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Antibiotic resistance and virulence genes in coliform water isolates

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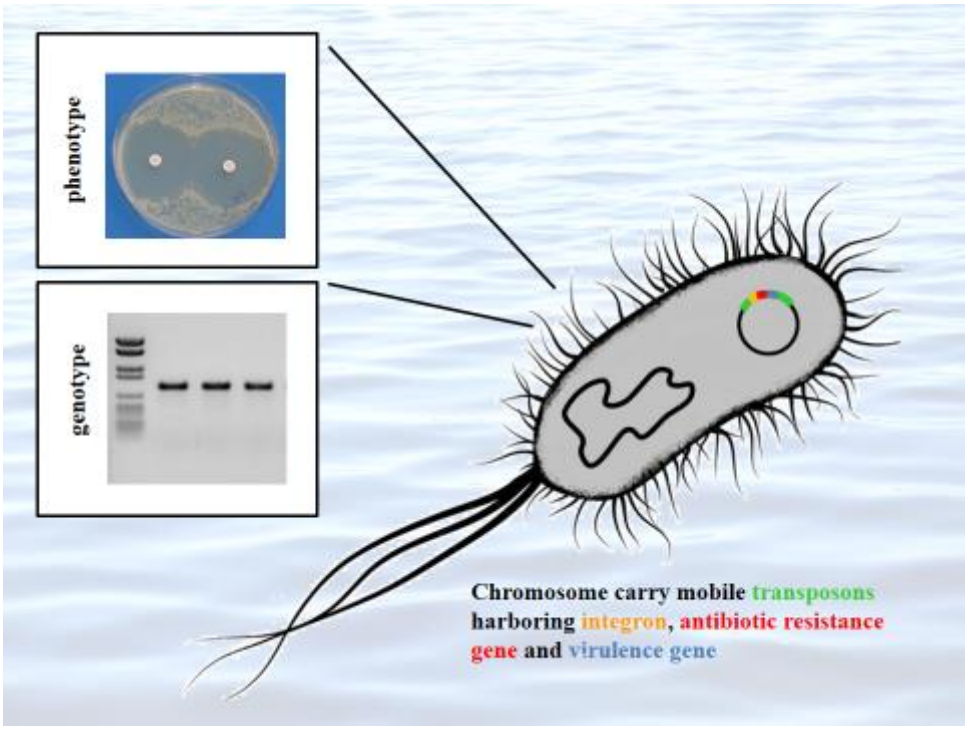
Graphical abstract



ABSTRACT

Widespread fecal pollution of surface water may present a major health risk and a significant pathway for dissemination of antibiotic resistance bacteria. The River Rhine is one of the longest and most important rivers in Europe and an important raw water source for drinking water production. A total of 100 coliform isolates obtained from River Rhine (Germany) were examined for their susceptibility to seven antimicrobial agents. Resistances against amoxicillin, trimethoprim/sulfamethoxazole and tetracycline were detected in 48 %, 11 % and 9 % of isolates respectively. The antibiotic resistance could be traced back to the resistance genes *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *ampC*, *sul1*, *sul2*, *dfrA1*, *tet*(A) and *tet*(B). Whereby, the *ampC* gene represents a special case, because its presence is not inevitably linked to a phenotypic antibiotic resistance. Multiple antibiotics resistance was often accompanied by the occurrence of class 1 or 2 integrons. *E. coli* isolates belonging to phylogenetic groups A and B1 (commensal) were more predominant (57 %) compared to B2 and D groups (43 %) which are known to carry virulent genes. Additionally, six *E. coli* virulence genes were also detected. However, the prevalence of virulence genes in the *E. coli* isolates was low (not exceeding 4.3 % per gene) and no diarrheagenic *E. coli* pathotypes were detected. This study demonstrates that surface water is an important reservoir of ARGs for a number of antibiotic classes such as sulfonamide, trimethoprim, beta-lactam-antibiotics and tetracycline. The occurrence of antibiotic resistance in coliform bacteria isolated from River Rhine provides evidence for the need to develop management strategies to limit the spread of antibiotic resistant bacteria in aquatic environment.

KEY WORDS

Antibiotic resistance, antibiotic resistance genes, virulence genes, integrons, coliform isolates, river water

INTRODUCTION

The discovery of antimicrobial substances as well as their large scale production and use in the modern medicine has revolutionized treatment of infectious diseases. However, widespread application of antibiotics in human and veterinary medicine has led to the emergence, selection, and dissemination of antibiotic resistant bacteria and genes encoding for antibiotic resistance in different environmental compartments such as surface water (Zhang et al., 2015; Stoll et al., 2012), groundwater (Li et al., 2014), drinking water (Schwartz et al., 2003; Guo et al., 2014) and sediments (Rosas et al., 2015) throughout the world. Antibiotic resistance genes (ARGs) and virulence genes (VGs) are emerging environmental contaminants (Pruden et al., 2006; Haack et al., 2009).

Bacteria can acquire antibiotic resistance by random DNA mutation or by horizontal or vertical gene transfer (Frost et al., 2005; Barlow et al., 2009). Gene transfer can result in the exchange of ARGs, in particular if the genes are located on mobile elements such as plasmids, integrons or transposons. Horizontal gene transfer is a major mechanism for spreading of ARGs among different strains or bacterial species (Frost et al., 2005) and beyond the habitat of original hosts (Moore and Linday, 2001). Integrons play an important role in the dissemination of ARGs as they carry determinants of site-specific recombination and an expression system, which integrates single or groups of mobile antibiotic resistance gene cassettes (Hall and Collis, 1995). Integrons have been widely associated with multiple antibiotics resistance (Lai et al., 2013; Ammar et al., 2016). Besides ARGs, VGs could also be located in genetic mobile elements (de la Cruz and Davies, 2000; Ochman et al.; 2000)

Fecal coliform bacteria which inhabit the gastrointestinal tracts of humans and animals are widely used as indicators of fecal pollution in the aquatic environment.

