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RESISTANCE TO TETRACYCLINE IN *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*: BRIEF OVERVIEW ON MECHANISMS OF RESISTANCE AND EPIDEMIOLOGY

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Abstract

In this work we briefly present the mechanisms of resistance to tetracyclines. Tetracycline's were introduced in clinical practice in 1948, and are used for the therapy in human and veterinary medicine or as growth promoters in livestock industry. There are three major mechanisms of resistance to tetracyclines. Gram negative bacteria utilize efflux pump system of proteinaceous transporters in eliminating the drug from the cell. This mechanism of resistance is encoded by *tet* genes that belong to the group 1. Gram positive bacteria promote resistance to tetracyclines by producing soluble cytoplasmatic ribosomal protection proteins and the most frequent once are TetM and TetO proteins. Enzymatic inactivation is not widespread mechanism and the responsible gene is termed *tetX*. Epidemiological importance of tetracyclines is well documented in number of research papers. We described few works showing that tetracycline's are provoking resistance to other classes of antibiotics or vice versa. This phenomenon is probably due to the fact that resistance determinants are often situated on mobile genetic elements. Withdrawal of the therapy does not exclude resistance in short time frame due to the various environmental factors and animal feeding habits. Most often resistance to tetracycline is reported in *Escherichia coli* isolates from pigs, chickens and turkeys. The TetM and TetK proteins are most often found in methicillin resistant *Staphylococcus aureus*.

Key words: resistance, tetracycline, *Escherichia coli*, *Staphylococcus aureus*, mechanisms of resistance

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REZISTENCIJA NA TETRACIKLINE KOD *ESCHERICHIA COLI* I *STAPHYLOCOCCUS AUREUS*: KRATAK PRIKAZ MEHANIZAMA REZISTENCIJE I EPIDEMIOLOGIJE

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Kratak sadržaj

U radu su prikazani osnovni mehanizmi rezistencije na antibiotike iz grupe tetraciklina. Njihova upotreba u kliničkoj praksi, u terapiji ljudi i životinja otpočela je 1948. godine, a široko su primenjivani i kao promotori rasta u stočarstvu. Bakterije su razvile tri glavna mehanizma rezistencije na tetracikline. Gram negativne bakterije uglavnom koriste mehanizam efluks pumpe preko proteinskih transportera, u cilju eliminacije leka iz ćelije. Mehanizam rezistencije efluks pumpe kodiran je *tet* genima grupe 1. Gram pozitivne bakterije produkuju rastvorljive proteine u citoplazmi koji štite ribozom od tetraciklina, a najčešći su TetM i TetO proteini. Enzimska inaktivacija tetraciklina je treći mehanizam rezistencije povezan sa prisustvom *tetX* gena, i u odnosu na prva dva, ovaj mehanizam nije često ustanovljavana pojava. U literaturi postoji veliki broj podataka o epidemiološkom značaju tetraciklina. Na ovom mestu ukazujemo na značaj tetraciklina u razvoju rezistencije na druge klase antibiotika i obrnuto. Nastanak takvog fenomena objašnjava se činjenicom da su determinante za rezistenciju locirane na mobilnim genetičkim elementima. Ukidanje terapije tetraciklinima ne isključuje pojavu rezistencije u kratkom vremenskom intervalu, zbog uticaja raznih faktora sredine i načina ishrane životinja (antibiotici kao dodaci hrani). Rezistencija na tetracikline najčešće je ustanovljavana kod izolata *Esherichia coli* poreklom od svinja, ćuraka i pilića, a proteini TetM i TetK kod meticilin rezistentnih sojeva *Staphylococcus aureus*-a.

Ključne reči: mehanizmi rezistencije, tetraciklini, *Escherichia coli*, *Staphylococcus aureus*

INTRODUCTION

The tetracyclines are one of the oldest antibiotics that are used in livestock industry. They have been discovered in 1948, and have been used under generic name chlortetracycline and oxytetracyline. Over the years tetracycline antibiotics have been improved and marketed under various trade names. The

range of activities for these antibiotics is very broad encompassing therapy of infections caused by Gram-negative and Gram positive bacteria but also chlamydiae, mycoplasmas, rickettsiae and protozoan parasites. Importantly, they are also used as growth promoters of farm animals worldwide. It is not surprising that resistance to these antibiotics has spread in various bacterial communities. Their mode of action requires inhibition of bacterial protein synthesis by preventing association of the aminoacyl t-RNA to the ribosome A site (Chopra and Roberts, 2001).

