PLASMID PROFILE ANALYSIS OF FECAL COLIFORMS ISOLATED FROM NATURAL WATERS

Jeffrey A. Evans, Noel P. D'mello, Krista K. Hamilton, and Robert D. Taylor
Departments of Chemistry and Biological Sciences
University of Southern Mississippi

INTRODUCTION

Water quality is of concern to its everyday consumers. One concern of the consuming public is the possible contamination of water supplies by disease-causing microorganisms. In order to meet the concern of the public, regulatory agencies at the local, state and federal levels are involved in the monitoring of water sources for potentially pathogenic organisms. Monitoring of water usually is accomplished by testing for indicator organisms instead of actual pathogens. These indicator organisms are usually in the coliform group of bacteria. Their presence in high levels in water is presumed to be caused by fecal contamination and indicates the potential presence of pathogens.

Routine screening of water for coliform indicator organisms has been of great health benefit, yet it does not actually reveal the source of bacterial contamination. A test that does reveal the source of bacteria would provide regulatory agencies a better "tool" for eliminating contamination. Other indicator organisms have been proposed for better determination of contamination sources, but have not been proven to be generally useful. Another method that has been used is the examination of fecal coliform to fecal streptococci ratios. A ratio of 4 or greater is considered evidence of human fecal contamination. Some believe this may be useful, while others argue that it can be affected by different die-off rates of the bacteria.

An alternative approach to locating sources of contamination is to determine if the same species of bacteria, e.g. Escherichia coli , originating in the gut of different animals differ in a detectable manner. If this were true, then bacteria from human sources could be traced in the environment by identification of its "human" specific character. One group of researchers has investigated hemagglutination in bacteria to see if it could identify specific cell surface antigens that would correlate with the source of the bacteria. They found over 60 different hemagglutination patterns, yet isolates from different animals shared many of these patterns. Thus hemagglutination patterns could not distinguish the source of the bacteria.

We have considered that bacteria from different sources may specifically differ in another way - their plasmid profiles. Plasmids are small circular extra-chromosomal DNA found in almost all bacteria. As an example of how analysis of plasmid profiles may distinguish sources of bacteria, research in other laboratories has shown that some E. coli possess specific plasmids that are required for that bacteria to colonize swine gut. This plasmid containing bacteria fails to achieve colonization in other animals. This implies that detection of this plasmid in bacteria from environmental samples would indicate that swine was the source of that bacteria. Our own work has demonstrated that differences exist in the size and number of plasmids found in bacteria isolated from different animals. This background led us to examine the use of plasmid profile analysis for environmental samples with the objectives described below.

The main goal of our work was to determine if plasmid profile analysis could be used to assess the bacteriological quality of water. To accomplish this goal we had four primary objectives. These were to:

1. Isolate coliforms above, at and below sewage outfalls into the Bouie and Leaf Rivers from sewage treatment lagoons near Hattiesburg, Mississippi.

2. Isolate plasmids from each coliform.

3. Characterize the plasmids from each isolate by size on agarose gel electrophoresis.

4. Compare the size profiles of the plasmids from coliforms found at the different sites.

The rationale and assumptions made in establishing these objectives are as follows. We assumed that sewage outfalls would contain a large number of coliforms originating from human fecal matter. We also assumed that samples obtained downstream from the outfall would have a predominance of human derived coliforms while upstream from the outfall, coliforms would be primarily from environmental sources, such as other animals (although the presence of human derived coliforms upstream could not be totally ruled out). Therefore, if plasmids were obtained from coliforms either at the outfall or downstream their sizes may be similar because of their similar source (human fecal material). On the other hand, plasmids obtained from coliforms upstream may differ from those below because of their dissimilar sources. Comparison of the sizes of plasmids obtained from the different sites would then provide a simple test of our idea.

METHODOLOGY

Sampling Procedure

Two different sewage outfalls close to Hattiesburg, Mississippi were chosen for sample collection. Beginning on September 23, 1987 and ending on November 11, 1987, water samples were taken on a weekly basis, but alternating weekly between the North Sewage Lagoon and the South Sewage Lagoon. The North Sewage Lagoon is located near North 31st Avenue and Lakeview road and empties into the Bouie River. The sample collection sites for the North sewage outfall were the outfall, about 25 feet upstream from the outfall and about 2 miles downstream from the outfall. The South Sewage Lagoon is located near East Hardy Street and empties into the Leaf River. The collection sites were at the outfall, about 30 feet upstream from the outfall and about 30 feet downstream from the outfall. The water upstream from the outfalls did not have any large sewage outfalls further upstream in close proximity to our sampling sites that were known to the investigators, but the presence or absence of smaller sources of human sewage upstream was not determined. In each case water collected at the downstream points were at sites where the river was well mixed. All water samples were collected in sterile Whirl-pack bags and transported to the laboratory on ice. The laboratory was located within 30 minutes driving time of the sampling sites.

