Antibiotic and Disinfectant Resistant Bacteria in Rivers of the United States

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Abstract

We examined natural water sources in the U.S. for the presence of bacteria resistant to natural and human-produced antimicrobial agents. The impact of these aquatic organisms on diseases in man and animals is unknown but resident bacteria in water represent a potentially important reservoir of resistance genes. Further, the presence of resistant organisms is usually an indication of some selective pressure in the past which has enabled these bacteria to arise. Usage of antibiotics and chemotherapeutic agents in veterinary medicine and agriculture can result in contamination of natural water sources. Clinical use in humans can also result in the introduction of such compounds into water. Since some studies have suggested a link between antibiotic resistance and resistance to disinfectants, we determined the nature of bacteria in water with respect to these two resistance traits. Our previous sampling of U.S. rivers demonstrated the widespread resistance of resident bacteria to a variety of synthetic and semisynthetic antimicrobial agents. We have investigated further the resistance to sulfa drugs which represent a class of compounds that are not found in nature except as a result of use in animals or humans. Bacteria resistant to sulfadiazine and sulfamethoxazole-trimethoprim were found in all rivers examined. In addition, these resistant organisms were not killed by other antibiotics and many contained plasmids which are common vehicles for transfer of genes among bacteria. Over forty percent of the organisms also contained integron sequences, DNA elements responsible for the transfer of blocks of genetic information. The sensitivity of sulfa-resistant bacteria to disinfectants was also tested. It was found that, in general, organisms possessing integrons were able to survive higher concentrations of benzalkonium chloride. The fact that resistance to these agents has been maintained in natural populations suggests that these traits may confer some additional advantage to bacteria who have received and maintained the respective genetic information.

Introduction

The presence of antibiotic resistant bacteria in groundwater has been documented (McKeon et al. 1995; Chee-Sanford et al. 2001) and previous work from our laboratory and others has demonstrated the widespread occurrence of such organisms in many rivers and streams in the U.S. (Ash et al. 2002; French et al 1987; Young 1993). We found, for example, that antibiotic resistant bacteria could be isolated from all 16 U.S. rivers tested. The presence of bacteria possessing resistance traits in nature may be the result of some selective pressure, i.e., previous exposure to a particular antibiotic. The selection could be the result of exposure to naturally-occurring antibiotics produced by organisms in soil or it may be due to human activity. Whatever the source of antibiotic resistance, such traits are apparently found in many bacteria in natural water sources. Further, these reservoirs of resistance may provide genetic material which can be passed from one organism to another.

In one study, contamination of groundwater with antibiotic resistant bacteria was shown to be the result of seepage from waste lagoons (Chee-Sanford et al. 2001). The source of the resistant bacteria was waste disposal from animal agriculture. It seems reasonable, based on our previous studies, to conclude that river water used for irrigation might also be a source of contamination for groundwater.

We have continued our studies on rivers of the U.S. with respect to bacteria containing resistance genes to therapeutic agents. Sulfa drugs have been used in human and veterinary medicine for over sixty years. This continuous use has resulted in the selection of resistant organisms. Despite this fact, the sulfonamides are still clinically relevant compounds when administered with other agents. Since these compounds are not found in nature, the only source in the environment must be from animal or human use. The present study was undertaken to evaluate the prevalence and properties of sulfa drug resistant bacteria in seven rivers in the Midwestern U.S. Our results indicate that organisms with genes specifying resistance to these compounds are found in U.S. rivers and that mechanisms for spreading these genes exist in these bacteria. Resistance to other antibacterial agents was also found in many of the isolates.

Because some studies have suggested that antibiotic resistance may be linked to disinfectant resistance (reviewed in

Russell 1997), we studied the patterns of sensitivity of the sulfa drug resistant bacteria to benzalkonium chloride (BC). This quaternary ammonium compound was chosen because the genes for sulfa drug resistance are often associated with a lack of sensitivity to these agents. Our results indicate that, on the whole, organisms possessing integrons were more resistant to benzalkonium chloride.

Materials and Methods

Sampling

Triplicate water samples were collected with a sterile pipette at a depth of approximately 15 cm. Undiluted samples and samples diluted in Luria-Bertani (LB) broth were plated immediately on LB agar plates +/- sulfadiazine (SD, 250 µg/ml). All plates were incubated at 30-32C. SD-resistant (SD^R) colonies were picked to fresh master plates containing SD and tested further. Initial characterization of the isolates as to gram character and lactose fermentation consisted of growth on MacConkey's agar and oxidase testing. Only gram negative bacteria growing on MacConkey's agar were studied further. Over 80% of the isolates were non-lactose fermenters. Enterotubes (Becton Dickinson and Co., Cockeysville, MD) were used for identification of the oxidase negative bacteria. Organisms identified most frequently include *Acinetobacter*, *Enterobacter*, *Serratia* and *Pseudomonas*.

