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GLOBAL EVALUATION OF ANTIMICROBIAL RESISTANCE:

'TIME TO GET SMARTER IN CYRPUS'

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APPROVAL OF REVISED THESIS:

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Nicosia, 2016

Cantas

ABSTRACT

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This thesis aimed to reveal the global picture of antimicrobial resistance and particularly focused on spread of the Extended-spectrum β-lactamases (ESBL) producing *Escherichia coli* in northern Cyprus. For many bacterial infections, drug resistant bacterial pathogens are increasingly more often found to be resistant, by subsequent testing, already by the time antimicrobial treatment starts. As is shown in this thesis, increased utilization of antimicrobials has contributed to greater resistance among pathogenic and environmental bacteria. The high prevalence of such organisms has created challenges for practitioners treating bacterial infections and antimicrobial resistance that has today become a major global health concern. Urinary tract infections (UTIs) caused by *E. coli* are one of the most frequently encountered infections in both in-patient and out-patient settings. We aimed to detect the antibiotic resistance rates of ESBL-producing *E. coli* and gain insight into the genetic basis - clonal evolution underlying the multi-drug resistace in clinical strains isolated from UTIs in Near East University Hospital, North Cyprus. A total of 389 *E. coli* strains were isolated between 2010 and 2014 and 50 of them molecularly analysed. Identification of bacteria and antibiotic susceptibility tests were performed by Phoenix (Becton Dickinson, USA) automated system, while gene specific Polymerase Chain Reactions (PCR), sequencing and molecular phylogenetic analysis were applied for genetic investigation. The ESBL-producing *E. coli* among hospitalized patients increased from 36% in 2010/2011 to 53% in 2014 with a significant increase up to 71% in 2013 ($p< 0.001$) in northern Cyprus. The CTX-M was the most predominant gene responsible for ESBL production and found in genetically diverse *E. coli* isolates (42 of 50) in this study. Predominantly 3 main clones have been found, whereas 37 of CTX-M positive isolates mostly isolated from in-patients (n: 21) gave high similarity with isolates from out-patiens (n: 16) which could result from the high antimicrobial exposure of this clone both in hospital and community. The CTX-M-15 sub-type was the most frequent (88%), in isolates followed by CTX-M-1 (4,8%), CTX-M-80 (4,8%) and CXT-M-36 (2,4%). To our knowledge, there are no known previous studies on

these issues in Cyprus. This thesis underline the importance of proper use of certain antimicrobials globally and particularly in Cyprus. The strong 'cross talk' between the drug, the bacteria and the environment is considered with multi-disciplinary perspective for controlling of antimicrobial resistance development. The immediate need for establishment of optimal therapy guidelines based on the country specific surveillance programs are emphasized. The need for urgent prescription habit changes and ban of over-the-counter sale of antimicrobials at each segment of healthcare services is suggested.

INDEX

SHORTEN WORDS AND SYMBOLS

AMPs	Antimicrobial Peptides
AMR	Antimicrobial Resistance
EARSS	European Antimicrobial Resistance Surveillance System
ECDC	European Centre for Disease Prevention
EU	European Union
ESBL	Extended-spectrum β-lactamases
MDR	Multi-Drug Reistance
MRSA	Methicillin/oxacillin-resistant *Staphylococcus aureus*
PRSP	Penicillin-resistant *Streptococcus pneumoniae*
ROS	Reactive Oxygen Species
US	United States
UTI	Urinary Tract Infection
VRE	Vancomycin-resistant *Enterococcus spp*

FIGURES and GRAPHICS

INTRODUCTION

Arguably one of the greatest examples of serendipity in science was between Alexander Fleming and Ernest Duchesne (Figure 1). Although Fleming generally holds the reputation of the discovery of penicillin since 1928 with his well know 'mold contaminated petri dish' story, a French medical student, originally discovered the antimicrobial properties of *Penicillium* earlier, in 1896.

He observed that Arab stable boys close to an army hospital kept their saddles in a dark and damp room to encourage mold to grow on them. When asked why, they told him the mold helped heal saddle sores on the horses. The curious Duchesne prepared a solution from the mold and injected it into diseased guinea pigs. All recovered. He immediately submitted his work as a dissertation to the Pasteur Institute, but it was ignored because of his young age and because he was unknown (Pouillard 2002). One way or another, the modern antimicrobial revolution began and antimicrobials have been very important corner stones of modern medicine during the last half of the previous century (Figure 1).

Figure 1. Ernest Duchesne (left) who noted that certain molds kill bacteria in 1896. Alexander Fleming (right) who discovered penicillin after noticing some mold accidentally contaminated a petri dish and prevented the growth of bacteria around in 1928 (Figure by Leon Cantas, 2014).

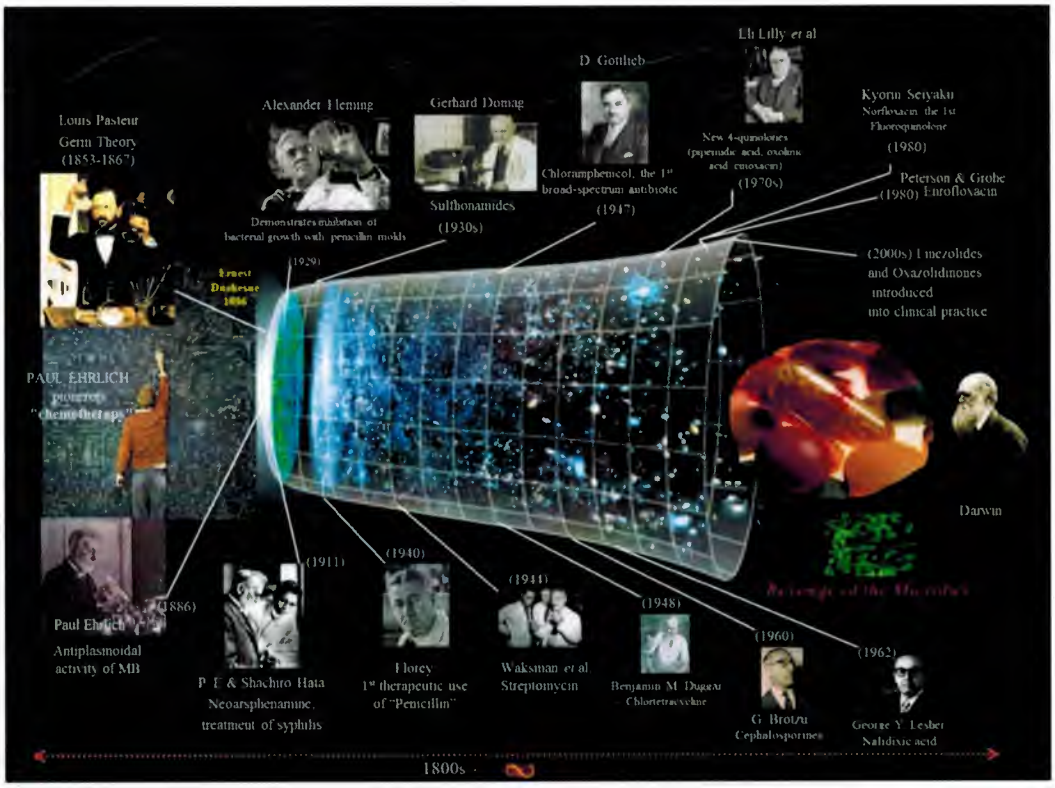


Figure 2. Historical milestones and discoveries that shaped the use of antibacterial agents. (Figure by Leon Cantas, 2014).

Antimicrobials have saved millions of lives and eased patients' suffering. But today, the treatment of bacterial infections is once again becoming increasingly complicated because microorganisms are developing resistance to antimicrobial agents worldwide (Aminov 2010).

1.1. ANTIMICROBIAL RESISTANCE: THE BACTERIA'S STRUGGLE FOR SURVIVAL

Antimicrobial resistance is as old as the clinical use of antimicrobials in medicine. Generally, resistant pathogens were observed soon after the introduction of new drugs in hospitals where antimicrobials were intensively used (Levy 1982).

In history, sulfonamide-resistant *Streptoccoccus pyogenes* emerged in military hospitals, in the 1930s (Levy 1982). Likewise, in the following decade, penicillin resistant *Staphylococcus aureus* appeared in British civilian hospitals very soon after the introduction of penicillin (Barber and Rozwadowska-Dowzenko 1948). It is important to note that a significant fraction of all human infections are caused by these two bacteria (i.e., strep-throat, pneumonia, scarlet fever, septicemia, skin infections, wound infections, etc.).

Similarly, streptomycin resistant *Mycobacterium tuberculosis* emerged in the community soon after the clinical usage of this antimicrobial started (Crofton and Mitchison 1948). Thereafter, resistance to multiple drugs started to be common. Especially, it is detected very frequent in enteric bacteria such as *Escherichia coli*, *Shigella* spp. and *Salmonella enterica* in the late 1950s to early 1960s (Levy 2001; Olarte 1983; Watanabe 1963).

Over the years, and continuing into the present almost every known bacterial pathogen has developed resistance to one or more antimicrobials in clinical use. The antimicrobial resistance (AMR) problem remains a growing public health concern because infections caused by resistant bacteria are increasingly difficult and expensive to treat.

The consequences of this problem are: longer hospital stay, longer time off work, reduced quality of life, greater likelihood of death due to inadequate or delayed

treatment, increases in private insurance coverage and an additional costs for hospitals when hospital-acquired infections occur in addition to the increased overall health care expenditure (Korczak and Schöffmann 2010; Roberts 1996; Wilke 2010). Thus, in order to calculate the full economic burden of AMR we have to consider the burden of not having antimicrobial treatment options at all, which at the extreme would probably lead to collapse of the entire modern medical system (Falagas and Bliziotis 2007; Pratt 2010;). Otherwise everyone is at risk when antimicrobials become ineffective but the threat is greatest for young children, the elderly, and immune-compromised individuals (including cancer patients undergoing chemotherapy, organ transplant patients, cancer patients) (CDC 2004; Kingston 2008).

It is estimated that each year 25.000 people die in Europe directly due to mutli-drug resistant (MDR) infection (European Centre for Disease Prevention and Control, Stockholm, 2012). In the same report the economic impact was estimated at € 1.5 billion per year (Figure 3).

25,000 deaths directly attributable to multidrug-resistant infections				
Extra in-hospital costs	Extra outpatient costs	Productivity losses due to absence from work	Productivity losses due to patients who died from their infection	TOTAL
€ 927.8 million	€ 10 million	€ 150.4 million	€ 445.9 million	€ 1.5 billion

Figure 3. Human and economic impact of antimicrobial resistance in Europe. (Source: The bacterial challenge: time to react, Joint Technical Report from ECDC and EMA, Stockholm, September 2009).

1.2. DEVELOPMENT AND REGULATION OF ANTIMICROBIAL RESISTANCE

Antimicrobial resistance usually refers to the acquired ability of a microbe to resist the action of an antimicrobial agent to which it is normally susceptible.

Natural selection is the driving force for the appearance of resistant bacterial strains. Bacteria may manifest resistance to antibacterial drugs through a variety of adoptions (Dwyer et al 2009; Hegreness et al 2008, Livmore 2003, McKenzie and Sosenberg 2001) including horizontal transfer of resistance genes (Davies 1994), drug-specific selection of naturally occurring resistant variants within a population, and increased chromosomal mutagenesis in hypermutator strains (Andersson 2003 and Chopra et al 2003).

Antimicrobial resistance may develop as follows:

I) Intrinsic: Intrinsic antimicrobial resistance occurs naturally in a verity of strains of that species and is normally a result of the normal composition of the bacterial cell. An example of intrinsic resistance is naturally vancomycin resistant Gram negatives.

II) Acquired: Acquired antimicrobial resistance results from a mutation in the existing DNA of an organism or acquisition of new DNA. An example of mutational acquired resistance is the development of quinolone resistance seen in a large amount of genera including *E. coli* strains where a mutation in the chromosomal *gyrA* gene results in an amino acid alteration in the target enzyme DNA gyrase, which generate high levels of resistance to this antimicrobial (Lingren et al 2003).

Antimicrobial treatment can also result in multidrug resistance (Cohen et al 1989), which has been associated with mutations in multidrug efflux pumps (Ma et al

1993). These drug efflux pumps can be regulated by a number of transcription factors, including the superoxide-responsive SoxRS system (Greenberg et al 1990).

In addition, there is evidence that low level antimicrobial treatment can lead to mutations that cause resistance (Girgis et al 2009); however, the mechanisms underlying this effect are not well understood. Mutations will naturally occur at any time in a cell, and the mutational frequency is not influenced by the presence of the antimicrobial. Hence, a mutation leading to antimicrobial resistance can develop without a selective pressure (Kohanski et al 2010). But it will be registered in a bacterial population only after selection with antimicrobials.

Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms, where functional DNA from other bacteria is taken up by transformation, transduction or conjugation (Figure 4) (Furuya and Lowy 2006; McManus MC 1997). Such processes can be controlled by constitutively expressed regulatory genes. On the other hand it can be regulated by regulatory systems linked to the concentration of drugs in the environment of the bacterial cells (Baharoglu et al 2010). The central elements involved in such processes are genetic elements as plasmids, transposons and integrons.

Figure 4. Horizontal gene transfer between bacteria (Modified after (Furuya and Lowy 2006).

