

**MULTIPLE ANTIBIOTIC RESISTANT ESCHERICHIA COLI  
FROM A TROPICAL RAIN FOREST STREAM**

by

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## ABSTRACT

High densities of fecal coliforms were obtained from a pristine site and sewage contaminated site in a tropical rain forest watershed in Puerto Rico. Confirmation of fecal coliform isolates as Escherichia coli was significantly lower than for temperate waters. Antibiotic resistance and multiple antibiotic resistance were common for isolates at both sites; however, the site receiving sewage effluent had a greater proportion of multiple antibiotic resistant isolates. R plasmids were recovered from 4 MAR isolates, 2 from each site. All recovered plasmids were approximately 1 kilobase. The recovered plasmid were also capable of transforming E. coli HB101 in vitro. The high concentrations of enterobacteriaceae, small R-plasmid size, R-plasmid transformability, and long term survival of fecal origin bacteria in tropical freshwater environments give increasing importance to adequate sewage treatment, and better indicator monitoring methods for tropical areas.

## INTRODUCTION

Antibiotic resistant bacteria have been isolated from a large number of water sources around the world (4, 20, 32, 36, 37). Grabow et al. (21) has suggested that antibiotic resistant coliforms may also be more resistant to conventual sewage treatment including primary, secondary, and chlorination. Since antibiotic resistance (R plasmids) may be transferred to pathogenic bacteria via conjugation, transformation, or viral transduction (21, 30), indigenous or long surviving environmental coliform bacteria that harbour R plasmids could pose a serious health hazard. Especially if the surface water that these bacteria are found in is used for recreation and as a drinking water source.

It is common to find very high densities of coliform bacteria in tropical waters, even when no known human fecal source can be demonstrated (15-17, 24, 28, 33, 35, 40, 42). Several studies have even demonstrated that Escherichia coli, the target of the coliform assay, can survive for extended periods in tropical waters (10, 24, 29, 41) and may even be indigenous in tropical rain forests (8). DNA homology studies suggest that E. coli isolates from epiphytic plants 20 m above the ground in the rain forest are the same species as E. coli B, a clinical isolate (8). These strains were also found to have some resistance to antibiotics (34).

The present study examines the occurrence of multiple antibiotic resistant (MAR) plasmids in E. coli at a contaminated and at a pristine

1 site in a tropical rain forest watershed. These plasmids will be isolated  
2 and tested for their ability to be transferred to other bacteria via  
3 conjugation and transformation.

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## MATERIALS AND METHODS

**Study site.** The Mameyes River is located at the northeast of the island of Puerto Rico (Fig. 1). The river originates in cloud rain forest in a pristine portion of the Luquillo Experimental Forest, U.S. Forest Service. Samples were taken from two sites. Site 1 was a pristine area high in the rain forest, the highest point in the watershed. Site 9 was several km downstream, just below the outfall for a primary sewage treatment plant. For a more complete description of the Mamayas River watershed and sites 1 and 9, see Carrillo et al. (10).

**Water sampling.** Water samples were collected slightly below the surface with sterile 180 ml Whirl-Pak bags (NASCO, Ft. Wilkinson, Wis). Samples were brought to the lab at ambient temperature and analyzed within 3 h of collection.

Sample portions of 0.2, 1 and 10 ml from site 9, and 10 ml from site 1 were filtered through 0.45  $\mu$ m pore size, 47 mm diameter membrane filters (GN-6, Gelman Instrument Co., Ann Arbor, Mich.). Each filter was incubated at 44.5°C for 24 h on mFC agar (Difco Laboratories, Detroit, Mich.) Dark blue colonies were considered fecal coliform positive (3). Random isolates were isolated and streaked on trypticase soy agar (Difco), incubated at 37°C for 24 h and later kept at room temp.

**Characterization of Bacteria.** Colonies were characterized by Gram staining reaction, and API 20E strips (Analytab Products, Plainview, N.Y.). Antibiotic resistance was assayed on Mueller-Hinton

agar (Difco), using antimicrobial discs (Sigma Chemical Co., St. Louis, MO) with ampicillin (Amp), chloramphenicol (C), penicillin (PS), sulfathiazol (St), tetracycline (Te), rifampicin (Ra), and streptomycin (S2).

Susceptibility was determined according to the National Committee for Clinical Laboratory Standards, Approved Standards ASM2 (3).

Antibiotic resistant bacteria were identified from each site (Table 1). The strains chosen for R-plasmid extracts were E. coli strain 1 and 7 from site 1, and E. coli strain 14 and 15 from site 9.

