Tetracyclines in veterinary medicine and bacterial resistance to them

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ABSTRACT: Since their discovery in 1945, tetracyclines have been used extensively in the therapy and prophylaxis of infectious diseases and as growth promoters. These wide applications have led to the equally fast spread of tetracycline resistant strains of gram-positive and gram-negative bacterial genera, including strains belonging to pathogenic as well as nonpathogenic species. Nonpathogenic bacteria could act as a reservoir of resistance determinants, which can be disseminated by horizontal transfer into pathogens. More than thirty different tetracycline resistance genes have been characterized. They encode two major mechanisms of resistance: 1 – active efflux of the antibiotic, and 2 – protection of ribosomes. Further mechanisms of tetracycline resistance include enzymatic inactivation of antibiotic, permeability barriers, mutations or multidrug transporter systems. Effective horizontal spread is favoured by the location of tetracycline resistance genes on mobile genetic elements such as plasmids and transposons. Their exchange, enhanced by the use of tetracyclines, is observed between bacteria of the same or different species and genera as well. Thus, questions of reevaluating and global reducing of tetracyclines in human and animal healthcare and food production are extensively discussed.

Keywords: tetracycline resistance; *tet* genes; efflux pump; ribosomal protection; transposon; plasmid

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1. Introduction

Tetracyclines are broad-spectrum agents, which exhibit their activity against a wide range of gram-positive and gram-negative bacteria, chlamydia, mycoplasmas, rickettsiae and protozoan parasites (Katiyar and Elend, 1991; Chopra *et al.*, 1992; Roberts, 1996). They were the first major group of antibiotics to which the term "broad-spectrum" was ascribed (Roberts, 1997). Therefore, they have been extensively used in the therapy of human and animal infections, for prophylactic purposes in animals and plants and for growth promotion in food animals (Levy, 1992; IOM, 1998). Shortly after the discovery of tetracyclines, resistance to them has been detected. *Shigella dysenteriae* was the first tetracycline resistant bacterium to be discovered and was isolated in 1953 (Watanabe, 1963; Falkow, 1975). Since that time, a wide range of tetracycline resistant bacterial strains has been identified. Tetracycline resistance determinants can be found in the genomes of the physiological flora from humans, animals as well as from food and environmental sources. These bacteria can act as a reservoir of resistance genes, transfer these genes to the pathogenic genera and that leads to the increasing problems of the treatment of infectious diseases (Chung *et al.*, 1999a,b).

2. History and classification of tetracyclines

Tetracyclines were first discovered in 1945 (Duggar, 1948). Chlortetracycline and oxytetracycline were the first members of the tetracycline group to be described (Chopra and Roberts, 2001). They were produced by *Streptomyces aureofaciens* and *S. rimosus*, respectively. In the following years, other naturally occurring tetracycline molecules were discovered, e.g. demethylchlortetracycline from *S. aureofaciens* and tetracycline from *S. viridofaciens*. Subsequently, a number of important semisynthetic tetracyclines were developed, e.g. methacycline, doxycycline, minocycline, rolitetracycline, lymecycline and the most recently produced glycylcyclines (Goldstein *et al.*, 1994). All these compounds above mentioned belong to the first class of tetracycline antibiotics, also referred to as "typical tetracyclines". They exhibit bacteriostatic activity by means of interacting with bacterial ribosomes and blocking of the protein synthesis (Sum *et al.*, 1998). The second class, known as "atypical tetracyclines", includes chelocardin, anhydrotetracycline, anhydrochlortetracycline and thiatetracycline and these exhibit bactericidal activity by targeting the cytoplasmic membrane (Oliva *et al.*, 1992; Chopra, 1994). These compounds are of no interest for the therapy due to their low-level inhibition of the protein synthesis and their cytotoxicity and they are not licensed to be used in the European Union and in the Czech Republic (EMEA, 1999).