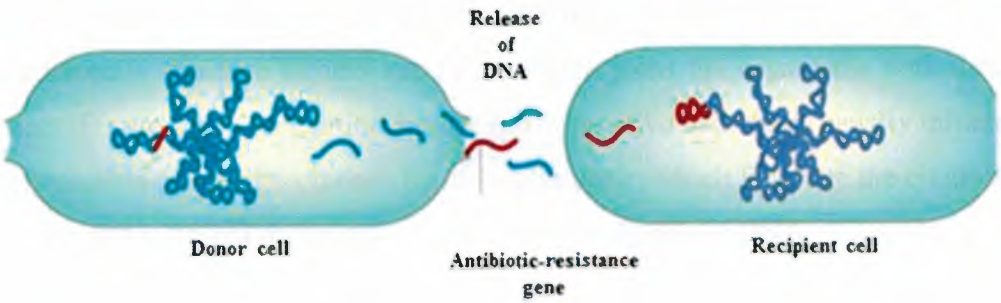


Figure 4.a. Bacterial transformation: Transformation is the process whereby bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis. Transformation can move resistance genes into previously susceptible strains.

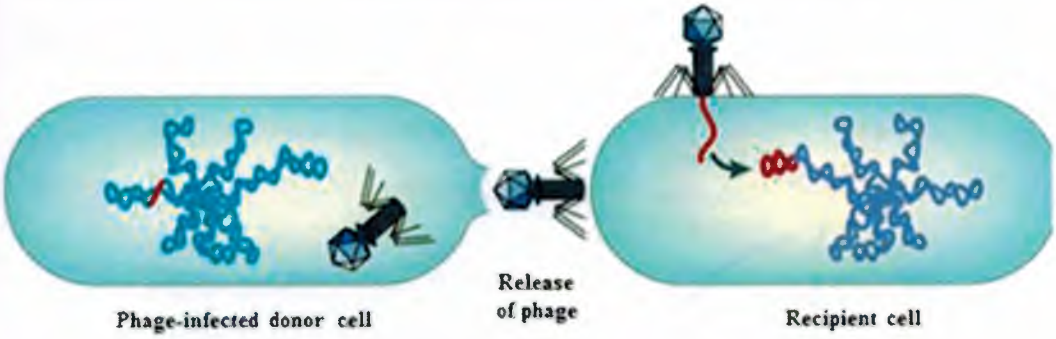


Figure 4.b. Bacterial transduction: During transduction, resistance genes are transferred from one bacterium to another via a bacteriophage (bacterial viruses) and, can be integrated into the chromosome of the recipient cell (lysogeny). This is now thought to be a relatively rare event (Figure 4b).

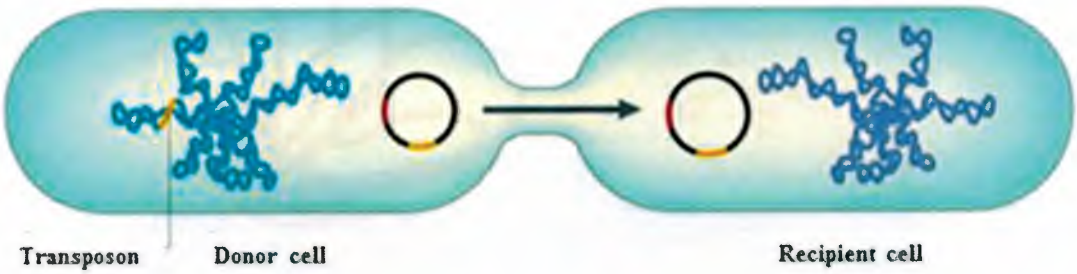


Figure 4.c. Bacterial conjugation: The Gram-negative bacteria transfer a plasmid-harboring resistance gene to an adjacent bacterium, often through an elongated proteinaceous structure termed sex *pilus*, which joins the organisms with direct contact (Figure 4c). Conjugation among Gram-positive bacteria is usually initiated by production of sex pheromones by the mating pair, which facilitate the clumping of donor and recipient organisms, allowing the exchange of DNA.

Several mechanisms have evolved in clinically important bacteria which confer them with antimicrobial resistance (Figure 5). These mechanisms can be rendering-inactivation of the antimicrobial (the most common mode), chemically modification, physical removal from the cell or modification of the target site so that it is not recognized by the antimicrobial.

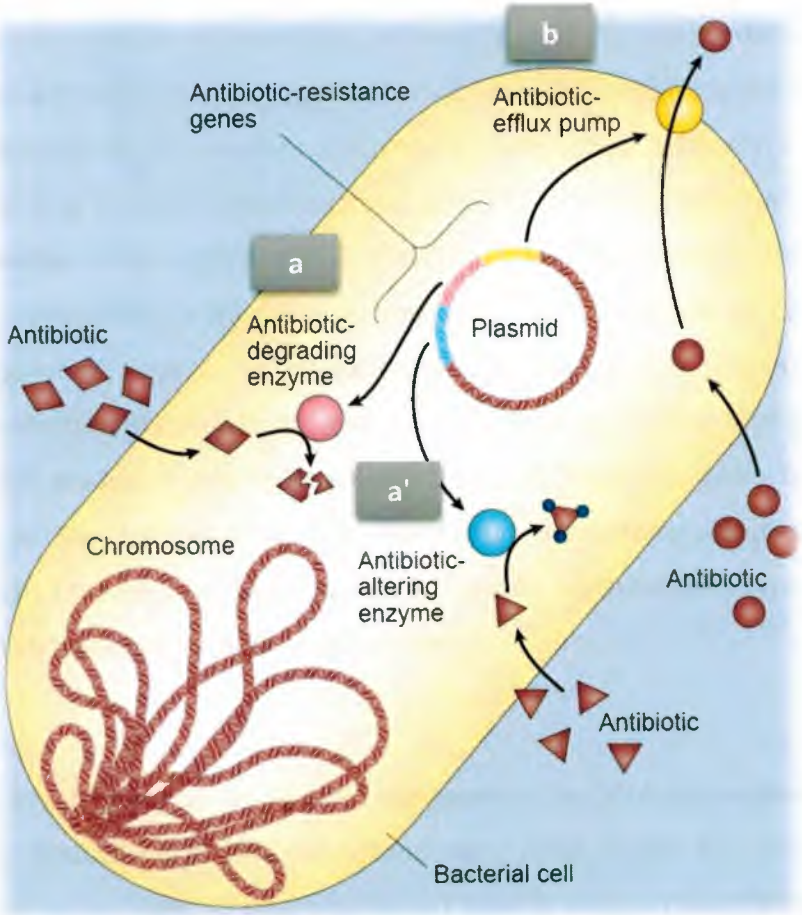


Figure 5. Various antimicrobial resistance mechanisms. **a-a')** Some are directed at the antimicrobial itself: enzymes such as β-lactamases destroy penicillins and cephalosporins, and modifying enzymes inactivate chloramphenicol and aminoglycosides such as streptomycin and gentamicin. **b)** Others target how the drug is transported; for example, an active efflux of drug mediates resistance to the tetracyclines, chloramphenicol and the fluoroquinolones (Levy 1992, McManus 1997, Nikaido 1996). **c)** A third type of mechanism (not shown) alters the intracellular target of the drug-for example, the ribosome, metabolic enzymes or proteins involved in DNA replication or cell wall synthesis making the drug unable

to inhibit a vital function in the microbial cell. Modified after (Levy and Marshal 2004).

There are several aspects to functional regulation that are important when considering antimicrobial resistance in bacteria and its evolutionary consequences. First, while many resistance systems act by modifying or eliminating the interaction between the antimicrobial and target molecule, some act by altering the regulation of the target molecule to prevent or overcome this interaction. Secondly, it is known (Shoemaker et al 1996) that the transmission of DNA between bacteria is affected by the presence of an antimicrobial. Finally, in systems where a unique gene product is responsible for allowing resistance, this product might be under some kind of regulatory control. An evolutionary prediction is that systems that can regulate production of a resistance gene product would be selected over systems in which a gene product would be constitutively made. Similarly, genes that confer resistance to the bacterium with a survivable deleterious effect would be evolutionarily favored if the bacterium could regulate when the wild-type gene and when the resistance gene would be transcribed.

The active involvement of the selected *tra*-genes in the DNA conjugation process is described (Kulinska et al 2008 and Schroder et al 2002) (Figure 6). The *traD/traG* genes (putative conjugative coupling factor) encode an inner membrane protein with putative ATPase activity for DNA transport during bacterial conjugation. This protein forms a ring-shaped structure in the inner membrane through which DNA is passed to the transferosome (Kulinska et al 2008 and Lu er al 2008). However, it has been shown that the *virB4* and *virD11* genes may, in addition, mediate conjugative transfer via a C-terminal ATPase function during pili assembly which is more efficient on surfaces than in liquids (Lin and Kado 1993; Porter et al 1987).

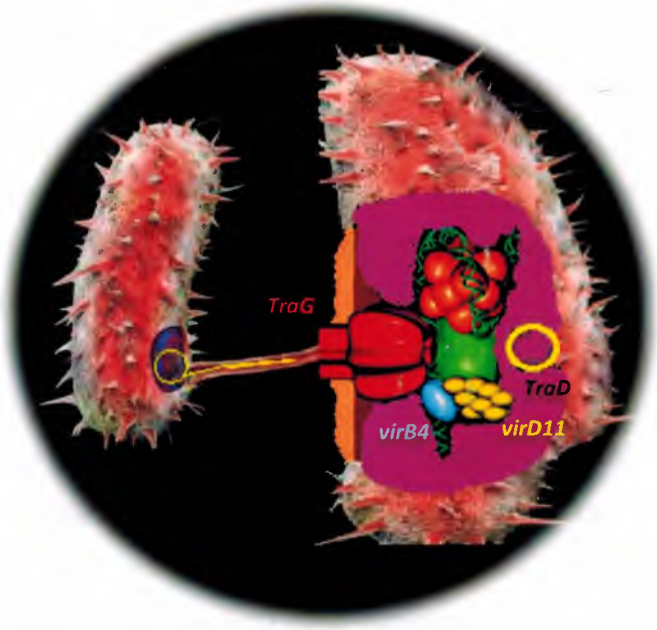


Figure 6. Illustration of the *tra* conjugation machinery as described by (Schorder et al 2002). The *traD, traG, virB11* and *virD4* genes play an important role in encoding the pilin protein from which the mating pilus is constructed. (Figure by Leon Cantas, 2014).

Recent research has shown that the SOS pathway may be essential in the acquisition of bacterial mutations which lead to resistance to some antimicrobial drugs (Cirz et al 2005). Miroslav Radman discovered and named the SOS response in 1975 (Cirz et al 2005; David and Lehninger 2005; Michel 2005). The SOS response is a global response to DNA damage in which the bacterial cell cycle is arrested and DNA repair and mutagenesis are induced. The increased rate of mutation during the SOS response is caused by three low-fidelity DNA polymerases: Pol II, Pol IV and Pol V (Cirz et al 2005). Researchers are now targeting these proteins with the aim of creating drugs that prevent SOS repair. By doing so, the time needed for pathogenic bacteria to evolve antimicrobial resistance could be extended, and thus improve the long term viability of some antimicrobial drugs (Lee et al 2005).

It is shown that the conjugative transfer of plasmids triggers a bacterial stress response, the SOS response, in recipient cells and can impact the cassette content of integrons. The SOS response is already known to induce various genome modifications. Human and animal pathogens cohabit with environmental bacteria,

in niches which will favor DNA exchange. SOS induction during conjugation is thus most probably able to impact a wide range of genomes. The bacterial SOS response could then be a suitable target for co-treatment of infections with dump-inhibiting the SOS response in order to prevent exchange of antimicrobial resistance/adaptation genes.

Quinolones, which are DNA-damaging antimicrobials, can stimulate the emergence of drug resistance via SOS-independent recombination (Lopez et al 2007) and through the induction of RecA-mediated processes, including homologous recombination (Drlica and Zhao 1997) and SOS-regulated error-prone polymerases (Cirz et al 2005). β-lactams can also induce the SOS response via RecA (Kohanski et al 2010) and the DpiAB two-component system (Miller et al 2004), and these drugs have been shown to induce DinB in an SOS-independent fashion, resulting in increased frame shift mutations (Perez et al 2005).

2. AIM & HYPOTHESIS

The purpose of the project was to review the global and nationwide picture of antimicrobial resistance and its genetic basis in northern Cyprus, factors that favor its spread, strategies, and limitations for its control. To our knowledge, there are no known studies on these issues.

This have achieved by:

I) Collection and review of multi-disciplinary data on antimicrobial resistance in medicine and global environmental microbiota.

II) Investigation of the phenotypial and molecular antimicrobial resistance profile in the indicator bacteria *E. coli* from the urine samples of the hospitalized and out-patients in northern Cyprus.

The hypothesis were:

I) Minor attention has been paid on the evolution of global antimicrobial resistance in environment (water and soil) related to its broad survey in human and veterinary medicine.

II) Misuse and overuse of antimicrobials lead to high and clonally diverse antimicrobial resistance emerge among bacteria in hospitalized and out-patients in northern Cyprus.

3. MATERIAL AND METHODS

This thesis was based on global evaluation of antimicrobial resistance and phenotypical and molecular investigation of antimicrobial resistance in clinical isolates cause UTIs in Cyprus.

The change in number of global antimicrobial resistance related published research papers in different multi-disciplines and covering different environment were searched by screening the ISI web of science.

Following keywords systematically were used that match with publication titles on global databases: (antibioti* OR antimicro*) AND resistan* AND the following specific terms: Hospital, (hospital* OR patient* OR clinic*); Animal, (animal* OR veterinary* OR livestock* OR pig* OR cow* OR chicken* OR poultry); Wastewater, (wastewate* OR sewage); Natural water, (water* OR lake OR river OR ocean OR sea); Soil, (soil* OR sediment* OR rhizosphere*). (Source: *http://apps. isiknowledge.com/*)

Retrospective phenotypical antimicrobial resistance patterns were conducted at the Microbiology Laboratory of Near East University Hospital (Nicosia, North Cyprus) between 2010-2014. Antimicrobial resistance in northern Cyprus was assessed

in indicator *E. coli* isolates cultured from urine samples of hospitalized and out-patients (Figure 7).