**Plasmid DNA Extraction.** Plasmid DNA was isolated according to Dillon et al. (13). Bacterial cells were grown on TSB (Difco) at 37°C.

At midlog phase, plasmids were amplified through the addition of 150 µg/ml chloramphenicol (Sigma) and further incubated for 3-5 h. The cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. The pellet was digested with lysozyme (10 mg/ml) in TES buffer (30 mM Tris HCl, pH 8, 50 mM EDTA, 5 mM NaCl) and 20% Triton X-100 (Sigma). The lysate was centrifuged at 18,000 rpm for 20 min at 4°C. The supernatant was adjusted to 4 ml with TES buffer, 3.65 g CeCl<sub>2</sub> (Sigma) was gently dissolved into the solution along with 100 ml Ethidium bromide (10 mg/ml, Sigma). This final mixture was centrifuged in ultra-clear tubes at 44,000 rpm at 5°C for 42 h.

The plasmid band was located in the resulting CsCl<sub>2</sub> gradient under a longwave ultraviolet light source and removed with a syringe. The plasmids were washed several times with aqueous isobutanol to remove the ethidium bromide before being precipitated in 2.5 vol 95% EtOH, and NaAc to a final concentration of 300 mM and stored at -20°C.

1 The precipitate was centrifuged at full speed in an Eppendorf  
2 microcentrifuge, pelleted and dried. The pellet was dissolved in 1 ml TE  
3 buffer, and dialyzed for 24 h as described in Maniatis et al. (31).  
4 Sample concentration was determined in a Beckman uv/vis  
5 spectrophotometer at 254 nm (1 OD = 50  $\mu$ g/ml of double stranded  
6 DNA). Ten microliter samples (#1 - 0.65  $\mu$ g; #7 - 2.55  $\mu$ g; #14 - 2.25  $\mu$ g;  
7 #15 - 1.15  $\mu$ g) were electrophoresed in a Bio Rad mini Sub DNA Cell  
8 through an 0.5% agarose gel in TBE (89 mM Tris / 89 mM boric acid / 2  
9 mM EDTA) at 6v for 15 h at room temperature in 1 x TBE buffer and 0.5  
10  $\mu$ g/ml Ethidium bromide. Whole DNA (48 kb) and Hind III DNA digests  
11 (23.1, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56 kb fragments. BRL) were used as  
12 markers.

13 **Transformation Experiments (Competent Cells).** The  
14 plasmids extracted from E. coli strain 14 were chosen to transform  
15 HB101 E. coli from Te sensitive to Te resistant. The recipient HB101  
16 cells (9) were made competent with  $\text{CaCl}_2$  following Maniatis et al. (31).

17 HB101 cells were grown in L broth at 37°C for 2-4 h with vigorous  
18 shaking, chilled for 10 min on ice and centrifuged at 4,000 x g for 5 min  
19 at 4°C. The cells were resuspended in 50 ml of ice cold, 50 mM  $\text{CaCl}_2$   
20 and 10 mM Tris HCl pH 8, chilled for 15 min and centrifuged as before.  
21 Cells were resuspended again in 7 ml of the same  $\text{CaCl}_2$  / Tris HCl  
22 solution, dispensed in 200  $\mu$ l aliquots into prechilled tubes and stored  
23 for 24 h at 4°C.

24 **Transformation.** The plasmid DNA (40 ng / 100  $\mu$ l) from strain  
25 14 was added in TE buffer to competent HB101 cells mixed and stored

1 on ice for 30 min. The mixture was heat shocked at 42°C for 2 min and  
2 incubated at 37°C for 30 min after the addition of 1 ml of L broth.

3 Transformed cells were tested for tetracycline resistance on LBA  
4 plates (L broth + 15 g Agar / l according to Hanahan (22)) as follows:

5 1. Positive control: HB101 E. coli cells in LBA.

6 2. Negative control: HB101 E. coli cells LBA plus tetracycline 100  
7 µl / 20 ml of LBA. Te stock used was 12.5 mg / ml in 50 / 50 EtOH  
8 water.

9 3. Second positive control: 50 ml HB101 plus Te plasmid mixture  
10 in LBA.

11 4. Experimental plates: HB101 plus Te plasmid mixture in LBA  
12 with Te; different amounts of the mixture, from 40 µl to 200 µl were  
13 spread on the plates.