3. Applications of tetracyclines in veterinary medicine

Tetracyclines exhibit the activity against a broad spectrum of pathogenic microorganisms; they are well absorbed, exhibit low toxicity and are relatively inexpensive (Moellering, 1990; Standiford, 1990). These attributes led to the wide use of tetracycline antibiotics in the therapy of human and animal bacterial and non-bacterial infections as well as for the prophylaxis of infections in food animals and pets. World production of tetracyclines is estimated to be in thousands of tonnes per year. The amount of tetracyclines used in the therapy in animals was quantified as 2 294 tonnes in the European Union in 1997 (Boatman/FEDESA, 1998), or as 3 000 and 3 200 tonnes in the U.S. in 2000 and 2001, respectively (AHI, 2002).

Tetracyclines are widely used in veterinary medicine mainly for the treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis (Prescott *et al.*, 2000).

For use in veterinary medicine, tetracycline preparations registered in the European Union and in the Czech Republic contain the following substances: tetracycline, doxycycline, chlortetracycline and oxytetracycline (EMEA, 1999; AISLP, 2003). Target animal species for the application of these preparations are beef cattle, pig, sheep, goat, horse, dog, cat, poultry, rabbit and fish.

Oral preparations of tetracyclines cannot be administered to ruminants for reasons of the destruction of the ruminal microflora and the attenuation of the digestive processes. Risk of the disruption of the gut flora exists also in horses (Cook, 1973). As a result of the intramuscular application of tetracyclines, swellings can occur (Immelman *et al.*, 1978). Rapid intravenous administration of tetracyclines

can result in cardiovascular dysfunction and collapse in any species (Gyrd-Hansen *et al.*, 1981). Medication with tetracycline antibiotics is also contraindicated in pregnant and young animals, because tetracyclines form chelates with calcium on the surface of teeth and bones, which results in the discoloration of teeth and in a retarded development of the skeleton (Moffit *et al.*, 1974). Another adverse tetracycline drug reaction is increased photosensitivity, mainly in animals with low level of skin pigmentation but it is of particular importance (Segal, 1963). The above mentioned limitations are also valid for the medication of humans and tetracyclines cannot be used for the treatment of pregnant women and young children (Standiford, 1990).

Subtherapeutic amounts of tetracyclines are used in certain countries as feed additives for the growth promotion in animal husbandry, e.g. in calves, chickens, turkeys, sheep and pigs (Dupont and Steele, 1987; Schnappinger and Hillen, 1996; Schwarz *et al.*, 1998). Growth-promoting properties of tetracyclines were first discovered in 1949 for chickens being fed chlortetracycline supplemented feed (Stockstad *et al.*, 1949). Subsequently, they were widely applied in animal husbandry thanks to improving the growth rate to feed intake ratio (IOM, 1998; Anonym, 1999; JETACAR, 1999). Positive growth effects of subtherapeutic doses of tetracyclines on humans were also demonstrated (Snelling and Johnson, 1952; Scrimshaw *et al.*, 1954; Jolliffe *et al.*, 1956). In 1969, the Swann report published in Great Britain, recommended the exclusion of the antimicrobial agents from animal feed that were used in human and/or animal therapy (Swann, 1969). Numerous studies described the effect of long-term usage of subtherapeutic doses of tetracyclines resulting in the increased level of resistant gut bacteria or pathogens (Smith and Tucker, 1975; Hooper and Hirsh, 1977; Langlois *et al.*, 1984; Hinton *et al.*, 1985). In contrast to the United States, the application of tetracyclines as growth promoters is not allowed neither in the European Union nor in the Czech Republic (Council Directive 70/524 EEC, 1970; Prescott *et al.*, 2000), and since 1975, no tetracycline has been used for the growth promotion in Europe (Schwarz and Chaslus-Dancla, 2001).

