

**Influence of Sex, Age, and Antibiotic Soap Use on the Number and Antibiotic
Resistance of Bacteria**

**Anna Bramucci
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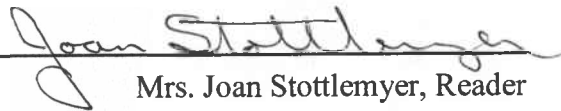
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Abstract

This research dealt with factors that may impact the number of enteric and fecal coliform bacteria and the antibiotic resistance of the bacteria present on the hands of children ages one to 15. The targeted bacteria were the subset enteric bacteria, which cause potentially lethal cases of diarrhea in the United States and worldwide. Further exacerbating the mortality rate associated with enteric bacterial infections is the degree of antibiotic resistance acquired by the bacteria. The sterile bag method was used to acquire bacteria from the hands of children; the bacteria were counted and tested for degrees of antibiotic resistance using antibiotic disks. I investigated the relationship between the quantity and antibiotic resistance of the bacteria colonies and the sex, age, and antibacterial soap use of the subject. I found that there was a weak correlation between age and number of bacteria per hand: $R^2 = 0.3654$, while for fecal coliforms per hand, the correlation was stronger: $R^2 = 0.8464$. These data indicated that age was a factor in the number of fecal coliforms per hand, while age was less of a factor in the number of bacteria per hand on children. There was no correlation between the sex or age of children and the antibiotic resistance that each bacterium had acquired. This study indicated that a high percentage of bacteria sampled were multi-drug resistant, which hints at a relationship in the mechanism of acquiring antibiotic resistance.

Introduction

The Problem.

Diarrhea is an illness attributed to bacterial flora residing in the intestinal tract. The number of diarrheal diseases that stem from infectious pathogens may be as high as 324 million cases annually in the US, 25 to 99 million of which are attributed to bacterial infections by enteric bacteria (Garthright *et al.*, 1988). The death rate due to resultant disease remains high in the young, elderly, and immunodeficient subsets of the population. In the US, 456 children under the age of 5 die each year due to complications of diarrhea or related intestinal diseases (Peterson and Calderon, 2003).

Pathogenic enteric bacteria and fecal coliforms such as *Escherichia coli*, *Shigella*, and *Salmonella* are transmitted through the fecal-oral pathway. One subset of enteric bacteria are fecal coliforms, which are gram-negative bacteria that ferment lactose in 48 hours at 35°C (Madigan, 2006). Enteric pathogens of animal and human origin can be carried via fecal-contaminated water systems (Chambers, 2005), the air, ingested on food matter, or transmitted via skin-to-skin contact with infected substances.

The numerous mechanisms of bacterial transmission account for the high degree of infections spread throughout animal and human populations worldwide. Although these diseases are currently treatable, and even largely avoidable in developed nations, they are still a threat in poor areas and third world countries; this is due in part to lack of sanitation and the cost of treatment, which is \$20 billion per year in the United States alone (Garthright *et al.*, 1988; Franco, 1997).

Exacerbating the Problem—Antibiotic Resistance.

Since the discovery of penicillin by Alexander Fleming in 1928, a widespread use of various antibiotics has occurred, leading to increased antibiotic resistance in clinical, agricultural and environmental settings. The first penicillin-resistant bacterial isolate was documented in 1947 (Demerec, 1948). Furthermore, there has been a documented 2.5-fold increase in the hospitalization rate of people suffering from bacterial-caused illnesses and diarrhea since 1985 (Frost *et al.*, 1988). Causes for this increase must be studied further, and some possibilities include the following: use of antibiotics in treatment plans, the particular hospital system (Frost *et al.*, 1988), or other untested variables. In one study of *Streptococcus pneumoniae*, 38% of patients suffering from antibiotic resistant strains died, while only 24% of those infected with antibiotic-susceptible strains died ($p = 0.001$) (Pallares *et al.*, 1995). This study demonstrated that while the naturally virulent strains of bacteria maintain potency, their antibiotic-resistant counterparts proved to be a much greater threat to the lives and well-being of humans.

The Spread of Antibiotic Resistance.

Since bacterial resistance to antibiotics is such a serious problem in the human community, the mechanism by which increased resistance occurs must be studied thoroughly. Overuse of antibiotics in humans and especially in animals is the most common explanatory theory for increased antibiotic resistance in bacteria (Van den Bogaard and Stobberingh, 1999). The magnitude of this problem is increasing drastically due to the quantity and number of antibiotics in use. In the US around 23×10^6 kg of antibiotics, half of which are for human consumption, are in circulation annually, and the estimate of penicillin and tetracycline usage is around 7×10^6 kg annually (Levy, 2002).