14 **Resistance to Nalidixic Acid.** Strain 14 E. coli and HB101

15 E. coli were grown on LB with nalidixic acid 100 µl / 20 ml LB.

16 Nalidixic acid stock used was 20 mg / ml in water.

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## RESULTS AND DISCUSSION

The density of fecal coliforms at both sites was high for natural waters, > 80 CFU/100 ml (Fig. 2), but typical for tropical areas (35). Densities of fecal coliforms were significantly higher at site 9 (the sewage contaminated site). However, since site 9 is lower in the watershed and previous studies have shown a long survival time for Escherichia coli in these waters, a concentration phenomena can not be ruled out (10, 29, 41). Only 70% of the fecal coliform positive isolates were confirmed as E. coli, this is considerably lower than temperate waters but typical for tropical waters, and undoubtedly due to the dominance of mesophilic background flora in tropical waters (34, 35). Previous studies in this watershed have demonstrated that E. coli isolated from epiphytes in trees 10 m above the ground has the same %mol G+C as E. coli B, a clinical strain (8). In addition, these rain forest isolates were shown to have at least 70% homology with E. coli B, further verifying that they were the same species. Thus, as suggested by Hazen (23) E. coli continues to appear as a part of the indigenous microbial flora of this and other tropical waters and an inappropriate indicator of recent human fecal contamination.

The most common isolate resistance was to penicillin (16/17, 94%), followed by ampicillin (47%); the most common sensitivity was to tetracycline (94%) and chloramphenicol & chloromycetin (88%) (Table 1). Multiple antibiotic resistance (MAR), to 3 or more antibiotics, was most common in bacteria from site 9 (17.6%) when compared to site 1

(6%) (Table 2). The most common pattern of resistance was, again, to penicillin only (41%), followed by penicillin, ampicillin (23.5%). Resistance to penicillin was widespread (94%) among the environmental isolates obtained. Moreover, all the fecal coliforms isolated from the highest point in the watershed (site 1), were penicillin resistant (Table 1). Multiple resistance to antibiotics was common, particularly in bacteria growing in sewage contaminated waters, site 9 (Table 2). The percentage of environmental *E. coli* strains which were resistant to two or more antibiotics (53%) was higher than the range previously reported (21, 27, 38).

Four strains (1, 7, 14, 15) were selected on the basis of possible R plasmid antibiotic resistance. According to their behavior in an agarose gel, the R plasmid DNA in these bacteria are approximately 1 Kb (ca.  $6 \times 10^5$  mw based on a standard of 1.5 Kb =  $1 \times 10^6$  mw, 1). Kabori et al. (1984) detected a plasmid with a molecular weight of ca. 1.0 Mdal (ca. 1.5 Kb) and they have suggested that this may be the smallest plasmid so far reported in natural environments. Plasmids ranging in molecular weight from  $2.7 \times 10^6$  to  $79 \times 10^6$  have been previously reported (25). Since Hanahan (22) has demonstrated that the probability of transformation increases linearly with decreasing plasmid size, these small plasmids have a very high transforming potential. Glassman et al. (19) suggested that there is a preponderance of small plasmids in isolates from clean sites but larger ones ( $> 30$  Mdal) in isolates from polluted sites. The isolation of small plasmids in this study would suggest that this site was unpolluted. Site 9 was the only site in the

watershed known to receive sewage contamination. Low concentrations of phosphorus and nitrogen reported for this watershed by other studies (10, 14, 29, 41) supports this finding.

Resistance transfer results to E. coli HB101 are given in Table 2. Tetracycline sensitive (Tes) HB101 were cultured with Te-R-plasmid DNA from E. coli strain 14 (Table 2). All resistance acquisition occurred through transformation since the DNA preparation excluded all other possibilities (i.e. conjugation and transduction). Resistance to Te was transferred in all cases, but was clearly evident after 24 h in those cultures where, at least, 100  $\mu$ l of the mixture (HB101 plus Te-R-plasmid DNA) was plated. Where smaller amounts were plated (20 to 75  $\mu$ l), growth was barely evident at 24 h; by 72 to 96 h higher counts were obtained. The assay was successful in as much as untransformed HB101 cells did not grow on the LBA Te medium (Table 3). Growth on the experimental plates were scarce, as would be expected if only transformants were to grow.