Antibiotic Sensitivity Testing

National Committee for Clinical Laboratory Standards (NCCLS 1997) methods and criteria for evaluation were used. Briefly, isolates were grown in LB medium until the turbidity of a 0.5 Macfarland standard was reached. Such cultures were swabbed on Mueller Hinton (MH) agar and antimicrobial discs (Becton Dickinson and Co.) were added. Plates were incubated overnight at 30-32C and zones of inhibition were measured.

Plasmid Isolation

Two methods were employed for each isolate. Boiling (Holmes and Quigley 1981) and alkaline lysis (Birnboim and Doly 1979) procedures were both employed to ensure that as many plasmids as possible were identified. Plasmids preparations were electrophoresed on 0.7% GTG agarose (FMC Corp. Rockland, ME) gels and stained with ethidium bromide.

Polymerase Chain Reaction

Class 1 integron sequences and *sul*1 sequences were amplified by PCR using the primers and conditions specified by Rosser and Young (1999). Primers were synthesized by Integrated DNA Technologies, Coralville, Iowa. PCR reagents were from Qiagen, Inc., Valencia, CA. Gel electrophoresis in 4% NuSeive agarose (FMC Corp.) was used for separation and identification of amplicons.

Disinfectant Sensitivity Testing

Tubes containing 3 ml of MH broth +/- disinfectant were inoculated with 0.1 ml of each isolate. Cultures for inoculation were prepared by suspending a few fresh colonies in MH medium. All tubes were incubated overnight at 22C. The minimal inhibitory concentration (MIC) of disinfectant was determined by plating 0.1 ml of each tube on a fresh LB agar plate and estimating whether survivors were present after incubation of the inoculated plates. Benzalkonium chloride was obtained from Sigma, St. Louis, MO.

Results and Discussion

Isolation of SD^R Bacteria from U.S. Rivers

The presence of sulfadiazine (SD) resistant organisms in U.S. rivers is indicated in Table 1. In the seven rivers shown, the total number of bacteria and SD^R organisms varied considerably. The fraction of the population in each river showing

SD resistance also displayed a wide variation. This is, perhaps, not surprising since sulfa drugs have been identified as frequent low level contaminants in U.S. streams (Kolpin et al. 2002), thereby providing selective pressure to maintain resistance. The sources of the sulfa drugs are not known. It is also unknown whether the sources are continuous or intermittent.

The resistance of gram negative isolates to other antibiotics is presented in Table 2. Gram positive bacterial isolates were not studied. Nearly half of the SD^R organisms were also resistant to one other antimicrobial agent with a smaller number being resistant to two or more antibiotics. The incidence of specific resistances are found in Table 3. Over one fourth of the SD^R were not inhibited by teracycline or chloramphenicol. A smaller fraction of the isolates were resistant to kanamycin and the beta-lactam antibiotics cefotaxime and ceftazidime. Many of the SD^R are probably intrinsically resistant to some of these other agents but the tetracycline resistance, in particular, may be acquired horizontally from other organisms.

Resistance to Sulfamethoxazole-Trimethoprim

The combination of sulfamethoxazole and trimethoprim (SXT) is used clinically for the treatment of a number of infections (Yao and Moellering 1999). Trimethoprim (TMP) at higher concentration is also a useful chemotherapeutic agent. These compounds, which are chemically-synthesized, inhibit the production of dihydrofolic acid but at different sites in the metabolic pathway (Skold 2000). Both sulfamethoxazole and TMP have been identified in U.S. streams (Kolpin et al 2002). The SD^R bacteria were tested for sensitivity to SXT and TMP at a concentration which was 4 times higher than that found in the combination (5 μg vs. 1.25 μg). Over 60% (149/238) of the isolates were resistant to the SXT combination while only 13% (31/238) demonstrated resistance to the high concentration of TMP alone. The genes responsible for enzymes which give rise to sulfa resistance may be chromosomal or carried on plasmids as part of integrons.

