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Antibiotic resistance and virulence genes in coliform water isolates

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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_{TEM}, bla_{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.

1. 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, 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 Lindsay, 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,

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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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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_{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 and Obst (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 *intI1* and *intI2* respectively was also investigated using previously published primers sets (Ibekwe et al., 2011). PCR amplification of ARGs, *intI1* and *intI2* 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 *intI1* and *intI2* 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.

2.5. 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