Swimming Pool & Spa International Conference 2009

Paper 5.2

Study of the diversity of *E.coli* strains isolated from swimming pool waters

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Abstract

The aim of the study was to assess the impact of the water's chlorination on the diversity of *E.coli* and to determine the genetic diversity and the possible clonal similarities among *E.coli* strains isolated from chlorinated and nonchlorinated pool waters. Total genomic DNA was extracted from 37 *E.coli* bacterial cells using QIAampDNA stool Mini Kit (Qiagen). From the 37 strains, 28 were typeable and were successfully fingerprinted by ERIC-PCR. The remaining 9 strains (8 from chlorinated and 1 from non-chlorinated samples) were considered non-typeable. For the clustering, the 28 typeable strains were observed visually and using Phylip 3.6.1 analysis software. The combination of the visual and the Phylip observation concluded in 8 ERIC , 4 consisting exclusively of chlorinated strains (E2, E3, E4, and E6). In 3 genotypes (E1, E5, E8) the majority of the strains is from non-chlorinated samples, with one exception in each type (28, 5γ and 32 respectively). Finally, in the last type (E7) only one non-chlorinated strain is clustered. A grouping of the strains isolated from chlorinated waters and non chlorinated waters in different ERIC-PCR types seems to appear. The paper presented here contains preliminary results and conclusions as the study is still in progress.

Introduction

The quality of swimming pool and spa water is known to affect the transmission of infectious diseases (1, 2). It has been proved that there is a correlation between the degree of faecal contamination, as well as contamination from bathers, and the risk of illness resulting from swimming activities (3, 4). European countries' regulations include various microbial indicators for pool water quality, reflecting different official views on the advantages and the disadvantages of these indicators (5, 6). Nevertheless, the presence of *E.coli* in water is a microbiological indicator of high valued for faecal contamination and for the evaluation of a pool's sanitary quality. WHO guidelines require absence of *E.coli* per 100 ml of pool water in order to be suitable for swimming (2). The *E.coli* cells present in water are mainly non-pathogenic strains) (7). In some cases, pathogenic strains such as enterotoxigenic (ETEC) and shiga-toxin producing *E.coli* (STEC) can also be present (8, 9, 10, and 11).

Standard disinfection procedures, which are used in various countries in line with national regulations, include chlorination, ozonation, ultraviolet light irradiation and ionisation. Chlorination is still a very popular method for the control of *E.coli* particularly in pool waters in many countries (12) and especially in Greece (5). There is a number of studies discussing the tolerance of *E.coli* strains in chlorine (13, 14). Studies have been conducted on a comparative analysis of efficacy through the effect on *Escherichia coli* membranes after chlorination and ozonation (15). Another study has demonstrated that chlorine treatment of *E.coli* damages cell surfaces, as evidenced by significant changes in surface topography and morphology (16). In a number of studies the question was raised on the benefit of the creation on a protective system against *E.coli* strains in specific environments (14).

The use of molecular methods in the study of bacterial diversity has unveiled new insights in the composition of *E.coli* microbial communities. In this study, *E.coli* isolates from swimming pools were biotyped using the enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR) as a molecular typing method, since typing defines the phenotypic and/or genetic analysis of bacterial isolates, below the species or subspecies level (17). Furthermore, the clustering of the strains enables an assessment of the impact of the water's chlorine concentration on the diversity of the isolated strains. Enterobacterial repetitive intergenic consensus (ERIC) sequences, also described as intergenic repetitive sequenses(18), are short, highly conserved 126-bp noncoding regions (19), and are located in extragenic regions of the bacterial genome, (20). ERIC sequences differ from most other bacterial repeats in being distributed across a wider range of species. ERIC sequences were first described in *Escherichia coli*, *Salmonella typhimurium* and other members of Enterobacteriaceae (21). The number of copies of the ERIC sequence varies among species: it was initially estimated by extrapolation that there may be about 30 copies in *E.coli* K-12 (18).