Fecal Coliform Isolation and MPN Analysis

Both presumptive and confirmed tests were performed for coliforms to generate a Most Probable Number (MPN) Index. For the presumptive tests, tubes of lauryl tryptose broth were inocu- lated with serial dilutions of the water samples. The tubes were incubated for 24 hours at 37° C. Positive tubes were transferred to culture tubes containing EC broth media and incubated at 44.5°C for 24 hours for the confirmed tests. Additional primary fermentation positive tubes, indicative of late fermenters, were transferred to EC broth after 48 hours incubation. The MPN Index was generated from scoring the positive EC broth tubes. The MPN's were then determined from a MPN table.

E. coli colonies were obtained by streaking positive EC broth tubes onto lauryl tryptose agar plates. The agar plates were supplemented with 100 ug/ml of 4-methylumbelliferone glucuronide (MUG). After the inoculated plates were incubated for 24 hours at 37°C, they were examined under long wavelength UV light for fluorescent colonies. Fluorescent colonies indicate they are E. coli since these are the only fecal coliforms that hydrolyze MUG. The fluorescent colonies were transferred to nutrient agar slants, grown up and stored at 4°C until ready for plasmid isolation. It should be noted that only one E. coli colony per EC tube was used for plasmid isolation to prevent the picking of clonal colonies from the plates that would skew the data.

Plasmid Isolation

Plasmids were isolated from the E. coli by our modification of the procedure of Kado and Liu. The procedure was as follows. E . coli were inoculated from nutrient agar slants into 10 ml of L medium and incubated overnight at 37°C. A 1.5 ml aliquot was removed and centrifuged for 30 seconds in a Fisher microcentrifuge (approx. 8,000 RPM). The supernatant fluid was decanted off and discarded (failure to decant off all the fluid results in poor results). The cells were resuspended in 0.1 ml of E buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.9). Bacteria were lysed by the addition of 0.2 ml of lysing reagent (3% sodium dodecyl sulfate, 0.61% Tris, pH 12.6). The solution was mixed by vortexing and heated for 30 minutes at 37°C. This was followed by the addition of 0.6 ml of a 1:1 solution of phenolchloroform. After mixing, the solution was centrifuged for 2 minutes in the microcentrifuge. The aqueous layer (upper layer) was aspirated off for use in the further analysis of the plasmids by electrophoresis.

Marker plasmids from two different E . coli strains were isolated as described above. Marker plasmids were also isolated from the Streptococcus lactis strain ML3 by the method of Anderson and McKay. The ML3 strain was a gift from Anderson and McKay.

Agarose Gel Electrophoresis

Each sample of isolated plasmids was run on agarose gel electrophoresis in the following manner. Gels, approximately 5 mm thick, containing 0.7% agarose (DNA grade, BioRad) were poured in a Pharmacia GNA-200 cell (20 by 20 cm). The electrophoresis buffer E (40 mM Tris-acetate, 2 mM EDTA, pH 7.9) was added to the cell. Twenty uls of each sample were mixed with 5 uls of track- ing dye (0.25% bromophenol blue, 50% glycerol, 50 mM Tris- acetate, pH 7.9. Twenty uls of each solution were then applied to the submerged gel wells and electrophoresed at 80 volts for about 5 hours. Gels were removed and stained with a 1 ug/ml solution of ethidium bromide for at least 30 minutes. Gels were then rinsed free of adhering dye and photographed under 302 nm illumination using a Kodak Wratten filter #23A to exclude UV light from the lense. Polaroid type 665 film was used to provide a relatively instant record of the electrophoresis. Plasmid DNA bands fluoresce bright orange under these conditions and show up as white bands on the Polaroid film.

Up to three different marker plasmid isolates were used for the electrophoresis standards. One isolate from S. lactis had plasmids of 60, 33, 5.5, 2

and 1 Megadaltons (Mdal) with the 60 and 1 Mdal bands often faint or unclear. Plasmid sizes were almost always based on the S. lactis strain. Another isolate was the pUC 18 plasmid from E . coli strain JM83 which had a plasmid of 1.7 Mdal and a dimer band of 3.4 Mdal. The final isolate was a animal derived strain of E. coli which had plasmid bands of 81, 43, 26 and 4.5 Mdal.