Plasmids in SD^R Bacteria

Since plasmids are frequently a means of horizontal genetic exchange among bacteria, we examined the SD^R isolates for plasmids by two methods. At least one plasmid was found in 45% (90/197) of the isolates tested. In some cases, plasmids isolated by the boiling method were not always observed with the alkaline lysis method, and vice-versa. This was probably due to differences among the bacterial genera. The high incidence of plasmids suggests that a mechanism for transfer of sulfa drug and antibiotic resistance genes is present in the isolates.

Integrons in SD^R Bacteria

Sequences specific to the integron integrase gene *(int*) and sufonamide resistance gene *(sul1*) were found by PCR in the SD^R bacteria (Table 4). The *sul*1 gene encodes an altered dihydropterate synthetase which accounts for resistance to sulfonamides. The presence of both of these genes provides good evidence for the presence of intact integrons. Over 40% of the SD^R bacteria contained both genes. Class 1 integrons also carry genes for resistance to quaternary ammonium compounds (quats) and may incorporate antibiotic resistance genes as well (Rosser and Young 1999).

Resistance to Disinfectants

The presence of integrons in the SD^R bacteria suggested that there may be a concomitant increase in resistance to quats. For this reason, the effect of benzalkonium chloride (BC) on killing of the SD^R organisms was examined. The range of MICs for the SD^R isolates from water is presented in Table 5. Those organisms possessing integrons tended to have higher MICs than those lacking *int* and *sul* genes. This is probably a reflection of the *qac* genes which are also present on integrons, but alternative explanations are possible.

Conclusions

The results of this study demonstrate the presence of bacteria resistant to sulfa drugs and trimethoprim in rivers. These SD^R bacteria were also less susceptible to some other antibiotics. Although some environmental bacteria are classified as intrinsically resistant to these agents, many acquire resistance genes through horizontal transfer by way of plasmids. This work has identified plasmids and the mobile DNA sequences known as integrons on a large fraction of the sulfa drug resistant organisms. This finding suggests that these bacteria are capable of transferring resistance traits to other organisms. The fact that so many organisms were identified suggests that this may be an ongoing process.

The presence of low concentrations of sulfonamides and TMP in water (Kolpin et al 2002) may be responsible for continued selection of resistant organisms. Alternatively, these gene clusters acquired by organisms may provide additional advantages such as resistance to other harmful agents. The trend toward greater resistance to quats associated with integrons indicates this may be the case. It is also possible that integron-bearing bacteria belong to groups of organisms which are innately more resistant to quats. The possibility should be considered that bacteria classified as "intrinsically resistant" to an agent may have, in fact, acquired the trait through lateral transfer at sometime in their history.

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Biographical Sketches

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Table 1
Isolation of Sulfadiazine-Resistant (SD^R) Bacteria from
U.S. Rivers

River	Location	CFU/ml* LB	LB+SD	% SD^R
Missouri	Parkville	1,960	123	6.2
Ohio	Cincinnati	25,200	3,001	11.9
Platte	Grand Island	1,666	540	32.4
Mississippi	St. Louis	9,700	256	2.6
Arkansas	Wichita	4,716	747	15.8
Kansas	Topeka	7,400	950	12.8
Des Moines	Des Moines	19,750	226	1.1

*CFU, colony forming units on LB medium +/- SD.

Table 2
Incidence of Resistance of SD^R Bacteria to Other Antimicrobial Agents

	Resistance to Other Agents*			
Total Tested	None	One	Two	Three
277	131 (47.3)	114 (41.1)	27 (9.7)	5 (1.8)

* Resistance to 0, 1, 2, or 3 of other agents tested: chloramphenicol, tetracycline, kanamycin, cefotaxime, ceftazidime, imipenem, ciprofloxacin. The number of isolates with the resistance is given and the % is in parentheses.

Table 3
Resistance of SD^R Bacteria to Specific Antibiotics

Antibiotic	% Resistant*
Chloramphenicol	27.8
Tetracycline	27.0
Kanamycin	8.3
Cefotaxime	1.8
Ceftazidime	0.7
Imipenem	0
Ciprofloxacin	0

*Total tested = 277

Table 4
Presence of Integron *(int*) and *sul*1 Sequences in SD^R Bacteria

Marker	No. Positive	% Positive
int	89/190	46.8

*sul*1	88/190	46.3
int + *sul*1	82/190	43.1

Table 5
Correlation between Integron Sequences and Resistance
to Benzalkonium Chloride

		Resistance to BC at:		
int/*sul**	No. Tested	1-10 µg	20-30 µg	40-50 µg
+	90	19	26	45
-	98	86	7	5

* Organisms shown to have *int* or *sul* sequences by PCR.