STUDY OF THE DIVERSITY OF *E. COLI* STRAINS ISOLATED FROM AQUATIC ENVIRONMENTS

Papatzitze O. [2,3,*], Pappa O. [4], Karagouni A.D. [3], Lymperopoulou D.S. [3], Kalkani E. [1], Papanastasiou A. [2], Spanakos G. [4], Keramydas D. [5], and Mavridou A. [1]

[1] Department of Medical Laboratories, Technological Educational Institute Athens
[2] West Attica General Hospital
[3] Laboratory of Microbiology, Faculty of Biology, Department of Botany, University of Athens
[4] National School of Public Health
[5] Public Health Inspector
* olgapapat@hotmail.com

ABSTRACT

E. coli strains are members of the human and animal gut's natural flora. They are often isolated from water sources contaminated by sewage outlets. Chlorination is one of the most commonly used disinfection procedures for drinking and swimming pool waters. The long term exposure to chlorine may cause changes in *E. coli* cells. The aim of the study is to isolate *E. coli* cells from chlorinated and non-chlorinated water samples, to assess the impact of water's chlorination on the diversity of *E. coli* and to determine the genetic diversity and the possible clone similarities among the strains. 101 water samples were collected from chlorinated swimming pool and mains waters situated all over the country and from the Marathon lake, (non-chlorinated samples). In total 117 strains were isolated. Standard methods were used for the isolation and identification of *E. coli* strains. Enterobacterial Repetitive Intergenic Consensus sequence polymerase chain reaction (ERIC PCR) was chosen as the molecular fingerprinting method. The fingerprinting patterns were analyzed statistically by visual observation and Phylip 3.6.1 Analysis Software. The Phylip program gave 15 genotypes. 11 Eric types consisted only or mainly of strains isolated from one water type (chlorinated or non chlorinated) and 4 ERIC types consisted equally of strains from chlorinated and non-chlorinated water sources. Some Eric types consisted of strains isolated from the same geographical area but others included strains geographically dispersed. 2 ERIC types consisted of strains isolated from chlorinated samples only, which originated from various geographical regions. This fact might be an indication of distribution of these strains in more than one area and possibly the formation of a new clone resistant to chlorine.

Key Words | *E. coli*, ERIC-PCR, Phylip 3.6.1, water samples, chlorination.

INTRODUCTION

E. coli strains are members of the human and animal gut's natural flora. They are often isolated from water sources (swimming pools, water supply networks, lakes, sea water) contaminated by sewage outlets. The presence of *Escherichia coli* in water can be used as a microbiological indicator for fecal contamination and as a measurement for sanitary quality (Tsen H.Y et al., 1998). The quality of swimming pool and spa water is known to affect the transmission of infectious diseases (Grabow W.O.K, 1991 and WHO, 2006). *E. coli* is a universal indicator of its fecal pollution. (Casarez A.E και συνεργτες, 2007). There is a relation between the degree of fecal contamination and the risk of numerous diseases (Baldy-Chuddzik K. et al., 2003). WHO guidelines require absence of *E. coli* per 100 ml of pool and drinking water in order to be suitable for drinking. (WHO, 2006). The *E. coli* cells present in water are mainly non-pathogenic

