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Antibiotic-Resistant Bacteria in Wild Primates: Increased Prevalence in Baboons Feeding on Human Refuse

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We examined three groups of wild baboons (*Papio cynocephalus*) in Amboseli National Park, Kenya, to determine the prevalence of aerobic antibiotic-resistant fecal bacteria in nonhuman primates with and without contact with human refuse. Using standard isolation and replica plating techniques, we found only low numbers of antibiotic-resistant gram-negative enteric bacteria in two groups of baboons leading an undisturbed existence in their natural habitat and having limited or no contact with humans. However, resistance was significantly higher among enteric bacteria from the third group of baboons living in close proximity to a tourist lodge and having daily contact with unprocessed human refuse. Conjugation studies and analysis of the cell DNA by gel electrophoresis showed that in many cases resistance was plasmid-borne and transferable. These data suggest that wild nonhuman primates in frequent contact with human debris have a higher proportion of antibiotic-resistant enteric bacteria than do conspecifics without this contact. The findings further suggest that such groups of wild animals may constitute a heretofore overlooked source of antibiotic resistance in the natural environment.

Direct and repeated exposure to antibiotics has been shown to produce strong selective pressures for the maintenance of antibiotic resistance in the enteric bacteria of humans and domestic animals (2, 5, 9). However, colonization of the intestinal tract by resistant coliforms can occur even in the absence of such selection pressures, particularly as a result of contact with resistant bacteria in food or on environmental fomites (8, 13–15). This latter process is not well understood and is, in fact, difficult to analyze systematically given the complex environment of humans and domestic animals in developed countries, as well as the numerous antibiotic-resistant bacteria already present in their intestinal tracts.

In contrast, most populations of wild animals have limited exposure to antibiotics or antibiotic-resistant bacteria, or both. Thus, studies of wild populations have the potential to provide unique insights into the factors affecting colonization of the intestinal tract by antibiotic-resistant bacteria. In this report we present the first quantitative data on the prevalence of antibiotic resistance in the aerobic enteric gram-negative bacteria of a wild nonhuman primate population, namely, yellow baboons, in the Amboseli National Park of Kenya. Specifically, we obtained samples of fecal microflora from most members of two large free-living and undisturbed groups of Amboseli baboons, as well as from the members of a third, much smaller group whose range encompassed human habitations and refuse dumps. Our results demonstrate that the members of this latter group have a significantly greater prevalence of antibiotic-resistant enteric bacteria than their completely wild conspecifics.

MATERIALS AND METHODS

Animals. The yellow baboon (*Papio cynocephalus*) population in the Amboseli National Park of Kenya has been the focus of long-term ecological, behavioral, and parasitological investigations (1, 3, 4). The members of three naturally formed and intact groups of Amboseli baboons served as the subjects for the present study. Two of these groups (the Alto group and the Hook group) ranged in portions of the Park unfrequented by humans, except for intermittent visits by tour vehicles (confined to specific routes), seasonal grazing by domestic stock of indigenous Maasai pastoralists, and near daily visits by members of present research team and co-workers (one to four total individuals). The range of the third group (the Lodge group) encompassed not only a variety of undisturbed habitats, but also the refuse pit (18 m^3 in volume) of a tourist lodge and several smaller pits and a latrine area used by campers. Although the Lodge group visited these pits on a near daily basis to scavenge for food scraps, its members continued to make extensive use of natural foods within their home range. As a result of long-term behavioral studies of the Amboseli baboon populations (1, 3), all three groups were habituated to the presence of observers on foot, and most subjects were individually identifiable and of a known genealogical affiliation.

Sample collection and processing. Amboseli baboons descended from their arboreal sleeping sites between 0700 and 0900 h each day and shortly thereafter moved into the open savannah, a habitat characterized through much of the year by hard-packed, sun-baked soils sparsely covered with a dry stubble of heavily grazed grass plants (1). During this morning time period and in this habitat, we collected 92 fecal samples from the members of three groups of Amboseli baboons over a 10-day period in June 1982. We attempted to obtain one sample from each individual over 1 year of age, although duplicate samples were collected from some individuals, particularly in the Lodge group. In terms of overall group composition, samples were obtained from 76.4 and 86.7% of the members of the Alto group (n = 55) and the Hook group (n = 30), respectively, and from at least 55.5% of the members of the Lodge group (n = 18).