Year	Study population	
	In-patient	Out-patient
2010/2011	29	46
2012	36	55
2013	40	66
2014	45	72
Sum	150	239

Figure 7. The number of *E. coli* isolates each year, cultured from urine samples of hospitalized and out-patients.

Urine samples collected in universal container, approximately 50 mL in amount. The samples inoculated using an inoculating loop of 10 µL volume calibration on blood agar and EMB mediums that incubated overnight at 37°C. Isolates were further examined with the BD Phoenix 100 Automated Microbiology System (Becton Dickson, USA). The inoculated Phoenix™ panels were placed into the Phoenix™ instrument for incubation and continuous reading. The following antimicrobial agents were used in the Phoenix™ ESBL test: Cefpodoxime-proxetil, Ceftazidime, Ceftriaxone with clavulanic acid, Cefotaxime/Clavulanate and Ceftazidime/Clavulanate. At the specific concentration of Ceftazidime or Cefpodoxime-proxetil, the growth or inhibition of these wells initiated a growthresponse check for the other test wells. The ESBL result was determined based on all the responses within 5–11 hours.

On the basis of colony morphology, gram staining, motility, and biochemical reactions, the organisms were identified as *E. coli.* Following criteria was accepted for identification of *E. coli* (Pamela, 2007).

- **Colony morphology:** circular shape, 2-3 mm diameter, regular margin, flat, smooth, lactose fermenting and translucent.
- **Gram staining:** Gram-negative rods, 1–3 × 0.3–0.5 μm in size, uniformly stained non-particular arrangement, non-sporing, and non-capsulated.
- **Motility:** motile bacteria in hanging drop preparation.
- **Biochemical reactions:** Oxidase (-), catalase (+), O/F test showed glucose fermentation, motility and gas production, reduces nitrates to nitrites, indole (+), methyl red (+) Voges–Proskauer (-), citrate (-), lactose fermenter, triple sugar iron agar showed both butt and slant yellow with gas production, lysine decarboxylase test (+).

The methods for rapid detection and identification of ESBL producing strains have been somewhat restricted. The National Committee for Clinical Laboratory Standards (NCCLS) has described standard broth microdilution (SBM) and disk diffusion screening and confirmatory tests for the detection of ESBL particullary in *E. coli*. Latest studies have recently reported that many clinical laboratories have experienced difficulty in detecting ESBL organisms (Turng et al 2000). In this study, the recently developed Phoenix™ Automated Microbiology System (BD Biosciences, Sparks, MD) was used to perform *in vitro* antimicrobial usceptibility testing. The test evolved from published data of known ESBL patterns in the current literature. The Phoenix™ ESBL test is based on the principle of a differential response between the inhibitory effects of selected second or third generation cephalosporins in the presence or absence of a β-lactamase inhibitor, clavulanic acid. The performance of Phoenix™ ESBL test previosly showed a sensitivity of 94% and specificity of 100% by Turng et al 2000. These results indicate that the Phoenix™ ESBL test can provide laboratorians and clinicians with a reliable and rapid means for the detection of ESBL production in clinically important gram negative bacteria.

BD Phoenix 100 Automated Microbiology System consisted of NMIC/ID-5 Phoenix panels which were combined with susceptibility and identification cards. Samples were inoculated and incubated according to the manufacturer's

recommendations. The Phoenix ESBL test uses growth response to selected expanded-spectrum (cefpodoxime) and broad-spectrum (ceftazidime, ceftriaxone, cefotaxime) cephalosporins, with or without clavulanic acid, to detect the production of ESBL. The result of this test is integrated into the antibiogram through the action of the BDXpert system.

Some of the antibiograms were also confirmed by Kirby-Bauer disk diffusion method as described by the Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standard Institute; 2012). Commercially available antibiotic disks (Oxoid, India) were used for antimicrobial susceptibility testing. The following antibiotic disks were used, amikacin (30 μg), amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), ciprofloxacin (30 μg), ertapenem (10 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), piperacillin-tazobactam (100/10 μg) and of trimethoprim/sulfamethoxazole (1.25/23.75 μg).

Diameter of zone of inhibitions were measured and recorded in millimeters with the help of sliding calipers and organism was labeled as sensitive, resistant, or intermediate. Isolates previously classified as intermediately susceptible to ampicillin and cefuroxime have been recategorised as susceptible according to 2014 EU/CAST guidelines.

Antimicrobial sensitivity records for each isolate yearly were coded in a Microsoft Excel 2013® spreadsheet and the mean antimicrobial resistance displayed as a histogram. We have calculated an overall pooled mean prevalence for each year. Changes in resistance prevalence over time within in-patients and out-patients were assessed by chi-square tests.

Randomly selected fifty strains ($n_{in\text{-}patient}$= 26 and $n_{out\text{-}patients}$= 24) among previously archived isolates have been sub-cultured on 5% sheep blood agar. Single colonies were afterwards selected on Brilliance ESBL agar (OX; Oxoid, Basingstoke, United Kingdom) for optimal purification prior to further molecular characterization of ESBL-Producing *E. coli.*

Purified Blue or Pink colour *E. coli* colonies on Brilliance ESBL agar (OX; Oxoid, Basingstoke, United Kingdom) () have been used for bacterial DNA extraction. QIAamp DNA isolation technique has been used according to the manufacturer's procedure. The concentration of extracted DNA was assessed by spectrophotometer. DNA extracts are then screened for the presence of *bla*CTX-M by Polymerase Chain Reaction (PCR). The PCR reactions were performed within a total volume of 25 µL. The mixture of reaction contained 1x buffer (10 mM TrisHCl, 50 mM KCl), 0.2µM of each deoxynucleoside triphosphate, 1 mM MgCl2, 0.5 µM of forward and reverse primers of CTX-M genes, and 1 Unit of Takara Taq (Takara Shuzo Co., Ltd., Shiga, Japan). Following primer sequences were used for detection of *bla*CTX-M genes in this study; CTX-M-F (5′-ACGCTGTTGTTAGGAAGTG-3′) and CTX-M-R (5′-TTGAGGCTGGGTGAAGT-3′) (Mansouri et al 2009).

PCR conditions for amplification of 857 bp fragment of the CTX-M gene were carried out by the thermocycler (AG 22331; Eppendorf, Hamburg, Germany) as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 30 second, and extension at 72°C for 1 min, were repeated for 36 cycles; a final extension at 72°C for 10 min.

Agarose gel electrophoresis have been done on a 1.2% agarose gel at 80V for 2 hours. After electrophoresis fragments were stained by Ethidium Bromide, and then visualized with ultraviolet light. PCR products were sequenced unidirectional. Editing and alignment of DNA sequences were performed by using the SeqMan II software package (DNAStar, Inc., Madison, WI).

Nucleotide sequences have been compared with sequences in the GenBank and EMBL databases using the BLASTN local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Dendrogram were obtained for CTX-M positive sequences by ClustalW analysis. Branch lengths were drawn to scale and show the proportional number of changes.

Following previously published referance *bla*$_{CTX-M}$ nucleotide sequences were used for phylogenetic analysis:

CTX-M-1 X92506	CTX-M-2 X92507	CTX-M-3 Y10278	CTX-M-4 Y14156	CTX-M-5 U95364	CTX-M-6 AJ005044
CTX-M-7 AJ005045	CTX-M-8 AF189721	CTX-M-9 AF174129	CTX-M-10 AF255298	CTX-M-11 AY005110	CTX-M-12 AF305837
CTX-M-13 AF252623	CTX-M-14 AF252622	CTX-M-15 AY044436	CTX-M-16 AY029068	CTX-M-17 AY033516	CTX-M-18 AF325133
CTX-M-19 AF325134	CTX-M-20 AJ416344	CTX-M-21 AJ416346	CTX-M-22 AY080894	CTX-M-23 AF488377	CTX-M-24 AY143430
CTX-M-25 AF518567	CTX-M-26 AY157676	CTX-M-27 AY156923	CTX-M-28 AJ549244	CTX-M-29 AY267213	CTX-M-30 AY292654
CTX-M-31 AJ567481	CTX-M-32 AJ557142	CTX-M-33 AY238472	CTX-M-34 AY515297	CTX-M-35 AB176534	CTX-M-36 AB177384
CTX-M-37 AY649755	CTX-M-38 AY822595	CTX-M-39 AY954516	CTX-M-40 AY750914	CTX-M-41 DQ023162	CTX-M-42 DQ061159
CTX-M-43 DQ102702	CTX-M-44 D37830	CTX-M-45 D89862	CTX-M-46 AY847147	CTX-M-47 AY847143	CTX-M-48 AY847144
CTX-M-49 AY847145	CTX-M-50 AY847146	CTX-M-51 DQ211987	CTX-M-52 DQ223685	CTX-M-53 DQ268764	CTX-M-54 DQ303459
CTX-M-55 DQ343292	CTX-M-56 EF374097	CTX-M-57 DQ810789	CTX-M-58 EF210159	CTX-M-59 DQ408762	CTX-M-60 AM411407
CTX-M-61 EF219142	CTX-M-62 EF219134	CTX-M-63 AB205197	CTX-M-64 AB284167	CTX-M-65 EF418608	CTX-M-66 EF576988
CTX-M-67 EF581888	CTX-M-68 EU177100	CTX-M-69 EU402393	CTX-M-79 EF426798	CTX-M-80 EU202673	CTX-M-81 EU136031;
CTX-M-82 DQ256091	*bla*$_{KLUC-1}$ AY026417	*bla*$_{KLUC-2}$ EF057432	*bla*$_{KLUG-1}$ AF501233	*bla*$_{KLUA-1}$ AJ272538	*bla*$_{KLUA-2}$ AJ251722
bla$_{KLUA-3}$ AJ427461	*bla*$_{KLUA-8}$ AJ427465	*bla*$_{KLUA-10}$ AJ427467	*bla*$_{KLUA-12}$ AJ427469	*bla*$_{KLUY-1}$ AY623932	*bla*$_{KLUY-2}$ AY623935
bla$_{KLUY-3}$ AY824948.					

4. RESULTS

4.1. GLOBAL ANTIMICROBIAL RESISTANCE: *ONE HEALTH*

Today, the most common multiple drug resistant organisms in human medicine are vancomycin-resistant *Enterococcus* spp. (VRE), *Enterobacter cloacae*, *Klebsiella pneumoniae*, methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA), ESBL-producing *E. coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Marshall and Levy 2011). Epidemiologically, MRSA and VRE are the most commonly encountered multiple drug resistant organisms in patients residing in non-hospital health care facilities, such as nursing homes and other long-term care facilities. Penicillin-resistant *Streptococcus pneumoniae* (PRSP) are more common in patients seeking care in outpatient settings such as physicians' offices and clinics, especially in pediatric settings including kinder gardens. ESBL-producing pathogens are most often encountered in the hospital (intensive care) setting, but MRSA and VRE also have significant nosocomial ecology.

The increasing cross-border and cross-continental movements of people plays a great impact on spread of (resistant) infectious bacteria. The transmission of resistant bacteria has been reported from person to person (Linton et al 1972). Among many others, the emergence and global spread of the international clone 1 of penicillin-resistant *Streptococcus pneumoniae* (Klugman 2002) and the recently occurring NDM-1 carbapenemase-producing *K. pneumoniae* (Kumarasmy et al 2010) are good examples of how multi-resistant bacteria have spread by the movement of people. NDM-1 inactivates all β-lactam antimicrobials and appears to have originated on the Indian subcontinent and subsequently could be found in North America, the UK and Europe. The global epidemiology and the origin of the antimicrobial-resistant microorganisms and its genetic elements involves complex and largely unpredictable systems that include several reservoirs (animals, humans, and the environment).

The veterinary medical antimicrobials were introduced soon after they became available for the treatment of human diseases from the mid-1940s and onward

(Gustafson et al 1997 and Mcewen 2006). The most of the exclusively used veterinary antimicrobials are structurally identical or very similar to those used in human medicine (Heuer et al 2009 and Swann 1969) (Figure 8).