Plasmid Size Profile Determination

Each photograph of an agarose gel electrophoresis run was examined to determine the distance the sample plasmids and the marker plasmids migrated. The markers were plotted with log Molecular Weight (Mdaltons) versus log distance migrated (centimeters) axes. The size of each sample plasmid was then determined from the standard curve. A new standard curve was prepared for every run due to slight differences in migration from run to run. Each band on the gel was assumed to caused by a plasmid presence. We are aware of the presence of different topological forms of the plasmids and plasmid multimers but in each case they seemed to be unique to that bacterial strain under our conditions of isolation and thus were counted as plasmid bands.

RESULTS

Sample Collection

The collection of water samples yielded different MPN values dependent upon collection site (see Table I) and provided excellent samples for studying plasmid profile analysis. A striking difference observed between the North and South Lagoon collection sites was the absence of impact that the North Lagoon's outfall had on the MPN's. This site resembled a site not affected by the presence of human derived fecal coliforms. In contrast, the South Lagoon site's MPN's were consistent with the outfall having the presence of human derived fecal coliforms. It had relatively low MPN's before the outfall and the effluent from the outfall increased the MPN's approximately 200 times. Furthermore, downstream from the outfall the MPN's were higher than before the outfall, yet some dilution effects were evident indicating mixing of the effluent with the stream as would be desired for our study. We concluded that the South Lagoon was best representative of the site for testing our hypothesis.

PLASMID PROFILES

Overall Plasmid Profiles

Plasmids were prepared from 344 E. coli isolates identified during the sample collection period from both the North and South Lagoon sites. From those isolates, 65% or 222 samples contained identifiable plasmids. From those 222 isolates we identified a total of 537 plasmids. The frequency of occurrence of plasmids of different sizes was determined. There was a wide range of plasmid sizes, with sizes from 1 to 187 Mdal. This is within the range of plasmids that have been found in bacteria. It must be noted that sizes above 100 Mdal should be considered only as relative, as this range was the least accurate under our conditions of analysis. Several plasmids were found to occur at least 10 times, while many were present in fewer numbers. Often one water collection sample yielded E. coli with multiple occurrences of the same plasmid sizes. This was presumably caused by clonal strains of E. coli within the water samples. In this case these possible clonal samples can not be ignored because they are representative of the actual sample. There were also repeat findings of the same plasmid sizes from different sample collections. Attempts to use cluster analysis in analyzing the data did not prove useful but other methods of analysis are described below.

Table I. Most Probable Numbers of Fecal Coliforms Found at Different Sample Sites

	Sample Site	Average NPN Per 100 ml
	North Lagoon	
1.	Above Outfall	2.10×10^2
2.	At Outfall	0.52×10^2
3.	Below Outfall	0.37×10^2
	South Lagoon	
1.	Above Outfall	5.80×10^2
2.	At Outfall	1.17×10^5
3.	Below Outfall	5.21×10^3

Site Dependent Plasmid Size Frequencies

The frequencies of different plasmid sizes found at each of the South Lagoon collection sites is shown in Figures 1A-C. In comparing the size profiles at the South Lagoon it is apparent that there is dissimilarity between each of the patterns. The patterns are different between plasmids above (Fig. 1A) and at the outfall (Fig. 1B), as one might expect because of the introduction of human- derived coliforms at the outfall. There appeared to be a greater variety of plasmid sizes in bacteria isolated at the outfall. There were 37 separate plasmid sizes found of 10 Mdal or greater from outfall derived bacteria. What was somewhat surprising was the overall differences between at the outfall (Fig. 1B) and below the outfall (Fig. 1C). Even though we assumed both sites contained coliforms of human origin, their plasmid size profiles differed. Below the outfall there was less variety of plasmid sizes (only

23 sizes) than at the outfall (37 sizes). The profiles from the North Lagoon also varied with sample site.

We can speculate as to why there are differences in profiles. One possibility is that there may be a wide variation in overall plasmid size profiles from E. coli from human sources. This would lead to the different profiles found for E. coli at the outfall compared to below the outfall. Indeed our data suggests that this is the case for the South Lagoon. Another possible explanation is that below the outfall, where the human- derived coliforms are less, the water samples also contain coliforms from other sources. The plasmids found in these samples would then reflect a mixture of human-derived and environmental sources. A third possibility is that upon introduction of the sewage borne E. coli into the river environment a rapid change may be induced in the plasmid profiles within the bacteria. We feel this is unlikely since sample collection and processing occurred at a more rapid pace than plasmid changes are thought to occur.