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TABLE 1. Fermentative characteristics and antibiotic resistance of coliform isolates from Amboseli baboons

Group	Total no. of colonies	Fermentative class (%)			Total no. of Mac plates	Antibiotic resistance (% of samples)[a]				
		Lac^+	LL^{+b}	Lac^-		None	Tet	Kan	Amp	Kef
Alto	2,988	86.8	7.6	5.6	40	52.5	7.5	47.5	2.5	2.5
Hook	1,652	78.3	5.3	16.4	25	64.0	32.0	4.0	12.0	0.0
Lodge	1,263	66.0	10.1	23.9	17	5.9	94.1	70.6	47.1	17.6

[a] Shown is the percentage of all fecal samples showing at least one colony resistant to the specific antibiotic tested. Tet, Tetracycline; Kan, kanamycin; Amp, ampicillin, Kef, cephalothin.
[b] LL^+, Late lactose fermenters.

Amboseli baboons typically defecated a single, well-formed, relatively dehydrated cylindrical bolus weighing 100 to 300 g (4). The baboons usually moved away from their own feces immediately after passage, at which time we collected materials for subsequent culturing at our field laboratory. Thus, fecal samples used in this study had generally been in contact with the ground for less than 5 min before collection, had little or no direct contact with vegetation, and had adherent soil particles only in a narrow band along their undersurface. In most cases, the entire sample was carefully removed to a sterile container and stored on ice for 2 to 4 h until returned to the laboratory. Alternatively, in the field, the fresh fecal bolus was sampled from its center by using a sterile swab which was then maintained in Stuart transport medium (Culturette; S/P) until plating. In all plating operations (as in the above use of sterile swabs), feces were sampled only from the soft interior of the bolus, with special care taken to assure that the sampling loop (or swab) did not contact any portion of the feces to which soil was adhering or which showed other signs of potential contamination.

Within 4 h of collection, each sample was streaked onto both MacConkey (Mac) agar (selective for aerobic gram-negative enteric bacilli) and Hektoen enteric agar (differential and moderately selective for *Salmonella* and *Shigella* spp.) plates by using a two-dimensional spreading technique (10). Additionally, for a total of 15 samples (10 from the Alto group and 5 from the Hook group), the number of aerobic gram-negative bacilli per gram was determined quantitatively by using a dilutional plating technique (12). All plates were incubated in air at ca. 36°C until there was sufficient growth to allow identification of colony types, ca. 24 to 36 h. The original Mac plates were then used as templates (or master plates) for replica plating (7) onto additional plates containing Mac agar and one of eight antibiotics. The antibiotics and dosage levels used in this phase of the study were tetracycline (10 μg/ml), kanamycin (10 μg/ml), ampicillin (30 μg/ml), cephalothin (30 μg/ml), streptomycin (30 μg/ml), chloramphenicol (25 μg/ml), nalidixic acid (30 μg/ml), and gentamicin (10 μg/ml). Plates were incubated as before, and the percentage of resistant colonies was tabulated by comparison with the master plate.

Transfer of resistance markers. Transfer of plasmid-mediated resistance was tested by the mating of 50 selected resistant strains with a nalidixic acid-resistant laboratory strain, *Escherichia coli* C600. Donors and recipient were grown separately to log phase in L broth, and ca. 10^9 bacteria of each were filtered by suction onto a 0.45-μm filter (Millipore Corp.), which was then placed on Mac agar. After overnight incubation at 37°C, the bacterial suspensions were washed from the filters by vigorous agitation in 5 ml of L broth, and 0.1 ml of the suspensions was plated on the appropriate selective agar to test for growth of transconjugants.

RESULTS

The numbers of bacteria in these samples which grew on Mac plates was highly variable, and an unexpected number of samples (10 to 20%) from each group yielded ≤10 CFU/g. Excluding these low-count samples, the mean titer for samples from the Alto and Hook groups was 3.3×10^5 CFU/g, with a range from 1.3×10^3 to 1.4×10^6. When three of the animals whose samples showed low titers were resampled, abundant growth of bacteria was seen; thus, day-to-day variability in bacterial numbers was suggested.