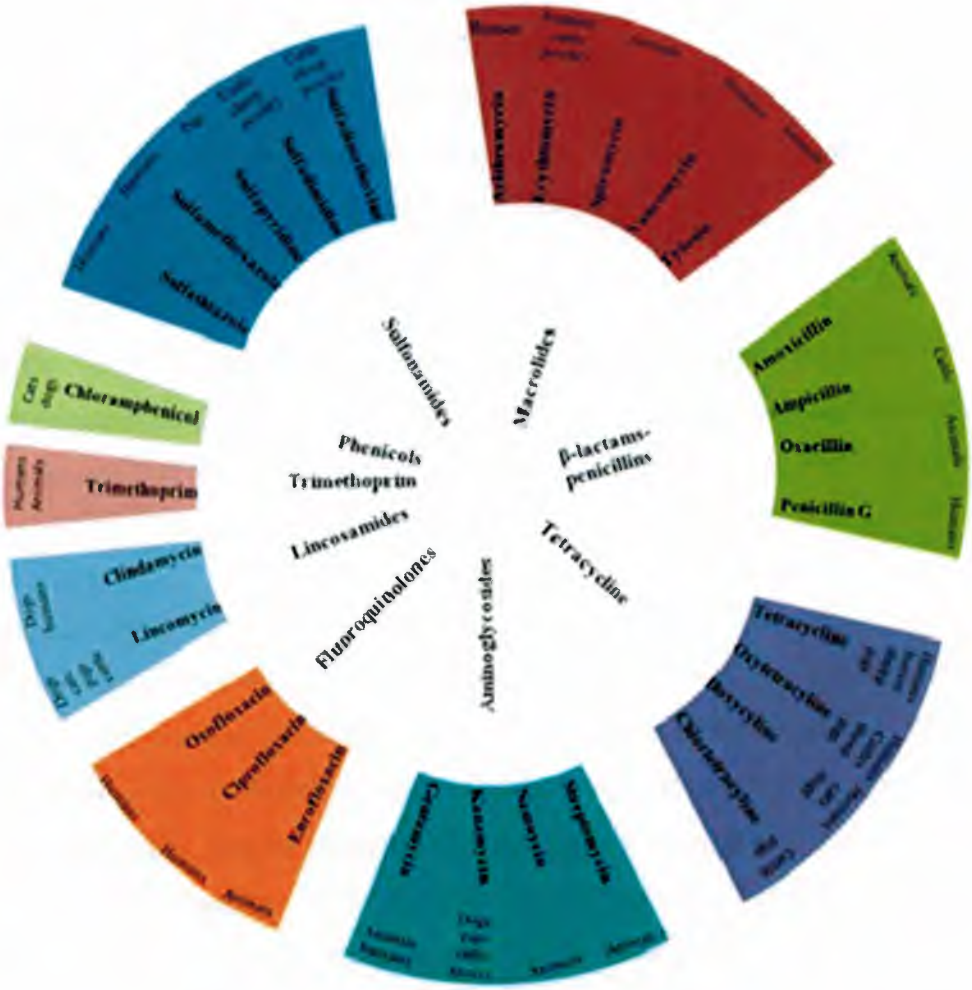


Figure 8. Antimicrobials used in veterinary and in human medicine (Figure by Leon Cantas, 2014).

In Europe, in 2012 the human population of the European Union (EU) was approximately 500 million (*http://epp.eurostat.ec.europa.eu/*). The number of pet owning households were estimated around 70 million in 2010 (*http://www.fediaf.org/facts-figures/*), while food producing animals in stock reached a total of more than 200 million (cattle, pigs, sheep, goats, and chicken) that lived on farms and were potential consumers of antimicrobials

(*http://epp.eurostat.ec.europa.eu/*). Annually a large amount of drugs are being used worldwide to sufficient quantities of food to feed a rapidly growing world human population (Vazquez-Moreno et al 1990; Roura et al 1992; Rassow and Schaper 1996; Martin et al 1996). The farm animals consume worldwide approximately 8 million kg of antimicrobials annually (70 % of which is used for non-therapeutic purposes such as growth promotion; forbidden in EU from January 2006, and disease prevention) compared with only approx. 1 million kg per year used in human medicine (Roe and Pillai 2003).

In the veterinary medicine antimicrobials are used differently compared to the human medicine. For example, growth promotion, prophylaxis and metaphylaxis (Andersson 2003; Anthony et al 2001; Cabello 2006; Casewell et al 2003).

Antimicrobials are routinely fed to livestock as growth promoters to increase profits and to ward off potential bacterial infections in the stressed and crowded livestock factory environment. In the EU the use of avoparcin was banned in 1997 and the use of spiramycin, tylosin and virginiamycin for growth promotion were banned in 1998. All other growth promoters in the feed of food producing animals were banned from January 1, 2006 after a few national bans the years ahead (*http://europa.eu*). In the United States (US) politicians are discussing to introduce a similar ban (S-742, 109th U.S. Congress. Preservation of Antimicrobials for Medical Treatment Act.). Despite the ban on the use of all antimicrobials as growth promoters in the EU and a ban on the use of quinolones as growth promoters in the poultry feed in the US medical important antimicrobials are still routinely fed to livestock prophylactically to increase profits and to ward-off potential bacterial infections in the stressed and crowded livestock and aquaculture environments in some parts of the world (Cabello 2006; Ndi and Braton 2012; Smith et al 2009). Because stress lowers the immune system function in animals, antimicrobials are seen as especially useful in intensive animal confinements (Halverson 2000). The non-therapeutic use of antimicrobials involves low-level exposure in feed over long

periods - an ideal way to enrich resistant bacterial population (Gullberg et al 2011; Kohanski et al 2010; Sharma et al 2008).

The use of antimicrobials in animal husbandry for many years has exerted an evolutionary effect ('Darwin-style' selective pressure) on the different types of bacteria which survive in farm animals. For years we have been actively selecting for bacteria which possess genes capable of conferring antimicrobial resistance (Sundin et al 1995; Bastinello et al 1995; Alexander et al 2011).

Despite large differences in methodology, most results demonstrate that not so long after the introduction of an antimicrobial in veterinary practice, resistance in pathogenic bacteria and/or the fecal flora increases. In particular, the wide-spread use of antimicrobials in animals has resulted in an increased emergence of bacterial resistance to antimicrobials, in zoonotic organisms such as bacteria in the *Salmonella*, *Campylobacter, Listeria* and *Enterococcus* genera, as well as the *E. coli* species. Some zoonotic bacterial are propagated primarily among animals and subsequently infect people (Corpet 1988; Levy 1984; Marshall et al 1990; Sundin et al 1995).

Moreover antimicrobial resistance has been detected in different aquatic environments (Ash et al 2002). Fish pathogenic bacteria often produce devastating infections in fish farms where dense populations of fish are intensively reared. Bacterial infections in fish are regularly treated with antimicrobials in medicated feed. So far most of the fish pathogenic bacteria with a history in diseased fish farms have developed drug resistance (Sørum 2008). Modern fish farming relies increasingly on vaccination procedures and improved management to avoid infections (Bowden et al 2003). For example, the Norwegian aquaculture industry has produced over one million tonnes farmed fish (*http://www.ssb.no/fiskeoppdrett_en/*) by using improved vaccines, management techniques, and only 649 kg of antimicrobials in 2011 (NORM/NORM-VET 2010).

Companion animals are increasingly treated as family members, also in the context of their infectious diseases. For instance, skin infections caused by staphylococci in dogs with or without underlying allergic reactions result in an increasing use of semi-synthetic penicillins because of the ineffectiveness of penicillin against penicillinase producing *S. pseudintermedius* (Yoon et al 2010).

Resistant bacteria can be transferred from animals to humans in three ways: i) the transfer may occur through the food chain. Bacteria originally from food animals can reach people through improper food handling and inadequate cooking (Roe and Pilai 2003), ii) livestock animals or animal health workers may also pick up resistant bacteria; and they could also become carriers of the drug resistant bacteria that can be spread to other humans in the community (Levy et al 1976), iii) environment which is contaminated with manure contains a great variety of bacteria, creating an immense pool of resistance genes that are available for transfer to bacteria that cause human disease (Schauss et al 2009).

On the otherhand, it is lately shown that soil and wastewater have a wide dispersion of AMR genes (Cantas et al 2013). Still, little is known about the diversity, distribution, and origins of resistance genes, particularly among the as yet noncultivable environmental bacteria.

Despite the importance of soil and wastewater sytstems as a reservoir for AMR genes, and the relevance of wastewater treatment to control resistance spread, to date the number of studies that have been published remains relatively low (Figure 9) compared to the human and animal focused surveys.

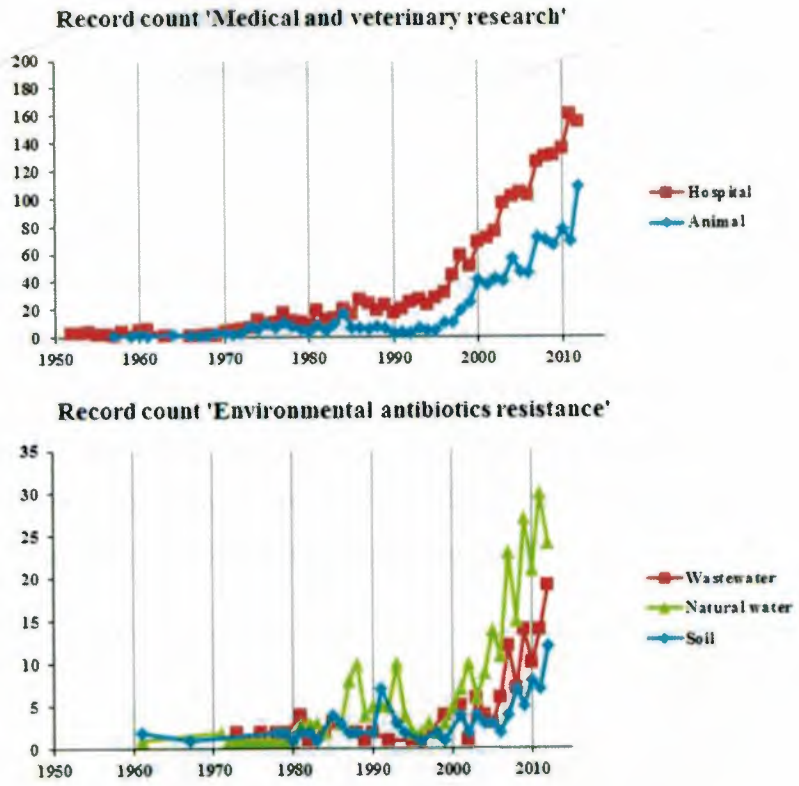


Figure 9. The change in number of antimicrobial resistance related published research papers in different subdisciplines and covering different environments (modified after Cantas L et al, 2013).

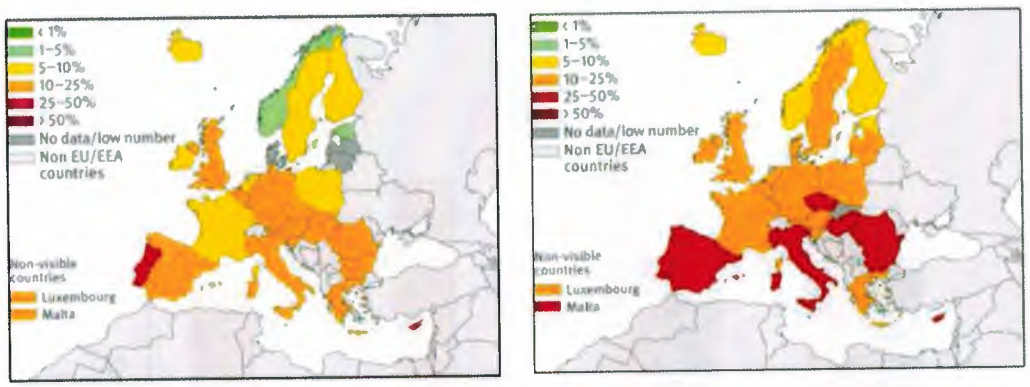
4.2. THE WORRYING RISE OF CYSTITIS SUPERBUGS IN CYPRUS

The major cause of urinary tract infections (UTIs) in humans is *E. coli* (Wirth et al 2006) that is most frequently used as an indicator bacterium for addressing antimicrobial resistance dissemination in different environments and host species. It is a frequent carrier of different antibiotic resistance genes and a prominent constituent of human and animals gut microbiota (Wirth et al 2006; Allan et al 2010; Guenther et al 2011).

The prevalence of multidrug-resistant *E. coli* strains is increasing worldwide principally due to the spread of mobile genetic elements, such as plasmids. Wide spread of multi-resistant *E.* coli, such as ESBL-producing *E. coli* certainly limits the infection therapeutic options which is a worldwide growing health problem.

In *E. coli*, β-lactamase production is the most important mediator of resistance to broad spectrum of β-lactams including third- and fourth-generation cephalosporins and monobactams.

The Europe-wide increase of resistance in *E. coli* to all antibiotic classes is under surveillance. Relatively increased resistance againts important antimicrobial, particullarly fluoroquinolones (one of the last resort antimicrobial), is found to be alarming. Based on data from human *E. coli*, gradient incresed resistance levels has been observed down from Scandinavian to Mediterranean countries (Cars et al 2001 and European Antibiotic Resistance Surveillance 2008).



A. Proportion of fluoroquinolone-resistant *E coli* in EU/EEA countries in 2003.

(Low number" = less than isolates reported 10 isolates reported)

Data source: EARSS (2008)

B. Proportion of fluoroquinolone- countries in EU/EEA countries in 2008.

Figure 10. Proportion of fluoroquinolone-resistant *E. coli* in EU/EEA countries in 2003-2008.

This trend is highlighted by the shift towards red which is evident comparing the maps of 2003 and 2008 (Figure 10-A and 10-B). However, percentage frequency of *E. coli* resistance profiles for four antimicrobial groups; fluoroquinolones, aminoglycosides, third-generation cephalosporins and carbapenems, for per country indicates a multi-resistant *E. coli* problem developing during the period of 2003–2005 (Figure 10).

As indicated on the map (above), 25-50% of *E. coli* isolates were reported to be MDR-resistant in Cyprus in 2008.

In this study, the UTIs rate caused by ESBL-producing *E. coli* among hospitalized patients found to be increased from 36% in 2010/2011 to 53% in 2014 with a significant increase up to 71% in 2013 ($p< 0.001$) in Cyprus. However, a gradual increase of ESBL-producing *E. coli* frequencies were also observed from 2010/2011 (14%) to 2014 (44%) ($p< 0.001$) in UTIs of out-patients (Figure 11).

