negative results (Fig. 1a).

In this study, we also observed that the MA antibody titers of the patients’ sera became negative after the sera were treated with 2ME, which strongly suggested that the antibodies detected using the MA assay belonged to the IgM class of immunoglobulins, which is the same finding reported previously (19, 25).

Distribution of MA titers measured for antibodies to E. coli O antigen. The MA titers measured for antibodies to the E. coli O antigen in the sera of the 60 patients are shown in Fig. 1b (Patients). The MA titers determined for antibodies to E. coli O111, O157, and O26 were in the range of ≤1:20 to 1:10,240, and the titers for antibodies to O1 and O18 were under 1:20 (data not shown). For E. coli O111, the number of patients with MA antibody titers of ≥1:160 was 45 (75.9%). The MA antibody titers of the sera collected from HUS patients and bloody diarrhea patients were high; the median values of the MA antibody titers were 1:640 in the HUS patients and 1:320 in the bloody diarrhea patients. However, the MA antibody titers of 9 serum specimens from one patient were under 1:20; this patient was a 70-year-old woman, one of the deceased patients who had the O111 stx2 and O111 stx-negative isolates (group B in Table 1). This patient suffered from rheumatoid arthritis and had been receiving immunosuppressive drugs, and therefore, she might have been in an immunocompromised state. In contrast, for E. coli O157, the number of patients with MA antibody titers of ≥1:160 was 10 (16.7%). The highest MA antibody titer in sera obtained from these 10 patients with O157 infection was 1:320. In this study, we defined sera with MA antibody titers of ≥1:160 as being positive for E. coli O111 and O157 antibodies (Table 1). The mean interval of sampling of the sera from the date of onset was 6.9 days (range, 2 to 14 days) in 13 of 15 patients negative for antibodies to O111 and O157; data for 2 patients with ambiguous dates of onset were excluded.

Patients positive for antibodies to E. coli O111 and O157. We determined that the rate of positivity for antibodies to O111 (45/60, 75.0%) was significantly higher than that for antibodies to O157 (10/60, 16.7%) (Table 1; P < 0.001). The rates of positivity were significantly different among the patients belonging to groups A to C (P < 0.001) but not among those belonging to group D. In group A patients, from whose stool specimens no EHEC or E. coli O111:H8 stx-negative isolates were obtained, the rates of antibody positivity were 12/19 (63.2%) for antibodies to O111 and 2/19 (10.5%) for antibodies to O157. Among HUS patient in the same group, the rates of antibody positivity were 10/10 (100.0%) for antibodies to O111 and 2/10 (20.0%) for antibodies to O157. The rates of antibody positivity in the HUS and bloody diarrhea patients were higher than those in the diarrhea patients in group A. In group B patients, who had only E. coli O111:H8 isolates, the rates of antibody positivity were 18/21 (85.7%) for antibodies to O111 and 3/21 (9.5%) for antibodies to O157. In group C patients, with both E. coli O111:H8 and O157:H7 isolates, the rates of antibody positivity were 13/15 (86.7%) for antibodies to O111 and 5/15 (33.3%) for antibodies to O157. Among HUS patients in this group, the rates of antibody positivity were 8/8 (100%) for antibodies to O111 and 4/8 (50.0%) for antibodies to O157. Group D contained only 5 patients, with only E. coli O157:H7 isolates, but the rates of antibody positivity were 2/5 (40.0%) for antibodies to O111 and 0/5 for antibodies to O157. Furthermore, the rate of positivity for antibodies to O111 increased with severe symptoms, from diarrhea to bloody diarrhea or HUS.

Relationship between MA antibody titers and the symptoms of patients. The relationship between MA antibody titers and patients’ symptoms is presented in Fig. 2. The median values of the MA titers measured for antibodies to O111 were 1:1,280 in 28 HUS patients, 1:1,280 in 13 bloody diarrhea patients, and 1:40 in 19 diarrhea patients. The MA titers for antibodies to O111 among the 3 symptom categories of the patients were determined to be significantly different by using the Kruskal-Wallis test (P < 0.001). Moreover, applying the Bonferroni method revealed that the MA antibody titers were significantly different between the
HUS and diarrhea patients \((P < 0.01)\) and between the bloody diarrhea and diarrhea patients \((P < 0.01)\). However, no significant difference in the MA titers for antibodies to O111 was detected between the HUS and bloody diarrhea patients. Conversely, the MA titers for antibodies to O157 among the 3 symptom categories of the patients were determined to be significantly different by using the Kruskal-Wallis test \((P < 0.01)\), and applying the Bonferroni method revealed that the MA titers for antibodies to O157 were significantly different between the HUS and diarrhea patients \((P < 0.05)\).

