JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1996, p. 977–979 Vol. 34, No. 4
0095-1137/96/$04.00+0

Indications of In Vivo Transfer of an Epidemic R Plasmid from *Salmonella enteritidis* to *Escherichia coli* of the Normal Human Gut Flora

EVANGELOS BALIS,[1] ALKIVIADIS C. VATOPOULOS,[1]* MARIA KANELOPOULOU,[2] EFSTRATIOS MAINAS,[1] GEORGE HATZOUDIS,[1] VASILIKI KONTOGIANNI,[1] HELEN MALAMOU-LADA,[2] SPIRIDULA KITSOU-KIRIAKOPOULOU,[3] AND VICTORIA KALAPOTHAKI[1]

Department of Hygiene and Epidemiology, Medical School, Athens University, 115 27 Athens (Goudi),[1] Department of Microbiology, Penteli Children's Hospital, Palea Penteli, 152 36 Athens,[2] and Department of Microbiology, Agia Olga General Hospital, N. Ionia, Athens,[3] Greece

Received 10 October 1995/Returned for modification 15 November 1995/Accepted 19 January 1996

The presence of conjugative R plasmids as well as the possible similarities among them were studied in nine ampicillin-resistant *Salmonella enteritidis* isolates and nine ampicillin-resistant *Escherichia coli* isolates from the normal fecal flora that were simultaneously isolated from nine epidemiologically unrelated outpatients. It was found that in eight patients, ampicillin resistance in *S. enteritidis* was encoded by ca. 34-MDa transferable plasmids very similar to those found in a recent study of the epidemiology of ampicillin-resistant *S. enteritidis* in Greece (A. C. Vatopoulos, E. Mainas, E. Balis, E. J. Threlfall, M. Kanelopoulou, V. Kalapothaki, H. Malamou-Lada, and N. J. Legakis, J. Clin. Microbiol. 32:1322–1325, 1994). Moreover, transferable R plasmids with the same molecular size and restriction pattern were found in the normal flora *E. coli* of two of these patients. This finding, if confirmed by further studies, is consistent with the hypothesis that normal flora *E. coli* could act as a reservoir of resistant genes and, consequently, as a factor in the dissemination of these genes among pathogens of human and animal origin such as *Salmonella* spp. and needs to be examined further.

During the last 6 years, a significant increase in the rate of isolation of ampicillin-resistant *Salmonella enteritidis* clinical strains has been noticed in Greece (27). In a recent study, this increase in resistance was found to be due to the spread of related 34-MDa R plasmids in a limited number of clones of *S. enteritidis* phage type 6a (27). However, the ultimate source of these *Salmonella* R plasmids and their possible relationship with resistance determinants of the normal human flora were not found.

In an attempt to study the role of the normal gut flora of humans as a potential intermediate in the flow of resistance genes among different genera of bacteria, the possible similarities among the R plasmids harbored by *S. enteritidis* and normal flora *Escherichia coli* strains isolated from individual patients were investigated. Nine outpatients from two Athens Hospitals (Penteli Children's Hospital and Agia Olga General Hospital), suffering from gastrointestinal illness caused by ampicillin-resistant *S. enteritidis* and consequently found to carry ampicillin-resistant *E. coli* in their feces, were selected for further study. All patients were epidemiologically unrelated and represented sporadic cases of food poisoning.

Ampicillin-resistant *Salmonella* and *E. coli* isolates were selected by direct plating of fecal samples on ampicillin-containing (10 μg/ml) MacConkey agar plates, and all isolates were identified by the API 20E test (API System, La Balme, France). The identification of *S. enteritidis* was confirmed by serotyping. For conjugation experiments, the *E. coli* K-12 strains 1R716 (Str^r), 20R764 (Rif^r), 26R793 (Rif^r), and 14R525 (Na^r) were used as recipients. *E. coli* 39R861, which harbors plasmids of 98, 42, 23.9, and 4.6 MDa, was used for the estimation of plasmid size (24). Antibiotic susceptibility was performed by a disk diffusion method on Mueller-Hinton agar (Oxoid Limited, Hampshire, United Kingdom) using the current recommendations of the National Committee for Clinical Laboratory Standards (1992) (15). Conjugation experiments were carried out in broth as previously described (27). Transconjugants were selected on MacConkey agar containing ampicillin (10 μg/ml) and, depending on the recipient, streptomycin (500 μg/ml) or rifampin (90 μg/ml) or nalidixic acid (40 μg/ml). Plasmid DNA was extracted by an alkaline lysis procedure described by Takahashi and Nagano (23). The isolated DNA was analyzed on 0.7% agarose gels, stained with ethidium bromide, and documented under UV illumination by the BIO-PROFIL (Vilber Lourmat, Marne La Valle, France) imaging analysis system.