During the last few years, activity of tetracyclines against the infective prion protein PrP^{Sc} was investigated. According to the study of Forloni *et al.* (2002), tetracyclines interact directly with PrP^{Sc} and render it susceptible to the proteolytic degradation, which is accompanied by the reduction in the prion infectivity.

Consumption of tetracycline antibiotics in veterinary medicine is relatively high as compared with other classes of antibiotics. Tetracyclines rank the first position in consumption statistics in the European Union and in the Czech Republic. According to the FEDESA (European Federation for Animal Health) sources, the consumption of tetracyclines in 1997 has reached the volume of 2 294 tonnes, that represented 66% of the total volume of antibiotics used for the therapy in animals in the European Union and Switzerland (Boatman/FEDESA, 1998; IFAH, 1999). During the period 2000 to 2002, the consumption of tetracycline antibiotics in the Czech Republic increased continuously from 22 093 kg in 2000 to 24 132 kg in 2001 and finally to 30 229 kg in 2002 (Monitoring USKVBL, 2000, 2001, 2002).

4. Mode of action

Tetracyclines permeate through the bacterial cell wall by the passive diffusion and through the cytoplasmic membrane by an energy-dependent process (Franklin and Snow, 1971; Yamaguchi *et al.*, 1991; Tsankov *et al.*, 2003). In contrast to mammalian cells, cells of most bacterial species actively concentrate them (Chopra *et al.*, 1992). Antibacterial activity of typical tetracyclines is associated with the reversible inhibition of the protein synthesis (Laskin, 1967; Kersten and Frey, 1972). Binding of the drug to the ribosome prevents the attachment of the aminoacyl-tRNA to the "A site" of the ribosome. Tetracyclines bind directly to the 30S-subunit protein S7 (Goldman *et al.*, 1983), other ribosomal proteins (S3, S14, and S19) are also involved (Franklin, 1966; Buck and Cooperman, 1990). Some bases in the 16S-rRNA, e.g. G_{693}, A_{892}, U_{1052}, C_{1054}, G_{1300} and G_{1138}, are also important for the binding of the tetracyclines to the ribosomes (Chopra *et al.*, 1992).

5. Mechanisms of tetracycline resistance

Three different tetracycline resistance mechanisms have been described: (1) active efflux of the antibiotic (Franklin and Snow, 1971), (2) ribosomal protection (Burdett, 1986), as the most common resistance mechanisms, and (3) enzymatic inactivation

of the drug (Speer *et al.*, 1991). All these mechanisms are based on the acquisition of one or several tetracycline resistance determinants, which are widely distributed among bacterial genera (Schnappinger and Hillen, 1996). Additionally, mutations in the rRNA, multidrug transporter systems or permeability barriers may be involved in the resistance to several antibiotics including tetracyclines.

Thirty-three different tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes have been characterized to date (Roberts, 2003). In this regard, a *tet* gene is considered as a new *tet* gene when its gene product shows less than 80% amino acid identity to any so far known Tet protein (Levy *et al.*, 1999). There is no essential difference between the *tet* and *otr* genes, but oxytetracycline resistance genes were first described in oxytetracycline producing organisms, which is reflected by the nomenclature (Ohnuki *et al.*, 1985; Doyle *et al.*, 1991).

The tetracycline resistance genes, known to date, the mechanism of resistance conferred by each gene, the distribution among gram-positive or gram-negative bacteria and the GenBank accession numbers for the known sequences are summarized in the Table 1.

Efflux pump coding determinants are represented by twenty-one *tet* genes: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(K), *tet*(L), *tetA*(P), *tet*(V), *tet*(Y), *tet*(Z), *tet*(30), *tet*(31), *tet*(33), *tet*(34), *tet*(35), *tcr*3; and by one *otr* gene: *otr*(B). Ten of *tet* genes: *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *tetB*(P), *tet*(32), *tet*(36), *tet*; and one *otr* gene: *otr*(A) code for ribosomal protection proteins (Roberts, 2003). Two genes, *tet*(X) and *tet*(37), code for enzymes which inactivate tetracyclines (Speer *et al.*, 1991; Diaz-Torrez *et al.*, 2003). Mechanisms of *otr*(C) and *tet*(U) encoded resistance are unknown (Levy *et al.*, 1999).