Resistance to tetracycline has been developed utilizing various mechanisms but the most prominent is the efflux pump driven by the proton motive force. Such efflux proteins belong to the major facilitator superfamily (MSF) of transporters. Bacteria can produce ribosomal protection proteins and over two decades ago, enzymatic inactivation of tetracycline has been discovered as well (Speer et al., 1991). Detection of the respective genes encoding resistance to tetracycline raises a possibility to analyze epidemiological aspects of the resistance and to discover mobile genetic elements that may have contributed in dissemination of the tetracycline resistance genes (*tet*) in nature. Indeed the *tet* resistant genes are found on mobile genetic elements the transposones. This is the case with the *tet*(A) gene that was found on transposone Tn*1721* while the *tet*M gene was detected on Tn*916* (Frech and Schwarz, 1999; Chopra and Roberts, 2001). Even though Tn*1721* is non-conjugative transposone it is inserted in conjugative plasmids and this is facilitating dissemination of the *tet* genes in the environment. On the other hand the Tn*916* transposone is conjugative so it could be self transferable.

Mechanisms of resistance

Efflux pump

The efflux proteins belong to a large group of transmembrane proteins encoded by *tet* genes. Based on sequence amino acid identity *tet* genes are placed in six groups (Chopra and Roberts, 2001). The *tet* genes belonging to the group 1, are most abundant and represented by *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), tet(H), tet(I), tet(J), tet(K), tet(L), tet(V), tet(Y), tet(Z), *tet*(30), *tet*(31), *tcr*3, *otr*(B), *tet*P(A). The amino acid identity between these proteins is 41 to 78% and for the repressor protein it is 37 to 88%. The TetZ protein is found also in Gram positive bacteria, but all other proteins from the group 1 belong to Gram-negative bacteria (Chopra, 2002). The structure of Tet proteins has been studied and it is evident that they consist of 12 predicted transmembrane

α-helices with nonconserved central loops connecting α-helicis 6 and 7. The efflux proteins are inserted in the lipid bilayer and their hydrophilic amino acid loops are protruding into the periplasm and cytoplasm. Efflux proteins utilize the energy by exchanging a proton for a tetracycline cation complex against a concentration gradient (Chopra and Roberts, 2001). Namely, upon entry to the bacteria cell by diffusion process tetracycline are releasing proton (H+) and become increasingly receptive for divalent cations (the Me+). The monovalent cationic reactive complex strives to binds to repressor - operator complex due to its own instability. This process disables the repressor binding to DNA by triggering conformational changes in the repressor protein. Even very small amount of tetracycline's are sufficient for separation of the TetR from the DNA. This mechanism leads to the constitutive expression of the *tet* genes encoding efflux proteins. In the absence of antibiotic, the repressor protein TetR negatively regulates *tet* gene expression by binding to the operator (TetO). It has been noted that tetracycline-metal complex present one of the most sensitive effector-inducible systems of transcriptional regulators. (Hillen and Barrens, 1994). In Gram negative bacteria two or more efflux genes may be commonly responsible for resistance to tetracycline while in Gram positive bacteria, the efflux mechanism is often coupled with the ribosomal protection mechanism which gives opportunity for development of new generation of drugs aiming at both resistance targets (Chopra, 2002).

Ribosomal protection proteins

Ribosomal protection proteins (RPP) are soluble cytoplasmic proteins and they are protecting ribosome from the tetracycline including doxycycline and monocycline. The best studied mechanisms of the RPP are encoded by *tet*(M) and *tet*(O) genes. TetM proteins induce release of tetracycline from the ribosome by using the energy from GTP hydrolysis. The analysis of the upstream and downstream sequence of the structural gene and GC content (<40%) of the tetM gene supports the postulate that *tet*(M) gene may have originated from Gram positive bacteria and indicate that these genes are been transferred to Gram negative microorganisms (Chopra and Roberts, 2001). TetO proteins are changing the architecture of the ribosome inducing alterations on tetracycline binding site. These alterations are transient but mechanisms involved to prevent rebinding of the tetracycline to the ribosome are presumptive. Two interesting postulates, yet to be experimentally proven, relay on a possibility that Tet proteins promote aa-tRNA binding to the A site or that Tet(O) acts successively before aa-tRNA binding. Even though the conformational

changes at the primary binding site of the tetracycline have been recognized, it is to be answered how ribosome's returns and sustains elongation cycles in the presence of tetracycline antibiotics (Connell et al., 2003).

