Carriage of Antibiotic-Resistant Fecal Bacteria in Nepal Reflects Proximity to Kathmandu

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Within Nepal, geographic, social, and economic barriers greatly limit access to allopathic health care. The country therefore offered the opportunity to evaluate the effect of antibiotic accessibility (as measured by allopathic medicine consumption) on antibiotic resistance in the normal intestinal flora. The aerobic gram-negative fecal flora of 33–34 healthy adults from each of 3 villages with different access to health care facilities in Kathmandu were examined for antibiotic susceptibility. The frequency of antibiotic resistance decreased significantly with increasing distance from Kathmandu and decreasing population density but did not reflect contact with health care providers or individual medicine consumption. The findings suggest that an individual's overall exposure to antibiotics and antibiotic-resistant bacteria (resulting from close proximity to other community members and to sources of accessible allopathic health care, such as in the vicinity of Kathmandu), has an equal or greater impact on an individual's carriage of antibiotic-resistant bacteria than does direct consumption of antibiotics.

Antibiotic resistance has emerged as a significant threat to public health worldwide [1]. Although studies have documented the correlation between the consumption of antibiotics and the frequency of indigenous resistant organisms in hospitalized individuals [2–4] and in countries [5–7], similar studies in communities lacking easy access to allopathic health care are minimal [8, 9]. Reports have asserted that major factors driving the selection pressures responsible for the increasing frequency of antibiotic resistance are volume and length of time of drug use [6, 10]. However, other studies have demonstrated that resistant organisms can be present in individuals and in animals not taking antibiotics [3, 7, 9, 11–15]. One explanation attributes resistance selection to the density of individuals being treated in a particular geographic setting [16].

This concept takes into consideration not only direct selection by antibiotics but also the person-to-person spread of resistant strains. For instance, antibiotic resistance appeared among individuals who were not taking antibiotics but were sharing the same home [17] or day care center [18–19] with those consuming antibiotics. In one such study, there was a significant correlation between the use of antibiotics in various communities and the frequency of penicillin-resistant pneumococci in children in those same communities [20]. Another study showed that individuals sharing a household with those taking antibiotics for acne harbored significantly higher numbers of drug-resistant staphylococci on the skin than did a control group of individuals in homes of nontreated individuals [17].

Populations that are isolated from the influences of Western drugs and medicine are difficult to access and to study, yet they may provide important information to help us understand the origins, evolution, and spread of antibiotic-resistance genes. Such an opportunity was offered by a study of the resident fecal flora of 3 different Nepalese villages, each exhibiting different population densities and levels of accessibility to allopathic health care because of their geographic distance and relative isolation from the capital city of Kathmandu or other major urban centers.

Materials and Methods

Demographics and target population. Three villages at various distances from Kathmandu were selected for study (table 1). Dhulikhel, which is accessible from Kathmandu via a 30-min bus trip, provides the 11,000 inhabitants access to numerous hospitals, pharmacies, clinics, and allopathic physicians. Dolakha is located 6 h from Kathmandu on the same bus route as Dhulikhel. There are 2 hospitals and 6 pharmacies available to the 3500 villagers in Dolakha, as well as bus access to Kathmandu and the health ser-

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Informed consent for participation in this study was obtained from all patients.

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Table 1. Demographics of Nepalese study populations in 3 Nepalese villages.

Village	Approximate population	Distance from Kathmandu, km	No. of hospitals[a]	No. of health posts[a]	No. of pharmacies[a]
Dhulikhel	11,000	31	6[b]	>10	>50
Dolakha	3500	133	2[c]	1	6
Simigaun	330	170	0	0	0

[a] Any facility within 1 h (walking or bus).
[b] Major hospital (⩾50 inpatient beds).
[c] Small hospital (<50 inpatient beds).

vices located there. Simigaun can be reached from Dolakha only by an additional 3-day walk. For this small village of 330 people, there were no hospitals, clinics, or pharmacies within a 1-day walk from Simigaun at the time of this study, and the nearest health post was a 5-h walk to the village of Lambagar. Since the completion of this study, a health post has been built in Simigaun through the efforts of the Nepal government and a private organization, United Hands to Nepal.

