EFFECT OF ANTIBIOTICS ON THE PREVALENCE OF ENTEROTOXIGENIC *Escherichia coli* IN TWO POPULATIONS IN THE PHILIPPINES

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OBJECTIVE : To determine if the selective pressure of antibiotics increases the prevalence of enterotoxigenic *Escherichia coli*.

BACKGROUND : Enterotoxigenic *Escherichia coli* (tox+ *E. coli*) are a common cause of diarrheal disease in developing tropical countries (7, 15, 17). Genes controlling the production of heat-labile (LT), and heat-stable (ST) enterotoxins are located on plasmids designated *ent* (11, 20). In the Far East, these enteric pathogens are often resistant to multiple antibiotics, and eighty percent of 31 of these isolates transferred antibiotic resistance in bacterial mating experiments (8). In 35 percent of the matings transferring antibiotic resistance, the ability to produce enterotoxin was also transmitted to the recipients. Antibiotic selection increases the probability of detection of *in vivo* conjugation, and provides for selection of R plasmid-containing bacteria in the intestinal flora (1, 18). Smith and Linggood, among others, have suggested that antibiotics may also increase the prevalence of *E. coli* containing plasmids which contribute to enteropathogenicity (8, 10, 19-21).

A more direct effect of antibiotics on selection of *ent* has been proposed by several recent observations. In an experiment with an *E. coli* isolated from a pig with diarrhea, Gyles et al (12) transduced via P1 coliphage both antibiotic resistance and enterotoxin production implying the genes controlling both traits were located on the same plasmid. Ampicillin resistance has also been found to transpose onto an *ent* plasmid in an *E. coli* of human origin (13). If genes encoding for antibiotic resistance and enterotoxin production are frequently carried on the same plasmid, antibiotic selective pressure should increase the prevalence of tox+ *E. coli* in the gastrointestinal tract of man. To test these hypotheses *in vivo* the prevalence of LT+ST+ and LT+ST- *E. coli* in relation to antibiotic consumption was compared in two populations in the Philippines.

METHODS :

Study Population : One thousand and thirty different hostesses attending the social hygiene clinic for cervical cultures to detect *Neisseria gonorrhoea*, and 628 different individuals working in restaurants in Angeles City, the Republic of the Philippines were cultured for enteric pathogens.

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Seventy-two percent of the restaurant workers (RWs) were women of the same age as the hostesses who lived and worked in the same areas of Angeles City. Specimens were collected within a ten day period in May, 1979. All of the hostesses and RWs had formed stools when the cultures were obtained.

Collection of Specimens : Rectal cultures were inoculated into Cary-Blair transport media, stored at 4°C, and processed within two months. One or two milliliters of urine were frozen at -70°C, and examined for antibiotic activity within five weeks of collection.

Processing of Specimens : Specimens were cultured on MacConkey, Heckteon, and Desoxycholate media after overnight incubation at 37°C (DIFCO, Detroit, MI). A subculture of the APW was taken after six hours, and cultured on TCBS media. *Salmonella*, *Shigella*, and Vibrio species were identified by standard methods (5), employing the API 20E system, and with the use of commercial antisera (DIFCO, Detroit, MI).

Identification of Enterotoxigenic Isolates : Ten lactose-positive colonies, with the typical appearance of *E. coli*, were selected from the MacConkey media, stored on nutrient agar slant cultures, and tested within two months of isolation for the production of LT. Organisms that were LT+ were retested for LT and ST. No attempt was made to test for LT-ST+ *E. coli*. Bacteria to be screened for LT were inoculated into one milliliter of Trypticase soy broth (BBL, Cockeysville, MD) with 0.6% yeast extract (DIFCO, Detroit, MI), and incubated stationary for 48 hours at 37°C. Isolates tested for both LT and ST production were inoculated into five milliliters of similar media, and incubated in a tissue culture roller tube apparatus at eight revolutions per minute at 37°C for 24 hours. Supernatants were tested for LT and ST in the Y-1 adrenal cell (16), and suckling mouse assays simultaneously (6). All enterotoxigenic strains were identified with the API 20E system.