The *tetM* gene was most frequently found in methicillin resistant *Staphylococcus aureus* (MRSA) from 24 clinical isolates, the *tetK* gene was represented by 21 isolate and the *tetKM* genotype was found in 21 isolates (Trzcinski et al., 2000). In their research, for the first time, a single *tetK* gene in heterogeneous MRSA isolates from Poland was discovered. It was established that all isolates possessing *tetM* gene are resistant to all tetracycline's while *tetK* gene does not induce resistance to monocycline. A high level of resistance to tetracycline's (MIC > 128 mg/L) was obtained when *tetKM* genes were present in *Staphylococcus aureus* isolates comparing to isolates with single determinants. It was elucidated that preincubation with subinhibitory concentration of tetracyclines or monocyclines (except for *tetK* positive isolates where preincubation with monocycline only slightly increased MIC) induced higher MICs in MRSA isolates hence involving improved screening method (the preincubation with antibiotics before MIC analysis).

The abundance of *tet*(M) genes in the collection of clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) was documented in the research of Schmitz et al. (2001). The *tetM* gene was detected in 76% of MRSA isolates while *tetK* gene was present in 73% of the isolates. Both genes the *tetM* and *tetK* were present in half of the MRSA isolates. They have revealed that in methicilin susceptible/tetracycline resistant *S. aureus* (MSSA) the *tet*(K) gene was the most frequently found (in 96% of isolates), while tetM gene was detected in 10% of isolates. In MSSA isolates combination of *tet*(M) and *tet*(K) genes were represented at 6%.

Enzymatic inactivation and other mechanisms of resistance to tetracyclines

TetX protein is responsible for the enzymatic inactivation of tetracyclines. The tet(X) gene encoding the relevant protein was found on transposon of the strict anaerobes of the genus *Bacterodies*. Less research was directed to reveal the actual presence of *tet*(X) genes in nature and because of that the *Bacteroides* group is the only, yet to be known, species having *tetX* genes. In their natural host, the *tetX* gene is probably not functional since it requires oxygen and NADPH to chemically modify tetracycline. There are some unknown mechanisms conferring low level of resistance to tetracycline's encoded by *tet*(U) gene. The sequence of the *tet*(U) gene is however not similar to

other sequences obtained for T*et* determinants and it is difficult to reveal their mechanisms of resistance. The *otr*(C) gene was found in *Streptomycetes*, but the whole sequence was not obtained and the resistance mechanisms are not yet explained (Chopra and Roberts, 2001).

Presence of tet resistance genes in humans and animals

Resistance to tetracycline is widely distributed and occurs because of the application of the antibiotics in human and veterinary medicine. This type of resistance is often found in multi drug resistant isolates and often is the most prevalent resistance in commensal and clinical isolates (Miles et al., 2006). It is not surprising that single resistance to TET is rare among family of *Enterobacteriaceae* (Dolejska et al., 2009). The clinical impact on resistance to tetracycline was estimated during the tigecycline phase 3 clinical trials. Patients all over the world were enrolled in the clinical trial. The incidence of resistance to tetracycline in *E. coli* was 39%. Multiple efflux pump determinants were represented by 33% of isolates. It was concluded that resistance rate to tetracycline depend on its presence in the environment, excessive use of biocides and genetic transfer of resistance elements among bacterial species (Tuckman et al., 2007). Cross selection of resistance to tetracycline occurs in patients treated with other antibiotics, but it is important to note that resistance to tetracycline in *E. coli* from stool specimens of infants that have not received any antibiotic was similar to the *E. coli* from children that were treated with β-lactam antibiotics. Colonization capacity of the TET^r versus susceptible strains of *E. coli* in infants was similar showing that resistance traits was not related to treatment with antibiotics. Rather it was influenced by the presence of virulence determinant, presumably the P fimbriae and aerobactin. Hence the colonizing capacity and invasiveness of the bacteria really on virulence factors in infantile intestinal microbiota (Karami et al., 2006). A longitudinal study on antimicrobial drug resistance in *E.coli* isolates from humans and food animals in the USA has revealed that the most prevalent was resistance to tetracycline. Also this type of resistance was commonly found with the resistance to streptomycin, sulfonamide, ampicillin and chloramphenicol (Tadesse et al., 2012). The withdrawal of antibiotics especially those that have been used as growth promoters, has led to a moderate decrease in resistance rate. So was the case when application of antimicrobial agents was terminated for subtherapeutic use in specific pathogen free Yorkshire herd of pigs, held at the Kentucky Agricultural Experiment Station Research farm in the USA. Decrease of resistance to tetracycline in lactose positive enteric bacteria was 82 to 24%, but this decrease did not show steady trends after 126 months of withdrawal, since