Smith et al. (39) reported that transformation has been found to occur in both gram positive and gram negative bacteria in nature. The latter have evolved mechanisms which favor the development of competent cells under conditions which inhibit cell division. Transformable Acinetobacter strains become competent when entering a stationary phase (26). Although homologous DNA is preferentially transferred in gram negative bacteria, there is evidence that transformation also occurs by uptake of non-homologous DNA though less efficiently (39). Thus, the transformation which we have evidenced

1 in vitro could actually be taking place in nature. Recent studies by  
2 Cruz-Cruz et al. (12) have shown that in situ genetic transfer can take  
3 place between Pseudomonas aeruginosa strains at these sites. Their  
4 study, using diffusion chambers placed in the stream, showed that  
5 transfer occurred within 3 h, and that transconjugants did not decrease  
6 in survivability. Considering that even under stressed conditions E. coli  
7 could survive in appreciable densities for 3 h, it is highly likely that R-  
8 plasmids that reach these tropical waters through E. coli could  
9 transform other E. coli, pathogenic bacteria, or other indigenous  
10 microbiota. Shaw and Cabelli (36) reported an outbreak of recreational  
11 waterborne enteric disease caused by MAR Shigella. Several other  
12 investigators have also reported a number of deaths in several parts of  
13 the world due to MAR containing bacteria (5, 18).

14       The fact that antibiotic resistance may be readily transferred  
15 among the genera that comprise the Enterobacteriaceae has been  
16 studied extensively in recent years (2, 6, 7, 11, 20, 22, 26). The current  
17 studies demonstrate that tropical waters may present an even greater  
18 danger to public health due the rapid genetic transfer rates of R-  
19 plasmids, and the long survival time to E. coli in these environments.  
20 Adequate indicators of recent human fecal contamination for monitoring  
21 water contamination in the tropics are desperately needed to increase  
22 the health of the 65% of the world's population that lives in tropical  
23 areas.