The transfer of enteric bacteria to humans from animals, continually fed “subtherapeutic doses” of antibiotics, led to an increase in antibiotic resistance acquired by fecal coliforms (Gibbs *et al.*, 2006). This research also showed that antibiotic resistance to penicillin increased 65%, while resistance to erythromycin and tetracycline increased by 82% and 94%, respectively (Gibbs *et al.*, 2006).

Due to the cost and life-threatening nature of these *super germs*, a current focus of microbial research is not only to determine the degree of increase in antibiotic resistance of the pathogens, but also to discover where antibiotic-resistant microbial reservoirs reside in animal and human populations. Gibbs *et al.* (2006) showed that one such reservoir was present near animal dwellings where livestock were continually fed subtherapeutic doses of antibiotics. Finally, another mechanism of transfer occurs naturally during plasmid transfer of the affected bacteria to surrounding bacteria.

The concern is that intestinal flora may serve as a reservoir for antibiotic-resistant genes, and the increased quantity may be caused by hyper-sanitation of hospital settings rather than antibiotic ingestion. Levy *et al.* (1988) found that 60 % of patients abstaining from antibiotic usage still had an increased antibiotic resistance to multiple drugs. This suggests that there may be other factors, aside from a history of antibiotic drug use, that play a role in the increasing resistance seen in patients who have extended hospital stays.

Whether this increase is linked to the animals with which humans come in contact (Gibbs *et al.*, 2006) or the amount or duration of antibiotics received (Levy *et al.*, 1988) neither have been studied extensively; however, the unexplained antibiotic resistance increase observed by Levy *et al.* (1988) emphasizes the need for further examination. For example, the number of antibiotic resistant bacteria may increase based on the

number of times a day a subject washes his or her hands, the type of soap used, or even the location of the subject at the time prior to sampling.

Objectives.

The goals of the present study were as follows: to determine the influence age and gender may have on the amount of bacteria (enteric and fecal coliforms) per hand, examine differences in the effectiveness of antibacterial soap versus generic soaps, and discuss possible factors affecting antibiotic resistance in children. In the current study, the normal hand flora of children ages 1-15 was sampled, for *E. coli*, coliforms, and total bacteria. The dominant colony type was screened for antibiotic resistance (Table 2). I hypothesized that there would be a relationship between antibiotic resistance of bacterial isolates from the hands of children and the sex, age, and antibiotic soap use of the subject.

Materials and Methods

Random Sample Population. A random population of children, ages one to 15, from Helena Montana were sampled in order to analyze the relationship between the number of antibiotic-resistant bacteria, the history of hand washing, use of antibiotic soap, and age of the child. The study population consisted. To ensure the population was random, children were sampled at different times and locations. Subject anonymity was maintained throughout this project by using number codes to track samples and corresponding survey data. This allowed for anonymous tracking of an individuals demographic information and the antibiotic resistance of their flora.

Every subject received a survey and submitted the required information prior to sampling. This information was used to divide the children into equal subsets of the whole sample population. The divisions were based on sex and age of the individuals (Table 1). Differences in the bacterial counts per hand of these groups should indicate the differences among the general activities of children that are at these stages.

| Table 1: Informational Survey Breakdown | |
|---|--------------------------|
| Variable | No. (%) of case subjects |
| Males | 74 (40.9) |
| 1-3 | 15 (28.4) |
| 4-7 | 33 (36.5) |
| 8-10 | 18 (24.1) |
| 11-15 | 8 (11.0) |
| Females | 107 (59.1) |
| 1-3 | 21 (22.4) |
| 4-7 | 32 (27.1) |
| 8-10 | 25 (23.4) |
| 11-15 | 29 (27.1) |
| Time and Location | |
| School (8-11am) | 43 (23.6) |
| Swimming (8-11am) | 33 (18.2) |
| CC b-ball camp (8-11am) | 13 (7.1) |
| Helena Fair (12-4pm) | 13 (7.1) |
| Alive at Five (4-8pm) | 65 (36.0) |
| Outside Vans (4-8pm) | 14 (8.0) |

Sampling Bacteria and Fecal Coliforms. Children washed both of their hands for 15-30 seconds in a *Ziploc* bag containing 10mL of sterile 1% saline solution. Most samples required a standard 10:1 dilution prior to plating 0.1 mL of 1% saline on nutrient agar plates for total bacterial counts. However, for coliform and *E. coli* counts 0.5 mL of undiluted sample were plated onto Bio-Rad *E. coli 2* agar: Marnes-La-Cequette, France. For this study Nutrient Agar plates were made using Nutrient Broth and 15 grams of a common agar base. The *E. coli* plates were made as prescribed by the manufacturer using Bio-Rad *E. coli 2* agar. The plated microorganisms were then incubated for 18-24 hours and maintained at a constant temperature of 35° C (Madigan, 2006).