These findings argue that a simple comparison of overall plasmid sizes in E. coli isolated in the environment can not be used to determine if the E. coli was of human origin. Although the overall profiles were not identical, overlap of plasmid sizes between sites was observed. This suggests that a more refined method of comparison of plasmids may yield a means of identifying plasmids of human origin.

Comparison of Plasmid Size Ranges

Another means of studying the plasmids at the different sites was to group the plasmids into arbitrary size ranges of about 10 Mdal each. The results after these groupings are shown in Figure 2 for the South Lagoon. The percent of the total plasmids in that size range from each collection site was plotted versus increasing size ranges. For the lagoon system there were size ranges where collection sites above, at and below the outfall yielded similar percents (e.g. 40-49 Mdal), while in other ranges (eg. 30-39 Mdal) dissimilar percents were found. Above the outfall only 38% of the plasmids were in the range of 0 to 9.9 Mdal while at and below the outfall the percents were 40 and 52, respectively. This is suggestive of a slight increase in plasmids of low molecular weight in waters impacted by the release of human derived fecal coliforms. Our earlier work demonstrated the large frequency of low molecular weight plasmids in fecal coliforms isolated from humans. By visual inspection of the figure, one does not see any trend towards sites at and below the outfalls as being more similar than before the outfall. Statistical analysis by Chi-square means was consistent with the size grouping patterns not being the same. Again, it seems that a relatively broad comparison of plasmids can not be used to detect "human" specific coliforms in the environment.

Comparison of plasmid size ranges from the North Lagoon collection sites again showed no overall correlation of the percent of plasmids in any size groupings between any two collection sites. Above the outfall only 27% of the plasmids were of low molecular weight (0-9.9 Mdal) while at the outfall 45% of the plasmids were of that size range. This was consistent with the introduction of human derived coliforms of low molecular weight at the outfall. Yet in other size ranges some similarities were more evident between the samples above and at the outfall than below (results not shown). This may be related to the long distance the samples collected below the outfall were from the outfall.

Comparison of Plasmid Numbers

The results were viewed in yet another manner by determining the number of plasmids per isolate. This was converted to percent of the total plasmids that had either 0, 1, 2, 3 or >3 plasmids and was used to generate Figure 3. At the South Lagoon the comparison of plasmid numbers per isolate (Fig. 3) did show similarity between the site at the outfall and the site below (e.g. plasmid groupings 1, 3, and >3, with the plasmid group 3 being identical. Yet the site above the outfall also had similarities to the outfall (e.g. plasmid group 2). The low number of isolates without plasmids at the outfall is consistent with what we have seen in earlier work with isolates from human feces. Below the outfall also had a lower percentage without plasmids than above the outfall. This may reflect the influence of human-derived coliforms on the isolates which harbored plasmids.

At the North Lagoon the similarity between plasmid numbers at and below the outfall was more impressive. The percent of isolates in each of the different groupings at and below the outfall were within 5 points, or less, of each other. The samples above the outfall varied from the other two between a range of 2 to 30 points. The question is what does it mean? Based on MPN data the North Lagoon samples did not seem to be impacted by known human-derived coliforms, yet the pattern before the outfall is quite dissimilar to the other two. It could mean that the effluent from the lagoon influenced the coliforms found in the river far downstream, as well as at the outfall. This evidence is only suggestive since it does not correlate with the size grouping data for the North Lagoon sites.

The North and South Lagoons were compared by analysis of collection sites in regard to plasmid numbers per isolate. The two lagoons were shown to differ above the sewage outfall for 1, 2, 3 and >3 plasmids per isolate. In both cases the 0 plasmids per isolate is relatively high (37% to 45%) compared to human derived isolates. The patterns observed at the outfalls of each lagoon are similar only in the 3 plasmids per isolate range, while below the outfalls showed similar percents in the 0 and 3 plasmids per isolate groupings, 35% and 10% respectively.

CONCLUSIONS AND SIGNIFICANCE

The main objectives of the project were met as fecal coliforms were collected at sites either affected by known sources of human derived bacteria or sites less affected. Plasmids were isolated from E. coli in these samples and were characterized by size. Finally, a comparison of size profiles of the plasmids was made and the value of such a comparison was then evaluated in assessing water quality.