Colonies on each Mac plate were counted and classified based upon lactose-fermentative characteristics: lactose fermenters (Lac^+), late lactose fermenters (LL^+), or lactose nonfermenters (Lac^-). The Lodge group samples showed a more diverse microflora than did samples from the other two groups (Table 1). There was a significantly higher proportion of Lac^- colonies in these samples (23.9%) compared with that found in the Alto and Hook groups combined (9.4%), (χ^2 = 219.4 for raw data, df = 2, $P \leq 0.001$). Furthermore, 20% of the fecal samples from the Lodge group yielded Lac^- H_2S^+ colonies on the Hektoen enteric agar, compared with only 2.7% of the samples from the other two groups combined. None of these isolates proved to be *Salmonella* sp., however, when tested with API-10 Enteric Strips (Scientific Products).

In an initial series of tests, 82 Mac plates from the three groups were assayed by replica plating for the presence of colonies resistant to four commonly used antibiotics: tetracycline, kanamycin, ampicillin, and cephalothin (Table 1). The prevalence of bacteria resistant to one or more of these antibiotics was significantly higher among the Lodge group samples than among samples from the other two groups. Whereas 52.5% of the Alto group samples and 64.0% of the Hook group samples demonstrated no resistant colonies, only 5.9% of the samples from the Lodge group failed to show any resistant colonies (Table 1; $\chi^2 = 13.8$, df = 1, $P \leq 0.001$). Moreover, Lodge group samples contained a much higher frequency of resistant colonies per plate for each individual antibiotic tested than did samples from either of the other two groups. Additionally, with the exception of the one Lodge group plate that produced no resistant colonies at all, each of the remaining Lodge group plates (94.1%) showed resistance to at least two different antibiotics, and this finding was statistically significant in comparison with samples from the Alto group (10.0%) or the Hook group (12%) ($\chi^2 = 46.0$, df = 1, $P \leq 0.001$) (Fig. 1A). The most common resistances seen in all samples were to tetracycline and kanamycin.

Lodge group samples also had a significantly higher proportion of resistant Lac^+ colonies per plate than did samples from either of the other two groups (Fig. 1B). Only 9 of 40 samples from the Alto group and 2 of 25 from the Hook group showed more than 20% of all colonies with resistance

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to one or more antibiotics. In contrast, 12 of 17 Lodge group samples contained at least 20% resistant bacteria (Fig. 1B; $\chi^2 = 18.9$, df = 1, $P \leq 0.001$). Thus, the Lodge group not only produced significantly more samples exhibiting antibiotic resistance than did the other two groups, but also showed a significantly higher proportion of resistant colonies among the total Lac^+ population in each sample.

Multiply resistant colonies were present in samples from the Lodge group at levels ranging from 1 to 6% of all CFU per plate, and a total of 64.7% of Lodge group samples contained colonies showing multiple resistance. In contrast, only two samples each from the Alto and Hook groups showed multiply resistant colonies, representing 5.0 and 8.0% of the total samples, respectively ($\chi^2 = 41.5$, df = 1, $P \leq 0.001$). Furthermore, multiple resistance in Lodge group samples involved six different combinations of antibiotics: tetracycline-kanamycin, tetracycline-ampicillin, kanamycin-ampicillin, kanamycin-cephalothin, tetracycline-kanamycin-ampicillin, and tetracycline-kanamycin-ampicillin-cephalothin. Multiple resistance in the Alto and Hook groups was to the single combinations of tetracycline-kanamycin and tetracycline-ampicillin, respectively.

In a second series of replica plating, 10 samples from the Alto group and all 17 from the Lodge group were examined for resistance to streptomycin, chloramphenicol, nalidixic acid, and gentamicin. Resistance to chloramphenicol and nalidixic acid was uncommon (<12% of the samples in both groups) and, where present, amounted to only a few colonies per plate. Resistance to gentamicin was not found. Of the 17 Lodge group samples, 15 demonstrated streptomycin resistance (at levels of 6 to 39% of all colonies per plate), compared with only 1 of the samples from the Alto group (17% of all colonies per plate) ($\chi^2 = 11.9$, df = 1, $P \leq 0.001$).