Plate Counts. Following incubation, the number of bacteria in the original sample was estimated by colony counting. A plate inoculated with a blank saline control was also

counted to verify contamination was not being introduced during the handling, dilution and plating of samples. Samples that produced plates too numerous to count were diluted further, plated again and examined as described previously. One colony representing the dominant colony type on each plate was selected for antibiotic resistance screening. A portion of the colony was transferred to 5 mL Nutrient Broth incubated overnight at 35° C. Following incubation, 0.5 mL of the culture solution were plated onto nutrient agar, and five antibiotic discs were placed in a pentagon shape on the plate (Table 2). The plates were then incubated again at 35° C.

Resistance. Antibiotic resistance for each bacterium was assessed using standard antibiotic susceptibility testing. The diameter of clearing around an antibiotic disk was measured to determine susceptibility/resistance to a given antibiotic (Microbiology, 2006).

Table 2: *Zones of Clearing for Various Antibiotics.* (Microbiology and Bacteriology: The World of Microbes; Figure 10-2).

| Antibiotic | Disk Potency | Inhibition Zone | Diameter (mm) |
|---------------------|--------------|-----------------|---------------|
| | | Resistant | Susceptible |
| Tetracycline (TET)* | 5 µg | <19 | ≥19 |
| Ciprofloxacin (CIP) | 5 µg | < 21 | ≥ 21 |
| Erythromycin (E) | 15 µg | < 18 | ≥ 18 |
| Penicillin G (P)* | 10 units | < 29 | ≥ 29 |
| Sulfisoxazole (G25) | 25 µg | < 17 | ≥ 17 |

* data from *Zone sizes for some antimicrobial disc susceptibility tests*, Table 24.4 (Madigan, 2006).

Statistical Analyses. For these analyses, I first determined that the data sets were skewed right of the normal distribution curve. To normalize the data sets for general bacteria and fecal coliforms per hand, I used the natural logarithm of all the points. Re-fitting those data points to the normal curve then allowed for use of the multivariate analysis of

variance (MANOVA), with the *Statistica* software program.

Results

Bacteria and Fecal Coliform Counts.

Of the 181 children sampled throughout this study only one tested positive for *E. coli*. For this reason general bacteria and fecal coliform counts became the main focus of the study. To determine the effect age had on these two variables a MANOVA statistical test was performed. This test showed a statistical correlation between age and general bacteria and fecal coliform counts on the hands of children: $p < 0.001$ (Table 3).

Table 3: Results of MANOVA for overall effects of age on the total number of bacteria and fecal coliforms per hand as measured by plate counts.

| | F | Age df | p |
|------------------------------|------|-----------|---------|
| MANOVA (Wilk's Criterion) | 0.80 | 6,166 | 0.005 |
| ANOVA | | | |
| general bacteria | 3.93 | 3 | < 0.001 |
| Fecal coliforms* | 6.49 | 3 | < 0.001 |

* Includes only samples positive for fecal coliform contamination; when the remaining samples were included, the statistics were not significant.

Given the statistical differences among the four predetermined age groups and general bacterial load. For these analyses, the log transformed data of general bacteria counts were compared to age. A statistical difference was observed between age groups C and D. Evident differences were observed between age groups B and D, and A and C (Table 4).

Table 4: P-values of post-hoc comparisons (Tukey HSD) among the age groups for the log of the bacterial load. Age groups in years: 1-4(a), 5-7(b), 8-10(c), 11-15(d).

| | A | Age B | C |
|---|-------|----------|-------|
| A | | | |
| B | 0.233 | | |
| C | 0.089 | 0.898 | |
| D | 0.963 | 0.067 | 0.022 |

Age group D has statistically more general bacteria per hand than age group C has. Due to decreased sample size and high variability of data age groups B and C are graphically different, but the change is not significant. The youngest age group A is shown to have increased number of bacteria per hand in comparison to age group B; however, again this difference is not significant (Figure 1).