Sample collection and processing. During a 6-week period in June and July 1997, 50 volunteers between the ages of 15–45 years in each of the 3 villages were selected for the study. Volunteers were solicited at a public meeting held in the morning during the traditional time when villagers congregate as they head out to the fields to work. Individuals were recruited in this fashion in all 3 villages, to avoid selection bias between villages. Villagers who thought they might be pregnant or whose occupations involved trekking or tourism were excluded. Each consenting participant was instructed on the proper collection of freshly voided feces: culture swabs containing Cary-Blair transport medium (CultureSwab Transport System, Cary-Blair media, L-VV0052-2; Difco) were used. Each volunteer also responded to an oral questionnaire regarding age, sex, ethnicity, occupation, history of hospital or doctor visits, and visits to Jhankri (traditional healers). Samples were submitted to the investigators within 15 h of collection, were sealed with parafilm, and were transported and stored on ice.

Samples were plated by 2-dimensional streaking onto MacConkey agar (for isolation of aerobic gram-negative enteric bacteria) and onto Hektoen enteric (HE) agar, for selective isolation of *Salmonella* and *Shigella* species. Samples from all 3 villages were plated between 72 and 96 h, to eliminate variability in isolate recovery secondary to differences in plating time. The plates were incubated at 37°C for 24 h, and the numbers and phenotypes of isolated colonies on each selective medium were evaluated. Quadrants were selected to provide a yield of 50–100 colonies, if possible. These isolation plates then were used as master templates for replica plating [3, 21, 22] onto Mueller Hinton (MH) agar containing cotrimoxazole (CMZ; 3 μg/mL trimethoprim and 57 μg/mL sulfamethoxazole) and onto a series of MacConkey agar plates containing tetracycline (Tet; 10 μg/mL), amoxicillin (Amox; 30 μg/mL), chloramphenicol (Chl; 25 μg/mL), cephalothin (Cthn; 30 μg/mL), gentamicin (Gm; 10 μg/mL), nalidixic acid (NA; 25 μg/mL), norfloxacin (Nfx; 1 μg/mL), and a plain MacConkey agar (control plate). Growth on any of these antibiotic-containing plates was recorded as resistance to the antibiotic.

The HE agar plates were likewise replica plated to MH agar plus CMZ and to HE agar plus Amox and HE agar plus Nfx. Plates were incubated as before, and the percentage of resistant colonies was calculated by comparing the numbers and phenotypes of colonies in the designated quadrants with the comparable quadrant on the original master and final control plates. Resistance at the ⩾10% frequency level was defined as ⩾10% of growth, compared with the master plate. High-frequency resistance or the presence of resistance in ⩾50% of an individual's flora was defined as ⩾50% of growth, compared with the master plate.

After return from Nepal to the Center for Adaptation Genetics and Drug Resistance, 5 swab samples from each village underwent confirmatory testing by repeat analysis, using the identical replica-plating method. Despite the fact that >1 month had elapsed since collection, there was no significant difference in either survival or resistance profile in organisms from the swabs that were subjected to repeat testing.

Statistical analysis. Data were entered and statistics were performed using the SPSS software program (SPSS) and Epi Info software package (version 6.0, Center for Disease Control and Prevention). Frequency of resistance in gram-negative aerobic fecal flora for each of 8 different antibiotics was analyzed by use of analysis of variance, with a Student-Neuman-Keuls test, where applicable. Two levels of resistance (⩾10% and ⩾50% of the aerobic fecal flora) were analyzed. Multiple resistance profiles were determined on the replica-plated samples, using χ^2 analysis. Potential confounding, such as the use of medicine and visits to health care providers, was evaluated with independent sample Student's *t* tests.

Results

Of the 50 swabs distributed in each village, 33–34 per village were returned to the investigators. This rate of sample return (66%–68%) may reflect that there was <24 h between distribution and collection. Not all volunteers may have produced a sample in that time period. In addition, some lack of return may be attributable to Nepalese cultural embarrassment concerning bowel habits. There was no difference in the time of return of the samples among the villages (15 ± 3 h).

The total population surveyed consisted of 38% males and 62% females, ranging in age from 15 to 47 years (mean, 25.5 years; table 2). The sex distribution among the participants was similar in Dolakha and Dhulikhel. The low percentage of males in Simigaun (9.1%) was presumably due to employment outside the village in the trekking industry or participation in regional political elections. There was no significant variation in the mean ages among villages.

About 61% of the samples showed only Lac^+ (lactose fermenters) phenotypes on MacConkey agar. Thirty-nine percent of samples contained both Lac^+ and nonfermenters (Lac^-), but the latter constituted <15% of any given sample. Since the Lac^- population comprised a minor component of the total, this analysis focused on the profiles of the predominant Lac^+ population. The recovery of *Salmonella* or *Shigella* species from HE was infrequent and occurred in ~1% of the samples recovered from the participants. Because of the low frequency, these isolates were not characterized further.