The presence of coliform bacteria especially *Escherichia coli* in water indicates the potential presence of disease-causing fecal microorganisms. Most *E. coli* strains are commensal, however, acquisition of specific VGs may lead to the development of virulence attributes causing a wide spectrum of intestinal and extraintestinal infections, such as diarrhea, urinary tract infection, meningitis, and septicemia (Kaper et al., 2004). Already in the 1980s, Fecal coliform genera members *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* in water samples have been shown to carry antibiotic resistance determinants (Niemi et al., 1983). *E. coli* is considered as a major carrier of resistance traits (Oppegaard et al., 2001).

E. coli can be divided into four main phylogenetic groups (A, B1, B2 and D) according to the combination of the three genetic markers *chuA*, *yjaA* and DNA fragment TspE4C2 (Clermont et al., 2000). The virulent extra-intestinal strains belong mainly to group B2 and to a lesser extent to group D (Picard et al., 1999), whereas most commensal strains belong to group A and B1 (Johnson et al., 2001).

Numerous virulence factors including adhesion, host cell surface-modifying factors, invasion, toxins, and secretion systems are involved in *E. coli* pathogenicity (Bekal et al., 2003). Diarrheagenic *E. coli* have been classified into five well-described groups: enterotoxigenic *E. coli* (ETEC) strains, enteropathogenic *E. coli* (EPEC) strains, enterohemorrhagic *E. coli* (EHEC) strains, enteroaggregative *E. coli* (EAEC) strains, and enteroinvasive *E. coli* (EIEC) strains (Kaper et al., 2004). At present, there is paucity of information on the prevalence of coliform bacteria including *E. coli* carrying ARGs, integrons and VGs in surface water which is often used for potable and non-potable purpose such as irrigation (Graham et al., 2014; WHO, 2014)

In this study, the occurrence of ARGs, integrons and VGs in coliform and *E. coli* bacteria isolated from River Rhine water was studied. The specific objectives

of the study were (i) to determine the frequency of occurrence of antibiotic resistance against 10 antibiotics and 13 specific antibiotic resistance genes in coliform bacteria isolated from the German river Rhine; (ii) to examine the ratio of ARGs presence and phenotypic antibiotic resistance (iii) to classify *E. coli* isolates and assess the distribution of the VGs.

MATERIALS AND METHODS

Surface water sample collection. Grab samples (n=17) were collected in sterile container from River Rhine near Düsseldorf, Germany, between June 2006 and June 2007. The collected samples were transported on ice to the laboratory for processing.

Culture methods. Coliform bacteria and *E. coli* were isolated from the collected water samples at 37°C for 24 h on lactose-TTC-agar (Heipha, Germany) according to National Committee for Clinical Laboratory Standards (NCCLS). (Single well isolated yellow-orange colonies were isolated and purified). The presumptive isolates were then identified with API 20E (BioMeriéux, Germany) biochemical test strips.

Antibacterial susceptibility of coliform isolates was examined according to a standard method recommended by the NCCLS, with Mueller-Hinton II agar (Heipha, Germany) and susceptibility test disks (Merck, Germany). The following antibiotic disks were used: amoxicillin, piperacillin, tetracycline, gentamicin, meropenem, ciprofloxacin and trimethoprim/sulfamethoxazole (Tiehm et al., 2009) Trimethoprim was utilized together with sulfamethoxazole as this combination is widely used to control infections due to their synergistic mode of action on inhibition of folate synthesis pathway in bacteria. The concentration of antibiotic used in this study and diameter of inhibition zones surrounding the antibiotic disks was interpreted as outlined in Table 1.

DNA extraction. Genomic DNA was isolated from purified single colonies using DNeasy tissue and blood kit (Qiagen, Germany) according to manufactures instructions. The integrity of the extracted DNA was checked by performing PCR with the eubacteria specific primers 27f and 1492r on a ten and 100 fold dilution of extracted nucleic acid, followed by gel electrophoresis. DNA extracts were stored at (temperature) until ARGs and VGs analysis.

PCR detection of ARGs, integrons and the genes *uidA*, *chuA*, *yjaA* and TspE4C2. Coliform isolates were screened for the presence of 13 ARGs against four antibiotic classes. The ARGs included sulfonamide resistance genes (*sul1* and *sul2*), trimethoprim resistance genes (*dfrA1*, *dfrA12*, *dfrA13*), beta-lactam resistance genes (*ampC*, bla_{TEM}, bla_{SHV} and $bla_{\text{PSE-1}}$) and tetracycline resistance genes (*tet*(A), *tet*(B), *tet*(C), and *tet*(M)). These ARGs were selected due to their reported presence in River Rhine samples (Stoll et al., 2012). Previously published primer sets were used for the PCR amplification of ARGs (Stoll et al., 2012).

Presumptive *E. coli* isolates were confirmed by PCR amplification of the *uidA* gene using primers published by Frahm et al. (2003). Verified *E. coli* strains were assigned to four phylogenetic groups using a triplex PCR based on the presence or absence of three DNA fragments: *chuA*, *yjaA*, and TspE4C2, as previously described (Clermont et al., 2000). The presence of class 1 and 2 integrons carrying the integrase genes *intl1* and *intl2* respectively was also investigated using previously published primers sets (Ibekwe et al., 2011). PCR amplification of ARGs, *intl1* and *intl2* as well as the genes *chuA*, *yjaA*, and TspE4C2 was performed in 20 µL reaction mixtures. Each reaction mixture contained 1x buffer with $MgCl_2$ (Molzym, Germany), 200 µM each dNTP (Roth, Germany), 0.5 µM each primer (Invitrogen, Germany), 2 U of Taq Polymerase (Molzym, Germany) and 2 µL of DNA template. The PCR amplifications were performed using Tpersonal thermocycler (Biometra, Germany). The

thermocycling parameters for the amplification of ARGs and *chuA*, *yjaA*, and TspE4C2 were: initial denaturing for 3 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, annealing at 55 °C for 30 s and extension for 30-120 s depending on the product length at 72 °C followed by a final extension for 10 min at 72 °C. For the detection of *intl1* and *intl2* annealing temperatures were 58 °C and 48 °C respectively. The amplified product (10 µL) was electrophoresed on a 1 % Tris-acetate-EDTA agarose gel containing 2 µg of ethidium bromide mL^{-1}. DNA molecular weight marker pBR 328 (Roth, Germany) was used as a standard DNA ladder.