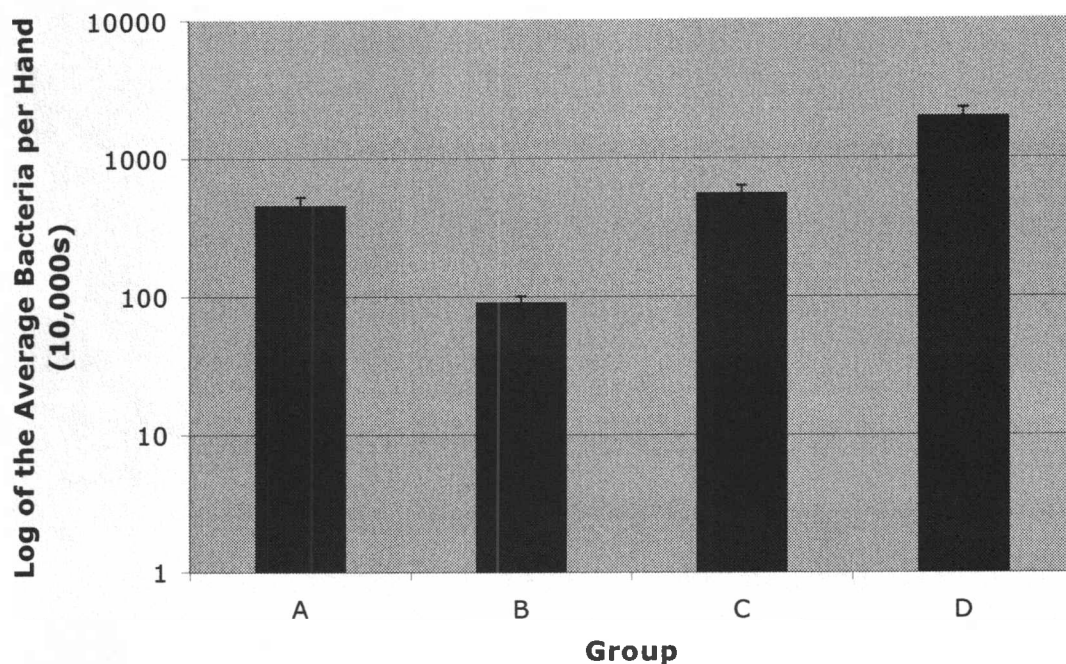


Figure 1. The association of age and the logarithm of the average bacteria per hand on children ages one to 15: $p = 0.00954$. Age groups in years: 1-4(a), 5-7(b), 8-10(c), 11-15(d).

Due to the large number of samples that tested negative for fecal coliform presence, fecal coliform counts were highly variable. Removing all of the points for samples that tested negative for fecal coliforms made the test more accurately represent the relationship between age and the number of fecal coliforms per hand. A relationship between fecal coliform counts and the age groups was evident in age groups A, B, and D; however the difference between the log transformed counts of fecal coliforms for age group C was statistically significant (Table 5).

Table 5: P-values of post-hoc comparisons (Tukey HSD) among the age groups for the log of the fecal coliform load. Age groups in years: 1-4(a), 5-7(b), 8-10(c), 11-15(d).

| | A | Age B | C |
|---|-------|----------|---------|
| A | | | |
| B | 0.998 | | |
| C | 0.021 | 0.006 | |
| D | 0.427 | 0.448 | < 0.001 |

The average log transformed counts for age groups A, B, and D decreased with age, while age group C had the highest average fecal coliforms per hand (Figure 2). All of the children in age group A were being supervised by adults at the time of sampling; however this number decreases with age: 89% (B), 39%(C), 37%(D).