Antibiotic Sensitivity Testing : Antibiotic sensitivities of tox+ and five tox- *E. coli*, isolated from each subject, were determined by the agar dilution method employing Mueller-Hinton (M-H) agar and broth. The minimum inhibitory concentration for resistant organisms by agar dilution was >16 μg/ml for tetracycline (Tc), streptomycin (Sm), ampicillin (Ap), and kanamycin (Km), and >8 μg/ml for gentamicin (Gm), and chloramphenicol (Cm). Antibiotic sensitivity to neomycin Nm (30 μg), and sulfisoxazole Su (250 μg), were determined by the agar diffusion method (3). Twenty-four hour cultures of the isolates were adjusted to 85% transmission at 550 mμ in a Coleman, Jr. II spectrophotometer (equal to the turbidity of 0.5 McFarland barium sulfate turbidity standard (2)). *E. coli* ATCC 25922, sensitive to all antibiotics, *Pseudomonas aeriginosa* ATCC 27858, resistant to Ap, Cm, Km, and Nm, and a clinical isolate from the Philippines, *E. coli* 203, resistant to Ap, Cm, Gm, Km, Nm, Sm, Su, and Tc were included as controls when clinical isolates were tested for antimicrobial sensitivity.

Urine Testing for Antibiotic Activity : A multisensitive *Bacillus subtilis* ATCC 6633 was grown overnight in M-H broth, adjusted to 85% transmission, and spread on a M-H plate. Ten microliters of urine were placed onto the plate which was incubated at 37°C for 24 hours. Clear zones indicated the presence of antibiotics in the urine (4).

To test the sensitivity and specificity of the urine test, random urines were tested from laboratory personnel who had not received antibiotics in the previous two weeks. Urines were also collected from individuals six, 24, and 48 hours after they had ingested either 250 mg of penicillin, 250 mg of tetracycline, 250 mg of ampicillin, or 500 mg of sulfaguanidine.

RESULTS :

Specificity and Sensitivity of Urine Tests : None of 23 random urines produced clear zones on M-H agar plates inoculated with *B. subtilis*. Antibiotic activity was detected in all urines collected, six and 24 hours, but not 48 hours after the antibiotics had been ingested.

Study Populations : Twenty-eight percent of 1030 hostesses, and four percent of 628 RWs gave a history of taking antibiotics within a week of being cultured ($p<0.001$) (chi-square). Ten percent (14/628) of RWs had antibiotics detectable in their urine ($p<0.001$). Sixty-five of 103 hostesses (63%) with antibacterial activity in their urine denied prior ingestion of antibiotics, which implies antibiotic use was greater than reported. Eighty percent of the hostesses, who admitted taking prior antibiotics, received ampicillin or penicillin. Three of 603 RWs (<1%), from whom urine was available, denied previous therapy, but had antimicrobial activity in their urine suggesting RWs were, in fact, taking antibiotics infrequently. RWs received a variety of different antibiotics which included ampicillin, chloramphenicol, neomycin, sulfaguanicine amd tetracycline.

There was no significant difference in the isolation rate of *Salmonella*, *Shigella*, and *Vibrio parahaemolyticus* between hostesses and RWs (8/1030 vs 7/628) ($p>0.50$) LT+ST+ and LT+ST- *E. coli* were isolated from 12/1030 (1.2%) hostesses, and 11/628 (1.7%) RWs ($p>0.40$). None of the individuals infected with this pathogen had antibiotics detectable in their urine. One hostess and one RW were infected with LT+ST+ *E. coli*. The remaining tox+ *E. coli* identified produced LT alone.

The percent of antibiotic resistance among tox- *E. coli* (five isolates/subject) isolated from cultures of 300 hostesses, selected in proportion to the total number cultured at the clinic on a specific day, and 136 random RWs (29 and 22 percent of the population respectively) are listed in Table 1. Forty-eight percent (722/1500) of tox- *E. coli* isolated from hostesses vs 33 percent (226/680) from RWs were resistant to two or more antibiotics ($p<0.01$). Fifty-seven percent (28/49) of tox+ *E. coli* isolated from hostesses and 32 percent (8/25) isolated from RWs were resistant to two or more antibiotics ($p<0.10$).

This study is complete.

Table 1. Antibiotic Resistance among Enterotoxigenic and Non-enterotoxigenic *Escherichia coli* isolated in Angeles City, Republic of the Philippines

Population	Percent Resistance							
	Ap	Cm	Gm	Km	Nm	Sm	Su	Tc
Hostesses								
tox+ *E. coli* (49*)	40	16	1	4	4	34	34	32
tox- *E. coli*	26	16	0	2	2	28	28	28
Restaurant Workers								
tox+ *E. coli*	17	22	0	0	0	35	35	26
tox- *E. coli*	17	14	0	3	3	27	27	43

* Number of isolates tested

REFERENCES :

1. Aserkoff, B., and J.V. Bennett. 1969. Effect of antibiotic therapy in acute Salmonellosis on the fecal excretion of Salmonellae. N. Engl. J. Med. 281: 636-640.

