PCR Targeting of Antibiotic Resistant Bacteria in Public Drinking Water of Lahore Metropolitan, Pakistan

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Objective To investigate the prevalence of kanamycin (*kan*) and ampicillin (*amp*) resistant bacteria in public drinking water. **Methods** Bacteria containing *kan* and *amp* resistant genes were amplified by PCR and further characterized by colony hybridization and transformation studies. The genus of *kan* and *amp* resistant bacteria was determined with standard methods. **Results** Among the 625 drinking water samples, 400 contained *kan* and *amp* resistant bacteria and the percentage was 42.5% and 57.5%, respectively, which was further confirmed by the amplification of a 810 bp *kan* resistant gene and a 850 bp *amp* resistant gene. Of the 170 *kan* resistant bacteria, 90 were Gram negative and 80 were Gram positive. Of the 230 *amp* resistant bacteria, 160 were Gram negative while 70 were Gram positive. *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and *E.coli* were detected as 13%, 11%, 17%, 30%, and 29%, respectively. Bacterial strain DH5α transformed with plasmids isolated from *kan* and *amp* resistant bacteria confirmed that the antibiotic resistant genes were mediated by plasmids. **Conclusion** Drinking water is contaminated with *kan* and *amp* resistant bacteria due to poor sanitary conditions.

Key words: Kanamycin; Ampicillin; PCR; Plasmid; Colony hybridization

INTRODUCTION

Frequent use of antibiotics as medicine in treatment of humans and in food of animals results in the prevalence of antibiotics resistant bacteria[1-2]. Although antibiotics help to improve the health of humans and animals, they also lose their effectiveness. Use of antibiotics in animal food contributes significantly to the development of antibiotics resistant bacteria[3-5].

A significant number of hazardous bacteria causing infections are resistant to the common antibiotics[4, 6]. Excessive use of antibiotics leads to evolutionary adaptation of bacteria to resist powerful drugs. Resistant bacterial strains spread in the environment through air and water and affect human health[7-8]. Waste of humans and animals also favors the development of antibiotics resistance in coliform and fecal coliform populations[6, 9]. Surface waters receiving runoff from lands also contain wastes of livestock[10], polluted estuaries[11], and contaminated water supplies[12-13].

Kanamycin is a member of aminoglycoside antibiotics which has been used for many years[14]. This drug, either bactericidal or bacteriostatic, is effective against both Gram negative and positive organisms. Nearly 18 genes have been characterized as aminoglycoside modifying enzymes through acetylation, adenylation, and phosphorylation. No structural similarity has been found among the different classes of genes and aminoglycosidase enzymes[15]. Kanamycin is less frequently prescribed because of its toxicity and development of bacterial resistance. Over time, many pathogenic bacterial strains can acquire resistance to kanamycin[16].

Ampicillin, one of the bactericidal antibiotics, is effective against Gram positive and negative bacteria. It is used in treatment of respiratory, gastrointestinal and genitourinary infections. The resistance to ampicillin has been investigated in many microbial flora[17-18]. Antibiotics resistant bacteria in drinking water may act as a vehicle to transfer virulent genes to other bacteria, leading to high morbidity, mortality, and failure of treatment of life threatening bacterial

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infections in humans and animals[13].

This study was to identify and characterize the kanamycin and ampicillin resistant bacteria in public drinking water, which helps to investigate the existence of antibiotic resistant microbes in our local habitats and to cure diseases during medical treatment or waste water treatment.

MATERIALS AND METHODS

Sample Collection

A total of 625 drinking water samples were collected from different areas of Lahore metropolitan. Sampling sites were selected based on the extensive use of water by the public. Of the 625 water samples, 280 were collected from different residential areas, 95 from colleges, and 250 from different hospitals of Lahore in 500 mL sterile bottles as previously described[19], stored at 4 ℃, and processed for analysis within 10-12 h.