**Relationship between MA antibody titers and isolates of the patients.** The relationship between MA antibody titers and the isolates obtained from the patients’ stool specimens is presented in Fig. 3. The MA titers measured for antibodies to O111 and O157 were significantly different in groups A, B, and C \((P < 0.01)\) but not in group D, although the MA titers for antibodies to O111 were higher than those for antibodies to O157 in group D. In group A patients, from whose stool specimens no *E. coli* O111 or O157 isolates were obtained, the median value of the MA titers for antibodies to O111 was slightly higher than that in patients in groups B and C (with *E. coli* O111:H8 and/or O157:H7 isolates). Furthermore, the MA titer for antibodies to O157 in group A was the highest among the 4 groups.

**DISCUSSION**

The remarkable features of the food-poisoning outbreak that occurred in Japan in 2011 are that 2 serotypes of EHEC O111:H8 and O157:H7 were isolated from certain patients, whereas no isolates of either EHEC O111:H8 or O157:H7 were obtained from samples of some patients with HUS, bloody diarrhea, or diarrhea. Only a few reports are available that have described outbreaks in which isolates of multiple serotypes of EHEC have been obtained from the stools of patients and that have also measured antibodies to the *E. coli* non-O157 serotype \((26)\). To the best of our knowledge, this study is the first to describe the measurement and evaluation of antibodies to the O antigen of *E. coli* O111 and O157 by using the MA assay.

In our serological study, the rate of positivity for antibodies to O111 \((75.0\%)\) was higher than that for antibodies to O157 \((16.7\%)\). The MA titers measured for antibodies to O111 were
significantly higher than those for antibodies to O157, regardless of the serogroups of *E. coli* isolated from the patients’ stool specimens. One key finding was that positivity for antibodies to O111 (40.0%) but not O157 was present in group D, which was composed of patients with the *E. coli* O157:H7 isolate only. Moreover, this characteristic of elevated antibody levels was also recognized, and the titers for antibodies to O111 were higher than those for antibodies to O157 in the patients from whose stool no *E. coli* isolates were obtained (group A). These results suggest that EHEC *O111:H8 stx*2 was the major pathogen in this outbreak. However, our findings do not exclude the possibility of a role of the O157 *stx*1 and *stx*2 isolates in the outbreak, because the MA antibody titers measured for O157 were significant in 10 patients, consisting of 8 HUS patients and 2 bloody diarrhea patients (Table 1 and Fig. 2). In this study, we also determined that the antibodies measured using the MA assay were IgM, because these antibodies lost their binding activity after treatment with 2ME. The antibodies detected are considered to have been elevated during this outbreak and were not from a past infection.

Previously, Paton et al. reported an outbreak caused by dry fermented sausages containing multiple serotypes of EHEC (26), and they detected antibodies to the LPSs (O antigens) of *E. coli* O111 and O157 by performing Western blotting. Paton and colleagues reported that the antibodies to *E. coli* O antigen detected in the patients’ sera were of the same serotype as those to the O antigen of *E. coli* isolated from the patients’ stool specimens (26).
In contrast, others have reported detecting antibodies to another type of E. coli O antigen which was distinct from the E. coli O antigen isolated from the patients’ stools (4, 10, 21). These studies reported that the antibodies may cross-react with pathogens of other serotypes (4) or that the serological test used might have been unsuitable for specifically detecting EHEC without O157 (10) and that the patients might have been concurrently infected with E. coli O157 and non-O157 strains (21). However, these studies investigated only a single patient in sporadic cases; they failed to evaluate the results for reaction of antibodies to the O antigen of E. coli. Our results showed that the antibodies to the E. coli O antigens in patients’ sera were not always consistent with those to the O antigens of E. coli isolated from the patients’ stools in the outbreak described here; this has not been reported in previous cases of EHEC infection.

In this outbreak, we found that no E. coli O111:H8 and O157:H7 isolates were obtained from the stool specimens of certain HUS patients and that certain patients had only isolates of O157, but their serum antibodies were determined to be positive only for E. coli O111. In the gastrointestinal tracts of these patients, both EHEC O111 and O157 were considered to contribute to the production of stx₂. Although EHEC O157 has previously been implicated in HUS, reports indicate that no patient was frequently detected in patients’ stools at the time of acute illness (4, 9, 10). In Japan, no causative pathogen has been detected in the stool specimens of approximately 30% of HUS patients (24, 25). Therefore, measuring antibodies by using the MA assay can facilitate the diagnosis of EHEC infection in patients from whose stool no E. coli isolates are obtained.

In conclusion, by using the MA assay to perform serodiagnosis, we have provided evidence that O111 stx₂ played a primary role in the pathogenesis of the food-poisoning outbreak in Japan described in this study. Furthermore, our results reveal that the isolates obtained from the stool specimens of patients were not always the major causative pathogen in multiple EHEC infections. Thus, antibodies to E. coli O antigens should be measured, especially in cases with EHEC infection, even with a non-O157 serotype.

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