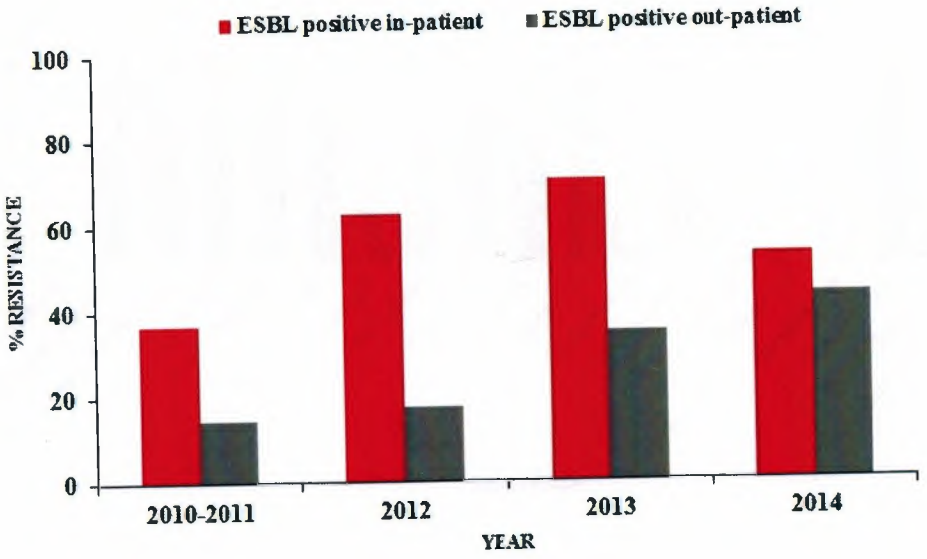


Figure 11. Percentage distribution of ESBL-producing *E. coli* in urine samples of hospitalized patients and out-patients (2010/2011-2014).

Many of the ESBL-producing *E. coli* isolates were resistant to quinolones (in-patients= 78%, out-patients= 79%), gentamicin (in-patients= 45%, out-patients= 61%), and trimethoprim/sulfamethoxazole (in-patients= 60%, out-patients= 62%), whereas all ESBL-producing *E. coli* remained susceptible to amikacin, carbapenems except ertapenem (in-patients= 6%, out-patients= 11%). Barely reduction (aprox 5%) in Ertapenem resistance from 2010 to 2014 was shown among

in-patients and out-patients. Partial resistance to nitrofurantoin (in-patients= 14%, out-patients= 11%) was also observed.

All other non ESBL-producing *E. coli* were sensitive to imipenem, meropenem, amikacin and mostly to nitrofurantoin (inpatient= 91%, out-patient= 93%). The highest resistance rates were against trimethoprim-sulfamethoxazole (inpatient= 69 %, out-patient: 41%) between 2010/2011 and 2014 (Figure 12 and Figure 13).

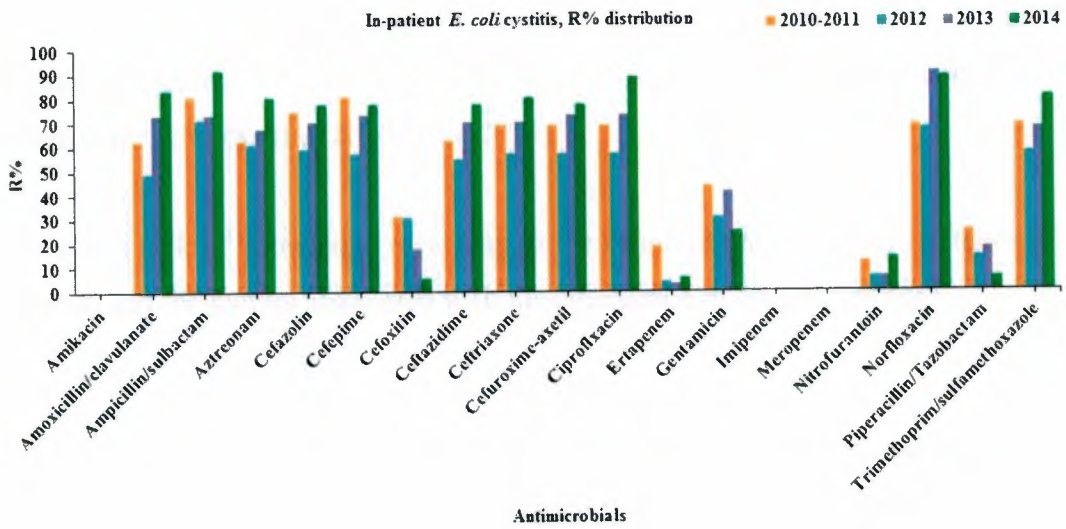


Figure 12. Antimicrobial susceptibility testing for all *E. coli* strains isolated from in-patients with cystitis (2010/2011-2014).

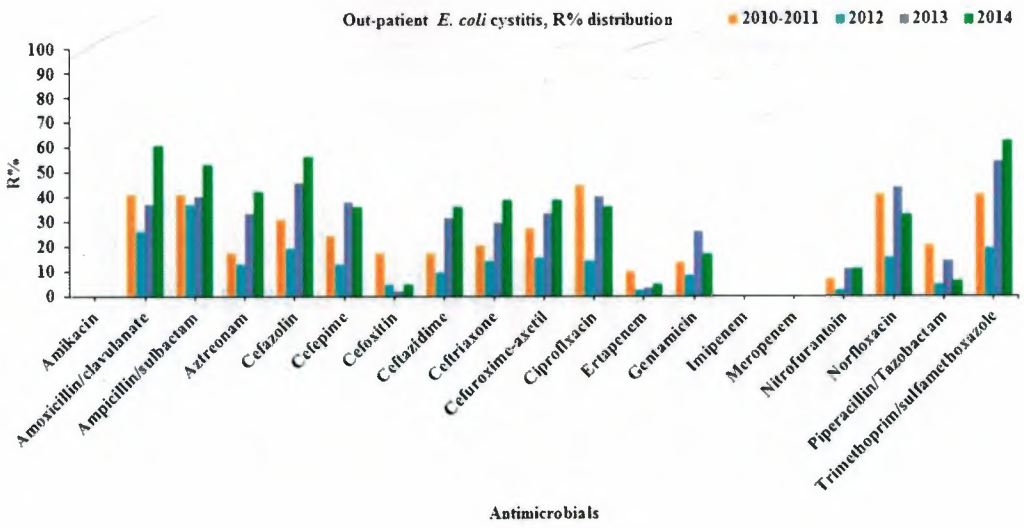
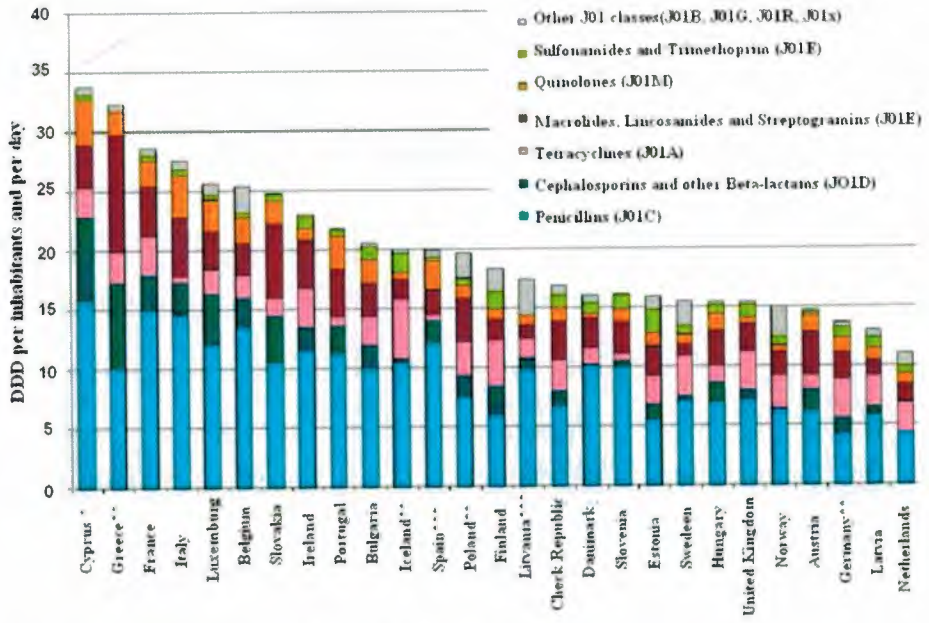


Figure 13. Antimicrobial susceptibility testing for all *E. coli* strains isolated from out-patients with cystitis (2010/2011-2014).

There is a lack of knowledge regarding to the origin of antimicrobial reistance in Cyprus, and the factors associated with the emergence of drug resistance.

The convincing evidence of strong relation between increased antimicrobial consumption and higher levels of antimicrobial resistance is globally known (Alanis 2005). According to the lates data that collected by the European Antimicrobial Resistance Surveillance System (EARSS) which is contracted by the European Centre for Disease Prevention and Control (ECDC), and funded by the EU, the Dutch Ministry of Health, Welfare and Sports and the Dutch National Institute of Public Health and the Environment; Cyprus has the highest out-patient antimicrobial use rates among the EU/EEA countries (Figure 14). These results may be linked to the the worrying rise of superbugs on the island.



*Total use, i.e. including inpatients, for Cyprus, Estonia, Greece and Lithuania.

**2006 data for Germany, Greece, Iceland and Lithuania; 2005 data for Poland and United Kingdom.

***Reimbursement data, which do not include over-the-counter sales without prescription.

Data source: The European Surveillance of Antimicrobial Consumption (ESAC) 2007.

Figure 14. Total outpatient antimicrobial use in EU/EEA countries.

In another independent cross-sectional study showed that 97,6% of community pharmacists were related to inappropriate antimicrobial dispensing without medical prescriptions in the north of Cyprus (Kaya Suer, Aslı Aykac, Sanda Cali unpublished survey, 2015). Furthermore, 60% of physicians are adhered to international antibiotic-prescribing guidelines (Personal notes, Leon Cantas, 2014) which may not be relevant to epidemiological essential of the island.

4.3. MOLECULAR CHARACTERIZATION OF ESBL-PRODUCING *ESCHERICHIA COLI* IN CYPRUS

In the literature a great genetic diversity of ESBLs were showed (Figure 15) and ESBL encoding genes mainly encoded by mobile genetic elements such as plasmids, transposons or integrons (Gniadkwoski, 2001), which facilitates transfer between organisms and the lower digestive tract of colonized patients has been

recognized as the major source of ESBL producing organism (Gniadkwoski, 2001; Lucet and Regnier, 1998).

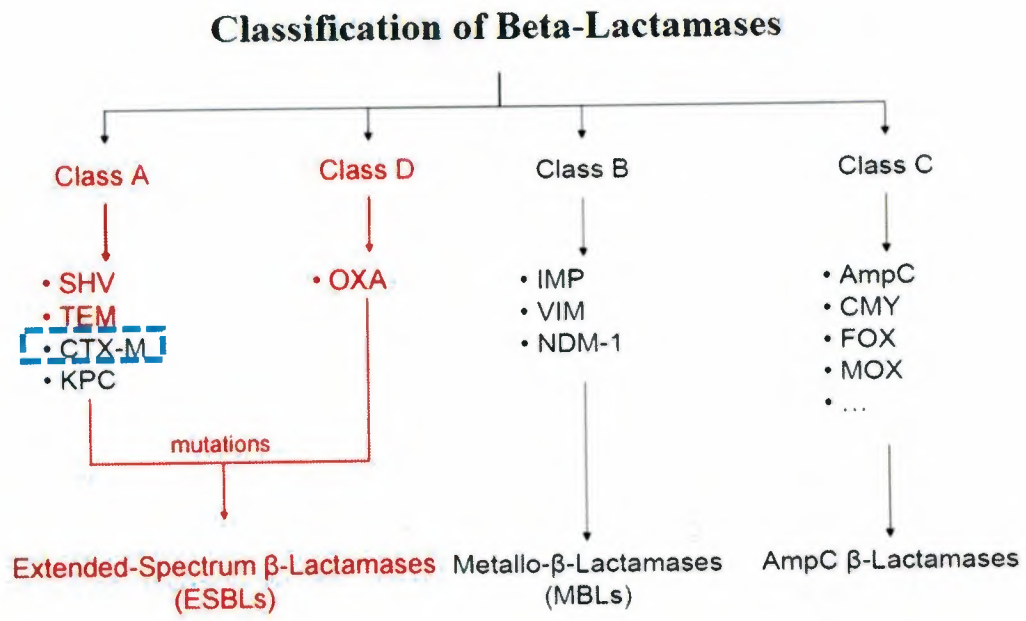


Figure 15. Classification of Beta-Lactamases. According to global distribution point of view, during the last 20 years, most of the ESBL found in *E. coli* has been of TEM or SHV lineage. Recently TEM and SHV types have been replaced by CTX-M-type ESBL, whose emergence and proliferation are significantly increased in group of class A (Bou G et al, 2002).

The first CTX-M-type ESBL (CTX-M-1) was isolated from enterobacterial strains in 1980s, in Europe (Bonnet R, 2004). Since then, over 50 variants have been described in 6 sublineages (Rossolini et al 2008).

In this study, forty-two strains (84%) were positive for *bla*CTX-M among fifty ESBL producing *E. coli* (Figure 16-a). Previously published 85 nucleotide sequences of *bla*CTX-M genes were included in recent sequencing data for phylogenetic analysis (Figure 16-b).