Kanamycin and Ampicillin Resistant Bacteria

For isolation of *kan* and *amp* resistant bacteria, 1mL drinking water sample was spread separately on LB agar plates (NaCl 1%, trypton 1%, yeast extract 0.5%, pH 7.0, 1.5% agar) containing kanamycin (30 μg/mL) or ampicillin (60 μg/mL) and incubated for 18 h at 37 ℃. Based on the same size, shape and colour, two colonies were picked up and made replica on a LB agar medium plate containing the same antibiotic concentration as described above.

PCR Amplification of kan and amp Resistant Genes

Primers for PCR amplification of *kan* and *amp* resistant genes were designed using the available nucleotide sequence of kanamycin and ampicillin in pET28a and pET22b vector (Novagen). For kanamycin, (FPkan, (sense) 5′-CATATGAGAAAAACTCATCGAGCATC-3′ and RPkan, (antisense) 5′-GAATTCAGCCATATTCAACGGGAA-3′) primers were synthesized. For ampicillin, (FPamp, (sense) 5′-CATATGCTTAATCAGTGAGGCACCT-3′ and RPamp, (antisense) 5′-GAATTCAGTATTCAACATTTCCGTGTCG-3′) were synthesized. Restriction site of *Nde*1 (CATATG) was added in both forward primers, and restriction site of *Eco*RI (GAATTC) was added in both reverse primers.

The *kan* and *amp* resistant genes were amplified separately in 50 μL reaction volume using pET28a and pET22b plasmid DNA as a template, respectively. PCR was performed in iCycler (Biorad) using 1 μL plasmid DNA (0.1 μg/μL) as a template containing 2.5 units of recombinant Taq DNA polymerase, 1× PCR buffer, 0.2 mmol/L each dNTPs, 2 mmol/L $MgCl_2$, 0.5 μmol/L of each forward and reverse primer. PCR conditions were as folows: an initial denaturation for 2 min at 95 ℃ followed by 35 cycles of denaturation at 95 ℃ for 1 min, annealing for 1 min at 59 ℃ (Tm of the primers) and elongation for 2 min at 72 ℃, a final elongation for 10 min at 72 ℃. The amplified PCR products were analyzed on a 1% agarose gel using 0.5×TBE buffer. The amplified *kan* and *amp* PCR products were purified by QIAquick gel extraction (Qiagen, Germany).

Preparation of Biotin-labeled DNA Probes

Biotin-11-dUTP-labeled *kan* and *amp* DNA probes were prepared as previously described[20] with minor modifications. Briefly, 10 μL (0.1 μg/μL) DNA template (PCR amplified *kan* and *amp* resistant genes) was mixed separately with 10 μL decanucleotide in 5×reaction buffer and 24 μL nuclease free water. The mixture was centrifuged, incubated in a boiling water bath for 10 min, immersed in ice water, mixed with 5 μL biotin-labeled dNTP mixture and 1 μL (5 units) Klenow fragment (exe^-), and incubated for 4 h at 37 ℃. Unincorporated biotin-labeled dNTP was removed by selective precipitation of labeled DNA with ethanol in the presence of ammonium acetate[21].

Screening by Colony PCR

The presence of *kan* and *amp* resistant genes in bacteria was confirmed by colony PCR. The reagents required for PCR were mixed in a 0.5 mL PCR tube as described above and a small number of colonies were added. The mixture was kept in a thermal cycler under the conditions as described above. At the end, 2-5 μL aliquot was analyzed on a 1% agarose gel using 0.5×TBE buffer.

Screening by Colony Hybridization

The existence of *kan* and *amp* resistant genes in bacteria was also determined by colony hybridization. Briefly, replica plates were made and growing colonies were transferred onto a nitrocellulose membrane and lysed as previously described[21]. The nitrocellulose membrane was baked for 2 h at 80 ℃ under vacuum to fix DNA on the membrane. Blots were exposed to biotin-labeled non-radioactive *kan* and *amp* DNA probes and detected using streptavidin-alkaline phosphatase as previously described[22] using nitroblue tetrazolium (5 mmol/L)

and 5-bromo-4-chloro-3-indolyl phosphate (5 mmol/L) as a substrate, respectively.

Species Identification

All *kan* and *amp* resistant bacteria were processed for Gram staining[19]. Genera of Gram negative and positive bacteria were further identified as previously described[19].