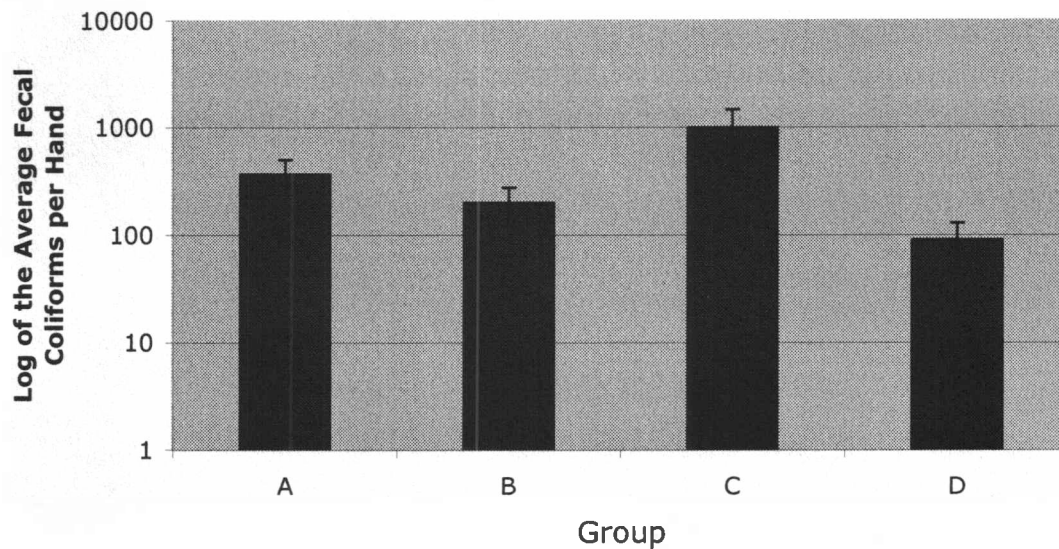


Figure 2. The association of age and logarithm of the average fecal coliforms per hand on children ages one to 15: $p=0.7498$. One outlier was removed from the 8-10 age range: 1,110,000 fecal coliforms per hand. Age groups in years: 1-4(a), 5-7(b), 8-10(c), 11-15(d).

Generic versus Antibiotic Soap Use.

Another objective of this study was to determine the influence of the use of antibiotic soap on quantity and percent of antibiotic resistance of hand bacteria. This was determined statistically using the standard errors (SE) of these data. The SE's for these

data portray that insignificance of the comparison of generic soap and antibacterial soap users at the 0.05 level for general bacteria (Table 6). There is a high significance in those data relating to the average number of fecal coliforms on the hands of generic and antibacterial soap users, as shown in Table 6 the generic soap users had many more fecal coliforms per hand than antibacterial soap users did.

Table 6: Difference in the average bacteria and fecal coliforms per hand for children who have used antibacterial soaps versus those who have used generic brands of soap.

| Population Subset | Average Number of Bacteria per Hand (10,000's) |
|--------------------------|---|
| Generic Soap Users | 568.15 (SE= ±240.03) |
| Antibacterial Soap Users | 556.34 (SE= ±260.63) |

| Population Subset | Average Number of Fecal Coliforms per Hand |
|--------------------------|--|
| Generic Soap Users | 723.0 (SE= ±230.1) |
| Antibacterial Soap Users | 98.0 (SE=± 33.7) |

While the users of generic soap had higher numbers of fecal coliforms per hand, antibacterial soap use did not effect multi-drug resistance of the bacteria. Frequency of MDR to two or three antibiotics is noticeable in both antibiotic and generic soap users (Figure 3).

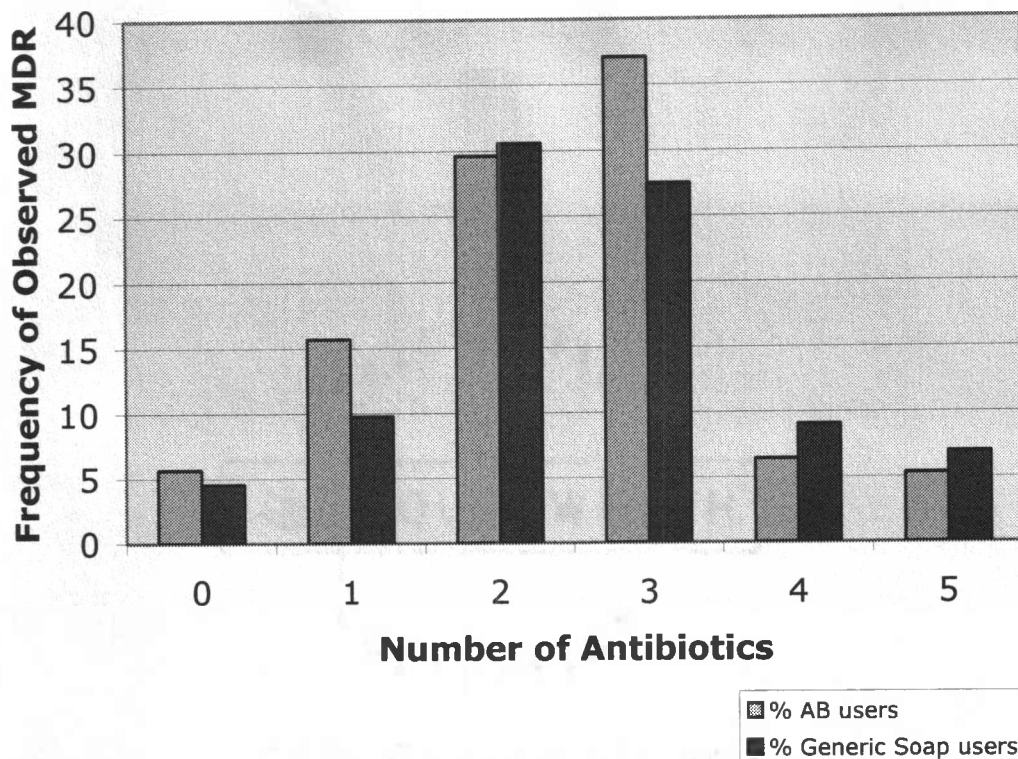


Figure 3. The effect of antibiotic soap use on enteric bacterial antibiotic resistance.

