Antibiotic Resistant Bacteria Found in Municipal Drinking Water

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Abstract: Multidrug resistant bacteria in water supply systems have been emerging as a growing public health concern. Many factors affect the source and fate of these bacteria. However, conditions in the plumbing systems may contribute in the dispersion of resistance genes among bacterial populations. Through the process of lateral gene transfer, resistance genetic material can be exchanged between species in the microbial population, intensifying the problem of resistance genes. The main aim of this study was to investigate the diversity of microorganisms in tap-water in Glasgow, Scotland, and the occurrence of certain antibiotic resistance genes and gene-transfer mechanisms. Results show that antibiotic resistant bacteria exist at the consumers' end of the distribution system, some of which also contain integrase genes, which can aid in the dispersion of resistance genes. Presence of such microorganisms indicates that further investigations should be taken to assess the risks to public health.

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Key words: antimicrobial resistance, water distribution system, *intI, qac, sul*

Highlights

- Plumbing systems contain antibiotic resistance bacteria
- *sul*1 and *sul*2 genes are found in bacteria which show resistance against sulfonamides drugs
- The presence of integrons suggests that resistance traits can be transferred to other bacteria

1 Introduction

Supply of safe drinking water to the population of the world remains one of the major concerns for public health. Many factors impact this provision, including increasing world population, limited freshwater resources, and pollution. Contemporary issues affecting supply include contamination with chemical compounds, e.g. pharmaceutical and personal care products, and biological agents, which can contribute to increase antimicrobial resistance in bacteria (Batt et al. 2006; Larsson et al. 2007; Fick et al. 2009; Pruden et al. 2013; Hartmann et al., 2014). The use of these chemicals has been increased to a point that propagation of antimicrobial resistance has become unavoidable, and it is now considered an emerging contaminant of concern in the environment (Pruden et al. 2006). In urban areas, water treatment plants utilise multiple technologies to remove many pollutants (Xu et al. 2007), while integrated constructed wetlands are used in rural areas for removal of pollutants and resistance genes (Chen et al. 2015a), however these systems are never entirely effective (Xu et al. 2007).

Various processes in the treatment plants (Armstrong et al. 1981), and the physical and chemical nature of the long distribution systems, facilitate the enrichment of bacteria and their genes. Attachment to particulates, capsule formation and increased tolerance of bacteria to chemical disinfectants help in the survival and spread of resistant organisms in water supply systems (Ridgway and Olson 1982; LeChevallier et al. 1984; LeChevallier et al. 1988; Bridier et al. 2011, Wingender and Flemming 2011).

The presence of antimicrobial resistant bacteria and their genes in water bodies is not limited to under-developed countries (Khan et al. 2013; Ahammad et al. 2014); they have been found in developed nations including Australia, Germany and the United Kingdom (Stoll et al. 2012). Many enteric bacteria with multiple drug resistance (MDR), e.g., *Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella* and *Shigella* species, have been found in drinking and recreational water resources (Kumar et al. 2013). Previously, both Gram-positive and Gram-negative bacteria,

including *Staphylococcus*, *Alcaligenes*, and *Acinetobacter* species, have been reported in drinking-water distribution systems (Armstrong et al. 1981). Antibiotic resistant *Pseudomonas* species have also been isolated from drinking water (Vaz-Moreira et al. 2012; Ribeiro et al. 2014). The presence of MDR in the clinical environment makes the treatment of diseases difficult (Ashbolt et al. 2013); their presence in water bodies, and increased exposure risk, may necessitate enhanced water treatment and increase expense.

Bacteria isolated from drinking water may not only contain antibiotic resistance, they may also express resistance to commonly used disinfectants, including chlorine and monochloramine (Ridgway and Olson 1982; Chiao et al. 2014, Khan et al, 2016). These disinfectants are indispensable in water treatment plants to eradicate bacteria and minimise the growth of bacteria in the distribution system. Resistance develops upon exposure to sub-inhibitory concentrations over time, and a number of bacterial species with monochloramine resistance have been reported in drinking water, including: *Coxiella*, *Desulfuromonas*, *Desulfomonile*, *Escherichia*, *Geobacter*, *Legionella*, *Mycobacterium*, and *Sphingomonas* species (Chiao et al. 2014).