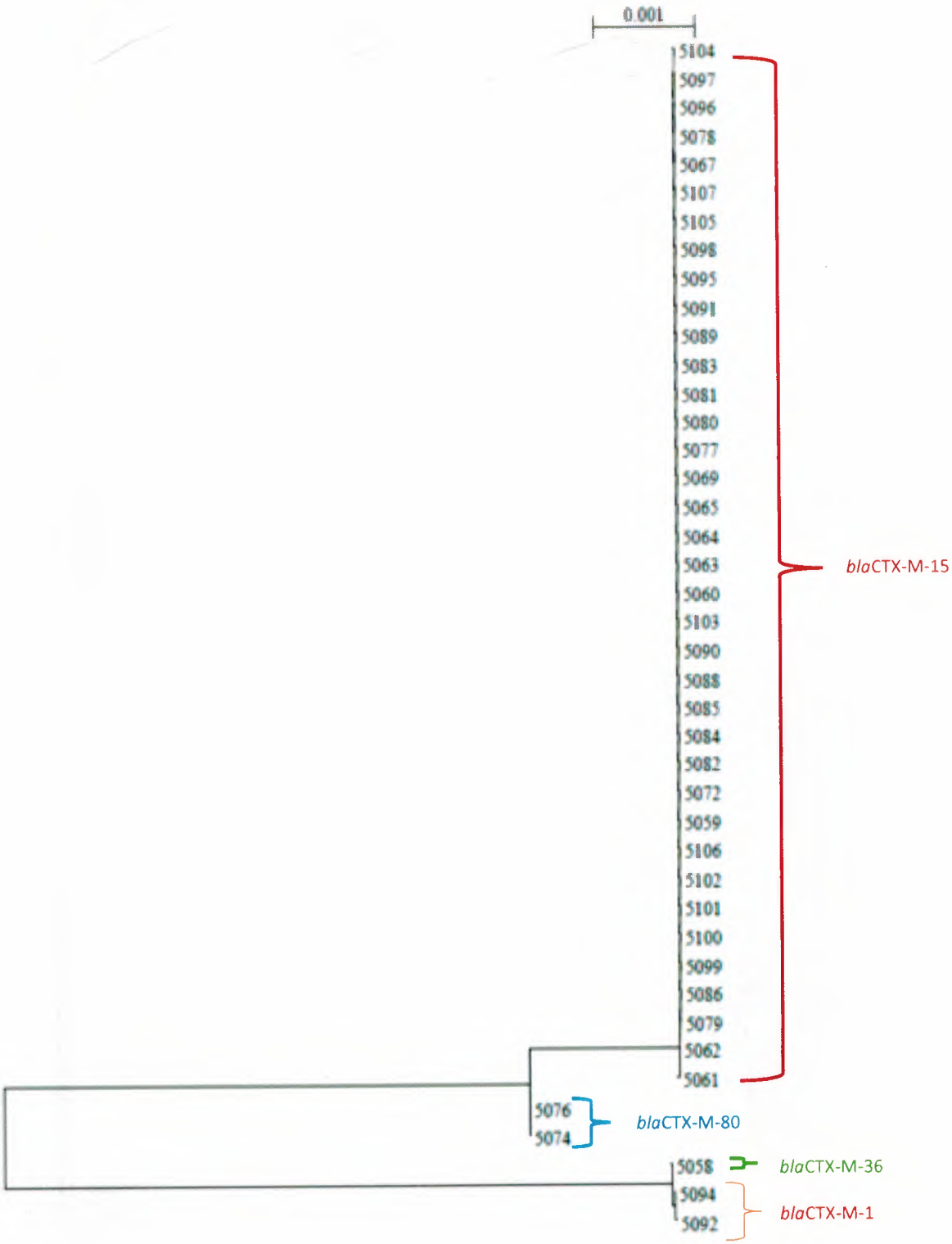


Figure 16 a. Phylogenetic tree of *bla*CTX-M sequences $n_{\text{this study}}=42$.

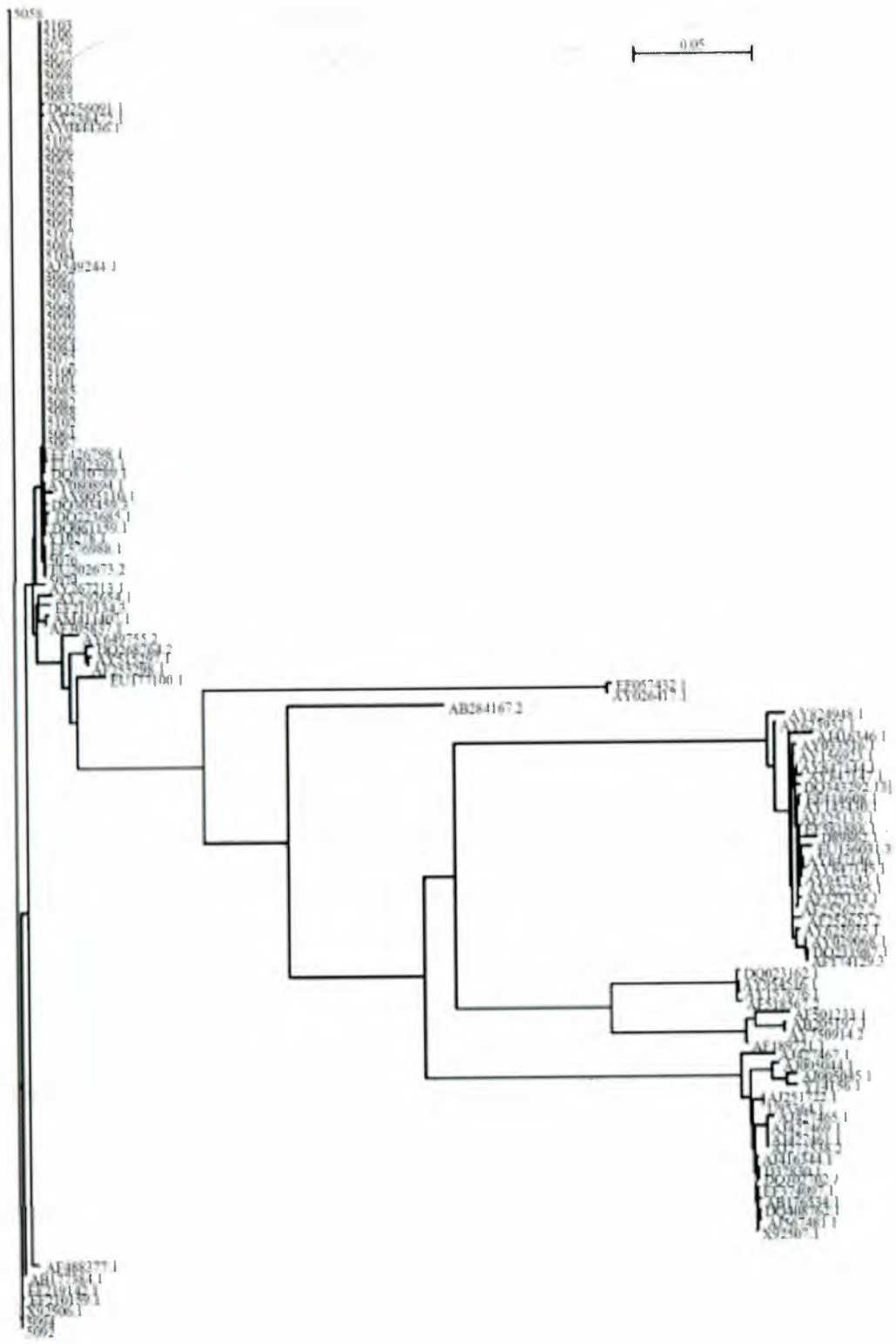


Figure 16.b. Phylogenetic tree of *bla*CTX-M sequences $n_{\text{this study}}=42$ and $n_{\text{previously published}}=85$.

The samples 5058 (in-patient/2012-3), 5094 (out-patient/2012-4) and 5092 (out-patient/2013-1) show identical CTX-M sequences (Figure 16a-b). Those genetic sequences were found to be in close phylogenetic relation with previously published CXT-M-1 (Germany, 2005) and CXT-M-36 (Japan, 2004) that isolated from clinical strains.

The sequences of the CTX-M genes were also identical in the samples 5074 (out-patient/2013-2) and 5076 (in-patient/2012-2) that revealed a clonal relation with CTX-M-80 first discovered in Chinese clinical isolates in the world (Figure 16a-b).

All remaining CXT-M PCR positive samples (n: 37) from 2011 to 2014 have also revealed an identical CTX-M gene (in-patient: 21, out-patient: 16) in the biggest cluster on the phylogenetic tree (Figure 16a-b). CXT-M-15 was the closest ESBL gene subtype in this cluster that initially found in India.

The CXT-M PCR negative samples were mainly from out-patients ($n_{CXT\text{-}M\ negative\ (OP)}=5$) rather than in-patients ($n_{CXT\text{-}M\ negative\ (IP)}=3$).

5. DISCUSSIONS

Emergences of resistance towards antimicrobials which are critically important for human therapy are the most worrisome. These include the recent emergence of ESBL producing and carbapenemase positive Enterobacteriaceae (Horton et al 2011), the emergence of farm associated MRSA ST398 (the main pig associated clone) (Cuny et al 2009 and Kluytman 2010) and of plasmid mediated quinolone resistance in animal isolates and food products (Nordmann et al 2011 and Poirel et al 2005). Unfortunately, there are several examples in the bibliography that show that these are already widespread in Europe and other parts of the world and have a large impact in human health (Nordmann et al 2011 and Heuer et al 2009).

The ecological niches of human, animal, water, and soil can easily be evaluated as separated small niches, but this evaluation might not give the entire truth. Bacteria are present in micro-ecological niches, but move between ecosystems from animals to humans, from humans and animals (faeces and manure) to water and soil and return to human and animals, through, for example, food (plants or vegetables).

In addition, the use of antimicrobial treatment in each small niche (humans, animals or plants) selects the resistant strains to become the reservoir of resistance genes. These antimicrobial resistance genes are present and can be transported within the bacteria from one niche to another. Figure 17 illustrates the interaction between the different reservoirs of bacteria in the food chain.

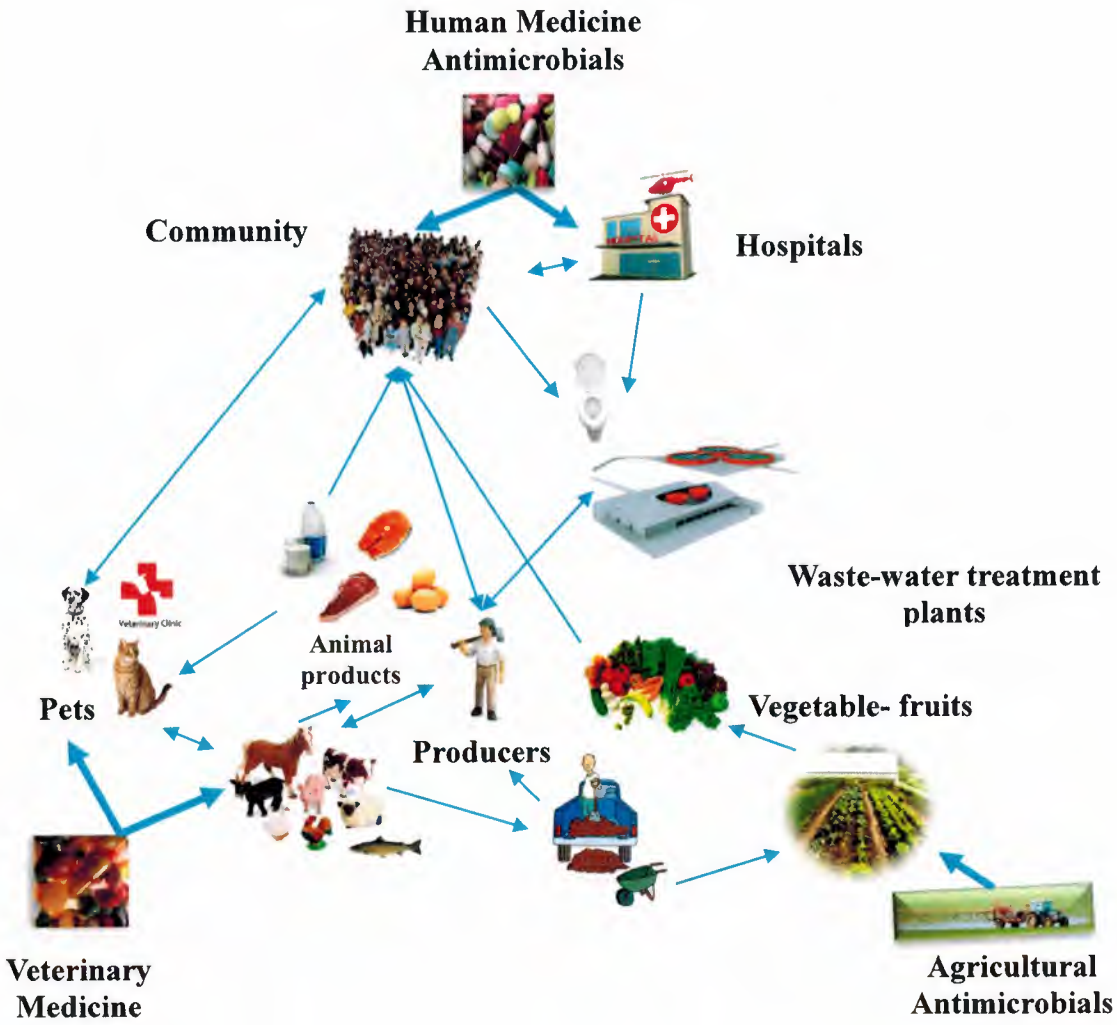


Figure 17. Schematic representation of the complexity of selection/development of antimicrobial resistant bacteria in different known reservoirs. The possible routes of transmission throughout the environment of these resistant bacteria are suggested. The reservoirs where antimicrobials are applied also suggested as 'hot spots' for horizontal gene transfer. Thick arrows show major selective pressures for selection of antimicrobial resistance genes, thin arrows show the significant directions of gene flow (Figure by Cantas et al 2013).