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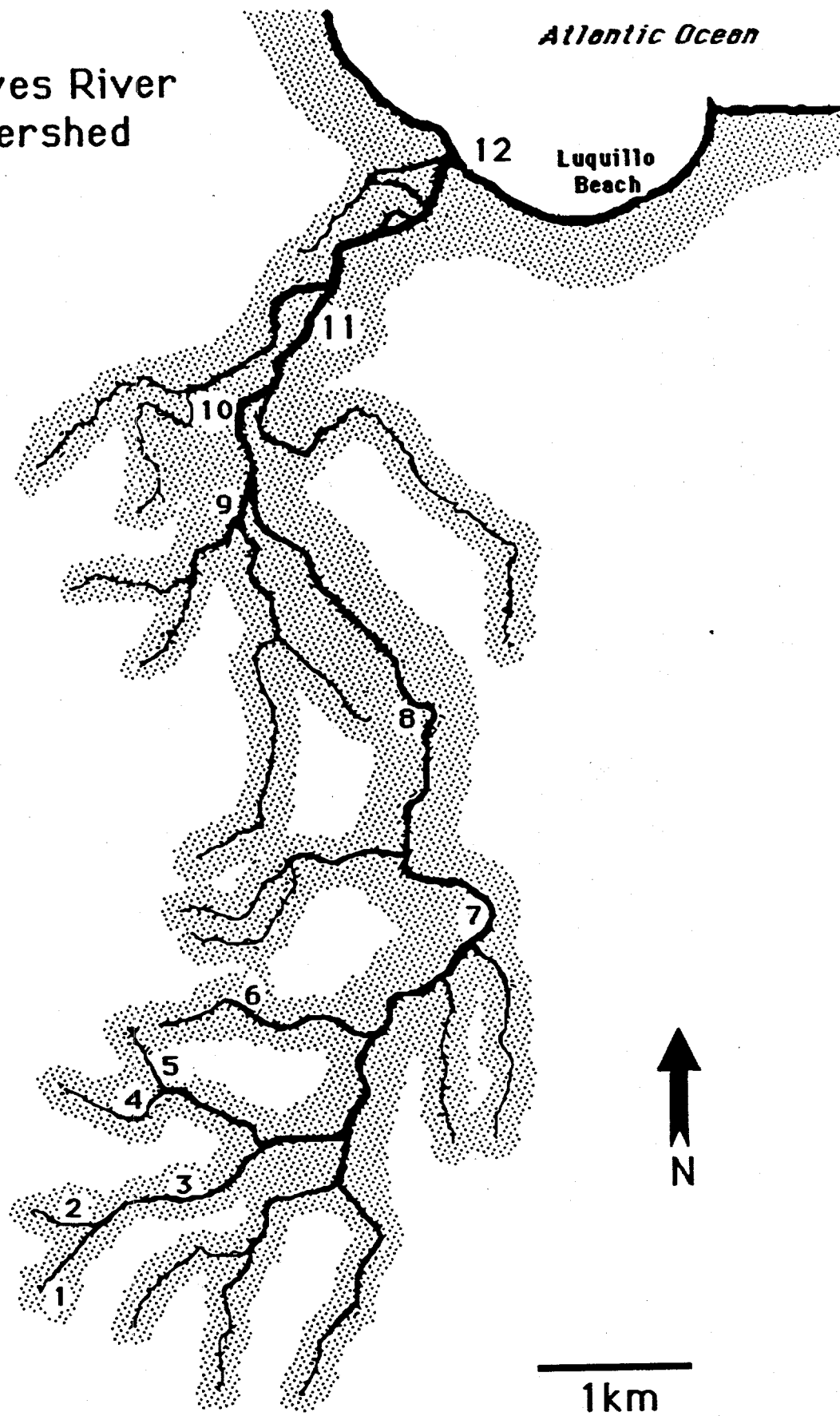
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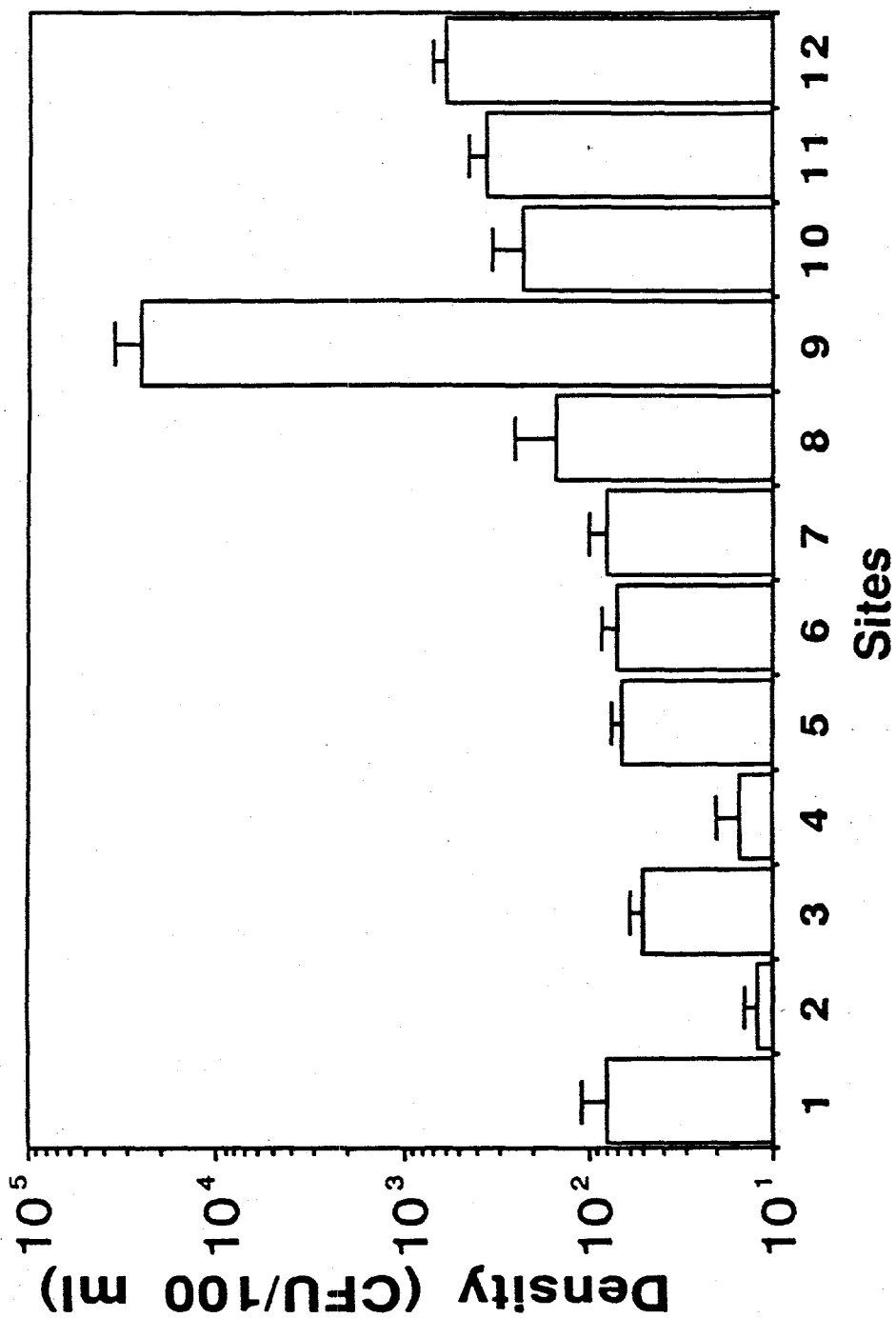
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# Mameyes River Watershed