PCR detection of *E. coli* VGs. Additionally, confirmed *E. coli* isolates were tested for the presence of VGs. The list of VGs and the corresponding pathotypes tested in this study is shown in Table 2. PCR confirmed *E. coli* isolates were screened for the presence of 11 diarrheagenic *E. coli* VGs by using previously published primer sets, *stx1* and *stx2*, *eaeA*, *ehxA*, LT, *bfp*, ST, *aggR*, *ipaH*, *astA* and *cdtB* (Sidhu et al., 2013) PCR reactions were performed on a Bio-Rad iQ5 thermocycler system (BioRad Laboratories, California), using iQ supermix (BioRad Laboratories, California). Each 25 µL PCR mixture contained 12.5 µL of supermix, 120-200 nM of each primer, and 3 µL of template DNA with thermal cycling conditions as outlined previously (Sidhu et al., 2013). For each PCR run, corresponding positive (i.e. target DNA) and negative (sterile water) controls were included. A melt curve analysis was performed after each PCR run to differentiate between actual products and primer dimers and to eliminate the possibility of false-positive results. The melt curve was generated using 80 cycles of 10 s each starting at 55 °C and increasing in 0.5 °C intervals to a final temperature of 95 °C. The midpoint melting temperature (T_m) for each amplicon was determined using the iQ5 software (Bio-Rad). *E. coli* O157:H7 (ATCC 35150) was used as a positive control (*uidA* gene) in PCR assays to confirm presumptive *E. coli* and for the *eaeA*, stx_1 and stx_2 genes. *Shigella sonnei* (ATCC 29930) was used as positive control

for the *ipaH* gene. *E. coli* strain belonging to serotypes O138 of porcine origin was used as positive control for ST and LT genes. For the remaining VGs, DNA extracted from clinical *E. coli* isolates were screened for the presence of appropriate size bands. The bands were excised from the gel and DNA was purified and subsequently used as positive controls.

RESULTS

Antimicrobial susceptibility (phenotypic results)

54 % of the coliform isolates were resistant to at least one antibiotic, and 46 % of the isolates were sensitive to the tested antibiotics. Some bacteria were multi-drug resistant against two, three or four antibiotics (4 %, 5 %, and 1 %). It is noticeable that all bacterial isolates that were resistant against the combination of trimethoprim and sulfamethoxazole were at least resistant to one more antimicrobial substance. Also eight of the 11 tetracycline resistant coliform bacteria were multi drug resistant. Five isolates were resistant to trimethoprim/sulfamethoxazole, tetracycline and amoxicillin and one isolate exhibited a quadruple resistance (trimethoprim/sulfamethoxazole, tetracycline, amoxicillin and piperacillin).

The distribution of coliform genera determined with API 20E strips in sensitive and resistant isolates is shown in Figure 1. *E. coli* represented 41.3 % of the sensitive and 20.4 % of the resistant isolates. *Klebsiella* was the second most often detected genera with 4.3 % sensitive and 33.3 % of the resistant isolates. 23.9 % of the sensible and 16.7 % of the resistant isolates could not be allocated to specific genera. None of the bacteria identified as *Leclercia* sp. showed an antibiotic resistance. By contrast, all *Pantoea* isolates were resistant against at least one antibiotic.

Distribution of ARGs

Resistance to the beta-lactam antibiotic amoxicillin was most frequently detected in coliform isolates. In total, four genes that encode for amoxicillin resistance encoding beta-lactamases were analyzed. Nearly 43 % of the isolates carried an *ampC* gene, but only 20.9 % (nine isolates) of the *ampC* positive isolates were amoxicillin resistant. In addition, the beta-lactamase gene bla_{TEM} was detected in most of these isolates. Moreover, *ampC* was found in many bacteria which were amoxicillin intermediate or sensitive (22 and 12 respectively). The appearance of bla_{TEM} or bla_{SHV} was often accompanied by presence of phenotypic resistance against amoxicillin (85.7 % and 77.8 % respectively).

The tetracycline resistant isolates were examined for the resistance genes *tet*(A), *tet*(B), *tet*(C) and *tet*(M). Only *tet*(A) and *tet*(B) genes were detected in 36.4 % and 54.5 % of the 11 tetracycline resistant isolates respectively (Figure 2). In only one isolate, phenotypic antibiotic resistance could not be associated to an according ARG.

The trimethoprim/sulfamethoxazole resistant isolates were tested for the genes *sul1*, *sul2*, *dfrA1*, *dfrA12*, and *dfrA13*. Phenotypic resistance in the test only is observed, if the bacteria are simultaneously resistant against both antibiotics. Nearly 33.3 % of trimethoprim/sulfamethoxazole resistant isolates carried the *sul1* gene and remaining 66.7 % carried *sul2* gene. Hence, this resistance could be assigned to the corresponding genes. Five isolates carried *sul1* gene, but no phenotypic resistance against trimethoprim/sulfamethoxazole was observed in the agar diffusion test. The genotypic background of the trimethoprim resistance was not fully elucidated. Only *dfrA1* gene was detected in 55.6 % of the resistant bacteria. Four non-resistant isolates carried either *dfrA1* or *dfrA12* gene (two each). However, given that the susceptibility against the combination of trimethoprim and sulfamethoxazole was tested, the presence of one gene mediating resistance to one antibiotic does not lead to resistance

against the combination. Possibly the isolates would have shown resistance to trimethoprim – as single antimicrobial substance – but this was not tested.

Occurrence of integrons

Isolates were screened for the presence of class 1 and 2 integrons carrying the integrase genes *intl1* and *intl2* respectively. The presence of class 1 integrons was observed in 10 % of the isolates. Half of these isolates showed resistance against two or more of the seven analyzed antibiotics. One isolate was resistant against one antibiotic and the remaining three isolates were sensitive against all tested antibiotics. Class 2 integron was detected in two isolates, one with resistance to two antibiotics with four ARGs and second isolate exhibited resistance to a single antibiotic with two ARGs. Overall, the occurrence of class 1 and class 2 integrons is accompanied by cumulative appearance of antibiotic resistance genes (Table 3, bottom rows).