strains (Tsen H.Y, 1998). In some cases, pathogenic strains such as enterotoxigenic (ETEC) and shiga-toxin producing *E. coli* (STEC) can also be present (Verma A., et al., 2007 and Kon T, 2007). 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 and especially in Greece (Mavridou et al., 2005). There are a number of studies discussing the tolerance of *E. coli* strains in chlorine (Zhao T. et al., 2001). Studies have been conducted on a comparative analysis of efficacy through the effect on *E. coli* membranes after chlorination and ozonation (Arana I. et al., 1999). Another study has demonstrated that chlorine treatment of *E. coli* damages cell surfaces, as evidenced by significant changes in surface topography and morphology (Wang H. et al., 2006). In a number of studies the question was raised on the benefit of the creation of a protective system against *E. coli* strains in specific environments (Saby S. et al., 1999). 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* isolated from swimming pools, water supply networks originated from all over Greece and from Lake Marathon, were molecular typed using the enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR). 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 sequences (Wilson L.A and Sharp P.M, 2006), are short, highly conserved 126-bp noncoding regions, and are located in extragenic regions of the bacterial genome (Olive D.M and Bean P., 1999,Hulton C.S.J et al., 1991,and Wilson L. and Sharp P.M, 2006 and Versalovic et al., 1991). ERIC PCR was chosen as the molecular typing method according to the references. Casarez E.A et al., studied the Genotype diversity of *E. coli* isolates in natural waters determined by PFGE and ERIC-PCR (Casarez E.A. et al., 2007). Baldy – Chudzik et al., reported the use of rep-PCR in the genomic fingerprinting of *E. coli* isolates from Wojnowskie Wschodnie and Wojnowskie Zachodnie lakes and aqueous/freshwater environment (Baldy-Chudzic et al., 2003). Kon T et al., studied the genetic relatedness of *E. coli* isolates in interstitial water from a Lake Huron (Canada) beach (Kon T., et al., 2007). ERIC-PCR uses any combination of primers designed to identify 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 (Meacham K.J. et al., 2003). ERIC-PCR has a moderately high ability to resolve different but closely related bacterial strains (Casarez E.A. et al., 2007). 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 (Matsumoto M. et al., 2001 and Stumph A.N et al., 2005). It is a relatively low cost method, for generating information about the genetic similarity of the bacterial strains (Meacham K.J. et al., 2003). On the other hand, the use of random primers creates certain constraints. The main disadvantage of the method is its low reproducibility (Meacham K.J. et al., 2003). The method is sensitive in the event of changes in PCR conditions. 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 for epidemiology. The fingerprinting patterns were analysed statistically with Phylip 3.6.1 Analysis Software.

The aims of the study were the isolation of *E. coli* strains from water samples, the assessement of the impact of the water's chlorination on the diversity of *E. coli* and the determination of the genetic diversity and the possible clonal similarities among *E. coli* strains isolated from water samples.

MATERIALS AND METHODS

Water samples were collected from swimming pools, water supply networks in various locations around Greece (chlorinated samples) and from Marathon Lake in Athens, (non chlorinated samples). Standard sampling and transport procedures were followed (ISO 19458:2006). From each sample, 100 ml of water was filtered twice and the membranes were incubated on MLSA, at 44± 0.5 °C for 21±3h.The isolates were identified as *E. coli* by oxidase reaction and indole production (Mavridou et al., 2010). After purification on Nutrient Agar the isolated *E. coli* colonies were resuspended in cryovials containing Nutrient Broth

with 20% glycerol and stored at −80 °C. Total genomic DNA was extracted from *E. coli* bacterial cells using the QIAampDNA stool Mini Kit (Qiagen). The cells were fingerprinted using ERIC–PCR. DNA amplification was carried out as described by Rademaker J.L.W and de Bruijn F.J., 1997. Phylogenetic analysis was carried out using PHYLIP 3.6.1 (Felstein J., 1989). The level of relative similarity indicates similarities in the DNA fingerprints and potential relationship among bacterial isolates. 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 (Matsumoto M. et al., 2001 and Van Belkum A., 2007). Five Reference Materials (NCTC 9001) were used for the control of the confirmation processes and the reproducibility of the method.

RESULTS

A total of 101 water samples were collected. 143 preliminary positive *E. coli* colonies were isolated and 117 isolates were confirmed as *E. coli*. 62 strains were isolated from chlorinated waters and 55 from non-chlorinated waters. The 117 *E. coli* isolated from water samples 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 (Tenover F.C. et al., 1995). From the 117 strains, 105 were typeable and were successfully clustered by ERIC–PCR. The remaining 12 strains were considered non-typeable because these strains either did not give band patterns or the patterns were of low intensity.

The typeability of the method is 90% and the discriminatory power 0.93.

Using Phylip analysis software the 105 *E. coli* strains were clustered into 15 ERIC types (Figure 1, Table 1).