With respect to the number of antibiotics an individual bacterium was resistant to it was more likely they would be resistant to 2-3 antibiotics and thus exhibit a MDR phenotype ($\chi^2 = 152$, $df = 5$, $p < 0.001$). The vast majority of the bacteria tested had multiple resistances for both bacterial sets. Furthermore, the largest group showed 39% antibiotic resistance to two different antibiotics for the general hand bacteria, and 38% antibiotic resistance to three antibiotics for the fecal coliform bacteria.

There was also a significant deviation from the expected frequency of observed MDR in the fecal coliform data sets for multiple resistances ($\chi^2 = 97$, $df = 5$, $p < 0.001$). The antibiotic resistance level for fecal coliforms was greater than that for general hand bacteria. However, the frequency of complete antibiotic resistance to all five antibiotics was similar for both data sets: 9% for general bacteria and 10% for fecal coliforms (Figure 4).

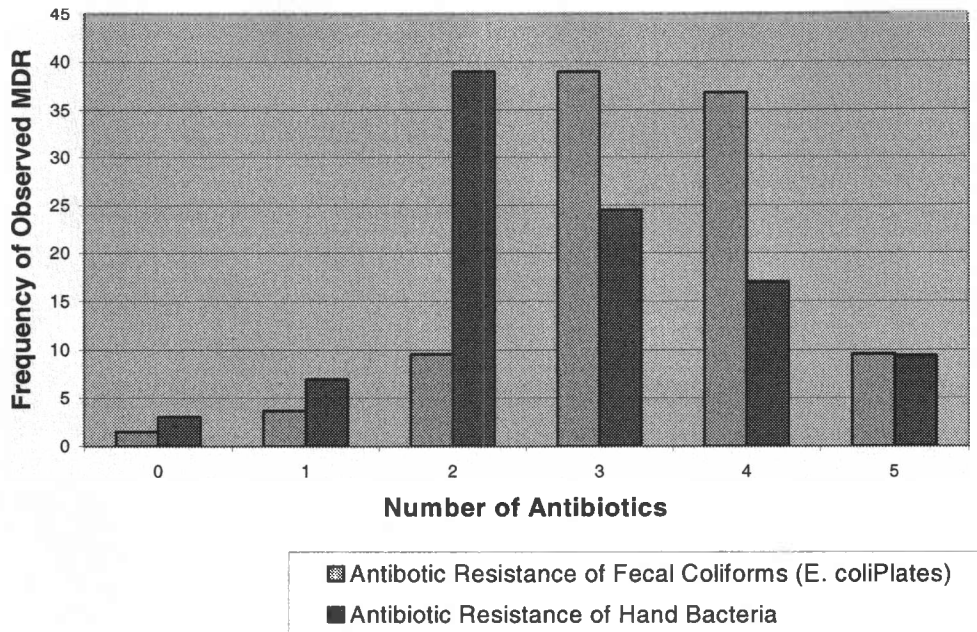


Figure 4: Frequency of MDR to various Antibiotics of tested fecal coliforms and enteric bacteria.

Inherent Antibiotic Resistance Levels.

Inherent antibiotic resistance to the five antibiotics was present in the tested bacteria (Figs. 5a and 5b). The inherent antibiotic resistance for penicillin and tetracycline was uncharacteristically high among the sampled bacteria. However, the frequency of antibiotic resistance for the other three antibiotics was present in around 30% of the tested general bacteria (Fig. 5a), while for the same three antibiotics frequency of antibiotic resistance approached 60% of the total tested fecal coliforms (Fig. 5b).