***E. coli* genotypes and virulence factors**

E. coli bacteria within the coliform isolates were verified using PCR. Out of 100 coliform isolates, 47 were confirmed as *E. coli.* In the next step, confirmed *E. coli* isolates were allocated into four phylogenetic groups (i.e. A, B1, B2 and D). In this study, the group A (46.8 %) was the predominant group followed by B2 (29.8 %), D (12.8 %) and B1 (10.6 %). Overall, *E. coli* belonging to phylogenetic groups A and B1 (commensal) were more predominant compared to B2 and D groups.

Multiple PCR assays were used to identify a spectrum of 11 VGs (Table 4). Out of 11 VGs tested, only six were detected in 47 *E. coli* isolates. The genes *ipaH* and *bfp* were found in one isolate each and for *eaeA, stx2, aagR* and *astA* two isolates each tested positive. Only one isolate carried two VGs (*aagR* and *bfp*). However, this isolate showed no resistance to any of the tested antibiotics. One bacterium positive for single gene *astA* showed multiple antibiotic resistances against three of seven antibiotics tested.

DISCUSSION

The analysis of coliform bacteria isolated from River Rhine revealed the prevalence of amoxicillin (48 %), tetracycline (9 %), sulfamethoxazole/trimethoprim (11 %) resistance. This could be due to the fact that these antibiotics are most commonly produced and prescribed in human and veterinary medicine in Germany (GERMAP, 2012). In 2005, amoxicillin was the single most commonly prescribed antibiotic in the human medicine with nearly 80 million defined daily doses per year which corresponds to 80 tons per year (GERMAP, 2012) In addition, beta-lactams are also among the largest group of antibiotics used in veterinary medicine in Germany (GERMAP, 2012). Tetracycline, sulfonamide and trimethoprim are also extensively used for the treatment of bacterial infections in humans and animals. Sulfonamide also has high prevalence in the aquatic environment (Watkinson et al., 2009; Gao et al., 2012) Therefore, detection of coliform bacteria resistant to amoxicillin, tetracycline and sulfamethoxazole/trimethoprim is not surprising in coliform bacteria isolated from surface water and the findings are consistent with published literature (Laroche et al., 2009; Ibekwe et al., 2011; Su et al., 2012; Koczura et al., 2012; Rosas et al., 2015; Kotlarska et al., 2015; Zhang et al., 2015). The assessment of antibiotic resistance in only water phase may lead to an underestimation of the health risk, as higher number of resistant *E. coli* have been reported in the sediment (Rosas et al., 2015).

In this study, 43 % of isolates carried *AmpC* gene however, only 20.9 % of isolates showed phenotypic resistance to amoxicillin. The primary mechanism for the formation of significant resistances against beta-lactam antibiotics is the acquisition of secondary beta-lactamases like *AmpC*. Bacteria expressing the AmpC beta-lactamase are typically resistant to penicillins, beta-lactam-beta-lactamase inhibitor

combinations, and cephalosporins (Bush et al., 1995). *AmpC* genes are widely distributed among bacteria of the family *Enterobacteriaceae* (Lindberg and Normark, 1987). *E. coli* and *Shigella* species have a chromosomal gene which codes for *AmpC* beta-lactamase enzymes at low levels, usually clinically insignificant and known as constitutive beta-lactamases (Bush et al., 1995). In other bacteria like *Citrobacter freundii* and *Enterobacter cloacae*, *ampC* can be induced by certain beta-lactam (Lindberg and Normark, 1987). In this study, most of the *ampC* positive isolates belonged to the genus *E. coli*, *Citrobacter* and *Enterobacter* (Table 3).

Beside the *ampC* gene, other beta-lactamase genes such as bla_{SHV} and bla_{TEM} are known to impart beta-lactam resistance. Enzymes of these families are able to inactivate most of the penicillins and group 1 and 2 cephalosporins (Bush et al., 1995). Both of these enzymes are important in clinical isolates as already in the beginning of this millenium studies have hypothesized that worldwide 50 % of clinical *E. coli* isolates carry a TEM-1-beta-lactamase (one variant of TEM beta-lactamases; Philippon et al., 2002). Furthermore, the $bla_{\mathrm{TEM\text{-}1}}$ gene has been reported to be transferred to many other Gram-negative species (Bush et al., 1995). In this study, a previously published bla_{TEM} gene primer set was used (Wiegand et al., 2007) which is capable of detecting all derivates of the TEM gene. However, prevalence of bla_{TEM} gene in coliform isolates from River Rhine water was relatively low. Only seven of the 100 analyzed coliform bacteria possessed a gene of the bla_{TEM} group but six of them expressed resistance against amoxicillin and were identified as *E. coli* via PCR. Frequent detection of bla_{TEM} gene in *E. coli* isolates obtained from surface water from creeks and channels along the middle Santa Ana River has been reported earlier (Ibekwe et al., 2011).

The TEM-1 and SHV-1 beta-lactamases encode for enzyme with the same hydrolytic profile (Matthew et al., 1979). SHV-1 is a species specific beta-lactamase

gene in *Klebsiella pneumoniae* that is chromosomally encoded (Babini and Livermore, 2000). Whereas, TEM-1 are plasmid-coded enzyme variants with identical substrate profile (Chang et al., 2001) Plasmid-coded SHV-1 lactamases are widely distributed in *Enterobacteriaceae* of clinical origin (Spanu et al., 2002; Fernandes et al., 2014). In this study, 77.8 % of the isolates carrying a bla_{SHV} gene were identified as *Klebsiella*. In addition, all of these *Klebsiella* isolates were found to be resistant to amoxicillin (Table 3). Altogether, antibiotic resistant *Klebsiella* spp. seems to be more prevalent in the River Rhine water (Stoll et al., 2012). Whereas, in a Chinese study, beta lactamase genes (bla_{TEM}, bla_{SHV}, bla_{OXY}, bla_{CTX}, and bla_{OXY}) were detected in ampicillin resistant *E. coli* isolates collected from Jinsha Lake (China). The most frequently detected gene was bla_{TEM} followed by bla_{SHV} (Wang et al., 2013).