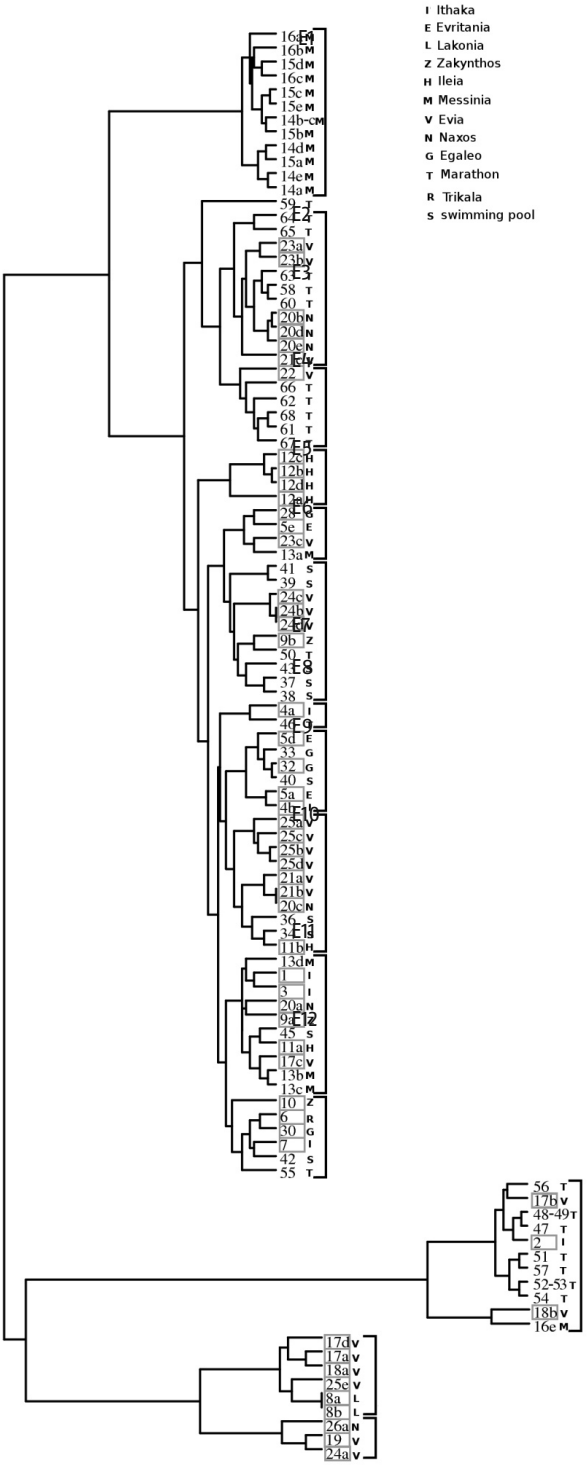
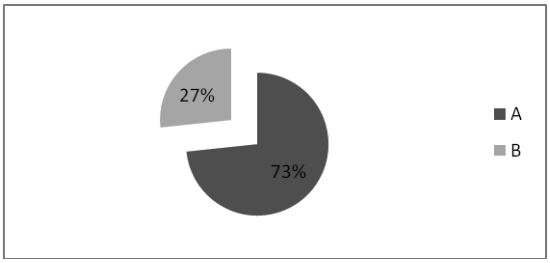


Figure 1 Cluster analysis of ERIC PCR fingerprints by Phylip 3.6.1 analysis software.

Table 1 Distribution of 105 *E. coli* strains on 15 ERIC types based on the phylogenetic analysis software Phylip 3.6.1

ERIC types	Strains isolated from chlorinated waters	Strains isolated from non-chlorinated waters	Number of strains
Eric 1		13	13
Eric 2		1	1
Eric 3	6	5	11
Eric 4	1	5	6
Eric 5	4		4
Eric 6	3	1	4
Eric 7	4	6	10
Eric 8	1	1	2
Eric 9	5	1	6
Eric 10	8	2	10
Eric 11	6	4	10
Eric 12	6	4	6
Eric 13	3	10	13
Eric 14	6		6
Eric 15	3		3

11 Eric types consisted only or mainly of strains isolated from one water type (chlorinated or non-chlorinated) and 4 ERIC types consisted equally of strains from chlorinated and non-chlorinated water sources.



A Group of types consisting only or mainly of strains isolated from chlorinated or non-chlorinated samples

B Group of types consisting equally of strains from chlorinated and non-chlorinated water samples

Furthermore, strains from the same ERIC type come from different geographical areas.

DISCUSSION

The study of bacterial diversity assesses the relationship 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 been 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 (Tenover F.C. et al., 1995, Van Belkum A. et al., 2007). The use of fingerprinting techniques that use total DNA provide more detailed and discriminatory similarity results than those using a single DNA region (Dos Angos Borges L.G. et al., 2003).