5.1 Active efflux

Efflux of tetracycline is mediated by energy-dependent efflux pumps. Their proteins are encoded by: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(K), *tet*(L), *tet*(Y), *tet*(30), *tet*(31), *tet*(34), *tet*(35) in gram-negative bacteria and by: *tet*(K), *tet*(L), *tetA*(P), *tet*(V), *tet*(Z), *tet*(33), *tcr*3 or *otr*(B) in gram-positive bacteria. Efflux proteins, (approximately 46 kDa), located in the cytoplasmic membrane, exchange a proton for a monocationic magnesium-tetracycline complex. They work as antiporters and thus reduce the amount of the antibiotic in the cytoplasm (Sum *et al.*, 1998).

There are differences between five groups of efflux pumps proteins:

Group 1 includes Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(Z) and probably Tet(I), Tet(J) and Tet(30) and these proteins consist of 12 transmembrane α-helices. With the exception of Tet(Z), they are exclusively found in gram-negative bacteria (Roberts, 1996). Compared to the others, Tet(B) of gram-negative genera is effective against tetracycline as well as against semisynthetic derivatives as minocycline (Mendez *et al.*, 1980; Chopra *et al.*, 1992). Tet(I) has not been sequenced, but phenotypic studies suggest it encodes an efflux pump (Jones *et al.*, 1992a). The protein Tet(33) found in gram-positive *Corynebacterium glutamicum* revealed amino acid sequence homology to the group 1 tetracycline efflux systems, especially to Tet(Z) (Tauch *et al.*, 2002).

Group 2 is represented by Tet(K) from *Staphylococcus aureus* (Mojumdar and Khan, 1988) and Tet(L) from *Bacillus subtilis* (McMurry *et al.*, 1987). Primarily found in gram-positive bacteria, they were occasionally identified in gram-negative genera and also among anaerobes (Miranda *et al.*, 2003; Roberts, 2003).

Group 3 includes Otr(B) and Tcr3, both found in *Streptomyces* spp. (Ohnuki *et al.*, 1985; Dairy *et al.*, 1995). Group 2 and 3 are characterized by 14 membrane-spanning α-helices (Levy, 1992; Guay *et al.*, 1993).

Group 4 includes TetA(P) from *Clostridium* spp. with 12 transmembrane α-helices (Sloan *et al.*, 1994).

Group 5 includes Tet(V) from *Mycobacterium smegmatis* (De Rossi *et al.*, 1998; Chopra and Roberts, 2001). Tet(V) differs highly from the other efflux proteins of gram-positive bacteria. It is highly hydrophobic with at least 10 transmembrane-spanning α-helices. It contains some motifs typical for members of the major facility class (MFS) of efflux proteins, which confer multi-drug resistance in *Streptococcus pyogenes* and *Mycobacterium tubeculosis* or *M. fortuitum* (De Rossi *et al.*, 1998).

Although the Tet(34) protein is rated among efflux pumps proteins, its deduced amino acid sequence shows a significant homology to xanthine-guanine phosphoribosyltransferases (XGPRTs) that catalyze the synthesis of GMP, XMP, and IMP from guanine, xanthine, and hypoxanthine, respectively, and thus supply the purine nucleotides for the translation.

The protein Tet(34) has probably similar function consisting in the activation of the Mg^{2+} dependent purine nucleotide synthesis, finally resulting in the protection of the protein synthesis. The gene *tet*(34) was localized on the chromosome in an oxytetracycline resistant isolate of *Vibrio* spp. (Nonaka and Suzuki, 2002).