Trimethoprim resistance is often imparted by dihydrofolate reductase enzyme encoded by *dfr* genes (Seputine et al., 2010). Whereas, for the sulfonamide resistance, genes that encode for dihydropteroate synthase are responsible. For all trimethoprim/sulfamethoxazole resistant coliform bacteria the genetic background of the sulfamethoxazole resistance was evident whereas, the genetic basis for the trimethoprim could not be fully resolved. This could be due to the fact that only three trimethoprim resistance genes were analyzed in this study but more than 30 trimethoprim resistance mediating dihydrofolate reductase genes are reported till date (Seputine et al., 2010).

Conversely, sulfomamide resistance in Gram-negative bacteria is mainly caused by just two resistance genes (Antunes et al., 2005). Ibekwe et al. (2011) demonstrated that the trimethoprim/sulfamethoxazole resistance in *E. coli* isolates obtained from surface water in USA was associated with the presence of the *dfrA1* gene sequence. In this study, prevalence of *sul1* gene (33.3 %) was much lower than *sul2* gene (66.7 %) in trimethoprim/sulfamethoxazole resistant isolates. In contrast, Su

et al. (2012) showed highest prevalence for *sul2* (89%), followed by *sul3* (42%) and *sul1* (40%) in trimethoprim/sulfamethoxazole resistant *E. coli* isolates from Dongjiang River in South China. In another Chines study *sul1*, *sul2*, *sul3* and *sulA* were detected in 60.2, 52.0, 33.7 and 57.1 % of 98 resistant isolates from Jinshia Lake (Wang et al., 2013).

Out of the 100 coliform bacteria isolates, 11 showed resistance against tetracycline and the genetic origin could be allocated to *tet*(A) and *tet*(B) in 10 isolates?. Since we tested for only four tetracycline resistance genes, it is therefore, likely that the remaining one isolate might have been resistant to one of other *tet* genes not tested. Previously published studies investigated the phenotypic and genotypic resistance of *E. coli* isolate from creeks and channels along the Santa Ana River (USA) and Jinsha Lake, China (Ibekwe et al., 2011; Wang et al., 2013). In the USA 4 % of the tetracycline resistant isolates were positive for *tet*(A), 19 % for *tet*(B) and 38 % for *tet*(C) (Ibekwe et al., 2011). In China *tet*(A) was the most prevalent ribosomal efflux gene (72 %) followed by *tet*(B) (15%) in tetracycline resistant *E. coli* strains (Wang et al., 2013). The discrepancies in our results may be due to differences in microorganisms tested (*E. coli* versus coliform bacteria) or geographical location.

There are several studies that deal with the occurrence of genotypic resistance characteristics of coliform bacteria (Bryan et al., 2004; Tadesse et al., 2012; Schwaiger et al., 2012); but these studies are focused on bacteria isolated from human and animal feces or food. This fact makes direct comparison between the results of this study and previous studies difficult. In our recent published study, total DNA extracted from surface water samples was screened for the same ARGs (Stoll et al., 2012). Differences between the spectrum of antibiotic resistance genes for the coliform bacteria and the range of resistance genes in aquatic bacterial communities were evident. In contrast to the coliform bacteria, *tet*(C) proved an important

antibiotic resistance determinant in surface water which was potentially carried by bacteria other than coliform group. This information suggests that the possibility to measure the whole ARGs spectrum of water bodies on the basis of coliform bacteria is limited.

Some of the coliform bacteria in this study showed multiple antibiotic resistances. Integrons have been widely associated with capture, integration, and expression of gene cassettes encoding antibiotic resistance in enteric bacteria, such as *Enterobacteriaceae and Campylobacter* sp. (Lucey et al., 2000; Maguire et al., 2001). In this study, 70.0 % of all isolates with two or more antibiotic resistances carried class 1 or 2 integrons. In clinical *E. coli* isolates, reported prevalence of integrons range from 22 to 59 % (Maguire et al., 2001; Sallen et al., 1995; Koczura et al., 2012) which increase with the presence of multiple antibiotic resistances (Koczura et al., 2012). In total, 10.0 % of coliform isolates carried class 1 integrons. A similar incidence of class 1 integrons in *E. coli* isolated from wastewater (9.6 %), and surface water of the River Seine, France (11 %) and surface water of the Rio Grande, Mexico (13 %) have been reported previously (Laroche et al., 2009; Ferreira da Silva et al., 2007; Roe et al., 2003). In a study form China, a higher occurrence of class 1 integrons in *E. coli* isolated from Minjiang River (41 %) was reported however, the authors concluded that the high frequency was probably due to the widespread use of numerous antimicrobial agents in human therapy, livestock, poultry, and fish production in the study area (Chen et al., 2011). High prevalence of *E. coli* isolates carrying integrons in surface water from China has been reported in a number of studies. Class 1 integron was detected in at least 57% of multi-drug resistant *E. coli* isolates from rivers and Tai Lake (Zhang et al., 2015). Similarly, Su et al., 2012 reported presence of class 1 and 2 integrons in 83.2 % of 3456 *E. coli* strains isolated from the Dongjiang River catchment whereas, the occurrence of class 2 integrons in

this study was very low (2 %). However, the frequency of occurrence was similar to that of class 2 integrons in *E. coli* isolated (1.43 %) from the Seine estuary in France (Laroche et al., 2009). The prevalence of class 2 integrons is generally low compared to class 1 in the reported literature (Koczura et al., 2012; Kotlarska et al., 2015; Su et al., 2012).

Confirmed *E. coli* strains (47) were separated into four main phylogenetic groups (A, B1, B2 and D). The presence of strains from group D (6 isolates) and from group B2 (14 isolates) deserves attention since the strains from these groups are usually pathogenic. The strains from group B2 are usually responsible for extraintestinal infections (Picard et al., 1999; Johnson and Stell, 2000). The phylogenetic group D includes pathogenic strains such as *E. coli* O157:H7, which is highly virulent and can cause diarrhea, hemolytic uremic syndrome and hemorrhagic colitis (Parry and Palmer, 2000). Previous studies have demonstrated that group B2 *E. coli* strains are less resistant to antibiotics (Laroche et al., 2009; Moreno et al., 2006) and it is assumed that they tend to carry fewer integrons than the others phylogenetic *E. coli* groups (Skurnik et al. 2005).