Plasmid DNA was extracted from the transconjugants and digested with restriction endonuclease *Eco*RI (Boehringer Mannheim Biochemicals, Mannheim, Germany) according to the instructions of the manufacturer. The digests were subjected to electrophoresis through 0.8% agarose. *Hin*dIII digests of bacteriophage lambda provided linear molecular size markers. DNA-DNA hybridizations were performed with a nonradioactive DNA labelling and detection kit (DIG DNA Labelling and Detection Kit, Boehringer Mannheim Biochemicals) under high-stringency conditions as previously described (27). Plasmid pBR322 was used as the TEM-type β-lactamase probe (17).

Ampicillin resistance was easily transferred at high frequency to *E. coli* recipients from both *S. enteritidis* and fecal flora *E. coli* in the case of five patients, whereas in three patients ampicillin resistance was transferable only from *S. enteritidis* (Table 1). In the last patient, ampicillin resistance was not transferable from any isolate (Table 1). Susceptibility tests showed that the transconjugants from both *S. enteritidis*

* Corresponding author. Mailing address: Department of Hygiene and Epidemiology, Medical School, Athens University, 115 27 Athens (Goudi), Greece. Phone: 30-1-7771165. Fax: 30-1-7704225.

TABLE 1. Molecular sizes and antibiotic resistance phenotypes of plasmids transferred to *E. coli* recipients

Patient	Characteristics of transconjugants					
	S. enteritidis			Normal flora *E. coli*		
	Molecular size (MDa)	Phenotype[a]	RE pattern[b]	Molecular size (MDa)	Phenotype[a]	RE pattern[b]
1	34	AM	I	34	AM SXT TE	I
2	34	AM	I	34	AM	I
3	34	AM	I	34	AM	II
4	34	AM	I	60	AM	III
5	34	AM	I	60	AM	IV
6	34	AM	I	NT[c]		
7	34	AM	I	NT		
8	34	AM	I	NT		
9	NT			NT		

[a] AM, ampicillin resistant; SXT, cotrimoxasol resistant; TE, tetracycline resistant.
[b] Restriction enzyme pattern.
[c] NT, not transferred.

and *E. coli* from all patients were resistant only to ampicillin, except for one that originated from a fecal flora *E. coli* isolate, which was also resistant to cotrimoxasol and tetracyclines (Table 1).

Agarose gel electrophoresis of the transconjugants revealed that in all *S. enteritidis* transconjugants, ampicillin resistance was encoded by the ca. 34-MDa plasmid. Plasmids of the same molecular size were found in transconjugants from normal flora *E. coli* from three patients, whereas transconjugants from the same species from two patients carried ca. 60-MDa plasmids (Table 1). Plasmid fingerprinting through cleavage patterns generated after digestion with restriction endonuclease *Eco*RI showed that the 34-MDa R plasmids from all of the *S. enteritidis* transconjugants displayed extensive homology (Fig. 1). Moreover, very similar restriction patterns were displayed by two of the three transconjugants originating from the normal flora *E. coli* (Fig. 1). Interestingly, these plasmids seem to be very similar in their restriction pattern to the R plasmids isolated in our previous study from *S. enteritidis* clinical isolates in Greece (27) (Fig. 1). Hybridizations of the *Eco*RI restriction fragments of the 34-MDa plasmids with the TEM-type probe revealed the locus of the β-lactamase gene to be on a ca 6.6-MDa fragment in all *S. enteritidis* transconjugants and in the two similar *E. coli* transconjugants (Fig. 2).

Although antibiotic consumption is believed to be the main factor associated with the increase in antibiotic resistance in human fecal flora in both outpatients and hospitalized patients (12, 20, 21), animals could also be an important reservoir of resistant bacteria (14). Evidence that strains of animal origin can colonize the human gut has been demonstrated (13), and transmission of resistant *E. coli* from animals to humans has been shown to occur via the food chain (10, 11) or after direct contact with livestock (9, 16). Similarly, the spread of antibiotic-resistant *Salmonella* strains from animals to humans has previously been shown (8, 25).