The gene *tet*(35) was discovered on the plasmid pATJ1 of *Vibrio harveyi* and was suggested to code for a new putative efflux pump protein with 9 transmembrane α-helixes. In 2003, *tet*(35) was for the first time described in *Stenotrophomonas maltophilia* (Miranda *et al.*, 2003)

The regulation of *tet* gene expression differs in gram-positive and gram-negative bacteria. In gram-negative bacteria, each determinant consists of two genes coding for an efflux protein and a repressor protein, both regulated by tetracycline. They are originated divergently and share the central regulatory region (Hillen and Berens, 1994). In the absence of tetracycline, the repressor protein TetR binds to the operator of the structural efflux gene and thus blocks its transcription. Induction occurs when the Mg^{2+}-tetracycline complex formed in the cell binds to the repressor and conformation changes of the repressor lead to its release from the operator allowing the transcription of the structural efflux gene. The repressor binds again to the operator if the intracellular amount of tetracycline decreases (Roberts, 1996).

In case of the gene *tet*(35), this was found to be transcribed convergently with the second gene in the opposite direction, designed *txr* and coding for the protein Txr, a putative transcriptional regulator. However, subsequent analysis has revealed that Txr did not act in this way and was probably only required to interact with Tet(35) and thus to enable its function (Teo *et al.*, 2002).

Efflux coding genes *tet*(K) and *tet*(L) of gram-positive bacteria seem to be regulated by a process referred to as translational attenuation (Schwarz *et al.*, 1992). In contrast, *tet*(Z) from *Corynebacterium glutamicum* is the first example of a repressor-regulated *tet* gene found in gram-positive bacteria (Tauch *et al.*, 2000).

5.2 Ribosomal protection

Ribosomal protection is the second most important mechanism of the tetracycline resistance in bacteria and has been discovered first in streptococci (Burdett, 1986). Ribosome protective proteins ensure the resistance to tetracycline, doxycycline as well as to minocycline. These proteins are approximately 72.5-kDa cytoplasmic proteins structurally similar to elongation factors EF-Tu and EF-G and also having a ribosome-dependent GTPase activity (Sanchez-Pescador *et al.*, 1988; Taylor and Chau, 1996). Ribosomal protection proteins might confer resistance by means of the reversible binding to the ribosome (Schnappinger and Hillen, 1996).

Genetic determinants can be divided into three groups based on the amino acid sequence of encoded proteins. Group 1 includes: *tet*(M), *tet*(O), *tet*(S) and *tet*(W); group 2: *tetB*(P) and *otr*(A), and group 3 is represented by: *tet*(Q) and *tet*(T) (Chopra and Roberts, 2001).

The gene *tet*(32) was identified in the *Clostridium*-related human colonic anaerobe bacterium K10, which also carried *tet*(W) (Melville *et al.*, 2001).

The gene *tet*(36) was originally identified in *Bacteroides* spp. strain 139 from swine manure pits and its presence than confirmed in other attending anaerobes (Whittle *et al.*, 2003). The encoded protein shares the most important sequence homology with the protein Tet(Q).

5.3 Enzymatic inactivation

Up to 2003, the gene *tet*(X) was the only example of tetracycline resistance due to the enzymatic modification and inactivation of the antibiotic. This gene codes for a 44-kDa cytoplasmic protein that chemically modifies tetracycline in the presence of oxygen and NADPH (Speer *et al.*, 1991). This gene has been discovered on two *Bacteroides* transposons, Tn*4351* and Tn*4400*, and has been found to share considerable amino acid homology with a number of NADPH-requiring oxidoreductases (Speer *et al.*, 1991). Recently, a novel gene, *tet*(37), was isolated as a part of the oral metagenome (Diaz-Torrez *et al.*, 2003). Similarly to *tet*(X), this gene also codes for an enzyme inactivating tetracycline requiring the presence of NADPH, but no sequence homology has been observed between the proteins Tet(37) and Tet(X).