For further estimation of pathogenicity of the *E. coli* isolates the presence of 11 VGs was investigated in this study. However, it should be mentioned that the presence of a single or multiple VGs in an *E. coli* strain does not necessarily indicate that a strain is pathogenic unless that strain has the appropriate combination of VGs to cause disease in the host (Boerlin et al., 1999). The pathogenic *E. coli* use a complex multi-step mechanism of pathogenesis involving a number of virulence factors depending upon the pathotype, which consists of attachment, host cell surface modification, invasion, variety of toxins and secretion systems which eventually lead toxins to the target host cells (Kaper et al., 2004). Thus VGs are ideal targets for determining the pathogenic potential of a given *E. coli* isolate (Kuhnert et al., 2006).

EAEC cause persistent diarrhoea in children and adults and are defined by the presence of heat-stable enterotoxin-1 (east1) along with *aggR* (Kaper et al., 2004). In this study, *astA* gene encoding the *east1* enteroaggregative *E. coli* heat-stable enterotoxin-1 *astA* was detected in two *E. coli* isolates. In contrast, other studies found a high prevalence of *E. coli* carrying *astA* gene in fresh and estuarine water (Masters et al., 2011) and storm water (Sidhu et al., 2013) probably due to its presence in many commensal *E. coli* isolates (Table 4). All *E. coli* isolates were screened for the presence of *aggR* gene to determine if they belong to the EAEC pathotype (carrying both *astA* and *aggR* genes). The *aggR* gene was detected in two isolates but not in the ones positive for the *astA* gene. Hence, no confirmed EAEC pathotype was among the *E. coli* isolates.

The *eaeA* gene which codes for intimin protein was also detected in only two out of 47 *E. coli* isolates which shows it's extremely low prevalence in the River Rhine water. This gene is necessary for intimate attachment to host epithelial cells in both the EHEC and EPEC pathotypes. This observation also contrasts with previous investigations where high prevalence of the *eaeA* gene (up to 96 %) was reported in surface water (Sidhu et al., 2013; Masters et al., 2011; Shelton et al., 2006).

EHEC causes hemorrhagic colitis and hemolytic uremic syndrome in humans and key virulence factors include intimin (*eae*A gene) and shiga-toxins (*stx*1 and *stx*2 genes, Boerlin et al., 1999). The *stx*2 gene was detected in 4.3 % of the *E. coli* isolates whereas the *stx*1 was not found in any isolate however; *eaeA* and *stx*2 genes were not detected in the same isolates.

All 47 *E. coli* isolates were also tested for the presence of the *bfp* gene to determine if they belong to the EPEC pathotype. Typical EPEC strains carry the LEE pathogenicity island, which encodes for several virulence factors, including intimin (*eaeA*) and the plasmid encoded bundle forming pilus (*bfp*) which mediates adhesion

to intestinal epithelial cells (Kaper et al., 2004; Hamilton et al., 2010). In this study, the *bfp* gene was not detected along with *eaeA* in any of isolates. Overall, the *bfp* gene was detected in just one isolate. Similarly, prevalence of invasion plasmid antigen H (*ipaH*) usually carried by EIEC strains (Guion et al., 2008) was also very low (4.3 %).

In total, nine *E. coli* strains carrying VGs were detected. Out of these nine isolates, five could be allocated to the phylogenetic groups B2 and D (non-commensal). In accordance to these findings, recent studies on the phylogenetic distribution of *E. coli* strains showing the pathogenic diversity indicate that pathogenic *E. coli* strains associated with severe acute diarrhea are distributed outside of the B2 and D groups (Escobar-Páramo et al., 2004a and b). Overall, the results of this study show that diarrheagenic *E. coli* pathotypes did not occur at the sampling sites. Pathogenic *E. coli* seem to play a subordinate role in the German River Rhine near Düsseldorf. In contrast to this finding a number of other studies have demonstrated the presence of *E. coli* carrying VGs in aquatic environments and in particular in surface water systems (Sidhu et al., 2013; Chen et al., 2011; Hamilton et al., 2010; Koczura et al., 2013). Antibiotic resistant shiga toxin producing *E. coli* (STEC) and ETEC were detected in surface water samples from River Ganga and River Gomti in India (Ram et al., 2008). Frequent detection of potential EPEC strains from contaminated marine recreational water in Avalon Bay, California USA has also reported (Hamilton et al., 2010). In another study, about a quarter of the *E. coli* isolated from Minjiang River in China carried at least one of the 11 virulence genes evaluated (Chen et al., 2011). Similarly, presence of EPEC, EAEC, EIEC, EHEC and ETEC pathotypes has also been reported in sub-tropical surface water collected in Brisbane, Australia (Sidhu et al., 2013). Another Australian study found 56 % out of 264 *E. coli* isolates collected from the surrounding environmental waters of sewage treatment plants identical to strains isolated from sewage treatment plant's final

effluent (Anastasi et al., 2012). Among these isolates the appearance of virulence genes like the siderophore gene *iroNE. coli* and the toxin genes *eaeA*, *hylA*, *cnf1*, *estII* was common (Anastasi et al., 2012). A Polish study showed that the occurrence of heat-stable toxin gene of ETEC, *S. fimbriae* subunit gene *sfaS*, and siderophore receptor genes, *fyuA* and *intA* in *E. coli* isolated from Warta River was associated with the presence of class 1 integrons (Koczura et al., 2013). In South Africa 171 out of 278 confirmed *E. coli* strains collected form Kat River and Fort Beaufort abstraction water were positive for at least one pathogenic determinant and these include EPEC, ETEC and EHEC (Nontongana et al., 2014)

Summarizing the above, it can be said that diarrheagenic pathogenic *E. coli* are potentially more prevalent in warmer surface waters of India, USA, China and Australia than in cold waters of Germany. This could potentially be due to either better survival/growth or both of *E. coli* carrying VGs in warmer water. Tropical aquatic ecosystems with bio-available nutrient have been reported provide ideal habitat for *E. coli* (Winfield and Groisman, 2003). However, to confirm this assumption further investigation would be required.