In our study, *E. coli* strains were isolated using standard techniques and genotypes 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 phylogenetic program Phylip clustered the 105 strains into 15 genotypes. In some ERIC types, the clustering of the strains has proven to be a result of the geographical distribution of the samples. For example E1, E2 and E5 types consisted mainly of strains originating in the same geographical area. ERIC type 1 consists of strains isolated from water samples from Messinia, E2 consists only of one strain from Marathon Lake and E5 of strains from the Hleia area. Another grouping has been made according to the water sample type (chlorinated or non-chlorinated waters). For example E1 and E2 ERIC types consist of strains isolated from non-chlorinated water samples, while E5, E14 and E15 ERIC types consist of strains isolated from chlorinated waters. In addition, the majority of the strains on ERIC types E6, E9, E10 and E12 were isolated from chlorinated water samples. Thus in total 7 ERIC types (E5, E6, E9, E10, E12, E14 and E15) consist only or mainly of strains isolated from chlorinated waters located in many geographical areas around Greece. This fact might be an indication of distribution of a new clone resistant to chlorine which spreads around the country. According to references there is an indication that the presence of chlorine causes stress conditions to *E. coli*, and possibly creates new strains resistant to chlorine. Zhao T. et al., proved the chlorine tolerance of *E. coli* 0157:H7 strains (Zhao T. et al., 2001). Saby et al., proved the *E. coli* resistance to chlorine and reduced Glutathione (GSH) synthesis in response to oxygenation and starvation (Saby S. et al., 1999). Furthermore, studies compared the efficacy through the effect on *E. coli* membranes after chlorination and ozonation. Arana I. et al., 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 (Arana I. et al., 1999). In addition, chlorine treatments in *E. coli* damaged cell surfaces, as evidenced by significant changes in surface topography and morphology (Whang H. et al., 2006).

REFERENCES

Anon (ISO 9308-1: 2000) Water quality- Detection and enumeration of Escerichia coli and coliform bacteria – Part 1: Membrane filtration method

Anon (ISO 19458: 2006) Water quality-Sampling for microbiological analysis

APHA, 2005. Standard methods for the examination of water and wastewater. American Public Health, Association

Arana I., Santorum P., Muela A. and Barcina I., 1999. Chlorination and ozonation of waste water: comparative analysis of efficasy through the effect on *Escherichia coli* membranes. Applied and Environmental Microbiology, 86: 883-888

Baldy-Chudzik K., Niedbach J. and Stosik M., 2001. Application of rep-PCR fingerprinting for genotyping of *Escherichia coli* strains in Wojnowskie Wschodnie and Wojnowskie Zachodnie lake. Acta Microbiol Pol 50(3-4): 233-42.

Baldy-Chudzik K., Niedbach J. and Stosk M., 2003. REP-PCR fingerprinting as a tool for the analysis of genomic diversity in *Escherichia coli* strains isolated from an aqueous/freshwater environment. Cellular and Molecular Biology letters, 8: 793-798

Casarez E.A., Pillai S.D., and Di Giovanni G.D., 2007. Genotype diversity of *Escherichia coli* isolates in natural waters determined by PFGE and ERIC PCR. Water Research, 41: 3643-3648

Dos Anjos Borges, L.G., Vechia V.D. and Corcao G., 2003. Characterisation and genetic diversity via REP-PCR of Escerichia coli isolates frompolluted waters in southern Brazil. FEMS Microbiology Ecology, 45: 173-180

Felsenstein, J., 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166

Grabow W.O.K., 1991. New trends in infections associated with swimming pools. ISSN, 17, 2, 173-177

Hulton C.S.J., Higgings C.F. and Sharp P.M., 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, Salmonella typhimurium and other bacteria. Molecular. Microbiology,.5: 825-834

Kon T, Weir SC, Howell ET, Lee H and Trevors JT, 2007. Genetic relatedness of *Escherichia coli* isolates in interstitial water from a Lake Huron (Canada) beach., Appl Environ Microbiol. 2007 Mar, 73(6): 1961–7

Matsumoto M., Suzuki Y., Miyazaki Y., Tanaka D., Yasuoka T., Mashiko K., Ishikita R. and Baba J., 2001. Enterobacterial repetitive intergenic consensus sequence–based PCR (ERIC–PCR); its ability to differentiate Streptococcus pyogenes strains and applicability to the study of outbreaks of Streptococcal infection. The Tohoku Journal of experimental medicine., 194,4: 205–212