Isolation and Analysis of Plasmid DNA

Colonies positive for *kan* and *amp* resistant genes in colony hybridization and PCR were selected for extraction of plasmids by alkaline lysis with SDS method[21]. Molecular weight of plasmid DNA was estimated on a 1% agarose gel using 0.5×TBE buffer.

Transformation Study

Chemical competent cells of *E.coli* strain DH5α were transformed with isolated *kan* and *amp* resistant plasmids as previously described[21]. Transformed DH5α was cultured on antibiotic ampicillin (60 µg/mL) or kanamycin (30 µg/mL) and the results were recorded. Plasmids present in *kan* and *amp* resistant bacteria were extracted from the agarose gel and used to transform the DH5α independently.

RESULTS

This study was to isolate and characterize the antibiotic resistant bacteria in public drinking water. Of the 625 drinking water samples, 400 contained *kan* and *amp* resisitant bacteria, 170 contained kanamycin resistant bacteria, and 230 contained ampicillin resistant bacteria. The overall percentage of kanamycin and ampicillin resistant bacteria was 42.5% and 57.5%, respectively. The prevalence of kanamycin resistant bacteria in drinking water from a residential area was 70.58% (120 out of 170 samples), 42.85% from college drinking water (30 out of 70 samples) and 12.5% from hospital drinking water (20 out of 160 samples). The prevalence of ampicillin resistant bacteria in drinking water from a residential area was 54.70% (93 out of 170 samples), 73.75% in hospital drinking water (118 out of 160 samples), 27.14% in drinking water samples from colleges (19 out of 70 samples) (Table 1). Kanamycin and ampicillin resistant strains were also processed for Gram staining. Of the 170 kanamycin resistant bacterial strains, 110 were Gram negative (64.70%) and 60 were Gram positive (35.30%). Of the 230 ampicillin resistant bacterial strains, 180 were Gram negative (78.26%) and 50 were Gram positive (21.74%) (Table 2).

TABLE 1

PCR-identified *kan* and *amp* Resistant Bacteria in Drinking Water Samples

Total Samples	Sites	Samples Containing *kan* and *amp* Resistant Bacteria	Resistance (%)	
n=625	Residential Area 170	*n*=400	*kan*	70.58
			amp	54.70
	Hospital Area 160		*kan*	12.50
			amp	73.75
	Colleges Area 70		*kan*	42.85
			amp	27.14

Note. *kan* (kanymycin), *amp* (ampicillin), *n* (number).

TABLE 2

Gram Staining and Identification of *kan* and *amp* Resistant Bacteria

Resistant Bacteria (*n*=400)	Gram Staining (%)		*Salm* (13%)	*Shig* (30%)	*Staph* (11%)	*Strep* (17%)	*E. coli* (29%)
	Negative	Positive					
Kanamycin 170	64.70	35.30	4.5	9	2	7.3	9
Ampicillin 230	78.26	21.74	8.5	21	8	9.7	20

Note. *kan* (kanymycin), *amp* (ampicillin), *n* (number), *Salm* (*Salmonella*), *Shig* (*Shigella*), *Staph* (*Staphylococcus*), *Strep* (*Streptococcus*), *E. coli*.

The genera of Gram negative and positive bacterial strains were also identified. *Salmonella, Shigella, Staphylococcus, Streptococcus*, and *E.coli* accounted for 13%, 30%, 11%, 17%, and 29%, respectively, in *kan* and *amp* resistant bacteria. The *kan* and *amp* resistant *Salmonell*, *Shigella, Staphylococcus*,

and *E.coli* accounted for 4.5% and 8.5%, 9% and 21%, 3% and 8%, 7.3% and 9.7%, 9% and 20%, respectively (Table 2), indicating that kanamycin and ampicillin resistant genes are more prevalent in Gram negative bacteria.

