**Short Communication**

**A Survey of Pathogenic Genes from Diarrhea Stool Samples Obtained in Nara Prefecture**

Takeshi Sakai*, Akifumi Nakayama, Kiyoshige Nakazawa and Shunsuke Imai

Division of Bacteriology, Nara Prefectural Institute for Hygiene and Environment, Nara 630-8131, Japan

(Received June 23, 2006. Accepted January 18, 2007)

**SUMMARY:** We conducted a survey of diarrhea stool samples in which no virulent agents had previously been detected at clinical laboratories. DNA extracted directly and purified from the diarrhea stool was tested for bacterial pathogenic genes by polymerase chain reaction. The test results for 85 specimens were as follows: one sample was positive for *lt*, *ipaH*, and *eae*; two were positive for *aggR*; and eight were positive for *astA*. Inoculation with the stool specimens led to the isolation of a strain of *Escherichia coli* possessing *eae*, three strains of *E. coli* possessing *astA*, and a strain of *Klebsiella* possessing *astA*.

We often experience cases in which no pathogenic organisms are isolated from the stool specimens of patients with diarrhea. There are several reasons for the failure to identify responsible pathogens in such cases, including the use of antibiotics and the presence of both pathogenic strains and nonpathogenic strains. Such cases prompted us to search for diarrheagenic bacteria by the direct detection of bacterial pathogenic genes from stool specimens. In this trial study, target genes were restricted to virulent genes related to *Escherichia coli*.

A survey of diarrhea stool samples in which no virulent agents had yet been detected in clinical laboratories was conducted from May 2004 to March 2005. From four hospitals in Nara Prefecture, a total of 95 refrigerated diarrhea stool specimens were submitted, from which no virulent bacteria except for EAST1 were isolated. It is therefore suspected that EAST1 gene-positive bacteria contributed to the diarrhea in those cases.

For use in polymerase chain reaction (PCR) tests, the DNAs were extracted from the specimens and purified using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). Ready-made primers (Takara Bio, Inc., Tokyo, Japan) were used to test the purified DNA for *vt-1*, *vt-2*, *lt*, *ipaH*, and *invE*. Using previously reported primers (1), the DNAs were also tested for *eae*, *bfpA*, *aggR*, and *astA*. The PCR-test-positive specimens were inoculated onto MacConkey agar (Eiken Chemical, Co., Ltd., Tokyo, Japan) and incubated at 37°C for 24 h. Approximately 10 colonies were selected from the incubated agar plates and were tested for the virulent genes. Strains possessing the same virulent genes as those identified in the stool specimens were tested for microbial identification with the Vitek system (bioMerieux SA, Marcy l’Etoile, France) using GNI+ cards.

Results from clinical laboratories clarified that virulent bacteria had been detected in 10 specimens. Therefore, 85 specimens were considered to be worthy of further analysis. Of the DNAs of those 85 specimens, none were positive for *vt-1*, *vt-2*, *invE*, or *bfpA*. One specimen was positive for each of *lt*, *ipaH*, and *eae*. In addition, two specimens were positive for *aggR*, and eight were positive for *astA*.

Inoculation with the stool specimens did not lead to the detection of any bacteria possessing *lt*, *ipaH*, or *aggR*. One strain of *E. coli* possessing *eae* was isolated. As regards the eight *astA*-positive stool specimens, no *astA*-positive bacteria were detected in three of them, but strains of *astA*-positive *E. coli* were isolated from four specimens, and a strain of *astA*-positive *Klebsiella* was isolated from one specimen. Additionally, the latter strain was identified as *Klebsiella planticola* using the API-20E biochemical system (bioMerieux SA) and other biochemical tests.

The sequences of the PCR products generated from the *Klebsiella* strain using the primer sets were examined for comparison with the *astA* gene, which is the open reading frame encoding enterotoaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), although, to the best of our knowledge, EAST1-producing *Klebsiella* strains have not been reported to date. The following PCR primer sets were used for sequencing: EAST1-1 and EAST1-2 reported by Osek (2), generating a 111-bp product; EAST12a and EAST12b, generating a 203-bp product; and EAST13a and EAST13b reported by Yamamoto and Echeverria (3), generating a 393-bp product. The three PCR products were analyzed directly for both DNA strands with Takara Bio. The determined nucleotide sequence generated with the primer set EAST13a and EAST13b is shown in Fig. 1, and is compared with the EAST1 gene sequence (GenBank accession no. SR1691) reported by Yamamoto and Echeverria (3). The nucleotide sequence on the PCR product corresponding to EAST1 was 86% (98/114) identical to the reported EAST1 gene sequence. In addition, the nucleotide sequence of the PCR product was 88% (344/393) homologous to the reported gene sequence. Nevertheless, the possibility for production of the peptide remained unclear, because the start codon ATG was replaced by ACG.

Although EAST1 has not been well accepted as a virulence factor, an outbreak of gastroenteritis in Osaka, Japan in 1996 was reportedly caused by *E. coli* that had a coding gene for EAST1. In their report, Nishikawa et al. suggested that EAST1E (a strain of *E. coli* that possesses no diarrheagenic characteristics except the EAST1 gene) plays important roles in sporadic diarrheal illness (4,5). In the present study, *astA* genes were detected from 9.4% (8/85) of the diarrhea stool specimens examined, from which no virulent bacteria except four strains of *E. coli* and one strain of *K. planticola* had been isolated. It is therefore suspected that EAST1 gene-possessing bacteria contributed to the diarrhea in those cases.

* Corresponding author: Mailing address: Division of Bacteriology, Nara Prefectural Institute for Hygiene and Environment, Ohmori-cho 57-6, Nara 630-8131, Japan. Tel.: +81-742-20-2888, Fax.: +81-742-27-0634, E-mail: tsakai@ihe.pref.nara.jp

---

The following results were obtained for the 10 stool specimens in which virulent bacteria had been detected in clinical laboratories: four samples were positive for *Campylobacter jejuni*, two were positive for *Shigella sonnei*; and the remaining four cases were positive for each of *Salmonella* sp., *Vibrio parahaemolyticus*, *enterohemorrhagic E. coli*, and *E. coli* serogroup O114, respectively. In the two *S. sonnei*-positive stool specimens, *ipaH* and *invE* were detected, whereas *vt*-1 and *vt*-2 were detected in the *enterohemorrhagic E. coli*-positive stool specimens, and *astA* was detected in the *Salmonella*-positive stool specimen; moreover, a strain of *Salmonella* possessing *astA* was isolated from the latter stool sample.

The strains of *Salmonella enterica* carrying phage-encoded EAST1 genes were investigated by Bacciu et al. (6) as bacteria other than *E. coli* possessing an *astA* gene.

Important clues can be obtained from PCR tests for bacterial pathogenic genes using DNAs extracted and purified from diarrhea stool samples. In tests of indigenous bacteria such as *E. coli*, this evidence is useful for detecting virulent strains among nonvirulent strains. In cases in which diarrheagenic bacteria are nonculturable, such clues are helpful for arriving at a differential diagnosis of the cause of diarrhea. Furthermore, direct PCR tests of diarrhea specimens for virulence genes may aid in the accumulation of evidence regarding genes that are not been well accepted as virulence factors, and may help characterize the dissemination of pathogenic genes among bacterial species.

**ACKNOWLEDGMENTS**

This work was supported by a grant from the Daido Life Welfare Foundation, Japan.

**REFERENCES**