In the present study, R plasmids with restriction patterns very similar to the ones responsible for ampicillin resistance in *S. enteritidis* in Greece (27) were found in the normal flora of two of the nine patients studied.

The mechanisms of dissemination of resistance genes in nature are complex, and direct evidence of in vivo transfer of resistance or of the direction of this transfer is not available in our study. Nevertheless, in cases where epidemiological association with a common exposure is absent, ampicillin resistance plasmids in the normal flora *E. coli* are expected to be different; ampicillin resistance in normal flora is found to be due mainly to the independent acquisition of the TEM-1 gene by different plasmids rather than to the presence of epidemic strains or plasmids (20). Moreover, a diversity of R plasmids encoding TEM-type β-lactamase is found in the fecal flora of children in Greece and a 34-MDa plasmid was not identified in any case (26). Similarly, many studies of the epidemiology of ampicillin resistance in clinical isolates identified a wide distribution of plasmids of many different types (1). In that respect, the demonstration of similar plasmids among normal flora *E. coli* and *Salmonella* isolates in epidemiologically unrelated patients, if confirmed by further studies, is consistent with the hypothesis of in vivo transfer of resistance genes among *Sal-*

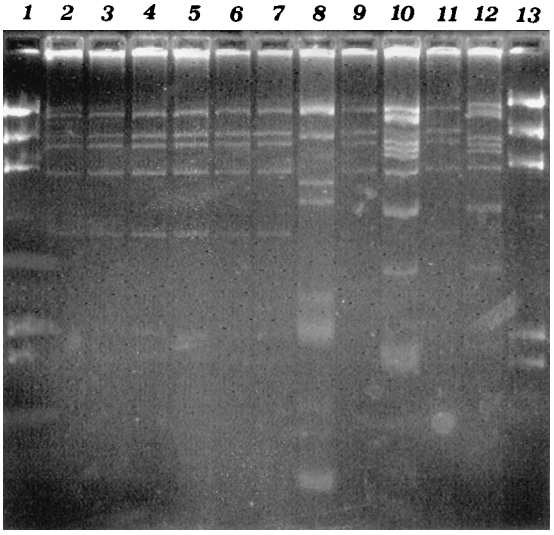

FIG. 1. *Eco*RI restriction enzyme patterns of the R plasmids isolated from *S. enteritidis* and normal flora *E. coli* transconjugants. Lanes: 1 and 13, DNA of bacteriophage λ digested with *Hin*dIII; 2, the 34-MDa plasmid from our previous study (27); 3, 5, 7, 9, and 11, restriction enzyme patterns of the 34-MDa plasmids from *Salmonella* transconjugants from patients 1, 2, 3, 4, and 5, respectively (Table 1); 4, 6, 8, 10, and 12, restriction enzyme patterns of the 34-MDa plasmids from the normal flora *E. coli* transconjugants from patients 1, 2, 3, 4, and 5, respectively (Table 1). It can be seen that the *Eco*RI restriction patterns of the 34-MDa plasmid from our previous study, all *Salmonella* transconjugants from the present study, and the transconjugants from the normal flora of patients 1 and 2 are identical (lanes 2, 3, 4, 5, 6, 7, 9, and 11).

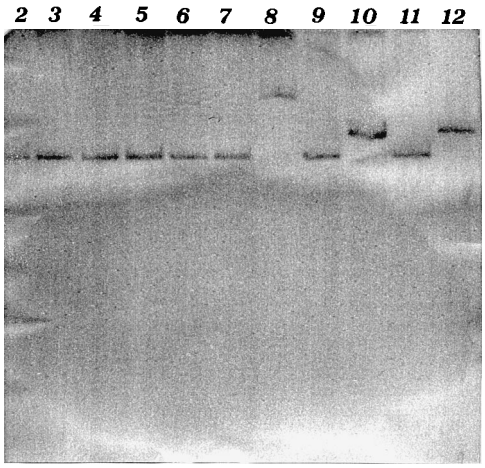

FIG. 2. Southern blots of the agarose gel shown in Fig. 1 (lanes 2 to 11) following hybridization with the digoxigenin-labelled TEM-type probe. Lanes: 2, the 34-MDa plasmid from our previous study (27); 3, 5, 7, 9, and 11, restriction enzyme patterns of the 34-MDa plasmids from *Salmonella* transconjugants from patients 1, 2, 3, 4, and 5, respectively (Table 1); 4, 6, 8, 10, and 12, restriction enzyme patterns of the 34-MDa plasmids from the normal flora *E. coli* transconjugants from patients 1, 2, 3, 4, and 5, respectively (Table 1).

monella spp. and normal flora *E. coli* and consequently of the possible role of normal flora as a reservoir of resistance genes.