resistance to tetracycline remains at the level of 40% (Langlois et al., 1983). Resistance to tetracycline's was the most commonly found by Miles et al. (2006) in *E. coli* isolates from humans (in 43.8% of isolates) and poultry (in 82.4% of isolates) in Jamaica. The cross resistance of tetracycline with kanamycin and nalidixic acid was limited to avian isolates, while isolates from humans were frequently cross resistant to the aminoglycosides, ampicillin and quinolones. The *tetB* and *tetD* genes, encoding the active efflux proteins, were detected on transferable plasmids in *E. coli*. Authors have discussed that *E. coli* from humans and poultry in Jamaica may not have a common source because they have different resistance patterns. The levels of resistance to tetracycline's was tested on 1263 isolates of *E. coli* from humans, domestic and wild animals in a research work of Bryan et al. (2004). The highest resistance was found in chickens, turkeys and pigs while other species like goats, horses, ducks, geese and deer have shown low level of resistance. The multiplex PCR assay aimed to detect *tet* resistant genes was performed in isolates having MIC to TET ≥ 93 mg/L. The obtained MIC represented high resistance to tetracyclines and presumably offer a possibility of resistance gene detection. In total 325 strains were analyzed by PCR and 97% have been found to contain one or more *tet* resistance genes. The distribution of genes was within the following order: *tetB* gene was detected most abundantly i.e. it was found in 63% of isolates, *tetA* gene was detected in 35% of isolates followed with the various frequencies of *tetC*, *tetD* and *tetM* gene detection. The *tetM* gene was found in *E. coli* from pigs and chickens only. Tetracycline genes in pair were found in 30% of isolates originated from turkey, pigs and horses while 4.5% of isolates from pigs have carried three *tet* resistant genes. The presence of several genes comparing to one *tet* resistant determinant was not correlated to the MIC values.

Even though it is a well established opinion that treatment of humans and animals with antibiotics select for antimicrobial resistance in pathogenic and commensal bacteria, there is a plausible lines of evidence that even in circumstances when antibiotics are not provided on farms or in cases when long withdrawal period of antibiotic use was established, resistance to tetracycline's and/or ampicillin is still documented. This is explained by the interchange of mobile genetic elements among bacteria that present the normal flora of the gastrointestinal tract of animals, age of animals and the diet provided. Another important fact is that resistance to some antibiotics coselects resistance to other and this is usually related to transferable nature of resistance mechanisms common in some bacterial species such as *E. coli* (Mirzaagha et al., 2011).

CONCLUDING REMARKS

The application of antibiotics for the therapy and for growth promotion in livestock industry is the main reason for resistance development in commensal and pathogenic bacteria. In human medicine problems occur if antibiotic treatment is required in life threatening diseases caused by multiresistant bacteria that are difficult to treat with standard antibiotics. The prominent examples now days are resistance of *Staphylococcus aureus* to vancomycin, but also resistance to metallo-β-lactamases of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* as well as *Enterobacter* spp. and *Klebsiella* spp. from the hospital environment. In developed countries patients infected with such microorganism are isolated in special units and this practice has shown to be adequate to restrict spreading of multiple resistant bacteria in the hospital settings (Levy and Marshall, 2004). Because of the broad antimicrobial activity of tetracycline's it is important to continue developing new generation of antibiotics, such as glycylcyclines, competent to efflux and ribosomal protection mechanisms, for treatment of infections caused by Gram positive and Gram negative bacteria (Chopra, 2002).

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