In this study, only one isolate was detected to contain two VGs (*aagR* and *bfp*). The occurrence of this unusual combination could potentially be explained on the basis of horizontal gene transfer between cells which enables the exchange of genetic material located on mobile elements (transposons, integrons or plasmids) among related or unrelated bacterial species. But, this assumption could not be confirmed by the detection of a class 1 or 2 integron. In this study, only one of the nine *E. coli* isolates, carrying VGs, also carried class 1 integron. Also an association between the occurrence of antibiotic resistance and the presence of VGs could not be demonstrated.

CONCLUSIONS

The bacteriological quality of the river water investigated in this study is good, as suggested by long term monitoring programs including total and fecal coliform counts. In-depth investigation of coliform bacteria appears obvious, since isolates are obtained from routine water monitoring. Such investigations can contribute to a better understanding of antibiotic resistance, pathogenicity and mobility of genes. This study demonstrates that coliform bacteria occurring in surface water is an important reservoir of ARGs for a number of antibiotic classes such as sulfonamide, trimethoprim, beta-lactam-antibiotics and tetracycline in Germany. The isolates showed a high level of resistance to antimicrobial substances and multiple resistances was common among the 100 isolates. The antibiotic resistances could be traced back to resistance genes. An exception represents the beta-lactamase gene ampC – its occurrence is often not linked with antibiotic resistance, because it could be an intrinsic chromosomal gene. The sulfonamide resistance genes *sul1* and *sul2*, the trimethoprim resistance gene *dfrA1* and the tetracycline resistance genes *tet*(A) and *tet*(B) were identified as prevalent genes in coliform isolates. This spectrum of prevalent ARGs differs from that found in total DNA extracts from surface waters. However, multiple antibiotic resistances were often accompanied by the occurrence of class 1 or 2 integrons. Only a few *E. coli* isolates obtained from the River Rhine water samples harbored VGs, like *eaeA*, *stx*$_2$, *ipaH*, *aagR*, *bpf* and *astA*, but no diarrheagenic *E. coli* pathotypes were detected. Overall, pathogenic *E. coli* strains occur at very low frequency in the German River Rhine near Düsseldorf.

Overall, the investigations contributed to a better understanding of antibiotic resistance, pathogenicity and mobility of genes in the aquatic environment. Nevertheless there is a need to further develop understanding on the public health

significance of occurrence of multiple antibiotic resistant bacteria, ARGs and VGs in the surface water.

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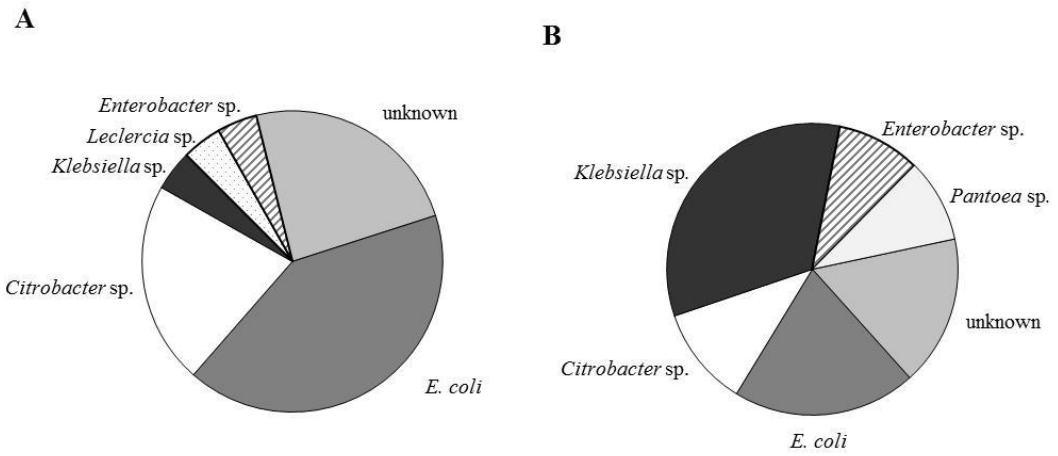

Figure 1. Distribution of bacteria genera (identified via API strips) in sensible isolates (A) and isolates with one or more phenotypic antibiotic resistances (B)

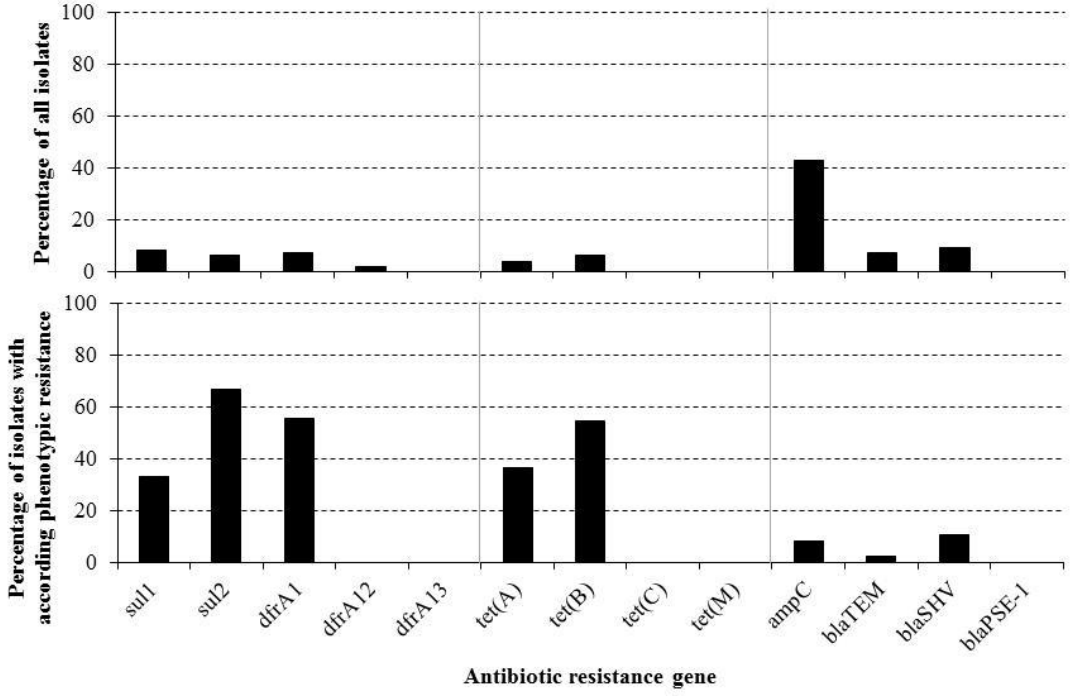

Figure 2. Occurence of antibiotic resistance genes in coliform bacteria and phenotypic resistant coliform bacteria