One of the most remarkable result of this current study was the relatively less published environment and antimicrobial resistance related research data in literature databases. According to recent metagenomic studies, the environment is the largest gene pool which is closely related to those conferring resistance in human pathogens (Cantas et al 2013, Cantas and Suer 2014). It is also known that precence of antimicrobials in a nich can enhance the drug resistance development among various bugs. Herein, it is wise to underline that the degeneration rate of antimicrobials in the environment varies from several weeks to months. For example, 0-50% of Macrolides degenerate in up to 30 days, while tetracyclines take 3 months in soil (Cantas et al, 2013).

Therefore, there is an immediate need for this area in the coming years. (1) the identification of the conditions that may enhance the horizontal gene transfer and selection of AMR (temperature, pH, etc.); (2) the classification and quantification of risk, e.g., the likelihood that an AMR bacterium or gene from wastewater habitats reach humans and causes issues for human health; (3) the improvement of wastewater treatment processes in order to minimize the loads of antimicrobial-resistant bacteria and genes in the final effluent (Dodd, 2012).

Another breakthrough result within the concept of this thesis was the identification of high frequent MDR and ESBL-producing *E. coli* isolate rates from hospitalized patients than out patients during last four years in urine culture isolates in Cyprus. ESBL-producing *E. coli,* was observed in 53% of hospitalized and 44% of out-

patients, latest in 2014. Variable but high emerge of ESBL-producing *E. coli* from urine samples in hospitalized patients and gradually increasing percentages among out-patients during the last four years are extremely worrisome signs of development of untreatable infections in close future on the island.

The frequent isolation of ESBL-producing of *E. coli* has been occurred typically in the hospital settings (Livermore 2007; Rodriguez-Bano et al 2004). Hospitalization, recurrent urinary tract infections, catheter applications (biofilm formations) and previous antimicrobial treatment (especially with third-generation cephalosporins) or previous international travel are previously described as great risk factors for the acquisition of these organisms (Topaloglu et al 2010; Kang et al 2012; Laupland et al 2008: Woodford et al 2004; Rodriguez-Bano et al 2004). Inevitably, these microbes today have begun to disseminate in the community worldwide (Rodriguez-Bano et al 2004, Colodner et al 2004).

We have witnessed a recent 2,5-fold increase in community-onset UTIs due to ESBL-producing *E. coli* in our area since 2010/2011. These results are in keeping with other recent reports (Topaloglu et al 2010) highlighting the rapid spread of these strains in the community. However, the proportion of ESBL-producing *E. coli* from out-patients with cystitis were found to be only 2.1% in Norway, which has the lowest levels of antimicrobial consumption rates among the other European countries (NORM/NORM-VET, 2013).

The high resistance rate among out-patients in this study seems to be result of widespread antimicrobial usage in the community without prescription demands on the island (Kaya Suer, Aslı Aykac, Sanda Cali unpublished survey, 2015). The actual defined daily doses (DDDs) of antimicrobials were not known *per se* during this study. However earlier sale trends of systemic anti-infective agents in Cyprus revealed that we had one of the highest values in comparison with other European countries (ESAC 2009, Hadjimichael 2006). Inappropriate antimicrobial dispensing without medical prescriptions in the north by community pharmacists

(97,6%) (Kaya Suer, Aslı Aykac, Sanda Cali unpublished survey, 2015) and adherence of international antibiotic-prescribing guidelines (60%) by the physicians (Personal notes, Leon Cantas, 2014) may be directly linked to the high frequency of MDR bactreria isolates on the island. As resistance is becoming more widespread, prudent use of antimicrobials has to be limited on the island. Prescribers should prioritize diagnostics to make more targeted antimicrobial treatment decisions.

None resistance to meropenem and imipenem might be due to the limited usage of these antibiotics in our population. Non-significant reduction in Ertapenem resistance is related to relatively lower sample during the establishment of the Laboratory in 2010.

Nitrofurantoin is one of the oldest urinary anti-infective drug that has been widely used on the island. It has multiple mechanism actions on bacteria and demand several mutations in order to antimicrobial resistance development that might explain its low prevalence in our region.

Antimicrobial resistance to cephalosporins (>50 %, except cefoxitin) in our country was significantly higher than Scandinavian countries (Sweden, Norway and Finland; 3-5.1%) which have more restricted antimicrobial consumptions compared to the other eastern European countries Bulgaria (22.9%), Slovakia (31%) (Allocati et al 2013). Cephalosporins have been frequently used for the empirical treatment of UTIs that is a clear evidence of strong relationship between prescribing habits and antimicrobial resistance (Allocati et al 2013, Lindbäck et al 2010).

Reduced susceptibility to both ciprofloxacin (53%) and gentamicin (26%) leaves clinicians only carbapenem in such serious cystitis treatment on the island. On the other side, fluoroquinolone resistance range found to be significantly lower in Sweden (8%) and Norway (9%) (ECDPC 2011), while they were similarly

predominant in Italy and Slovakia had almost similar predominance of fluoroquinolone resistance (42%) like us. Furthermore, the prevalence of isolates resistant to aminoglycosides were around 4% in Sweden and similarly higher in Romania, Slovakia and Greece (17%). However, the prevalence of multi-resistant (≥3 drugs) non-ESLB producing *E. coli* isolates were 24%, which recently ranged from about 1% in Sweden to more than 10% in Romania and Slovakia.

The sub-typing of the ESBL isolates based on the phenotypical antimicrobial susceptibility pattern of Beta-lactams with isolates producing ESBLs is often unreliable and can be difficult, due to great genetic heterogenocity (Branger et al 2005). For that reason, we have omitted to sort ESBL-producing *E. coli* isolates in detailed sub-groups by using antimicrobial resistance patterns.

The all genetic clonal typing of the previously isolated ESBL-producing *E. coli* types were not possible to perform in this study due to limited financial resources. Only 16% of the genetically analysed samples remained CTX-M PCR negative in this study. The remaining 42 out of 50 samples were found to be PCR positive for CXT-M genes with a minor genetic among uropathogens. This close clonal relation among different strains originated from in-patients and community might be linked to the frequently administered empirical antimicrobial therapies that cause complicated UTIs in Cyprus. This suprising result correlates with the global statement of Pitout et al 2005 '*ESBL sproducing strains are now emerging also in the community*'. It is an alarming issue that have direct major implications in bacterial infection therapy on island.

The source of ESBL genes are known, unlikely for the most of other acquired AMR genes in globe. The origins of CXT-M-type ESBL are resident in members of the *Kluyvera sp.* (non-pathogenic enviromental species) (Rossolini et al 2008). CTX-M-1 and -2 groups have been detected in *Kluyvera ascorbata* while CTX-M-8 and -9 groups occured in *Kluyvera ascorbata* (Bonnet et al 2004).

As mentioned CTX-M genes are found all over the world and CTX-M-15 is found to be the most dominating genotype world-wide. In this study, 88% of the ESBL production was originated from CTX-M-15 that was consistent with previous global studies (Canton and Couque, 2006).

➢ The genetic link between food borne CTX-M-15 and clinical isolates have been previously shown that food-environment microbioata is major reservoir of plasmid mediated CTX-M-15 (Upadhyay S et al. 2015). Uncontrolled and broad spectrum antimicrobial use in livestock without veterinary prescription contributes to selection of such AMR genes that inevitably cause MDR infections directly and/or indirectly in Cyprus public health. It is also known that individual farm animal treatment or pet treatment with antimicrobials are often made and administered by the owner who may get antimicrobials from pharmacies without prescribtion in Cyprus. There is immediate need for ban of over-the-counter sale of antimicrobials at each segment of healthcare services is immediately needed.

However, local variations in the global spread of ESBL subtypes may occur. In Europe, where the TEM- and SHV-type ESBLs were first detected and played a major role as ESBL determinants, the CTX-M-type ESBL have lately achieved a siginificant diffusion. One of the most sriking examples of rapid dissemination of these genes were reported in United Kingdom (UK), mostly CTX-M-15 (Livermore and Hawkey, 2005).

CTX-M-15 is a variant of CTX-M-3 that originally described in India, has also been observed in Poland (Baraniak et al 2002) as well as Bulgaria, Romania, and Turkey (I. Schneider, E. Kueleyom, R. Makovska, et al., 12th Congr. Clin. Microbiol. Infect. Dis., abstr. P430, 2002).

Particullarly, high-rates of CTX-M enzymes have also been reported in Turkish Hospitals. The CTX-M gene was the most prevalent enzyme (71%), followed by TEM (49%) and SHV (47%) derived enzymes. The CTX-M-15 sub-type was the most frequent, 69.4%, in isolates followed by CTX-M-3 (29%) and CTX-M-1 (2%) (Gur et al 2008).

A remarkable diffusion of CXT-M-type ESBL in *E. coli* has also been observed in other mediterranean countries, Spain (52 %) and Italy (54%). The CTX-M-9, CTX-M-10 and CTX-M-14 were found to be the most frequent types in Spain, where as CTX-M-1 and CTX-M-15 were predominant in Italy (Hernandez et al 2005 and Luzzaro et al 2006).

In the Scandinavian countries, the distribution of CXT-M genotypes is homogenous. In Sweden, almost half of the ESBL-producing *E. coli* harbor CTX-M-15 followed by *bla* CTX-M-14 found in 10–15% (Brolund A et al 2014). In Norway and Denmark, about 50% and 20% of the genotypes are CTX-M-15 and CTX-M-14 respectively (Naseer et al 2009 and Olesen et al 2013).

It is important mention that *E. coli* strains with CTX-M enzymes have also been detected in samples from livestock (Brinas et al 2005, Kojima et al 2005) and companion animals (Caratolli et al 2005) that act as an important resevior in todays close relation between animals-and-humans. More and more studies show that there is dissemination of bacteria between food, animal (both food-production animals and pets) and humans (Cantas et al 2010). There is no information on such surveillance in Cyprus to link.

Patients with severe infections are highly dependent on receiving right andadequate treatment early in the course of infection for a benign outcome. The negativ clinical impact of CTX-M-type ESBLs is devisteting. It is known that combination of antimicrobials particularly cephalosporin with clavulanic acid has been used to treat

UTIs caused by CTX-M ESBL-producing *E. coli* in clinical practice (Livermore et al 2008), which are unlicensed in our country. This is not recommended in this thesis as empirical therapy due to induction of AmpC enzymes in Enterobacteriaceae which attack the cephalosporin. UTIs may get even more severe and course with bacteraemia. Herein, delay in adequate therapy will lead to adverse outcomes and potentially increased mortality and morbidity (Kumar et al 2006). Intravenous antimicrobial therapy (chosen according to the susceptibility pattern of the organism) should be administered.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Bacterial infections are becoming increasingly resistant to existing antimicrobials and this is one of the world's most pressing public health threats. These drugs are a precious resource in our lives, like energy i.e. oil-gas, and we have a moral obligation to ensure that they are available for future generations. But, antimicrobial overuse increases the development of drug–resistant germs and there are unfortunately no better magic bullets in the *antimicrobial pipeline* to eradicate these superbugs in close future. Therefore, we continuously need to improve the use of antimicrobials that are currently available and the understanding of the drug resistance development in bacterial infections.

It is known that bacteria inevitably have evolved multiple mechanisms for the efficient evolution and spread of antimicrobial resistance. The new developments of molecular diagnostic techniques for the identification and epidemiological surveillance of genetic determinants of drug resistance in different hosts and in the environment showed that the soil microbiota represents one of the ancient evolutionary origins of antimicrobial resistance (Forsberg et al 2012). It might be considered as a huge reservoir of resistance genes ('resistome') which are available for exchange with clinical pathogens (Figure 18).

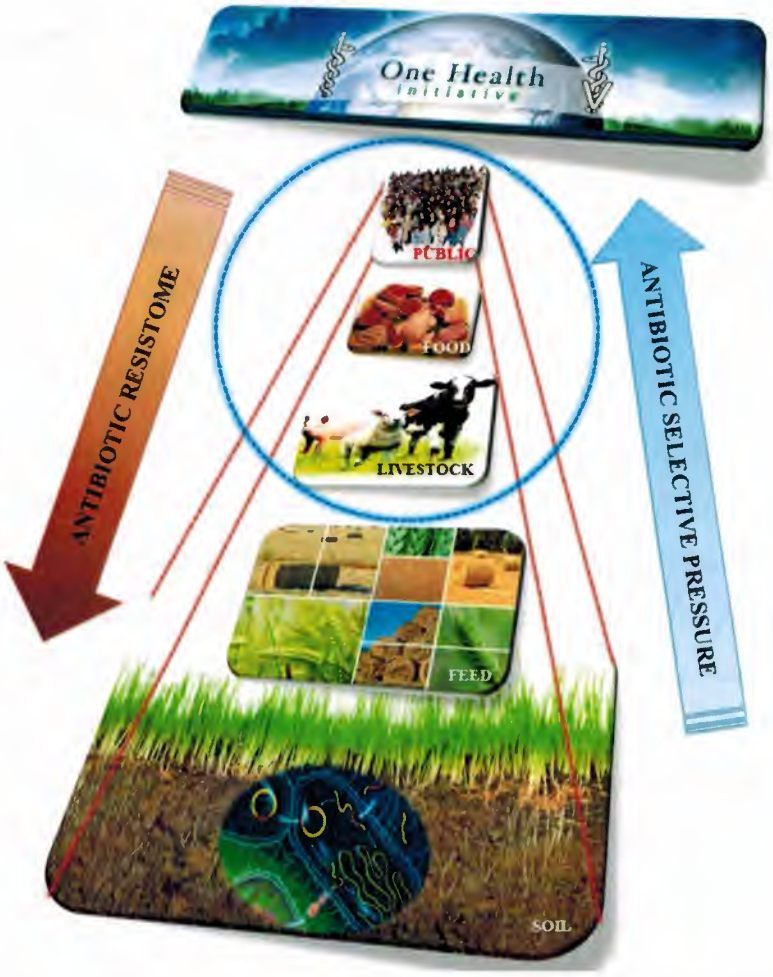


Figure 18. Antimicrobial selective pressure and development of bacterial resistance in human bacteria as a result of communication with the soil microbiota. Increased selective pressure and overuse of antimicrobials in animals and humans (blue circle) accelerate horizontal gene transfer between different bacterial species. Within the *one health* concept all prescribers need to be careful with the antimicrobial choice (Figure by Leon Cantas, 2014).