5.4 Mutations, multidrug transporters and permeability barriers

In addition to the specific mechanisms of tetracycline resistance encoded by tetracycline resistance

Table 1. Tetracycline resistance determinants

Gene	Protein	Mechanism of resistance	Distribution	GenBank Accession No. of complete gene sequences*
tet(A)	Tet(A)	efflux	G^-	X00006, X75761, AJ313332 , AF502943, AJ307714
tet(B)	Tet(B)	efflux	G^-	V00611, J01830, AF223162, AJ278685, AB084245, AB089585, AB089586, AB089587, AB089588, AB089589, AB089590, AB089591, AB089592, AB089593, AB089594, AB089595
tet(C)	Tet(C)	efflux	G^-	AB023657, AB089596, AB089597, AB089598, AJ132716, J01749, Y19114
tet(D)	Tet(D)	efflux	G^-	AB089599, AB089600, AB089601, AB089602, D16172, L06798, X65876, Y19115
tet(E)	Tet(E)	efflux	G^-	L06940, Y19116
tet(G)	Tet(G)	efflux	G^-	AB089603, AF119247, AF133139, AF133140, S52437, Y19117, Y19118
tet(H)	Tet(H)	efflux	G^-	AJ245947, AJ487672, AJ487674, U00792, Y15510, Y16103
tet(I)	Tet(I)	efflux	G^-	unsequenced
tet(J)	Tet(J)	efflux	G^-	AF038993
tet(K)	Tet(K)	efflux	G^-, G^+	M16217, S67449
tet(L)	Tet(L)	efflux	G^-, G^+	AY081910, D00006, D26045, M11036, M34478, U17153, X08034, X60828, X51366
tet(M)	Tet(M)	ribosomal protection	G^-, G^+	AB039845, AF491293, M21136, M85225, U08812, U58985, U58986, X04388, X56353, X75073, X90939, X92947
tet(O)	Tet(O)	ribosomal protection	G^-, G^+	AY190525, M18896, M20925, Y07780
tetA(P)	TetA(P)	efflux	G^+	AB001076, AB054981, AB054983, L20800
tetB(P)	TetB(P)	ribosomal protection	G^+	AB001076, L20800
tet(Q)	Tet(Q)	ribosomal protection	G^-, G^+	L33696, U73497, X58717
tet(S)	Tet(S)	ribosomal protection	G^+	L09756
tet(T)	Tet(T)	ribosomal protection	G^+	L42544
tet(U)	Tet(U)	unknown	G^+	U01917
tet(V)	Tet(V)	efflux	G^+	AF030344

tet(W)	Tet(W)	ribosomal protection	G^-, G^+	AF202986, AJ222769, AJ421625, AJ427421, AJ427422
tet(X)	Tet(X)	enzymatic inactivation	G^-	M37699
tet(Y)	Tet(Y)	efflux	G^-	AB089604, AB089605, AB089606, AB089607, AB089608, AF070999
tet(Z)	Tet(Z)	efflux	G^+	AF121000
tet(30)	Tet(30)	efflux	G^-	AF090987
tet(31)	Tet(31)	efflux	G^-	AJ250203
tet(32)	Tet(32)	ribosomal protection	G^+	AJ295238
tet(33)	Tet(33)	efflux	G^+	AF164956, AJ420072
tet(34)	Tet(34)	efflux (XGPRT)	G^-	AB061440
tet(35)	Tet(35)	efflux	G^-	AF353562
tet(36)	Tet(36)	ribosomal protection	G^-	unsequenced
tet(37)	Tet(37)	enzymatic inactivation	unkown	AF540889
otr(A)	Otr(A)	ribosomal protection	G^+	X53401
otr(B)	Otr(B)	efflux	G^+	AF061335, AF079900, M20370
otr(C)	Otr(C)	unknown	G^+	unsequenced
tet	Tet	ribosomal protection	G^+	M74049
tcr3	Tcr3	efflux	G^+	D38215

*Related references are accessible in the NCBI (National Center for Biotechnology Information) http://www.ncbi.nlm.nih.gov/

genes, other usually multidrug-resistance mechanisms can contribute more or less to the resistance to tetracyclines in certain bacterial genera. These common mechanisms include mutations, permeability barriers or multidrug transporter systems.