Table 2. History of health care exposure among study subjects in 3 Nepalese villages.

Village	No. of persons sampled	Sex, M/F	Age range, years	Visited doctor, %		Visited Jhankri, %		Used medication, %[a]	
				Within 1 year[b]	Ever[c]	Within 1 year[c]	Ever[c]	Within 1 year[d]	Ever[e]
Dhulikhel	33	16/17	15–47	42	88	21	42	48	91
Dolakha	34	19/15	15–44	65	88	47	68	68	94
Simigaun	33	3/30	15–44	18	48	61	85	42	76
Total	100	38/62							

NOTE. Jhankri, traditional healer.
[a] Any medication.
[b] The difference between Simigaun (the most rural village) and the other villages was significant ($P = .009$).
[c] The difference between Simigaun and the other villages was significant ($P < .001$).
[d] The difference between any 2 villages was not significant ($P = .098$).
[e] The difference between Simigaun and the other 2 villages approached statistical significance ($P = .059$).

The frequencies of resistance to each drug in Lac^+ colonies were compared among the samples from each of the different village populations. A comparison of results from males and females in Dolakha and Dhulikhel villages showed no significant difference between the sexes (data not shown). Consequently, the samples from males and females were combined. All samples showed some level of resistance to all the drugs tested, with the exception of Gm resistance, which was not found in Simigaun. The number of individuals with a ⩾10% frequency level of resistance (⩾10% of growth, compared with the master plate) to ⩾1 antibiotic was similar for all 3 villages: 31 for Dolakha and Simigaun and 32 for Dhulikhel. However, significant differences in resistance to specific drugs between villages were found for Tet, CMZ, Cthn, NA, and Gm (figure 1*A*). The most profound of these was a 2-fold higher resistance to Tet and a 5-fold higher resistance to NA in the closest village (Dhulikhel), compared with results from either of the further villages (Dolakha and Simigaun; $P < .05$). High-frequency resistance (i.e., resistance in ⩾50% of the organisms in an individual's Lac^+ flora) was significantly less prevalent in the distant villages than in Dhulikhel (figure 1*B*). Although 29 individuals (88%) from Dhulikhel demonstrated this high-frequency resistance to ⩾1 antibiotic, only 16 individuals from Dolakha (47%, $P = .001$) and 22 from Simigaun (67%) demonstrated such high-frequency resistance. Samples from Dhulikhel (the closest village to Kathmandu) had a 2–3-fold higher frequency of bacteria resistant to Tet, CMZ, Cthn, and NA, compared with samples from the further villages (figure 1*B*).

Coresistance to ⩾2 of the drugs within the same bacterial colonies of an individual's flora (at a level of ⩾10% of each sample) appeared in >90% of the samples from Dhulikhel and in <80% of samples from the other 2 villages (figure 2). Larger differences between the villages appeared with coresistance to ⩾3 drugs (Dhulikhel, >90%; other villages, <60%). Resistance to ⩾4 antibiotics was high in Dhulikhel (~70% of the sampled population), whereas it was markedly less in the other villages (<40%; figure 2). In fact, individuals from Dhulikhel had fecal organisms resistant to significantly more antibiotics, with a mean of 4.5 drugs (at ⩾10% of each sample), compared with a mean of 3.1 drugs for Dolakha and 2.5 drugs for Simigaun ($P \leqslant .001$; figure 2). At the ⩾50% level, individuals from Dhulikhel bore resistance to a mean of 3.1 antibiotics, compared with 1.2 antibiotics for Dolakha and 1.0 antibiotic for Simigaun (data not shown). These differences were also highly significant ($P \leqslant .001$).

Compared with reports from Dhulikhel and Dolakha, significantly fewer individuals in Simigaun had reported visiting an allopathic doctor within the previous year ($P = .009$) or within their lifetime ($P \leqslant .001$; table 2), and no Simigaun villagers reported visiting an allopathic physician within the past month. Among the subset of villagers who had ⩾50% resistance (as described above) to ⩾1 antibiotic, these differences persisted: 89% of inhabitants from 2 villages closest to Kathmandu had visited a doctor within their lifetime, compared with only 50% of those from Simigaun (data not shown). This difference remained statistically significant ($P \leqslant .001$).