ERIC sequences have been used as the basis of a technique for fingerprinting bacterial genomes (22). Baldy – Chudzik et al (23) reported the use of rep-PCR in the genomic fingerprinting of *E.coli* isolates from Wojnowskie Wschodnie and Wojnowskie Zachodnie lake and aqueous/freshwater environment (4). ERIC-PCR uses any combination of primers designed to the conserved ERIC region in order to generate an electrophoteric banding pattern based on the frequency and orientation of ERIC sequences in a bacterial genome (19). ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers (20). ERIC-PCR has a moderately high ability to resolve different but closely related bacterial strains (24). ERIC –PCR is a molecular genotyping method with several pros and cons. As far as the advantages are concerned, it is a fast and simple technique, appropriate for routine epidemiological investigation (25, 26). It is a relatively low cost method, compare to pulse field gel electrophoresis (PFGE) or multilocus sequencing analysis for generating information about the genetic similarity of the bacterial strains (19). ERIC-PCR makes no use of radioisotopes, a large number of fingerprint patterns per primer is generated to enable strains comparison, where no large DNA fractions are required, providing reliable results with high discriminatory power. No prior knowledge of the DNA sequences under study is required and theoretically it is a method that can be

performed on any microorganism. Furthermore, no prior DNA probe extraction is needed; hence the method can be automated and used for genetic analysis (27). For many organisms it possesses a higher discriminatory ability than that of quick-typing techniques, leading to its increased frequency of use (19). ERIC-PCR is faster and relatively simpler than PFGE, and it takes only one day to prepare and perform. Furthermore, PCR machines are available in clinical laboratories as well as in public health laboratories (25).

On the other hand, the use of random primers creates certain constraints. The main disadvantage of the method is its low reproducibility (19). The method is sensitive in the event of changes in PCR conditions, i.e. temperature, template concentration, concentration of magnesium ions, and so on (27). Nevertheless, ERIC-PCR is deemed a reliable genotyping method used widely for comparison and classification of strains of the same kind, for genetic mapping, for diagnostic purposes and epidemiology.

The aim of the study was

a) to assess the impact of the water's chlorination on the diversity of .*E.coli* and
b) to determine the genetic diversity and the possible clonal similarities among *E.coli* strains isolated from pool waters.

The paper presented here contains preliminary results and conclusions, as the study is still in progress.

Materials and Methods

1. Sampling

Water samples were collected during 2008 from swimming pools in various locations around Greece. At the same time, water sampling was carried out from non-chlorinated water sources (non-chlorinated drinking water and lake water from the Athens area). Standard sampling and transport procedures were followed (28). In the chlorinated samples the free chlorine level was measured in situ (Hanna Instruments: Multiparameter on Specificmeters Model HI 93710).

2. Bacterial isolation and identification

From each sample, 100 ml of water was filtered twice and the membranes were incubated on MLSA, at 36±2 and 44± 0.5 ^{0} C for 21±3h. .The isolates were identified as *E.coli* by oxidase reaction and indole production (6, 29). After purification on Nutrient Agar the isolated *E.coli* colonies were resuspended in cryovials containing Nutrient Broth with 20% glycerol and stored at -80^{o}C.

3. ERIC-PCR

Total genomic DNA was extracted from *E.coli* bacterial cells using QIAampDNA stool Mini Kit (Qiagen). The cells were fingerprinted using ERIC PCR.
Each 25 μl reaction mixture contained : 40 ng genomic DNA, 10 mM KCl (Biolabs, New England), 10 mM Tris – HCl (pH 9.0), 0.1% (w/v) Triton X-100 (Biolabs, New England), 1 Unit Taq DNA polymerase (Biolabs, New England), 0.2 mM dNTPs (Biolabs, New England), 3.75 mM $MgCl_2$ (Biolabs, New England), 4% (v/v) DMSO (4% (v/v) DMSO) and 20 nM of each ERIC primer (Invitrogen). A specific quantity