5.4.1 Mutations

In 1998, a mutation in the 16S-rRNA that conferred the resistance to tetracycline in gram-positive bacterium *Propionibacterium acnes* was discovered (Ross *et al.*, 1998). This mutation consisted in the change of a single base (G → C) at the position cognate with *Escherichia coli* 16S-rRNA base 1058. The base is localized in a conserved region called helix 34, which is potentially involved in the peptide chain termination and the translation accuracy (Moine and Dahlberg, 1994).

Another mutation in the 16S-rRNA was revealed in *Helicobacter pylori* strains showing a high-level resistance to tetracycline. Identical triple base-pair substitution ($AGA_{926\text{-}928}$ → TTC) (corresponding to the *E. coli* 16S-rRNA bases 965 to 967) located in the primary binding site of tetracycline was uncovered by several studies (Gerrits *et al.*, 2002; Trieber and Taylor, 2002). Recently, other substitution mutations of this triple-nucleotide at the same position in the 16S-rRNA have been detected: AGA → GTA or GGC; and AGA → GGA or AGC as the examples of double or single base change, respectively. However, these single and double substitutions mediated only low-levels of tetracycline resistance (Dailidiene *et al.*, 2002; Gerrits *et al.*, 2003).

5.4.2 Multidrug transporters

Multidrug transporters play an important role in the tetracycline resistance almost in gram-negative bacteria. On the basis of the energetic criteria, they can be divided into two classes separating multidrug transporters utilizing a proton motive force (PMF) for the exudation of drugs from the cell, and ATB binding cassette (ABC) multidrug transporters that gain the energy for the efflux from the ATP hydrolysis (Paulsen *et al.*, 1996a; Putman *et al.*, 2000). Within the class of PMF transporters, distinct families of proteins have been distinguished: the major facilitator superfamily (MFS) (Marger and Saier, 1993), the small multidrug resistance (SMR) family (Paulsen *et al.*, 1996b), the resistance-nodulation-cell division (RND) family (Saier *et al.*, 1994) and the multidrug and toxic compound extrusion (MATE) family (Brown *et al.*, 1999).

The EmrE multidrug transporter (also known as MvrC) of *Escherichia coli* was originally identified on the basis of its ability to confer resistance to ethidium bromide and methyl viologen (Purewal, 1991; Morimyo *et al.*, 1992). The EmrE protein is member of SMR family which unifies small efflux proteins (about 170 aminoacids) that function as drug/proton antiporters and export drugs to the periplasmic space (Nikaido, 1998). The EmrE protein is proposed to function as homotrimer (Yerushalmi *et al.*, 1996) and its overproduction through the multicopy plasmid carrying the gene *emrE* results in the low-level resistance to tetracycline and several other antibiotics (Ma *et al.*, 1994).