In evaluating the size comparison it was apparent that a simple visual inspection of size patterns was not adequate to distinguish water known to be impacted by human derived coliforms from other waters. Attempts to use cluster analysis on the plasmid profiles did not yield any useful information. Comparison of plasmids by size groupings also did not reveal any overall trend in similarity to water directly impacted by sewage (at a sewage outfall) and water downstream from the outfall. There was similarity in one important size grouping, as water impacted directly or, for the South Lagoon, indirectly (downstream from the outfall) had high values in the 0-9.9 Mdal range (from 40% to 52%). Above the sewage outfall this group ranged from 27 to 38%. The high number of low molecular weight plasmids was very similar to E. coli plasmids we found in human feces during the same season a year earlier and is much higher than that found in E. coli plasmids from animals.

Comparison of plasmids grouped by number of plasmids per isolate yielded the finding that water impacted by sewage had the lowest percent of E. coli isolates without plasmids. This is again comparable to what we found a year earlier for E. coli from humans. Examination of groupings of higher number of plasmids per isolate revealed a particular similarity in the plasmids per isolate range for water impacted by sewage but other groupings were not as similar.

The most significant finding of the research is that a simple view of overall plasmids profiles cannot be used to assess water quality. This is apparently due to a wide variation in plasmid profiles found in E. coli from any source. Some of our findings suggested that a more detailed look at the low molecular weight plasmids or looking at the frequency of isolates contain- ing plasmids may yield results that could be used in assessing water quality. Other techniques are available for looking for specific plasmids or plasmid genes and may prove useful in later work. These techniques are DNA hybridization and restriction fragment analysis. We have done preliminary work with the latter on this project and are currently developing a new simple means of DNA hybridization. Since the E. coli isolates from this project are archived we may be able to look at these techniques with these samples.

Of further significance is that the analysis provides for one of the few studies of plasmids profiles in freshwater. To our knowledge it is the only study where analysis was done on all the plasmids present in E. coli, the few other studies have looked only at plasmids that confer drug resistance to bacteria. This gives a baseline of comparison for other plasmid studies in water. For example, it could be used as reference for studying the effect of introduction of genetically engineered coliforms into the water environment or the effect of chemical pollution on plasmids in coliforms in freshwater.

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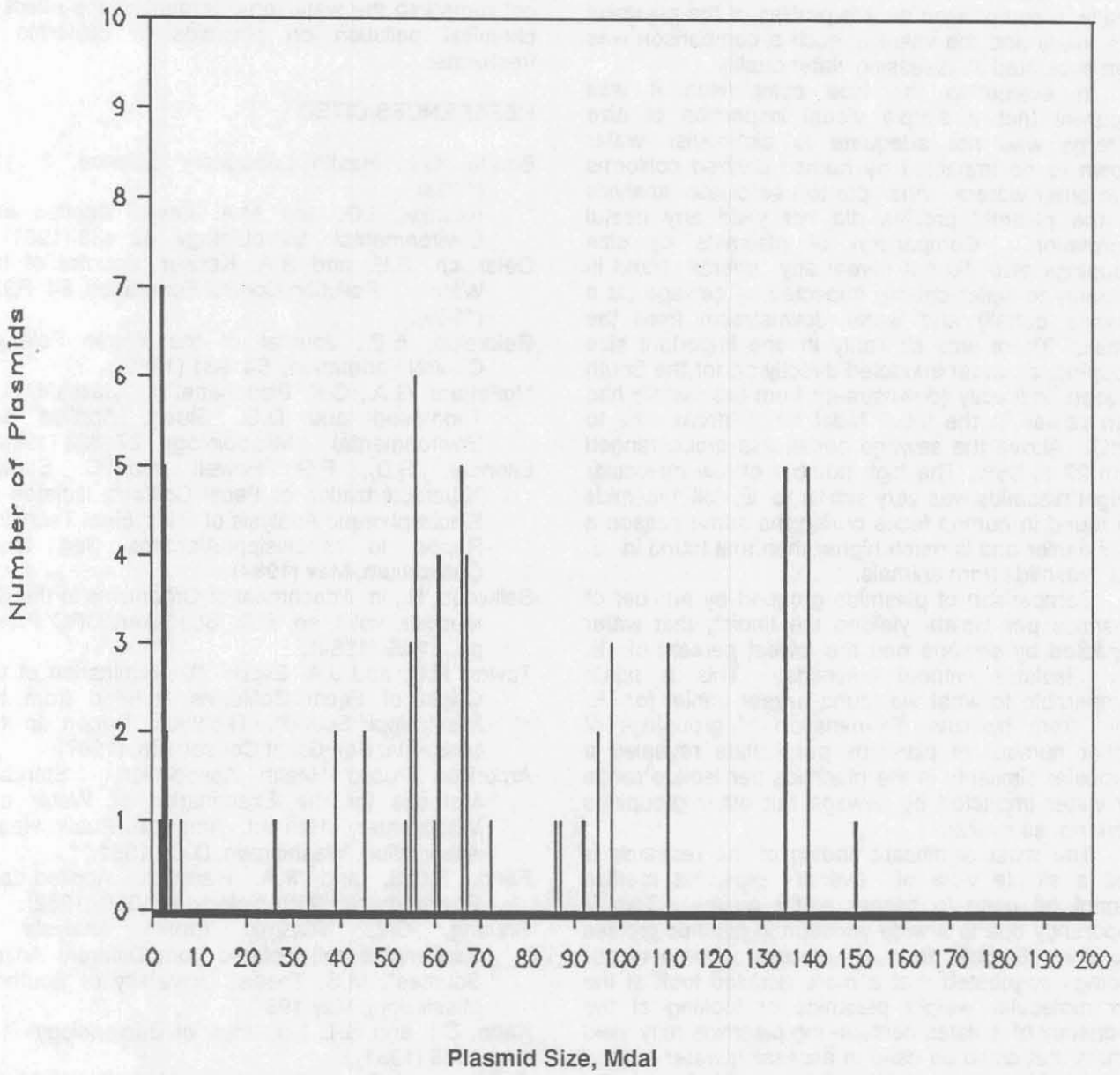