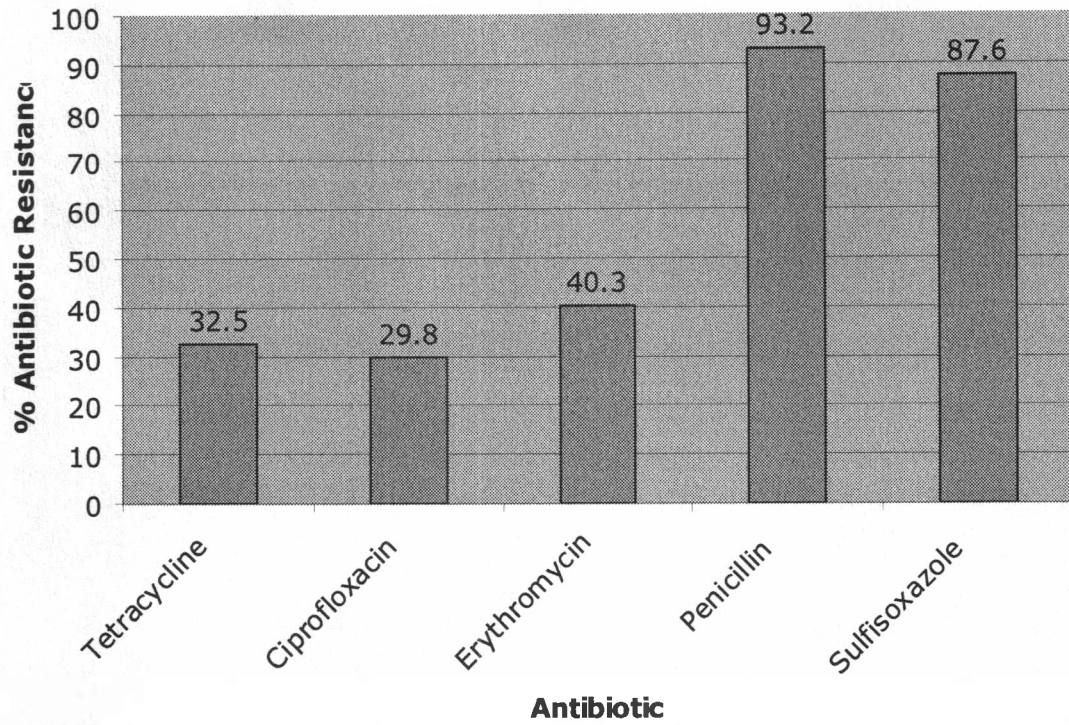


Figure 5a. Percentage of bacterial strains exhibiting antibiotic resistance.

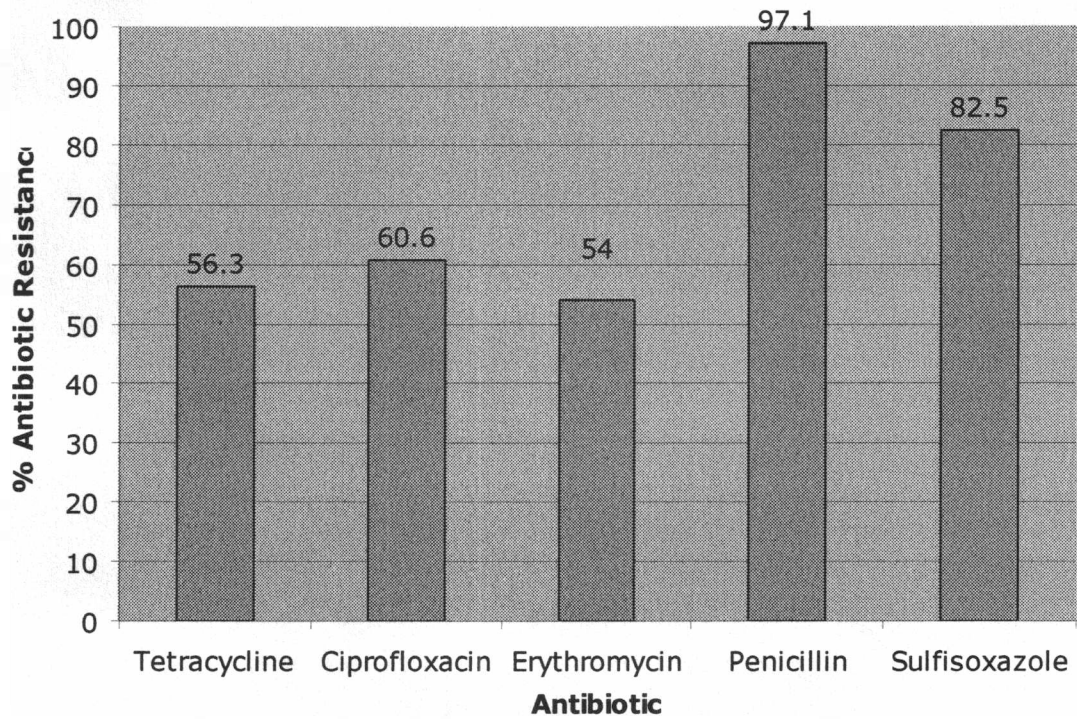


Figure 5b. Percentage of coliform strains exhibiting antibiotic resistance.