Besides physical and chemical processes, genetic factors contribute to the dispersion of resistance genes in drinking water distribution systems. Horizontal gene transfer (HGT) mechanisms move resistance genes from resistant to susceptible populations on mobile genetic elements like integrons (Mazel 2006; Gillings et al. 2008; Stalder et al. 2012; Jechalke et al. 2013). HGT spreads genetic elements and their genes among dissimilar groups of bacteria (Gaze et al. 2011), rapidly transferring antibiotic and disinfectant resistance genes (Boucher et al. 2007; Stokes and Gillings 2011; Mokracka et al. 2012). Integrons have a recombination system, which captures genes and serves as a reservoir of resistance genes (Stokes and Hall 1989; Demarre et al. 2007; Xu et al. 2011). The transfer of multiple genes on integrons via HGT intensifies the problem of resistance in bacterial communities, as it can link resistance

traits between environmental bacteria and human pathogens in drinking water systems (Ribeiro et al. 2014).

Among multidrug resistance bacteria, class 1 integrons are most common (Shearer and Summers 2009), and they are related to the presence of resistance to quaternary ammonium compounds (QACs), *qac*EΔ1 genes and sulphonamide resistance *sul*1 determinants (Kucken et al. 2000). Almost half of the class 1 integrons contain a *qac* resistance genes cassette (Gillings et al. 2009), and the prevalence of class 1 integrons in bacteria having previous exposure to QACs is greater than those without previous exposure (Gaze et al. 2005). Moreover, bacteria that possess or acquired plasmids, transposons or integrons, have greater QACs resistance than those which do not have any of these genetic elements (Bjorland et al. 2003). Bacteria often show cross resistance for QACs and antibiotics (Morente et al. 2013), including the use of efflux-pump systems to generate resistance to unrelated broad-range antimicrobials (Buffet-Bataillon et al. 2012). Enzyme based mechanisms of antibiotic resistance is also found in bacteria. In the presence of antibiotics, bacteria may acquire genes encoding enzymes which can destroy the antibiotic before reaching the target, resulting in the development of resistant in bacteria (Tenover, 2006).

This study aimed to determine the prevalence of bacteria and resistance genes in drinking water. Assays included genes responsible for resistance to a wide variety of quaternary ammonium compounds (*qac* genes) and sulphonamide antibiotics (*sul*1 and *sul*2 genes), as these genes commonly co-occur. Mobile genetic integrons were detected via their integrase gene *intI*1 and *intI*2. The co-occurrence of *sul* and *qac* genes in bacteria from the water distribution system was also examined.

2 Materials and Methods

2.1 Sampling and Processing

Tap-water samples were collected from residences in Glasgow in sterile screw capped bottles, and were processed within 2-4 hours of collection. One hundred millilitres water samples were vacuum-filtered through 0.22 μm pore size cellulose nitrate gridded membranes (Millipore, UK), which were then aseptically placed on Standard Plate Count Agar APHA (Oxoid, UK). The plates were incubated for 48 hours at 37 ± 2 °C. Selected bacterial isolates from the resultant growth were streaked on Nutrient Agar (Oxoid, UK) plates to isolate colonies; 4-5 colonies of each strain were preserved in glycerol using a Bacterial Beads Preservation Kit (Cryo vials TS/71-MX, Technical Service Consultants Ltd., UK) and stored at -80 °C.

2.2 DNA Extraction and PCR Amplification

DNA of the bacterial isolates were thermally extracted by mixing strains with 100 μL of PBS (pH 7.4) and undergoing a series of freeze thaw cycles at -80 °C and 70 °C with continuous shaking between each cycle. The contents were centrifuged at 10,000 rpm for 5 minutes at the end of the fourth thermal cycle, and DNA from the supernatant was stored at -80 °C.