Figure 1A. Frequency of Plasmid Sizes, Above Outfall

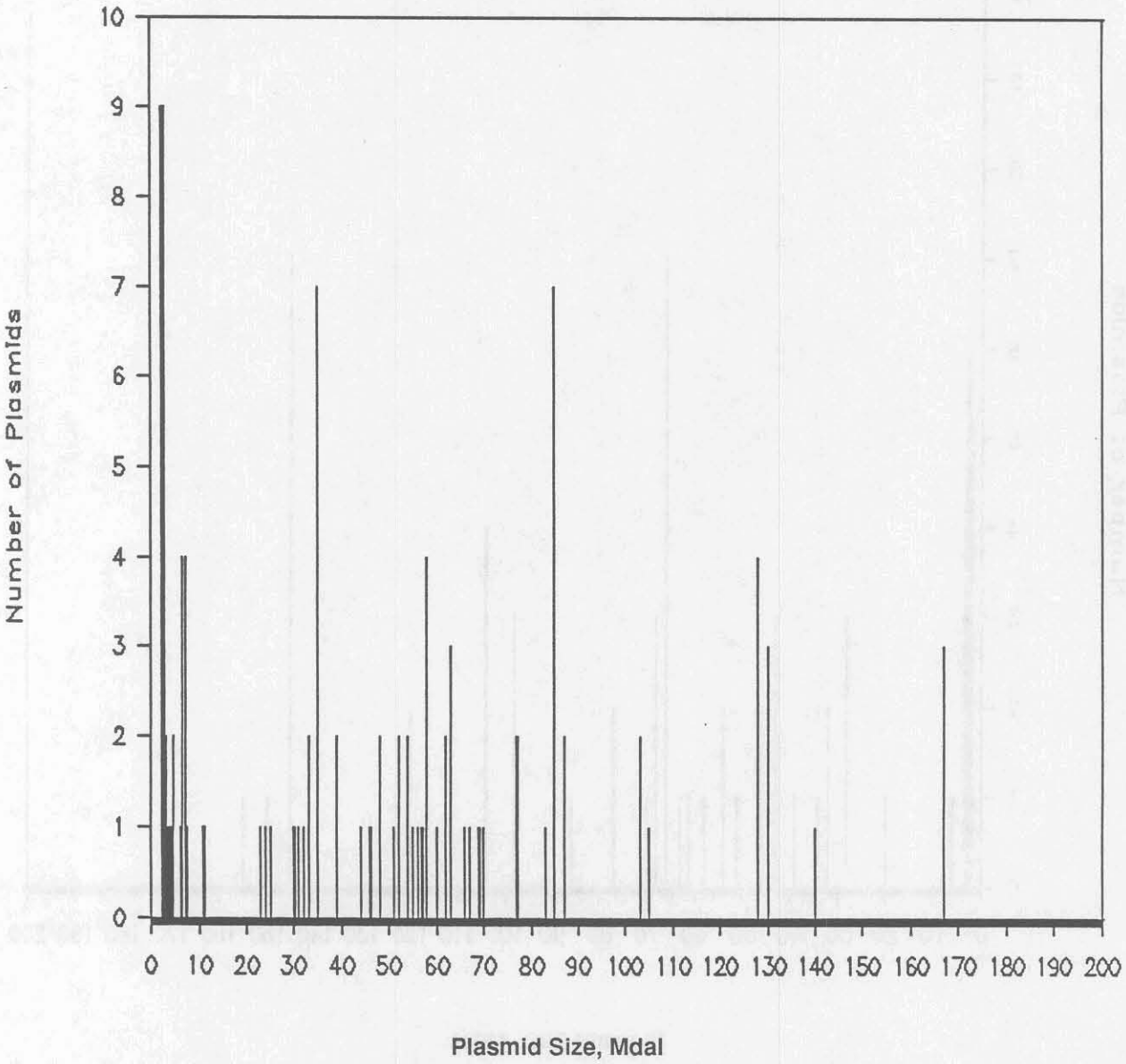


Figure 1B. Frequency