Discussion

This research investigated the influence of sex, age, and use of antibiotic soap on the number of bacteria and fecal coliforms per hand, and the antibiotic resistance of those bacteria. My null hypothesis was that none of the analyzed variables would have an effect on the number or antibiotic resistance of hand bacteria.

Influence of Age.

The relationship between age and the number of enteric bacteria/hand was highly statistically significant ($p < 0.001$). This suggests that the presence of general bacteria on hands increases with increasing age. Even more importantly the increase in number of general bacteria from age four to age 15 may be due to the related increase in hand size over those years of life. Thus, the disproportionately high number of bacteria on children from one to three years of age may be due to the cleanliness of children in that age range, while the elevated number of bacteria per hand for the oldest age group (11-15), may be due to increased hand size, and subsequently increase numbers of bacteria per hand.

The relationship between fecal coliforms and age, however, is more relevant to this study. Data for age groups A, B, and D suggest that the number of fecal coliforms per hand decreased as the age of the sampled child increased. This could possibly be due to the fact that social practices, which begin during preschool, stress upon children the importance of hand washing and cleanliness. This theory is supported by the decrease in the association of age and average fecal coliforms/hand. Children in age group C (eight-to-ten-year-old); however, had a disproportionate number of fecal coliforms per hand. The discrepancies in this group may be due to the percentage of children in this group that were sampled when under adult supervision. The percent of supervision decreased

with increasing age, but in this case supervision decreased drastically from age group B to C: 89%(B), 39%(C). This change in parental supervision of the children may be responsible for the sudden increase in fecal coliforms/hand in this age group.

Furthermore, the decrease in average fecal coliforms/hand in age group D (11-15) could be do simply to increased awareness about cleanliness habits, or due to the fact that though only 38%(D) were supervised by an adult 100% were with peers at the time of sampling. Societal pressure and positive peer pressure therefore may be a factor in the cleanliness habits of the older age group of children.

Generic versus Antibiotic Soap Use.

The decrease in the number of bacteria present on children who habitually use antibacterial soaps makes inherent sense, because those soaps are manufactured to kill 99.9% of hand bacteria.

Multi-antibiotic Resistance.

In cases of multiple antibiotic resistance, the increased degree of resistance can be caused by several different genes that, when mutated, control expression of antibiotic resistances in bacteria (Cohen *et al.*, 1993). Similar bacterial trends toward multiple antibiotic resistances were obtained in the present study for both general bacteria and fecal coliform bacteria. These results suggest that other factors may affect the acquisition of antibiotic-resistant bacteria, aside from the three hypothesized variables: age, sex, and antibacterial soap usage of the subject. Furthermore, a greater percentage of bacteria were resistant to three or more antibiotics, which statistically implies that some manner of linkage was unaccounted for in the transmission of antibiotic-resistant bacteria.

After first discovering forms of tetracycline-resistant *Shigella dysenteriae* in

1953, scientists have continually searched for the mechanism of increasing antibiotic resistance among bacteria (Falkow, 1975; Wasteson *et al.*, 1994). *Tet* genes impact the degree of tetracycline-resistant bacteria (Chopra and Roberts, 2001). Because tetracycline resistance is connected with the *tet* genes (Chopra and Roberts, 2001), and multi-resistant bacteria transfer is linked to *Mar* genes (Cohen *et al.*, 1993), it follows that those genes could possibly be genetically linked to other genes. If present, such a linkage would increase the frequency of multiple antibiotic resistances among bacteria. This possibility could explain the increase in antibiotic resistance of more than two of the tested antibiotics: penicillin, ciprofloxacin, tetracycline, erythromycin, or sulfisoxazole.

Summary

The present research tested whether sex, age, and antibacterial soap use affect the number of general bacteria and fecal coliforms present on the hands of children, and whether those factors also affect the antibiotic resistance of hand bacteria. Sex did not significantly affect any of the three parameters. Average general bacteria counts increased with increasing age, possibly because of the corresponding increase in hand size of the children. Age was inversely proportional to the average number of fecal coliforms present on the hands of children. This trend depicted that habits of cleanliness and societal mores influence older children to a higher extent than younger children. While antibacterial soap use did decrease the quantity of hand bacteria and fecal coliforms per hand, there was no correlation between the degree of antibiotic resistance and the soap use of the subject. Finally, there was increased likelihood that bacteria and fecal coliforms had acquired multiple antibiotic resistances among the tested children. This finding suggests that an external factor, aside from sex, age, and antibacterial soap use, influences the inheritance of antibiotic resistance in general hand bacteria and fecal coliforms.

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