In addition to avoiding contamination from the environment, we cultured potential contaminants for comparison with bacteria from feces. Ten Mac and ten Hektoen enteric plates were each streaked with ~10% slurry prepared from soil and dry vegetation that had been collected in those same habitats in which fecal samples were collected. Incubation of these plates according to the same procedure followed above produced limited bacterial growth, but in all cases the few resultant colonies were morphologically different nonenteric Lac^- or mucoid Lac^+, rather than the nonmucoid Lac^+ colonies characteristic of plates streaked with baboon feces.

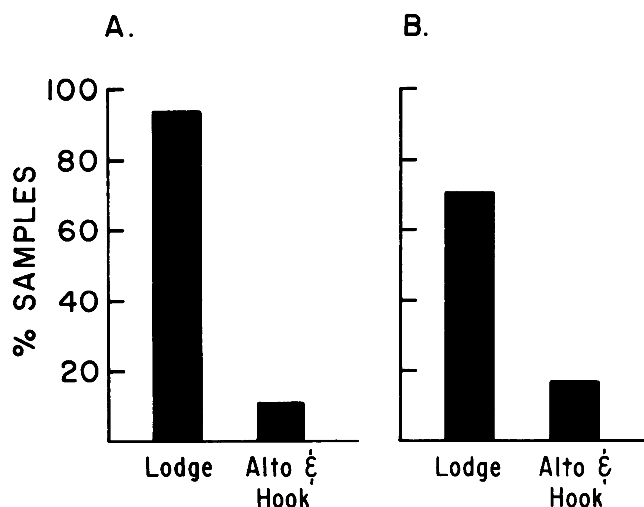

FIG. 1. Comparison of antibiotic resistance levels among the enteric bacteria of wild baboons with (Lodge group) and without (Alto and Hook groups) contact with human refuse. (A) Proportion of samples showing resistance to two or more antibiotics. (B) Proportion of samples meeting or exceeding a criterion level of 20% resistant colonies per plate. See the text for further details.

A group of 51 resistant strains were tested for the ability to transfer their resistances to a laboratory strain of *E. coli*, C600nal^r. None of the nine singly or doubly resistant strains from the Lodge group transferred their resistance markers; however, four of eight multiply resistant (≥ three markers) Lodge group strains exhibited transfer of two to five linked resistances, involving combinations of tetracycline, streptomycin, kanamycin, ampicillin, and chloramphenicol. In the Alto and Hook groups, 3 of 12 singly resistant strains transferred either tetracycline or ampicillin resistance, whereas 1 of 12 doubly resistant hosts transferred linked tetracycline-ampicillin resistance. Only 1 of 19 multiply resistant strains (≥ three markers) demonstrated transfer, and only of a single marker (streptomycin). Agarose gel electrophoresis showed multiple plasmids in donor strains and the transfer to recipients of unique plasmids associated with the resistances.

DISCUSSION

These data show that baboons feeding on human garbage and in contact with other forms of human detritus maintained significantly greater levels of antibiotic-resistant gut bacteria than did their wild counterparts. The latter, in fact, contained only very low numbers of resistant gram-negative intestinal bacteria, a finding in accord with other studies of humans and domestic animals not exposed to modern medicine (6, 11). At least some of the genes coding for this antibiotic resistance resided on transferable plasmids. It is highly unlikely that environmental contamination would explain these differences. First, our analysis of the soil and stubble showed no organisms similar to those found in the fecal samples. Second, previous studies of the Amboseli ecosystem have shown that daytime soil surface temperatures on the open savannah typically exceed 60°C, which makes it improbable that these dry, sunbaked soil surfaces would have supported prolonged survival of bacteria derived from previous fecal samples that may have fallen in the same location. Third, in one case we took samples recurrently (ca. every 12 h) from a bolus of feces left exposed to the environment over a 5-day period. The amount of bacterial growth that this sample produced on Mac plates diminished sharply over the first 36 h, and no growth was produced at all from plates streaked with feces that had been exposed to ambient conditions in Amboseli for longer than 60 h. In fact, the very size, shape, and density of baboon feces, together with the precautions we observed in our plating and sampling procedures, served to reduce even further the probability of such environment-derived contamination.

Our findings implicate food wastes and other forms of refuse as sources of resistant nonpathogenic bacteria in the intestine in the absence of known antibiotic selective pressure. Moreover, these data call attention to a previously unrecognized pathway by which antibiotic resistance plasmids may be transmitted to wild animals and subsequently spread to the natural environment.

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