PCR reactions were performed with a Bio-Rad iQ5 Real-Time PCR Detection System for the presence of 16S-rRNA, *intI*1, *intI*2, *sul*1, *sul*2 and *qac* genes using previously described primers (Table 1; Pei et al. 2006; Luo et al. 2010; Caporaso et al. 2011; Jechalke et al. 2013). Twenty microliter PCR reactions consisted of 10 μL of MegaMix-Blue-PCR Mastermix with dye (Microzone Limited, UK), 1 μL of each primer (500 ηM final concentration; Sigma-Aldrich Life Science, UK), 6 μL of nuclease-free water, and 3 μL of DNA sample. Each PCR run consisted of initial denaturation at 95 °C for 3 minutes; this was followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing for 30 seconds at annealing temperature (Table 1), extension at 72 °C for 30 seconds, and then a final extension at 72 °C for 10 minutes. PCR products were further verified with 2 % agarose gel in 1x Tris Acetate-EDTA buffer; the size of

amplified products was determined against a 50-bp incrementing DNA ladder (Fisher BioReagent, UK).

2.3 DNA Purification and Sequencing

PCR products from 16S-rRNA gene amplification were purified using the QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions. Purified and cleaned amplicon concentration was determined by the EPOCH™ Microplate spectrophotometer system (BioTek, UK). Amplicon was mixed with 5 µM forward primer solution used in PCR in a 1:1 ratio in a total volume of 10 µL and sent to LightRun Sequencing Service (GACT Biotech Ltd, London, UK) for sequencing. Bacteria were identified by comparing the sequences using the BLAST program through the National Centre for Biotechnology Information (NCBI).

3 Results

3.1 Identification of Bacteria by16S-rRNA Sequencing: Bacterial Community Structure

Bacteria were isolated from tap water by a membrane filtration method (n=148) and 87 colonies were identified up to genus level by sequencing the V4 region of 16S-ribosomal RNA gene. The water distribution system harboured three phyla of bacteria: Proteobacteria, Actinobacteria and Firmicutes. Among them, 54 (62.1 %) belonged to the phylum Proteobacteria. Sub grouping of this phylum indicated the presence of 10 alpha-proteobacteria (11.5 %), 38 beta-proteobacteria (43.7 %), 5 gamma-proteobacteria (5.7 %) and 1 epsilon-proteobacterium (1.2 %). Firmicutes were the second largest phyla found in drinking water and 18 (20.7 %) bacteria belonged to this group, while 15 (17.2 %) bacteria were from Actinobacteria.

The presence of both Gram-negative and Gram-positive bacteria was confirmed in the tap water; among them some of the bacteria can be pathogenic. Species of *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Kocuria*, *Staphylococcus*, *Arthrobacter*, *Comamonas*, *Acidovorax*, *Blastomonas*, *Variovorax*, *Escherichia* and pathogenic *Burkholderia* were found in the water distribution system.

3.2 Presence of Antibiotic Resistance Genes

Detection of the *sul*1 and *sul*2 genes specific for sulfonamide resistance was performed on 148 isolates and these genes were detected in 12 (8.1 %) isolates, thus confirming the presence of antibiotic resistant bacteria in the water distribution system; none contained both genes. *Sul*1 genes were detected in 8 (5.4 %) bacteria, while *sul*2 genes were present in 4 (2.7 %) isolates (Table 2). In two (1.4 %) isolates, these genes were also positive for integrons, while in 10 (6.8%) isolates they were found singly without integrase genes indicating that *sul* genes did not always correspond to *intI* genes as expected. This suggests that the *sul* genes in these bacteria might either be present on chromosome, or associated with other genetic elements (Gundogdu et al. 2011) other than *intI* genes. The distribution of bacteria containing antibiotic resistance genes (ARGs) was widespread among organisms including *Bacillus*, *Cupriavidus*, *Variovorax*, *Kocuria*, *Ralstonia*, *Dermacoccus*, *Micrococcus* and *Staphylococcus* species from the samples.