Table 1. Parameters of the antibiotic disc test.

antibiotic	concentration	zone of inhibition		
		sensitive	intermediate	resistant
trimethoprim/ sulfamethoxazole	1.25 µg/ 23.75 µg	>16 mm	15-16 mm	<15 mm
tetracycline	30 µg	>21 mm	17-21 mm	<17 mm
amoxicillin	10 µg	>22 mm	16-22 mm	<16 mm
piperacillin	30 µg	>21 mm	13-21 mm	<13 mm
gentamicin	10 µg	>20 mm	15-20 mm	<15 mm
meropenem	10 µg	>21 mm	14-21 mm	<14 mm
ciprofloxacin	5 µg	>22 mm	19-22 mm	<19 mm

Table 2. *E. coli* pathotypes and associated virulence genes tested in this study

Pathotypes	**Virulence genes (VGs)**			
	Adhesion/ invasion gene	**Function**	**Toxin**	**Function**
EHEC	*eaeA**	Intimin	stx_1 stx_2 *ehxA**	Shiga toxin I Shiga toxin II Enterohemolysin
ETEC			LT1 ST1	Heat labile toxin 1 Heat stable toxin 1
EPEC	*eaeA** *bfp*	Intimin Type IV bundle-forming pili	*cdtB*	Cytolethal distending toxin
EAEC	*aggR*	Transcriptional regulator for chromosomal gene	east1 (*astA*)	EaggEC heat-stable enterotoxin
EIEC	*ipaH*	Invasion plasmid antigen		

* genes shared by more than one *E. coli* pathotype

Table 3. Occurrence of antibiotic resistance genes and integrons in resistant and sensitive/intermediate isolates

	E. coli	*Citrobacter* sp.	*Klebsiella* sp.	*Leclerica* sp.	*Enterobacter* sp.	*Pantoea* sp.	unknown	total	*E. coli*	*Citrobacter* sp.	*Klebsiella* sp.	*Leclerica* sp.	*Enterobacter* sp.	*Pantoea* sp.	unknown	total
	amoxicillin resistant (n=48)								**amoxicillin sensitive or intermediate (n=52)**							
ampC	6.3 %	4.2 %	-	-	2.1 %	-	6.3 %	*18.8 %*	46.2 %	9.6%	-	-	1.9 %	-	7.7 %	*65.4 %*
***bla*$_{TEM}$**	8.3 %	-	-	-	-	-	4.2 %	*12.5 %*	1.9 %	-	-	-	-	-	-	*1.9 %*
***bla*$_{SHV}$**	-	-	14.6 %	-	-	-	-	*14.6 %*	1.9 %	-	-	1.9 %	-	-	-	*3.8 %*
***bla*$_{PSE-1}$**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	tetracycline resistant (n=11)								**tetracycline sensitive or intermediate (n=89)**							
***tet*(A)**	27.3 %	-	-	-	-	-	9.1 %	*36.4 %*	-	-	-	-	-	-	-	-
***tet*(B)**	45.5 %	-	-	-	-	-	9.1 %	*54.5 %*	-	-	-	-	-	-	-	-
***tet*(C)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***tet*(M)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	trimethoprim/sulfamethoxazole resistant (n=9)								**trimethoprim/sulfamethoxazole sensitive or intermediate (n=91)**							
sul1	33.3 %	-	-	-	-	-	-	*33.3 %*	1.1 %	1.1 %	1.1 %	-	1.1 %	1.1 %	-	*5.5 %*
sul2	33.3 %	-	-	-	-	-	33.3 %	*66.7 %*	-	-	-	-	-	-	-	-
dfrA1	55.6 %	-	-	-	-	-	-	*55.6 %*	1.1 %	1.1 %	-	-	-	-	-	*2.2 %*
dfrA12	-	-	-	-	-	-	-	-	-	-	-	-	1.1 %	-	1.1 %	*2.2 %*
dfrA13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	resistant to one or more antibiotic (n=52)								**sensitive or intermediate to all antibiotics (n=48)**							
intl1	7.7 %	-	-	-	-	-	3.8 %	*11.5 %*	2.1 %	-	-	2.1 %	4.2 %	-	-	*8.3 %*
intl2	3.8 %	-	-	-	-	-	-	*3.8 %*	-	-	-	-	-	-	-	-

Table 4. Occurrence of virulence genes in *E. coli* isolates in this study in comparison with other publications (- = not analyzed)

Reference	**Study area**	***E. coli* isolates positive for**										
		eaeA	***stx*$_1$**	***stx*$_2$**	***ehxA***	***ipaH***	**LT**	**ST**	***aagR***	***bfp***	***astA***	***cdtB***
this study	River Rhine, Germany	4.3%	0.0%	4.3%	0.0%	2.1%	0.0%	0.0%	4.3%	2.1%	4.3%	0.0%
Sidhu et al., 2013	Brisbane, Australia	56%	6%	10%	13%	14%	2%	4%	29%	24%	69%	8%
Chen et al., 2011	Minjiang River, China	-	-	0.4%[a]	-	-	-	-	-	-	24.8%	-
Shelton et al., 2006	Baltimore, USA	96%[1]	26%[2, b]		-	-	-	-	-	-	-	-
Hamilton et al., 2010	Avalon Bay, USA	3.6%	0.0%	0.0%	-	-	0.0%	0.0%	-	-	-	-
Ram et al., 2008	River Gomti, India	26.7%	33.3%	22.2%	-	-	21.1%	21.1%	-	-	-	-

[a] *stx*$_{2e}$

[b] *stx*$_1$ or *stx*$_2$