A history of the past and present use of allopathic medications showed that, even in the most isolated village, there was exposure to these drugs, and most (>75%) individuals from each of the 3 villages had used them at some point in their lifetime. Neither the lifetime usage nor recent (past year) usage differed significantly among the villages ($P = .059$ and .098, respectively; table 2). In addition, a comparison of the subset composed of only those individuals who possessed highly resistant flora (i.e., had flora with ⩾50% resistance to any drug) showed variable levels of contact with medications. Although 93% of these individuals from the 2 villages closest to Kathmandu reported use of medicines, only 77% of such individuals from Simigaun reported having used them ($P \leqslant .001$; data not shown).

Discussion

Antibiotic resistance is prevalent even among populations of people or animals who have had no recent antibiotic consumption [3, 7, 9, 11–15, 23–26]. Why this phenomenon exists is not clear. Some studies suggest that it relates to an environ-

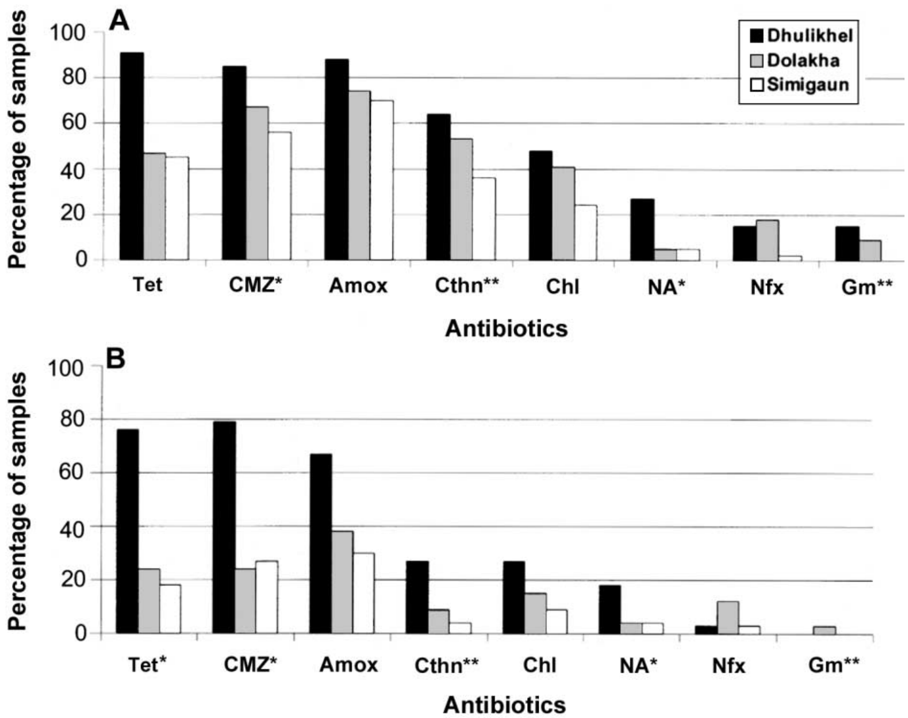

Figure 1. Percentage of fecal samples from each of 3 Nepalese villages bearing drug-resistant, gram-negative, lactose-fermenting fecal organisms. *A,* Resistance frequency at the 10% level (defined as ⩾10% of visible colonies able to grow when exposed to the antibiotics listed, using a replica-plating method). Statistically significant *P* values ($P \leqslant .05$) existed for values between the 2 distant villages of Simigaun and Dolakha and the most urban village of Dhulikhel (*) and for values between the most urban village of Dhulikhel and the furthest village, Simigaun (**). *B,* Resistance frequency at the 50% level (defined as ⩾50% of visible colonies able to grow when exposed to antibiotics as listed, using a replica-plating method). Statistically significant *P* values ($P \leqslant .055$) existed for values between the 2 distant villages of Simigaun and Dolakha and the most urban village of Dhulikhel (*); the differences between the 2 distant villages and the most urban village approached statistical significance ($P = .05$). Amox, amoxicillin; Chl, chloramphenicol; CMZ, cotrimoxazole; Cthn, cephalothin; Gm, gentamicin; NA, nalidixic acid; Nfx, norfloxacin; Tet, tetracycline.

ment conducive to the spread of resistant organisms among a population [11, 17, 27]. With the dramatic increase in population size and in local and global travel, person-to-person contact with relatively isolated communities has increased. Therefore, it becomes increasingly more difficult to determine true baseline levels of drug susceptibility in bacteria within a population that has had minimal contact with antibiotics. Moreover, the contribution of community exposure resulting from proximity to antibiotic sources and to other individuals has not been compared with the effects of individual antibiotic consumption on a population's carriage of drug resistance.