3.3 Presence of Disinfectant Resistance Genes

Disinfectant resistance genes *qac* were not found in any of the isolates. *Qac* genes associated with class 1 integrons have a high occurrence rate in the environment both in Gram-positive and Gram-negative bacteria (Jaglic and Cervinkova 2012); in this study, the presence of *qac* genes was not detected.

3.4 Presence of Transferable Markers

PCR amplification analyses indicated that class-1 integrons existed in eight bacteria (9.2 %), while *intI*2 genes were not detected in any isolate. In this study, *Dermacoccus* sp. had both *sul*1 and *intI*1 genes, while *Micrococcus* sp. had *sul*2 and *intI*1 genes simultaneously. Presence of *intI*1 genes confirms the presence of transferable genetic element integrons in the bacteria of the water supply system which could involve in the dispersion of antibiotic and disinfectant resistance genes in the environment.

4 Discussion

The presence of antibiotic resistant bacteria and their genes in water is a major public health concern and a global challenge (Pruden et al. 2012; Pruden et al. 2013). They are a major contributing factor in water pollution, found in natural water bodies (Ahammad et al. 2014), treated drinking water (Pruden et al. 2006), and drinking water distribution systems (Xi et al. 2009), can cause infectious diseases (Pruden et al. 2012), and are difficult to treat (Levy 2002). Currently, waterborne diseases are not only related to the presence of faecal bacteria, but also the occurrence of opportunistic pathogens in water systems (Wang et al. 2013). Around 30 different bacterial genera have been isolated from drinking water in different studies including genera of *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Escherichia* and *Proteus* (Norton et al. 2000; Allen et al. 2004; Gallego et al. 2005, Chiao et al, 2014). In developed countries, outbreaks of waterborne infections due to the drinking water have been reported (Kilvington et al. 2004; Brunkard et al. 2011). Antibiotic resistant infections are also not uncommon in these countries. In the USA, two million people suffered from antibiotic resistant infections every year and among them 23,000 died (CDC 2013). Drinking water distribution systems host a variety of microorganisms (Simoes et al. 2010), and bacteria having antibiotic resistance can find their way into drinking water in these systems. From drinking water, they can colonize the

gastrointestinal tract and serve as a potential health risk for the population, especially immune-compromise individuals (Lee et al. 2010).

Isolation of bacteria by membrane filtration demonstrates the presence of viable organisms, so it is a good indicator of the presence of living bacteria in the water environment, which can be actively involved in gene transfer among bacteria and spread of disease in humans. 16S-rRNA gene information is generally used for the identification of bacteria from different environments (Chakravorty et al. 2007). While a useful housekeeping genetic marker to classify bacteria, 1-14 % of organisms remain unidentified as it has low phylogenetic power at species level and cannot discriminate some genera properly (Drancourt et al. 2000; Woo et al. 2003; Mignard and Flandrois 2006; Janda and Abbott 2007). In this study, we identified 87 % of bacteria from the tap water, while 13 % of bacteria were not characterized as no significant similarity was found. These bacteria belong to 22 genera (Table 2), and also included *Burkholderia*, some species of which are waterborne pathogens and may cause melioidosis in humans (Howard and Inglis 2003). The isolation and identification of multiple types of bacteria at the point of use indicates that the distribution network or plumbing systems might be playing some role in the existence of these bacteria, and the ecology of the system could be a contributing factor in their incidence (Hong et al. 2010).