No single strategy can solve the issue of antimicrobial resistance; a multi-pronged approach is definitely required due to unavoidable broad interactions between different ecological niches. On the other hand, it is more obvious today than ever that the way we have managed our antimicrobials (Figure 17, within the blue circle) for the past 70 years has failed. The choice of antimicrobial treatment by prescribers (medical doctors, dentists and veterinary surgeons) in routine practice is commonly based only on the clinical experiences without precise diagnostic of pathogens.

Once a bacterial infection is suspected normally without identification of the causative microorganism, antimicrobials with a broader spectrum of antimicrobial activity has been frequently prescribed over the years, after brief evaluation of various patient related factors (such as allergy, pregnancy or severity of infection) to ensure theoretically the best clinical outcome.

It has been shown that administration of non-therapeutic levels of antimicrobials can interfere with DNA replication (e.g. quinolones) (Cirz 2005), folic acid synthesis (e.g. trimethoprim) (Yao and Moellering 2003), protein synthesis (e.g. tetracycline) (Skavronskaia 1988); as well as cell wall synthesis (e.g. β-lactams) (Miller 2004) and it may induce the so-called SOS response (Beaber et al 2004) which can promote acquisition and dissemination of antimicrobial resistance genes (Beaber et al 2004 and Guerin et al 2009). Thus, our results reinforce the need for great caution in the use of SOS-inducing antimicrobials to avoid induction of resistance transfer following antimicrobial therapy.

It is not possible to eradicate any multi-resistant genes posing bugs on the earth, but routine antimicrobial resistance screenings at least from gut microbes isolated from environment and animals will contribute to the better understanding and control of possible spread of 'superbugs' and resistance genetic elements. It is evident from the DANMAP (Danish integrated antimicrobial resistance monitoring and research program) and NORM/NORM-VET Report that reduction in antimicrobial usage with strict policies may still be the safest way to control the development and spread of AMR in the future.

The actual annual economic impact of the MDR infection in northern Cyprus is not known, but it is obvious that several costs associated with medical care and loss of productivity is high.

The rise of ESBL-producing *E. coli* may led to an increased consumption of more expensive drugs such as carbapenems, which facilitated the emergence and spread of carbapenemases. Hospitalisation of such patients in healtcare facilities creats a optimal opportunity to cross dissemination to other in-patients. It is a great threat for public health point of view and compels exploration of alternative therapeutic options. Herein, Cefoxitin is recently suggested as alternative to carbapenems for the treatment of UTIs caused by ESBL-producing *E. coli* (Raphaël L et al 2012, Guet-Revillet H et al 2014). Over 90% of the ESBL-producing *E. coli* isolates from hospitalized and out-patients found to be sensitive to cefoxitin for last two years that might be recommended for the therapy of complicated cystitis rather than carbapenems as first choice in our country.

There are not many practical solutions for the treatment of multi-resistant Gram-negative bacteria. No antimicrobial prescription is recommended to suppress bacteriuria in the elderly without clinical signs of UTI (Raphaël L et al 2012, Guet-Revillet H et al 2014). Extended courses of antimicrobials due to complicated cystitis should only be used in specific situations such as for men with a relapsing infection in prostate (Williams and Schaeffer, 2004).

The oral options available for the treatment of complicated UTIs caused by ESBL-producing *E. coli* with concurrent resistance to trimethoprim and quinolones are limited. In case of susceptibility nitrofurantoin treatment can be recommended for lower UTIs but resistance may develop on treatment (Personal notes-Leon Cantas 2014) (Pallet et al 2010). Rather, immune-modulationg some cranberry, pre-probiotic products are recommended to reduce the frequency of recurrent UTIs (Wagenlehner and Naber 2005, Williams and Schaeffer 2004, McMurdo et al 2009). Unfortunetly, our ESBL-producing *E. coli* isolates were not tested in term of resistance against fosfomicyn, which represents a current favorite choice among practicioners for MDR *E. coli* causing UTIs in northern Cyprus. The immediate inclusion of antimicrobial resistance test including fosfomicyn is needed.

The CTX-M-type ESBL have recently undergone a rapid and global dissmeination. Our molecular investigation was limited with the precence of the CTX-M but future studies need to determine type of *bla* genes responsible for ESBLs producing strains in entire Cyprus. However, likely spread of CXT-M genes in community and hospitalised patients with a minor genetic diversity is a matter of a major concern. This indicates that such super bugs are not restricted to the nosocomial settings. The spread og CTX-M-type ESBLs is causing fast, and unpredictable changes in the epidemiology of antimicrobial resistance.

No matter what, each year, over two million tourists visit Cyprus. Hypothetically, new resistance genes may travel in and out of the country with potential pandemics. Immadiate future studies are needed to establish ESBL libraries on the entire island.

High emerge of the ESBL-producing *E. coli* from in-patients is a worrisome problem. Hospital measures that limit risk of the spread of mobile genetic elements and MDR pathogens should be implemented. Prevention of the cross-contamination can be applied by the strict hygienic standard protocols, inlcuding proper hand hygiene, as well as control over the use of antimicrobial drugs.

Greater attention should be given to the risks associated with release of antimicrobials into environment (Cantas et al, 2013). Bacteria and mobile genetic element destroying apparatus should be integrated in the effluent of the hospital settings.

Besides prevention strategies new therapies need to be developed, such as phage therapy, antimicrobial peptide therapy and immunotherapy (Worthington et al 2013; Haq et al 2012).

The potential use of therapeutic bacteriophages was started over a centry ago that has been later on underestimaded by the discovery of the antimicrobials (Haq et al 2012 and Kutaleladze et al 2010). Mainly, Eastrean European medical doctors have used the phage therapy to treat the bacterail infecions (Kutateladze et al 2010 and Abedon et al 2011). Highly specific and bacteria lysing effective phages for different *E. coli* strains have been published (Brüssow 2005; Maura et al 2012; Tsonos et al 2012; Sillankora et al 2012; Merabishvii et al 2012).

The majority of the *E. coli* strains causing UTIs can produce biofilms which significantly increase resistance to antimicrobials and natural immune- system, whereas phages are able to pass through the extracellular matrix, to degrade the biofilm and kill the bacteria (Doolittle et al 1995; Lacroix-Gueu et al 2005).

There are also accumulating reports in the literature regarding activity of Antimicrobial peptides (AMPs) that contribute to innate immune responses and destroy the harmful pathogens such as *E. coli* (Corrales-Garcia et al 2013, Lira et al 2013). Those small peptides enter into membrane bilayer of the microbes and form channels resulting in cell death (Hassan et al 2012).

Surprisingly, the results obtained with phage treatment and AMPs so far paint a positive picture for the future prospects of MDR infection therapy in endemic countries, such as Cyprus.

In this thesis, a number of priorities have been reviewed which provide important results that can be used to direct actions relatively fast. Some of these priorities include the more frequent use diagnostic tools rather then empirical treatments which are mediating factors in spread-control of antimicrobial resistance.

Although there is still a great deal of exploring to do, the present state of knowledge is now such that it can serve as a base for policy changes and their implementation.

To improve and enforce such network more surveillance studies is need which can be extended globally. Such global initiatives can ensure that we do not return to the pre-1940s era.

However, it is becoming increasingly obvious that this approach will not be possible in the long run because bacterial resistance is developing faster than the marketing of new antimicrobials (Aminov 2010). Herein, exchange of horizontal DNA that encodes drug resistance between bacterial species is one of the greatest adaptive strategies for bacteria (Baharoglu et al 2010).

Future treatment recommendations should take into account the use of phage therapy and immune-modulatory supplements (i.e. AMPs) as a basis for optimal bacterial infection treatment to reduce the selective pressures (antimicrobials) on resistance development. We have since long learned that the use of antimicrobials inevitably entails the appearance of more resistant strains of bacteria. Until recently, new antimicrobials have been repeatedly created to combat the new strains of resistant bacteria.

7. MAIN CONCLUSIONS

➢ Antimicrobial resistance globally increasing, particularly in Gram-negative bacteria, with considerable fluctuation between countries that highlighs a serious public health problem.

➢ High emerging ESBL-producing *E. coli* from urine samples in hospitalized (53%) and out-patients (44%) is an extremely worrisome sign of development of untreatable infections in the near future on the island.

➢ The *bla*CTX-M genes were found as a predominant gene (84%) responsible for ESBL production in this study. Moelcular epidemiologic characteristics of *bla*CXT-M clones is result from the high antimicrobial exposure of this clone both in comunity and hospital.

➢ The CTX-M-15 sub-type was the most frequent (88%) in isolates followed by CTX-M-1 (4,8%), CTX-M-80 (4,8%) and CXT-M-36 (2,4%). By the literature, microbiota of common food products and close relation with pet animals may serve as a reservoir for some of the CXT-M-15 drug resistance genes prevalent in human pathogens.

➢ Most of the surveillances of antimicrobial resistance were focused on the both humans and animals. The increased resistance were linked to the over- and misuse of antimicrobials. Our review have revealed that environment plays a key role in the spread of antimicrobial resistance as an unlimited reservoir of antimicrobial resistance genes and more research is needed in this field of science.

➢ We underlined the need of a novel national and international approach to combating MDR infections.

- An immediate Global One Health Approach which requires interdisciplinary collaboration and communication in all aspects of health care for humans, animals and the environment will support public health.

- We defined the drug resistance profiles of clinical isolates in Cyprus for the first time and established a scientific support for clinical advices on how to use antimicrobials in the treatment UTIs to be able to diminish the development and spread of bacterial antimicrobial resistance to an optimal minimum.

- The need for urgent prescription habit changes and ban of over-the-counter sale of antimicrobials at each segment of healthcare services is immediately needed.

- Millions of patients get treated with antimicrobial agents by physicians every day; treatment of the bacterial infections becomes much more difficult ahead than in the past 50 years. Random antimicrobial choice without diagnostic image of the bacterial infection obviously has accelerated the spread of the resistant infections in a global view. The wise treatment of such a challenge has already been postulated by Dr. Paul Erlich. He has for the first time addressed the art of the infection treatment at 17th International Congress of Medicine just a century ago by suggesting 'Hit hard and fast-against bacterial infections'. Today the results of this thesis underlies the importance of the routine bacterial identification, quick and optimal antimicrobial choice according to resistance testing and correct dosage during the treatment of an individual MDR-infection to avoid further resistance development in the microbiom.

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8. PUBLICATIONS

Paper I

Review: the important bacterial zoonoses in "One Health" concept.

Cantas, L., and Suer, K. (2014). *Front. Public Health* 2:144.

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Abstract

An infectious disease that is transmitted from animals to humans, sometimes by a vector, is called zoonosis. The focus of this review article is on the most common emerging and re-emerging bacterial zoonotic diseases. The role of "One Health" approach, public health education, and some measures that can be taken to prevent zoonotic bacterial infections are discussed.

KEY POINTS:

- A zoonotic bacterial disease is a disease that can be very commonly transmitted between animals and humans. Global climate changes, overuse of antimicrobials in medicine, more intensified farm settings, and closer interactions with animals facilitate emergence or re-emergence of bacterial zoonotic infections.
- The global "One Health" approach, which requires interdisciplinary collaborations and communications in all aspects of health care for humans, animals, and the environment, will support public health in general.
- New strategies for continuous dissemination of multidisciplinary research findings related to zoonotic bacterial diseases are hence needed.

High Emergence of ESBL-Producing *E. coli* Cystitis: Time to Get Smarter in Cyprus.

Cantas, L., Suer, K., Guler, E., and Imir, T. (2016). *Front. Microbiol* 6:1446.

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Abstract

Background: Widespread prevalence of extended-spectrum βeta-lactamase producing Escherichia coli (ESBL-producing E. coli) limits the infection therapeutic options and is a growing global health problem. In this study our aim was to investigate the antimicrobial resistance profile of the E. coli in hospitalized and out-patients in Cyprus.

Results: During the period 2010-2014, 389 strains of E. coli were isolated from urine samples of hospitalized and out-patients in Cyprus. ESBL-producing E. coli, was observed in 53% of hospitalized and 44% in out-patients, latest one being in 2014. All ESBL-producing E. coli remained susceptible to amikacin, carbapenems except ertapenem (in-patients = 6%, out-patients = 11%).

Conclusion: High emerging ESBL-producing E. coli from urine samples in hospitalized and out-patients is an extremely worrisome sign of development of untreatable infections in the near future on the island. We therefore emphasize the immediate need for establishment of optimal therapy guidelines based on the country specific surveillance programs. The need for new treatment strategies, urgent prescription habit changes and ban of over-the-counter sale of antimicrobials at each segment of healthcare services is also discussed in this research.