of Plasmid Sizes, At Outfall

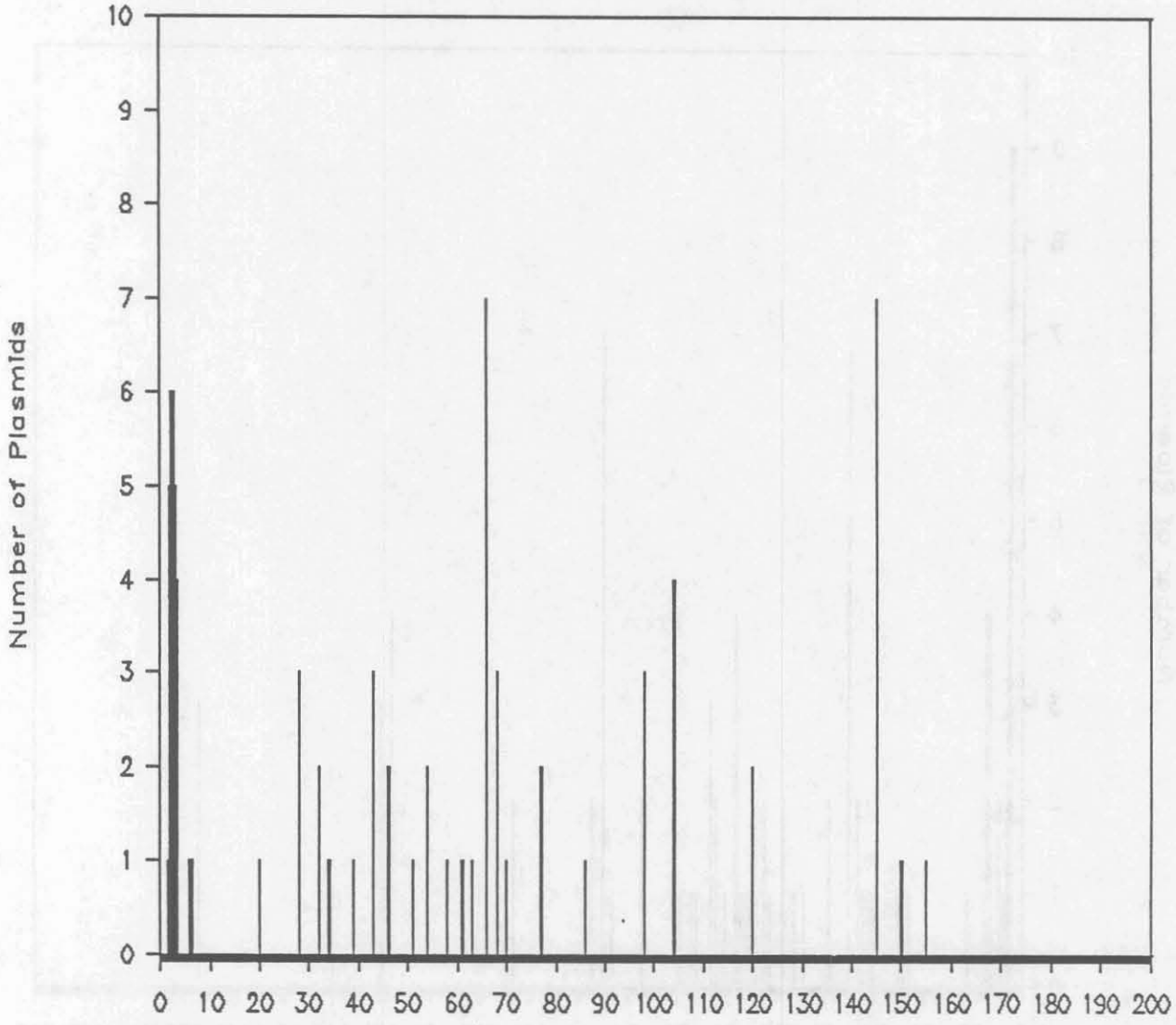
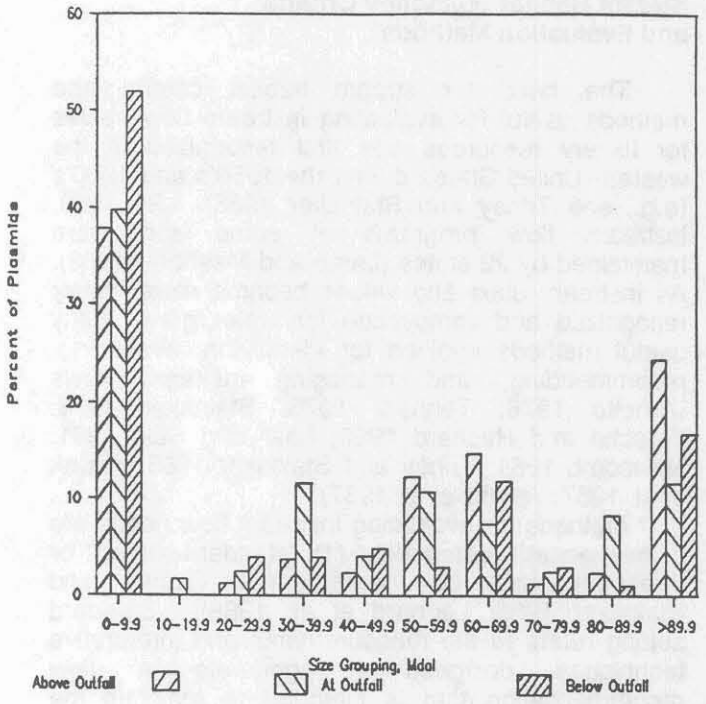