A well-recognized factor which could contribute to the existence of antibiotic resistance bacteria at the consumer end is the presence of biofilms in the distribution system or plumbing system of the buildings from where water samples have been taken. Prevalence of antibiotic resistant bacteria in a water supply system, even in the presence of disinfectant, could be exacerbated by aging infrastructure, in which biofilms have formed on surfaces (Abe et al. 2012). The multiple layers of microorganisms in these biofilms decrease the residual disinfectant levels to the inner layers; further, bacteria in these layers could develop and transfer resistance traits against antimicrobials to other susceptible populations present in these biofilms (Molin and Tolker-Nielsen 2003; Bridier et al. 2011). Water storage in tanks and

cisterns also causes a decrease in the amount of residual disinfectant as water retention time increases, thus allowing bacteria to grow in water. Disinfection itself could concentrate the antibiotic resistant bacteria and their genes in drinking water, as was found in the work of Shi et al. (2013), where chlorination caused the enrichment of *tet*, *amp* and *erm* genes.

The emergence of antibiotic resistance in the environment is not only due to physiological factors, but also depends on genetic factors like horizontal gene transfer (HGT) rate (Andersson and Hughes 2010). Co-selection of two different antibiotic resistance genes occurs through HGT due to the genetic linkage of these genes. For example, sulfonamide resistance genes are plasmid borne and often linked with other antibiotic resistance genes. It has also been found that *sul* and *intI* genes co-exist in water, which might be due to the presence of *sul*1 genes on the class 1 integrons (Chen et al., 2015b). This contributes to the reason that sulphonamide resistance has not declined even when the use of the antibiotics has been reduced (Enne et al. 2004).

Quaternary ammonium compounds resistant genes are also present on mobile genetic elements, such as class 1 integrons (Chapman 2003). Bacterial strains, which acquire genetic units like plasmids, transposons or integrons, show a higher resistance to QACs (Bjorland et al. 2003). The selective pressure by quaternary ammonium compounds (Stalder et al. 2012) disperses *qac* genes and antibiotic resistance genes associated with the integrons (Paulsen et al. 1993; Paulsen et al. 1996; Jeong et al. 2009). This indicates that cross resistance for QACs and antibiotics is possible (Morente et al. 2013). Other mechanisms like a multidrug efflux pump and modification of the cell wall also induce resistance in bacteria (Jaglic et al. 2012), which allow them to survive in the presence of disinfectant. This could be a reason that in the current study, *qac* genes were not detected in any of the bacteria despite the fact that *intI*1 genes were present with antibiotic resistance genes. This suggests that in the absence of disinfectant resistant genes, other mechanisms of resistance might help bacteria persist in the water distribution system.

5 Conclusions

Isolation of antibiotic resistant bacteria from drinking water demonstrates a need for greater awareness of ecological interactions in drinking water and increased monitoring of distribution systems and plumbing. Presence of those genera, some species of which could cause human diseases, indicates that water quality could not be guaranteed at the consumer end, and future studies should focus on treatment considerations at point of use to guarantee safety.

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Table 1. PCR Primers for targeting different genes

Primer	Sequence (5′-3′)	PCR annealing temperature (°C)	Amplicon size	Reference
V4-16S-515F V4-16S-806R	TGTGCCAGCMGCCGCGGTAA GGCTACHVGGGTWTCTAAT	50	312	(Caporaso et al. 2011)
qacEaIIF qacEaIIR	CGCATTTTATTTTCTTTCTCTGGTT CCCGACCAGACTGCATAAGC	60	Not detected	(Jechalke et al. 2013)
int1-F int1-R	GGCTTCGTGATGCCTGCTT CATTCCTGGCCGTGGTTCT	57	148	(Luo et al. 2010; Chen et al. 2015a)
int2-F int2-R	GTTATTTTATTGCTGGGATTAGGC TTTTACGCTGCTGTATGGTGC	56	166	(Luo et al. 2010; Chen et al. 2015a)
sul1-F sul1-R	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	56	163	(Pei et al. 2006; Chen et al., 2015a)
sul2-F sul2-R	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	60.8	191	(Pei et al., 2006; Chen et al., 2015a)

F, forward; R, reverse.

Table 2. Detection of *intI*1, *intI*2, *sul*1, *sul*2 and *qac* genes in bacteria isolated from the drinking water distribution system.