Ampicillin-resistant *S. enteritidis* and *E. coli* were selected in this study by direct plating fecal samples on ampicillin-containing MacConkey agar plates, a fact that makes the possibility of plasmid transfer between *E. coli* and *S. enteritidis* occurring in vitro during the isolation in the laboratory rather unlikely (3).

The acquisition of transferable resistance plasmids by *Salmonella* spp. from other enteric bacteria of the gut flora in the intestinal tract of individual patients has previously been reported (2, 5, 18, 19). Nevertheless, Tacket et al. (22) could not find evidence of spread of the *S. enteritidis* R plasmids to the normal flora of patients or their family members. Plasmid exchange between clinical isolates of animal and human origin was observed by Chaslus-Dancla et al. (4). Our report demonstrates the presence of similar transferable R plasmids in normal flora *E. coli* and *S. enteritidis* isolated from epidemiologically unrelated patients. Data on possible recent antibiotic consumption by the patients studied are not available, but it is well known that taking antibiotics may provide a selective advantage for resistant *Salmonella* spp. (3, 7, 8).

The potential of normal flora bacteria to cause endogenous infections or to transfer their resistance determinants to other pathogens, as well as the fact that virulence or pathogenicity factors such as toxins or adhesion factors are known to be associated with antibiotic markers encoded by plasmids (6), illustrates the public health importance of the transfer of resistance genes among bacteria of animal and human origin and underlines the need for further study of this complex matter.

We thank J. Pournou and M. Zarodimitraki for their excellent technical assistance.

This study was supported in part by a grant from the Greek Ministry of Health and Social Services (Dy5a/5297/27.5.1994).