Nepal, situated between India and Chinese Tibet, supports a population of >20 million people who are divided into >30 culturally diverse ethnic groups [28]. With 81% of the population occupied in farming, most Nepalese are primarily engaged in subsistence agriculture for their livelihood. The diverse geographical terrain of Nepal serves to demarcate the inhabitants into clearly defined urban (9%) and relatively isolated rural populations (91%), many of which are accessible only by footpath. Up to 85% of Nepalese, particularly in rural areas, rely primarily on traditional healing methods, such as those employed by Jhankri (traditional healers), who may use herbs but traditionally do not dispense antibiotics [29, 30]. Of all Nepalese who seek any type of medical care, ~10% consult first with a jhankri. This number reaches >20% in the Eastern Hill Region of Nepal [31]. The vast majority of allopathic health care services are centralized in major urban areas, such as Kathmandu, Pokhara, and Birgunj. For allopathic care, the inhabitants of rural areas often rely on health posts, which are minimally staffed and supplied.

Antibiotic use in Nepal is difficult to assess because of the lack of data on specific use. One study conducted in 1998 showed that 68% of drug prescriptions/recommendations for diarrhea and 70% of prescriptions/recommendations for acute respiratory infection symptoms were for antimicrobials. Of all encounters at health facilities and with private-sector pharmacists, 50.7% resulted in antimicrobials being dispensed [32]. With a physician-to-patient ratio of 1:16,667 (compared with a ratio of 1:420 in the United States), most Nepalese are forced to rely on health assistants and pharmacy shopkeepers as their

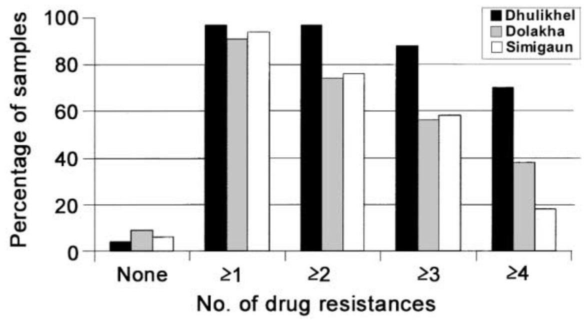

Figure 2. Total no. of antibiotics (0–8) to which individual samples (gram-negative, lactose-fermenting fecal flora) from each village were resistant at the 10% level (defined as ⩾10% of visible colonies able to grow when exposed to antibiotics, as listed in the figure 1 legend, using a replica-plating method). The comparison shows significant differences in the mean nos. of antibiotics to which organisms were resistant: Dhulikhel (mean, 4.5 antibiotics) and the other 2 villages (mean, <3.1 antibiotics; $P \leqslant .001$).

primary source of allopathic health care [28]. Studies have documented that ⩽97% of the medications distributed by these pharmacies for routine symptoms, such as diarrhea, are antimicrobials [19]. Given the enormous percentage of distributed medications that are antimicrobials, coupled with the lack of knowledge surrounding allopathic medications and disease names, we used distribution of any drug as a surrogate for antimicrobial distribution in this study.

The demographics of Nepal offered a unique opportunity to evaluate the frequency of antibiotic resistance in the aerobic, gram-negative, lactose-fermenting fecal flora from 3 village populations having different access to allopathic health care. Higher frequencies of resistance were noted (at both the ⩾10% and ⩾50% frequency levels) in people from Dhulikhel, the closest village to allopathic health care and urban centers, than in Dolakha or Simigaun. The differences in resistance to certain antibiotics (e.g., Tet, Amox, or CMZ) were highly significant (figure 1). The distribution of resistance to the different drugs coincided with what might be expected: higher frequencies among the older drug families and lower frequencies among the newer ones. This finding may reflect the fact that drugs such as beta-lactam antibiotics and Tet are still commonly available, whereas the newer and far costlier ones (e.g., Gm and the fluoroquinolones) are more difficult to obtain. Resistance to all of these newer antibiotics (Gm, NA, and Nfx) was almost completely absent (<5% frequency) in the furthest village of Simigaun (figure 1). The finding of higher levels of resistance to newer agents in the more urban populations suggests that antibiotics like the fluoroquinolones are available in these areas.