Mavridou A, Smeti E, Mandilara G., Boufa, Vagiona–Arvanitidou M., Vantarakis A., Vassilandonopoulou G.,Pappa O., Roussia V., Tzouanopoulos A., Livadara M., Aisopou I., MarakaV., Nikolaou E. and Karaouli V., 2010. Equivalency testing of TTC Tergitol 7 agar (ISO 9308–1: 2000) with five culture media for the detection of *E. coli* in watersamples in Greece. Water Science and Technology, IWA Publishing, 67–76

Mavridou A., Vagiona A., Boufa P., Vantarakis A., Roussia V. and Papapetropoulou M., 2005. Assessment of the quality of pool water in Greece using various microbial indicators. International Conference on health and Water Quality Aspects of the Man Made Recreational Water Environment. Budapest, 10–11 March 2005.

Meacham K.J., Zhang L., Foxman B., Bauer R.J. and Marrs C.F. 2003. Evaluation of genotyping large numbers of *Escherichia coli* isolates by Enterobacterial Repetitive Intergenic Consensus–PCR, Journal of Clinical Microbiology, 41,11: 5224–5226

Olive D.M. and Bean P., 1999. Principles and applications of methods for DNA–based typing of microbial organisms. Journal of Clinical Microbiology, 37, 6:1661–1669

Papapetropoulou, Mavridou., 1995. Introduction to water microbiology. Traulos Publications,Athens

Rademaker J.L.W. and de Bruijn F.J., 1997. Characterization and classification of microbes by rep–PCR genomic fingerprinting and computer assisted pattern analysis. In: DNA markers: protocols, application and overviews. (Caetano–Anolles G., Gresshoff P.M Eds) Willey–Liss Inc., New York, 151–171

Saby S., Leroy P. and Block J.C., 1999. *Escherichia coli* resistance to chlorine and glutathione synthesisin response to oxygenation and starvation. Applied and Environmental Microbiology, 65, 12: 5600–5603

Stumph A.N., Roggenkamp A. and Hoffmann H., 2005. Specificity of ebterobacterial repetitive intergenic consensus and repetitive extragenic palindromic polymerase chain reaction for the detection of clonality within the Enterobacter cloacae complex. Diagnostic Microbiology and Infectious Disease 53: 9–16

Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H. and Swaminathan B., 1995. Interpreting Chromosomal DNA restriction patterns produced by Pulsed–Field gel electrophoresis: Criteria for bacterial strain typing. Journal of Clinical Microbiology, 33, 9: 2233–2239

Tsen H.Y., Lin C.K. and Chi W.R., 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. Applied and Environmental Microbiology, 85: 554–560

Van Belkum A., Tassios P.T, Dijkshoorn, Haeggman S., Cookson B., Fry N.K, Fussing V., Green J., Gerner–Smidt P., Brisse S. and Struelens, 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clinical Microbiology and Infectious Diseases, 13: 1–46

Verma A., Bolton F.J., Fiefield D., Lamp P., Woloscin E, Smith N. and McCann R., 2007. An outbreak of *E. coli* 0157associated with a swimming pool: an unusual vehicle of transmission. Epidemiology and Infection,136, 2: 287

Versalovic J., Koeuth T. and Lupski J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res., 19: 6823–6831

Wang H., Feng H., Maclaren S. and Luo Y., 2006. Examination of cell morphological changes of *Escherichia coli* treated with acidic electrolysed water, peroxyacetic acid and chlorine using a MFP–3DTM atomic force microscope. Annual Meeting of the Institute of Food Technologists, Paper No. 003a –16.

WHO, 2006. Guidelines for safe recreational water environments, Volume 2: Swimming pools, spas and similar recreational environments. Geneva

Wilson L.A. and Sharp P.M., 2006. Enterobacterial repetitive intergenic ronsensus (ERIC) Sequenses in *Escherichia coli*: Evolution and Implications dor ERIC–PCR. Molecular Biology and Evolution, 23,6: 1156–1168

Zhao T., Doyle M.P., Blake P. and Wu F–M., 2001. Chlorine Inactivation of *Escherichia coli* 0157:H7 in Water, Journal of Food Protection, 64, 10: 1607–1