of crude DNA was used for amplification by ERIC-PCR with one set of primers (ERIC 1R: 5′ -ATGTAAGCTCCTGGGGATTCAC - 3′ and ERIC 2: 5′ -AAGTAAGTGACTGGGGTGAGCG – 3) (30). PCR amplification was performed using a peqLab thermal Cycler, advanced primus 25, as follows: initial denaturation at 94^{o}C for 10 min; 35 cycles of denaturation at 94^{o}C for 1 min; annealing at 52^{o}C for 1 min; and extension at 65^{o}C for 8 min; followed by a final extension at 65^{o}C for 16 min. (30). The amplification products were electrophorised in a 1% agarose gel and the gel was stained with ethidium bromide. A 100 bp-1500 bp ladder (Biolabs, New England), a reference *E.coli strain* and a no template control were used as DNA molecular weight markers to evaluate the method reproducibility and to assure lack of contamination (24).Electrophoresis was run according to previous study (30) modified as follows: 2h at 80V. The banding patterns of all lanes were visually compared. The intensity of the amplified bands was also considered when differentiating between strains (25).

4. Analysis of ERIC-PCR fingerprints

Phylogenetic analysis was carried out using PHYLIP 3.6.1 Distance-based analysis was undertaken using the neighbour-joining algorithm. The level of relative similarity indicates similarities in the DNA fingerprints and potential relationship among bacterial isolates (31). Furthermore, the banding patterns of all lanes were visually compared. Strains exhibiting differences of one or more bands were considered to be different ERIC types (25, 17).

5. Reference Materials

Five Reference Materials (NCTC 9001) were used for the control of the confirmation processes and the reproducibility of the method.

Results

Bacterial isolation and identification

A total of 100 water samples were collected from 47 pools, non- chlorinated water and lake waters situated all over the country. 163 preliminary positive *E.coli* colonies were isolated and 134 isolates were confirmed as *E.coli.*

Preliminary study of the selected Bacterial isolates

Among the 134 isolates confirmed as *E.coli*, 37 isolates were randomly selected for genomic fingerprinting by ERIC-PCR, 25 derived from chlorinated water samples 12 from non-chlorinated samples. The study of these strains was used as a pilot stage for the overall project and it should provide preliminary results.

ERIC-PCR and Gel electrophoresis

The 37 *E.coli* isolated from swimming pool waters were fingerprinted using the ERIC-PCR. Genomic DNA was successfully extracted from all isolates and analysed spectro-photometrically , to assess purity and quantify the amount of DNA extracted (32). From the 37 strains, 28 were typeable and were successfully clustered by ERIC-PCR. The remaining 9 strains (8 from chlorinated and 1 from non-chlorinated samples) were considered non-typeable because these strains either did not give band

patterns or the patterns were of low intensity. From the 28 typeable strains, 17 derived from chlorinated and 11 from non-chlorinated water samples (Table 1) (Figure 1).

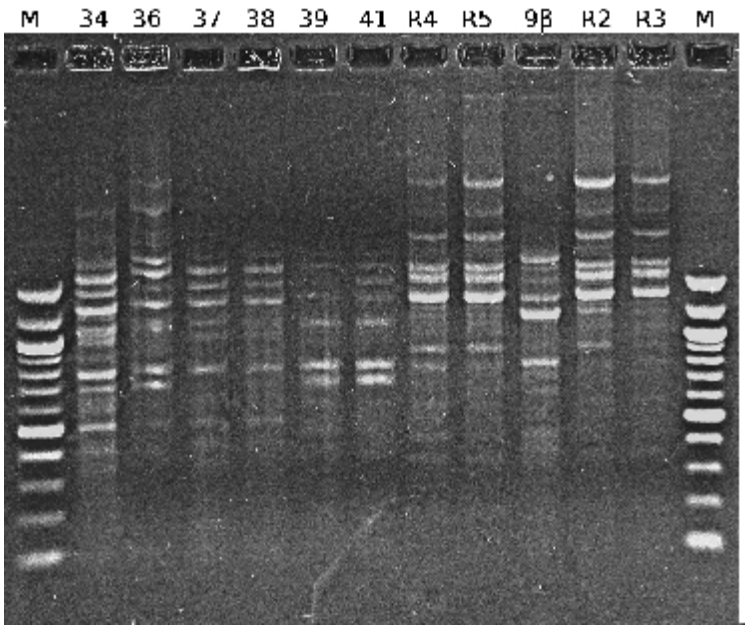

Figure 1. Electrophoresis on gel agaroze: Marker 100bp, 34, 36, 37, 38, 39, 41, R4, R5, 9β, R2, R3 and Marker 100bp.