Of particular note is the dramatic difference in frequency of resistance between Dhulikhel and Dolakha, which are of somewhat similar population size but very different in the number of health care facilities (figure 1 and figure 2). Here, however, neither doctor visits nor consumption of medications differed significantly. In fact, the village with less resistance (Dolakha) showed more recent doctor visits (65% vs. 42%) and more medication use (68% vs. 48%; table 2).

The levels of resistance found in the fecal samples from the furthermost village (Simigaun) were particularly interesting. The relative isolation of this village from hospitals and health posts makes contact with antibiotics through health care professionals relatively unlikely. In fact, none of the individuals from this remote village reported recent contact (within 1 month) with an allopathic physician, and most (52%) had never had such contact. Despite the lack of direct contact with health care workers, however, 87% of all villagers reported using allopathic medicines at some point in their lifetime, and 53% reported having used them within the past year, although the exact identifications could not be defined. This information suggests that drugs were obtained from other sources, such as trekkers, traveling contacts, or drug-distribution programs.

In addition, a relatively large number of men from Simigaun were involved in the trekking industry. These men are frequently absent from the village for extended periods and have contact with outside tourists and inhabitants of urban centers, such as Kathmandu. Our survey purposefully excluded these individuals as possible direct contributors. However, transfer of resistant bacteria occurs both in the presence and the absence of antibiotic-selective pressure [11, 27]. Thus, trekkers could potentially serve as transmission vectors to contacts in their home communities. In addition, more than half the 42 plant species recorded as widely available and commonly used in Nepalese herbal medicine are reported as having "antiseptic" or "disinfectant" qualities and may, therefore, contribute to the selection and propagation of drug-resistant bacteria [31]. Last, rural village dwellers frequently share a common water source for multiple purposes (i.e., drinking, bathing, laundry, and crop irrigation), and this may enhance the probability of shared bacterial strains among these populations. Unlike Dolakha and Dhulikhel, which have numerous water sources, Simigaun maintains a single common tap system, which is known to have many breaks with potential exposure to runoff.

Multidrug resistance (resistance to ⩾2 antibiotics) was significantly higher in Dhulikhel than in either of the more rural communities (figure 2). Although the low number of doctor visits could possibly explain the lower multiresistance found in Simigaun, it does not account for the similarly low multidrug resistance found in Dolakha (table 2). In addition, and more important, there was no significant difference in consumption of medicines that can explain the different multiresistance frequencies found in the 3 villages (table 2). There was not even a clear relationship between medicine use and resistance when comparing the subsets of all individuals who exhibited high-level resistance (i.e., ⩾50% of flora resistant to ⩾1 drug). Although 93% of those in Dolakha and Dhulikhel reported taking medication, only 77% of those in Simigaun reported doing so.

The finding of significantly higher proportions of multidrug resistance in the population(s) closest to clusters of health care centers and hospitals parallels our previous report of higher multidrug resistance frequencies in a hospitalized population with intense exposure to antibiotics [3]. It likewise lends support to the suggestion that resistance correlates with the environmental density of antibiotic emanating from focal points of drug distribution.

These findings support the hypothesis that it is not only medicine consumption or doctor visits but rather the environment in which the villagers live that dictates the level of antibiotic resistance. It is striking (see figures 1 and 2) that Simigaun and Dolakha resemble each other in resistance frequency, compared with Dhulikhel, despite the fact that they are of different sizes and at considerably different distances from the capital city. For the 11,000 inhabitants of Dhulikhel, there are >66 health care facilities (⩾1/166 persons), whereas Dolakha has only 9 for its 3500 villagers (1/389 persons), and Simigaun has none for 330 dwellers. Together, these data again support the hypothesis that it is the environmental density of antibiotics and antibiotic-resistant bacteria arising from foci of antibiotic availability that affects the net resistance frequency in the community population.

The higher frequencies of resistance to both single and multiple antibiotics in the fecal flora of villagers living closer to Kathmandu suggest that antibiotic availability was a key factor in determining the carriage of resistant fecal bacteria by an individual. However, the finding that this relationship between distance from Kathmandu and resistance frequency was independent of the individual's report of doctor visits and allopathic medicine usage suggests that the close community environment of antibiotic use and potential for spread of resistant bacteria contribute as much or more to the frequency of resistant bacteria in an individual's fecal flora than does the individual's own antibiotic usage.

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