The size of kanamycin and ampicillin resistant plasmids ranged 14 kb-18 kb (Fig. 1). In PCR, a 810 bp band was found in a kanamycin resistant gene (Fig. 2A) and a 850 bp band was detected in an ampicillin resistant gene (Fig. 2B), with no miss priming observed in PCR of *kan* and *amp* resistant genes. The presence of kanamycin and ampicillin resistant genes was also confirmed by colony hybridization. The deposition of blue formazan reaction products confirmed the reactivity of *kan* and *amp* resistant genes with their respective DNA probes (Fig. 3), which was further confirmed by transformation of the DH5α strain with purified *kan* and *amp* resistant plasmids. The survival of all transformants showed that *kan* and *amp* resistant genes are present in plasmids. The *kan* resistant bacterial strains were also cross checked against *amp* resistant bacterial strains and *vice versa*, showing that 50 bacterial strains are resistant to *kan* and *amp*. Of the 50 bacterial strains, 34 were Gram negative and 16 were Gram positive.

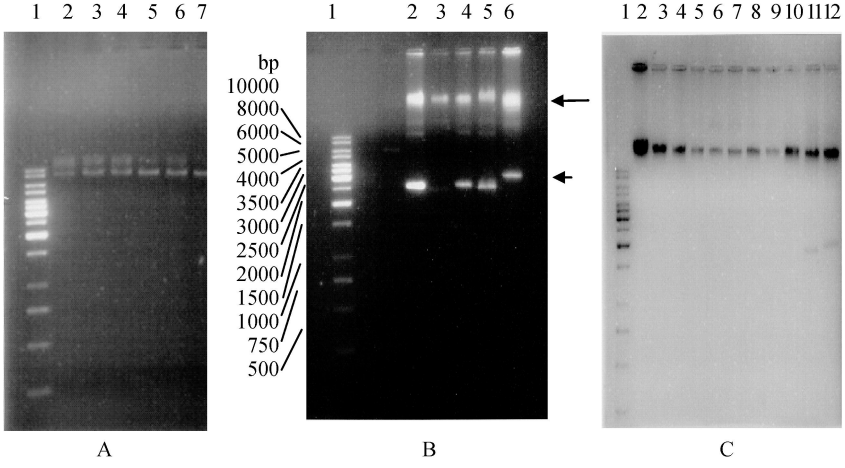


FIG. 1. Agarose gel electrophoresis analysis of plasmid DNA isolated from kanamycin and ampicillin resistant bacterial strains. A: Lane 1: DNA marker; lanes 2-7: plasmids isolated from *kan* resistant bacterial strains. B: Lane 1: DNA marker; lane 2-6: plasmids isolated from bacterial strains resistant to *kan* and *amp*. The *kan* and *amp* resistant plasmids (arrow) and amp resistant plasmids (arrow head) are shown in multiple drug resistance. C: Lane 1: DNA marker; lanes 2-10: plasmids isolated from *amp* resistant bacterial strains.

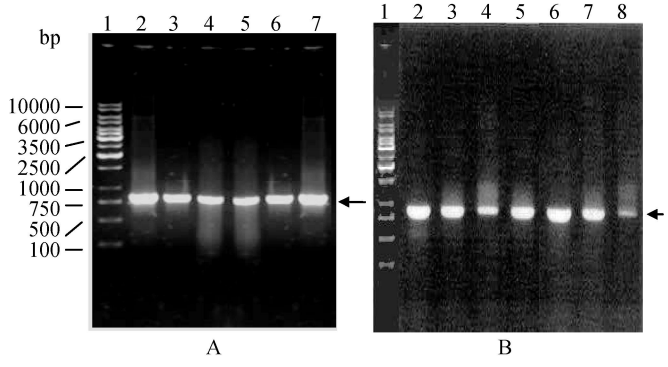
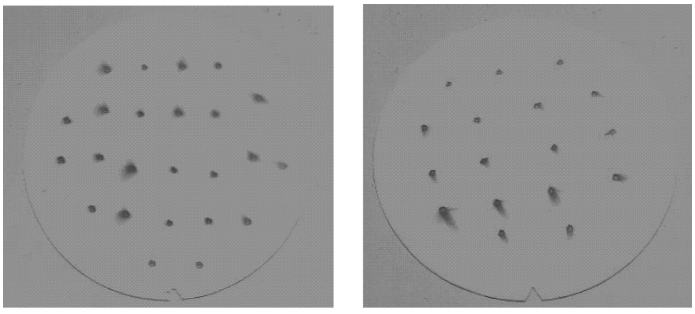


FIG. 2. Agarose gel electrophoresis analysis of PCR amplified kan and amp genes. A: Lane1: DNA marker; lane 2: PCR amplified standard amp resistant gene; lanes 3-8: PCR amplified a 850 bp amp resistant gene in samples (arrow). B: Lane1: DNA marker; lane 2: PCR amplified standard kan resistant gene; lanes 3-8: PCR amplified a 810 bp kan resistant gene in samples (arrow head).