Analysis of ERIC-PCR fingerprints.
For the clustering, 28 typeable strains were observed:

a) Visually (Table 1). When differentiating between strains the intensity of the amplified bands was considered (25). Strains differing by one or more bands were considered to be different ERIC types (17) and assigned the capital letter E followed by a number (25).

b) Using Phylip 3.6.1 analysis software. Distance-based analysis was undertaken using the neighbour-joining algorithm (31, 18) (Figure 2).

c) A combination of visual observation and dendrogram from the Phylip biostatistical program resulted in 8 ERIC genotypes (Table 2).

While with the visual observation the typeable *E.coli* strains were 28, with Phylip analysis software, they were 25 strains. Among the 25 typeable strains, 15 were from chlorinated samples while the other 10 were from non-chlorinated ones. Among the 12 non-typeable strains, 10 were from chlorinated samples, while the other 2 were from non-chlorinated samples (Figure 2).

The visual observation produced 15 ERIC types while the observation using Phylip analysis software gave 8 types. Finally, using the combination of the visual and the Phylip observation we concluded in 8 ERIC types (Table 3). The difference between

the groups produced by the Phylip and the combined observation is of minor importance (strain 32).Among the 8 ERIC types, 4 consist exclusively of chlorinated strains (E2, E3, E4, and E6). In 3 genotypes (E1, E5, E8) the majority of the strains is from non-chlorinated samples, with one exception in each type (28, 5γ and 32 respectively), which were isolated from chlorinated samples. Finally, in the last type (E7) only one non-chlorinated strain is clustered.

Table 1. ERIC-types deriving from visually observation.

ERIC-TYPES	**STRAINS**
E1	34, 36, 37, 38, 39, 41 , 45
E2	R1, **5β**
E3	35, 42
E4	43, **5ε**
E5	**32**, **33**
E6	R4, **9β**, **28**
E7	**4α**, **4β**, **5α**
E8	R2, R3, **5γ**
E9	**1**
E10	**2**
E11	40
E12	**6**
E13	**7**
E14	**30**
E15	**Mβ**, **Mγ**
Non Typeable	**3**, **5δ**, **8α**, **8β**, **9α**, **9γ**, **10**, **31**, 44, R5

*Chlorinated strains
**Non-chlorinated strains
*** Reference Materials : R1,R2,R3,R4,R5

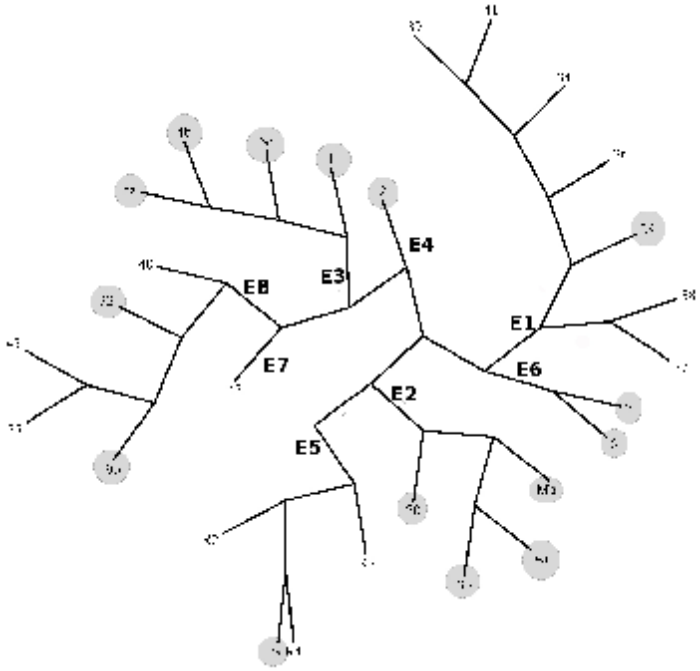

Figure 2: Cluster analysis of ERIC-PCR fingerprints. Distance-based analysis was undertaken using the neighbour-joining algorithm
* Grey circle strains deriving from chlorinated waters
** Blank strains deriving from non-chlorinated waters
***Reference Materials : R1, R2, R3, R4, R5