REFERENCES

1. **Amyes, S. G. B., D. J. Payne, and S. K. du Bois.** 1992. Plasmid mediated beta-lactamases responsible for penicillin and cephalosporin resistance. J. Med. Microbiol. **36:**6–9.
2. **Archambaud, M., G. Gerbaud, E. Labau, N. Marty, and P. Courvalin.** 1991. Possible in-vivo transfer of β-lactamase TEM-3 from *Klebsiella pneumoniae* to *Salmonella kedougou*. J. Antimicrob. Chemother. **27:**427–436.
3. **Bratoeva, M. P., and J. F. John, Jr.** 1994. In vivo R-plasmid transfer in a patient with mixed infection of shigella dysentery. Epidemiol. Infect. **112:** 247–252.
4. **Chaslus-Dancla, E., P. Pohl, M. Meurisse, M. Martin, and J. P. Lafont.** 1991. High genetic homology between plasmids of human and animal origins conferring resistance to the aminoglycosides gentamicin and apramycin. Antimicrob. Agents Chemother. **35:**590–593.
5. **Datta, N., H. Richards, and C. Datta.** 1981. *Salmonella typhi* in vivo acquires resistance to both chloramphenicol and co-trimoxazole. Lancet **i:**1181–1183.
6. **Gutmann, L., and G. S. Tillotson.** 1992. Is there any relationship between bacterial resistance and virulence? J. Med. Microbiol. **36:**20–22.
7. **Hefferman, H. M.** 1991. Antibiotic resistance among *Salmonella* from human and other sources in New Zealand. Epidemiol. Infect. **106:**17–23.
8. **Holmberg, S. D., M. T. Osterholm, K. A. Senger, and M. L. Cohen.** 1984. Drug resistant *Salmonella* from animals fed antimicrobials. N. Engl. J. Med. **311:**617–622.
9. **Hunter, J. E. B., J. C. Shelley, J. R. Walton, C. A. Hart, and M. Bennett.** 1992. Apramycin resistance plasmids in *Escherichia coli*: possible transfer to *Salmonella typhimurium* in calves. Epidemiol. Infect. **108:**271–278.
10. **Kapperud, G., L. M. Rorvik, V. Hasseltvedt, E. A. Hoiby, B. G. Iversen, K. Staveland, G. Johnsen, J. Leitao, H. Herikstad, Y. Andersson, G. Langeland, B. Gondrosen, and J. Lassen.** 1995. Outbreak of *Shigella sonnei* infection traced to imported iceberg lettuce. J. Clin. Microbiol. **33:**609–614.
11. **Levy, S. B.** 1984. Antibiotic-resistant bacteria in food of man and animals, p. 525–531. *In* M. Woodbine (ed.), Antimicrobials and agriculture. Butterworths, London.
12. **Levy, S. B., B. Marshall, B. Schluederberg, D. Rowse, and J. Davis.** 1988. High frequency of antimicrobial resistance in human fecal flora. Antimicrob. Agents Chemother. **32:**1801–1806.
13. **Linton, A. H.** 1977. Animal to man transmission of Enterobacteriaceae. Royal Soc. Health J. **97:**115–118.
14. **Linton, A. H.** 1986. Flow of resistance genes in the environment and from animals to man. J. Antimicrob. Chemother. **18**(Suppl. C)**:**189–197.
15. **National Committee for Clinical Laboratory Standards.** 1992. Performance standards for antimicrobial disc susceptibility tests. Publication no. M2A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
16. **Nijsten, R., N. London, A. Van Den Borgaard, and E. Stobberingh.** 1994. Resistance in faecal *Escherichia coli* isolated from pigfarmers and abattoir workers. Epidemiol. Infect. **113:**45–52.
17. **Paul, G. C., G. Gerbaud, A. Bure, A. M. Philippon, B. Pangon, and P. Courvalin.** 1989. TEM-4, a new plasmid-mediated β-lactamase that hydrolyzes broad-spectrum cephalosporins in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **33:**1958–1963.
18. **Platt, D. J., J. S. Chesman, and K. G. Kristinsson.** 1986. R-plasmid transfer in-vivo: a prospective study. J. Med. Microbiol. **21:**325–330.
19. **Schwalbe, R. S., C. W. Hoge, J. G. Morris, Jr., P. N. O'Hanlon, R. A. Crawford, and P. H. Gilligan.** 1990. In vivo selection for transmissible drug resistance in *Salmonella typhi* during antimicrobial therapy. Antimicrob. Agents Chemother. **34:**161–163.
20. **Shanahan, P. M. A., C. J. Thomson, and S. G. B. Amyes.** 1994. Beta-lactam resistance in aerobic faecal flora from general practice patients in the UK. Eur. J. Clin. Microbiol. Infect. Dis. **13:**760–763.
21. **Stark, C. A., C. Edlund, S. Sjostedt, G. Kristensen, and C. E. Nord.** 1993. Antimicrobial resistance in human oral and intestinal anaerobic microfloras. Antimicrob. Agents Chemother. **37:**1665–1669.
22. **Tacket, C. O., L. B. Dominguez, H. J. Fisher, and M. L. Cohen.** 1985. An outbreak of multiple-drug-resistant *Salmonella enteritidis* from raw milk. JAMA **253:**2058–2060.
23. **Takahashi, S., and Y. Nagano.** 1984. Rapid procedure for isolation of plasmid DNA and application to epidemiological analysis. J. Clin. Microbiol. **20:**608–613.
24. **Threlfall, E. J., B. Rowe, J. L. Ferguson, and L. R. Ward.** 1986. Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of *Salmonella typhimurium* phage type 204c isolated in Britain. J. Hyg. Camb. **97:**419–426.
25. **Threlfall, E. J., L. R. Ward, and B. Row.** 1986. R plasmids in *Salmonella typhimurium* in the United Kingdom. J. Antimicrob. Chemother. **18**(Suppl. C)**:**175–177.
26. **Vatopoulos, A. C.** 1995. Unpublished data.
27. **Vatopoulos, A. C., E. Mainas, E. Balis, E. J. Threlfall, M. Kanelopoulou, V. Kalapothaki, H. Malamou-Lada, and N. J. Legakis.** 1994. Molecular epidemiology of ampicillin-resistant clinical isolates of *Salmonella enteritidis*. J. Clin. Microbiol. **32:**1322–1325.