3 Figure 1. Map of study sites in Mameyes River.



4 Figure 2. Density of fecal coliforms by site in the Mameyes River (mean

5 ± one standard error, N = 36)

Table 1. Antibiotic sensitivity of fecal coliform positive environmental isolates.

| Isolate | Antibiotics |    |    |    |    |    |                |
|---------|-------------|----|----|----|----|----|----------------|
|         | C           | Am | PS | St | Te | Ra | S <sub>2</sub> |
| 1       | -           | +  | +  | +  | -  | ND | ND             |
| 2       | -           | -  | +  | -  | -  | ND | ND             |
| 3       | -           | -  | +  | -  | -  | ND | ND             |
| 4       | -           | -  | +  | -  | -  | ND | ND             |
| 5       | +           | -  | +  | -  | -  | ND | ND             |
| 7       | -           | -  | +  | -  | -  | ND | ND             |
| 8       | -           | +  | +  | -  | -  | ND | ND             |
| 9       | -           | +  | +  | -  | -  | ND | ND             |
| 10      | -           | -  | +  | -  | -  | ND | ND             |
| 11      | -           | -  | +  | -  | -  | ND | ND             |
| 12      | +           | +  | +  | -  | -  | +  | +              |
| 13      | -           | +  | +  | -  | -  | ND | ND             |
| 14      | -           | +  | +  | +  | +  | ND | ND             |
| 15      | -           | +  | +  | +  | -  | ND | +              |
| 16      | -           | +  | +  | -  | -  | ND | ND             |
| 17      | -           | -  | +  | -  | -  | ND | ND             |
| 18      | -           | -  | -  | -  | -  | ND | ND             |

Am = Ampicillin, St = Sulfathiazol, C = Chloromycetin, S<sub>2</sub> = Streptomycin,  
 Ps = Penicillin, + = Resistant, Te = Tetracycline, - = Sensitive, Ra =  
 Rifampicin, ND = not determined

Table 2. Antibiotic resistance patterns of rain forest isolates

| Antibiotic Pattern            | Number of Strains | %  | Site |
|-------------------------------|-------------------|----|------|
| Ps                            | 7/17              | 41 | 1, 9 |
| Ps, Am                        | 4/17              | 23 | 1, 9 |
| Ps, Am, St                    | 1/17              | 6  | 1, 9 |
| Ps, Am, St, Te                | 1/17              | 6  | 9    |
| Ps, Am, St, S <sub>2</sub>    | 1/17              | 6  | 9    |
| Ps, C                         | 1/17              | 6  | 1    |
| Ps, Am, C, Ra, S <sub>2</sub> | 1/17              | 6  | 9    |
| None                          | 1/17              | 6  | 9    |

Am = Ampicillin, Ra = Rifampicin, C = Chloromycetin, St = Sulfathiazol, Ps = Penicillin, S<sub>2</sub> = Streptomycin, Te = Tetracycline

Table 3. Transformed *E. Coli* HB101 with R-Plasmid DNA.

| Transformation<br>Mixture (ml) *                     | 24 h | 96 h |
|------------------------------------------------------|------|------|
| 20 (2) *                                             | 0*   | 1    |
| 40 (2)                                               | 0    | 0    |
| 50 (8)                                               | 0    | 60   |
| 75 (3)                                               | 0    | 14   |
| 100 (7)                                              | 3    | TNTC |
| 150 (1)                                              | TNTC | TNTC |
| 200 (3)                                              | 4    | TNTC |
| HB101 100 $\mu$ l (2)                                | 0    | 0    |
| (LBA-Te <sup>-</sup> ) 100 $\mu$ l (2)               | TNTC | TNTC |
| HB101 Mixture (LBA-Te <sup>-</sup> ) 100 $\mu$ l (2) | TNTC | TNTC |

\* number of *E. coli* HB101 cells transformed with R-plasmid DNA from *E. coli* strain 14 from site 9, TNTC = too numerous to count.