Table 2 The final ERIC-types based on a combination of visual observation and statistical analysis

E1	34,36,37,38,39,41,**28**
E2	**30**,**33**,**4$^{\alpha}$**,**Mβ**,**32**
E3	**5$^{\alpha}$**, **4β**, **5$^{\varepsilon}$**, **1**
E4	**2**
E5	R2,R3,R4,**5γ**
E6	**6**,**7**
E7	43
E8	40,35,45,**9β**
Non-typeable	**Mγ**, **3**,**5β**,**5δ**,**8$^{\alpha}$**,**8β**, **9$^{\alpha}$**,**9γ**,**10**,**31**,42,44, R1,R5

* Chlorinated strains
** Non-chlorinated strains
***Reference Materials : R1,R2,R3,R4,R5

Discussion

The study of bacterial diversity assesses the relationships between culturable bacteria and environmental factors. The use of molecular methods in the study of the diversity of microorganisms has revealed new insights in the composition of microbial communities. Molecular methods have also allowed characterization of many longtime recognized but poorly understood microorganisms. The importance of *E.coli* as a bacterial indicator of water quality has long being appreciated.Discriminatory power is an important attribute in any typing method and it is a feature of the method's ability to assign a different type to two unrelated strains sampled randomly from a population of a given species (32, 17). The use of fingerprinting techniques that use total DNA provide more detailed and discriminatory similarity results than those using a single DNA region (33).

In our study, *E.coli* strains were isolated using standard techniques and ribotypes were distinguished using the enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR **),** in order to assess the impact of the water's chlorine concentration on the diversity of the isolated strains. The visual observation of the *E.coli* strains revealed 15 types while the Phylip program gave 8 genotypes. The combination of the 2 methods gave 8 genotypes. Even though we have already studied only 37 out of 134 strains, which is a relatively small portion, ERIC-PCR types produced consisted exclusively of strains isolated from chlorinated water samples. One ERIC-PCR type consisted of a strain isolated from non chlorinated water, while 3 ERIC-PCR types consisted mainly of strains isolated from non chlorinated waters with only one strain each isolated from chlorinated samples. With the study of the 37 strains, a grouping of the strains isolated from chlorinated waters and non chlorinated waters in different ERIC-PCR types seems to appear. Certainly more strains are needed to be included in order to confirm these first results. Our results reveal a high level of discriminatory power. This could be attributed to the incorporation of a relatively small number of *E.coli* strains (37 strains). The observation of the total number of isolated strains (134 strains) might alter the present number of types.

A few discrepancies were obtained when isolates within computationally obtained clusters were visually inspected and compared with epidemiological data (34). For example, strain 32 according to the dendrogram belongs to genotype E8 but under visually observation it belongs to genotype E2. This observation is reinforced by the fact that this strain is very likely related to strain 33 due to the fact that they derived from a common source (the same swimming pool) and thus were both exposed to the same chlorination conditions (32). This indicates that visual inspection of clusters obtained from the analysis program is always necessary, and epidemiological data should always be taken into account when deciding whether genetically related strains are also epidemiologically related (34, 17, 32, and 25). Another interesting observation is that 3 out of 5 Reference strains cluster together with one strain isolated from chlorinated waters, while 2 reference strains are non typeable. The study of more strains may light on questionable results.

The generation of new *E.coli* types resistant to chlorine is mentioned in a number of studies. Zhao et al (2001) proved the chlorine tolerance of *E.coli* O157:H7strains (13) Saby et al (1999) proved the *E.coli* resistance to chlorine and reduced Glutathione (GSH) synthesis in response to oxygenation and starvation (14). Furthermore, studies compared the efficacy through the effect on *Escherichia coli* membranes after chlorination and ozonation. Arana et al, (1999) compared the effect of chlorine and ozone on *E.coli* cells resuspended in waste water. After ozonation, while no changes in cell surface hydrophobicity were observed, approximately 98.5% of cells displayed altered membrane permeability (15). In addition, chlorine treatments in *E.coli* damaged cell surfaces, as evidenced by significant changes in surface topography and morphology (16).

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