N#	Phylum	Genus	Genes					Isolate identification
			***intI*1**	***intI*2**	***sul*1**	***sul*2**	***qac***	
1	Actinobacteria	*Arthrobacter*	-	-	-	-	-	DW(518)
1	Actinobacteria	*Arthrobacter*	+	-	-	-	-	DW(509)
2	Actinobacteria	*Dermacoccus*	-	-	-	-	-	DW(597, 603)
1	Actinobacteria	*Dermacoccus*	-	-	+	-	-	DW(608)
1	Actinobacteria	*Dermacoccus*	+	-	+	-	-	DW(607)
1	Actinobacteria	*Dietzia*	-	-	-	-	-	DW(625)
1	Actinobacteria	*Janibacter*	-	-	-	-	-	DW(644)
1	Actinobacteria	*Kocuria*	-	-	-	-	-	DW(565)
1	Actinobacteria	*Kocuria*	-	-	+	-	-	DW(620)
1	Actinobacteria	*Kocuria*	+	-	-	-	-	DW(513)
2	Actinobacteria	*Micrococcus*	-	-	-	-	-	DW(505, 637)
1	Actinobacteria	*Micrococcus*	+	-	-	-	-	DW(638)
1	Actinobacteria	*Micrococcus*	+	-	-	+	-	DW(512)
9	Alphaproteobacteria	*Blastomonas*	-	-	-	-	-	DW(525, 526, 547, 551, 553, 554, 556, 559, 599)
1	Alphaproteobacteria	*Sphingomonas*	-	-	-	-	-	DW(576)
8	Betaproteobacteria	*Acidovorax*	-	-	-	-	-	DW(516, 521, 537, 539, 540, 541, 544, 569)
5	Betaproteobacteria	*Burkholderia*	-	-	-	-	-	DW(530, 615, 617, 626, 643)
1	Betaproteobacteria	*Comamonas*	-	-	-	-	-	DW(503)
10	Betaproteobacteria	*Cupriavidus*	-	-	-	-	-	DW(501, 502, 504, 511, 522, 570, 578, 580, 587, 591)
1	Betaproteobacteria	*Cupriavidus*	-	-	-	+	-	DW(515)
2	Betaproteobacteria	*Cupriavidus*	-	-	+	-	-	DW(610, 622)
1	Betaproteobacteria	*Cupriavidus*	+	-	-	-	-	DW(604)
5	Betaproteobacteria	*Ralstonia*	-	-	-	-	-	DW(609, 613, 616, 618, 619)
1	Betaproteobacteria	*Ralstonia*	-	-	-	+	-	DW(614)
2	Betaproteobacteria	*Variovorax*	-	-	-	-	-	DW(546, 549)
2	Betaproteobacteria	*Variovorax*	+	-	-	-	-	DW(557, 600)
1	Epsilonproteobacteria	Not identified						DW(533)
5	Firmicutes	*Bacillus*	-	-	-	-	-	DW(514, 527, 529, 531, 640)
1	Firmicutes	*Bacillus*	-	-	-	+	-	DW(507)
1	Firmicutes	*Bacillus*	-	-	+	-	-	DW(532)
1	Firmicutes	*Brevibacillus*	-	-	-	-	-	DW(535)
5	Firmicutes	*Paenibacillus*	-	-	-	-	-	DW(552, 623, 634, 635, 641)
1	Firmicutes	*Paenibacillus*	-	-	+	-	-	DW(536)
3	Firmicutes	*Staphylococcus*	-	-	-	-	-	DW(538, 542, 632)
1	Firmicutes	*Staphylococcus*	-	-	+	-	-	DW(631)
2	Gammaproteobacteria	*Enhydrobacter*	-	-	-	-	-	DW(506, 508)
2	Gammaproteobacteria	*Escherichia*	-	-	-	-	-	DW(560, 611)
1	Gammaproteobacteria	*Pantoea*	-	-	-	-	-	DW(595)

Conflict of Interest

No conflict of interest is found.

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