Figure 1C. Frequency of Plasmid Sizes, Below Outfall

Figure 2.
Comparison of Plasmid
Size Ranges



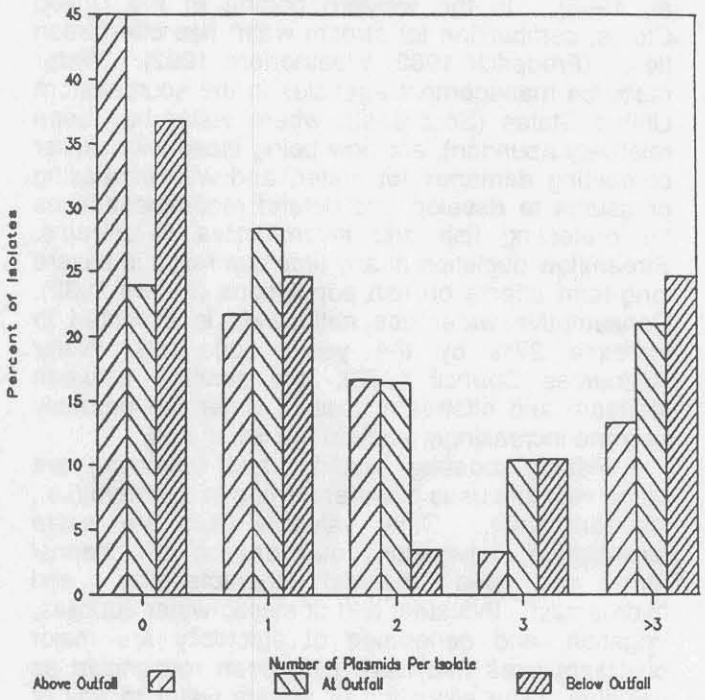


Figure 3.
Comparison of Plasmid Numbers
Per Isolate