A B

FIG. 3. Colony hybridization showing the presence of *kan* and *amp* resistant bacteria detected with non-radioactive biotin-labeled DNA probes (A), and *amp* resistant bacteria or *kan* resistant bacteria (B) confirmed by blue formazan reaction products.

DISCUSSION

Antibiotics resistance in bacteria is a major health problem in developing countries. Excessive use and increasing antibiotic dependency are a major concern in developing countries. The drinking water samples collected from Lahore metropolitan contained 42.5% and 57.5% of *kan* and *amp* resistant bacteria, respectively. The resistant bacterial strains were *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus* and *E.coli.*

The findings in this study suggest that *kan* and *amp* resistant bacteria are widespread in our local ecosystems, which is serious matter of pollution and health. The majority of *kan* and *amp* resistant bacteria were found in drinking water of residential and college areas. It was reported that the *kan* and *amp* genes are transmitable and common in bacteria, suggesting that many bacteria are capable of withstanding high antibiotic resistant levels to *kan* or *amp*[23-24]. *Kan* and *amp* resistant bacteria were detected in our metropolitan drinking water, which is consistent with the reported antibiotics resistant bacteria found in drinking water.

In early 1980, *kan* and *amp* resistant bacteria were found in bacteria isolated from commercial swine and drinking water and about 10%-50% *kan* and *amp* resistant bacteria could grow on a medium containing kanamycin or ampicillin[25-28]. It was reported that the prevalence of *kan* and *amp* resistant bacteria can increase to 20%-30% and 58.3%-67.4% in hospital drinking water[29-31]. Kikuvi *et al.*[32] showed that the average prevalence of *kan* resistant *E. coli*, *P. aeruginosa*, and *S. aureus* is 42.5% in drinking water, depending on the sample site. Ash *et al.*[33] have isolated 20 unique *kan* resistant strains from rivers in the United States.

The ratio of *amp* resistant bacteria has increased in the past 2 decades. The *amp* resistant bacteria account for 23.5% in drinking water samples from 4 states of India showed[26] and have increased to 37.9%[34]. Mulamattathil *et al.*[27] reported that the *amp* resistant bacteria account for 93% in effluents of chicken processing plants. Another study showed that the *amp* resistant bacteria account for 76% and 2%, respectively, in river water and traveling patients suffering from diarrhea while the β-lactamase gene has also been found in *E.coli* and *salmonella* isolated from animal food[35-37]. It has been shown that the *kan* and *amp* resistant genes are mainly mediated by plasmids and that they can be found on chromosomes[38-39].

In this study, the ratio of *amp* resistant bacteria was higher than that of *kan* resistant bacteria, the public drinking water was highly contaminated with these resistant bacteria, and microorganisms such as *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and *E. coli* also contained *kan* and *amp* resistant genes.

In developing countries, it is necessary to take care in using antibiotics, and use of antibiotics in food of animals should follow the guidelines to minimize the development of antibiotics resistant bacteria. The sanitary and slaughter hygiene conditions and proper disposal of pharmaceutical wastes should be improved to minimize the risk of transferring antibiotics resistant bacteria to humans. These antibiotics-resistant bacteria may pass through water distribution systems or through underground damaged pipes. Detection of *kan* and *amp* resistant genes by PCR and colony hybridization is equally valuable and can be used for rapid detection of antibiotic resistant bacteria in environment and food samples. Antibiotics-resistant bacteria in drinking water may increase the mortality and morbidity, and reduce the effect of antibiotics during chemotherapy.

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