2. Bailey, W.R., and E.G. Scott. 1970. Diagnostic Microbiology 3rd ed. The C.V. Mosby Company, St. Louis, MO.

3. Bauer, A.W., W.W.M. Kirby, J.C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disc method. Amer. J. Clin. Path. 45: 493-496.

4. Blair, J.E., E.H. Lennette, and J.P. Truant. 1970. Manual of Clinical Microbiology. American Society of Microbiology, Bethesda, MD.

5. Byers, P.A., and H.L. DuPont. 1979. Pooling method for screening large numbers of *Escherichia coli* for production of heat-stable enterotoxin and its application in field studies. J. Clin. Microbiol. 9: 541-543.

6. Dean, A.G., Y.C. Ching, R.G. Williams, and L.B. Harden. 1972. Test for *Escherichia coli* enterotoxin using infant mice : application in a study of diarrhea in children in Honolulu. J. Infect. Dis. 125: 407-411.

7. Echeverria P., M.T. Ho, N.R. Blacklow, G. Quinnan, B. Portnoy, J.G. Olson, R. Conklin, H.L. DuPont, and J.H. Cross. 1977. Relative importance of viruses and bacteria in the etiology of pediatric diarrhea in Taiwan. J. Infect. Dis, 136: 383-390.

8. Echeverria, P., L. Verhaert, C.V. Uylangco, S. Komalarini, M.T. Ho, F. Orskov, and I. Orskov. 1978. Antimicrobial resistance and enterotoxin production among isolates of *Escherichia coli* in the Far East. Lancet 2: 589-592.

9. Ericsson, H.M., and J.C. Sherris. 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta. Pathol. Microbiol. Scan. Suppl. (B) 217.

10. Falkow, S. 1975. Infectious Multiple Drug Resistance. Pion Ltd, London.

11. Gyles, C., M. So, and S. Falkow. 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. 130: 40-49.

12. Gyles, G.L., S. Palchaudhuri, and W.K. Maas. 1977. Naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. Science 198: 198-199.

13. McConnell, M.M., G.A. Willshaw, H.R. Smith, M.S. Scotland, and B. Rowe. 1979. Transposition of ampicillin resistance to an enterotoxin plasmid in an *Escherichia coli* of human origin. J. Bact. 139: 346-355.

14. Merson, M.H., R.B. Sack, A.K.M. Golom, K.A. Almahmood, Q.S. Adamed, and I. Hug. 1979. Use of colony pools for diagnosis of enterotoxigenic *Escherichia coli* diarrhea. J. Clin. Microbiol. 9: 493-497.

15. Ryder, R.W., D.A. Sack, A.Z. Kapikian, J.C. McLaughlin, J. Chakraborty, A.S.M.M. Rahman, M.H. Merson, and J.G. Wells. 1976. Enterotoxigenic *Escherichia coli* and reovirus-like agent in rural Bangladesh. Lancet 1: 659-667.

16. Sack, D.A., and R.B. Sack. 1975. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in mini-culture. Infect. Immun. 11: 334-336.

17. Sack, R.B., S.L. Gorbach, J.G. Banwell, B. Jacobs, B.D. Chatterjee, and R.C. Mitra. 1971. Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. J. Infect. Dis. 123: 378-385.

18. Smith, H.W. 1970. The transfer of antibiotic resistance between strains of enterobacteriacae in chickens, calves and pigs. J. Med. Micro. 3: 165-180.

19. Smith, H.W., and M.A. Linggood. 1970. Transfer factors in *Escherichia coli* with particular regard to their incidence in enteropathogenic strains. J. Gen. Microbiol. 62: 287-299.

20. Smith, H.W., and M.A. Linggood. 1971. Observations on the pathogenic properties of the K88, Hly, and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhea. J. Med. Microbiol. 4: 467-486.

21. Smith, H.W. 1973. The exploitation of transmission plasmids to study the pathogenesis of *E. coli* diarrhea. Proc. Roy. Soc. Med. 66: 2-3.