The AcrAB efflux system of gram-negative bacteria belongs to the RND family of multidrug transporters. The members of this family interact with a membrane fusion protein (MFP) and an outer membrane protein and this complex thus allows the efflux of drugs across the inner (cytoplasmic) and outer membrane into the surrounding medium (Putman *et al.*, 2000). The MFP proteins probably induce the fusion of the inner and the outer membrane, and thus form a channel-like structure through the periplasmic space (Zgurskaya and Nikaido, 1999). The *acrAB* locus consists of two genes: the gene *acrA*, coding for a MFP protein and the gene *acrB*, coding for a 12-transmembrane α-helix RND protein (Dinh *et al.*, 1994; Zgurskaya and Nikaido, 1999). Together with the membrane protein, TolC, they form the AcrAB efflux system (Fralick, 1996). The AcrAB efflux system of *E. coli* is essentially regulated by the global regulator locus *marRAB* (multiple-antibiotics resistance) (Ma *et al.*, 1995). The overexpression of the *acrAB* locus in *E. coli* occurs in case of mutation in *marR* (coding for the MarR repressor protein of the *marRAB* locus) or under the stress conditions affecting the cells (e.g. the presence of tetracycline in the environment), generally when the overproduction of the global activator protein MarA is induced (Hachler *et al.*, 1991). In addition to the positive regulation of the *acrAB* operon, the MarA protein down-regulates the synthesis of the major porin OmpF, through the increased production of the antisense RNA, encoded by *micF* and that leads to the decreased accumulation of tetracycline (Cohen *et al.*, 1988, 1989).

In *Pseudomonas aeruginosa*, resistance to a variety of antimicrobial agents is conferred by the synergy between low permeability of the outer membrane and several RND multidrug efflux systems, of which MexAB-OprM (Poole, 1993; Li *et al.*, 1994, 1995b) and MexCD-OprJ (Masuda *et al.*, 1995, Poole *et al.*, 1996) contribute to the resistance to tetracycline. They both consist of three components: MexA or MexC (periplasmic MFP proteins), MexB or MexD (inner membrane RND proteins) and OprM or OprJ (outer membrane channel proteins) and they pump drugs from the cytoplasm into the medium.

Homologs of AcrAB and MexAB-OprM systems are widespread among gram-negative bacteria, e.g. the MtrCDE system in *Neisseria gonogrhoeae* (Hagman *et al.*, 1995), or a multidrug efflux system in *Haemophilus influenzae* (Sanchez *et al.*, 1997).

In many bacterial species, various multidrug transporters contributing to different levels of tetracycline resistance have been identified: LmrP, a major facilitator superfamily (MFS) protein, or LmrA, an ABC multidrug transporter, both found in *Lactococcus lactis* (Bolhuis *et al.*, 1994, 1995); MdfA, a MFS protein of *E. coli* (Edgar and Bibi, 1997); Tap and LfrA proteins, both members of MFS proteins found in *Mycobacterium* spp. (Liu *et al.*, 1996; Ainsa *et al.*, 1998); YkkCD, a SMR family protein of *Bacillus subtilis* (Jack *et al.*, 2000).

5.4.3 Permeability barriers

The outer membrane of gram-negative bacteria represents the first effective barrier to the various compounds and thus plays a role in the antimicrobial resistance. Porins, the major outer membrane proteins, form channels in the outer membrane and allow the nonspecific passage of small polar molecules, amino acids or nutrients (Nikaido, 1994). The rapid passage of tetracycline into the cell occurs preferentially via the OmpF protein (outer membrane protein F) and in the magnesium-bound form of tetracycline. Whereas, in the porin-defficient cells the influx of the drug is slow, mainly in its uncharged form (Thanassi *et al.*, 1995). Thus, the decreased level of OmpF synthesis (e.g. in *mar* mutants, cells under stress conditions) leads to the increased level of tetracycline resistance (Cohen *et al.*, 1988). In addition to the decreased number of porin channels in the outer membrane, several studies have revealed mutations and amino acid changes that influence the structure and the function of porin (De *et al.*, 2001; Olesky *et al.*, 2002).

6. Methods of determination of bacterial resistance to tetracyclines

Phenotypic antimicrobial susceptibility testing can be performed reliably by either dilution or diffusion methods (Jorgensen *et al.*, 1999).

6.1 Dilution method

Dilution test results in quantitative MIC (Minimal Inhibitory Concentration) value, in micrograms per milliliter, and this is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (NCCLS, 2002a). The dilution method is based on the inoculation and growth of the microorganism in media containing different concentrations of an antimicrobial agent. This procedure can be carried out by the agar-based or the broth-based method and the concentration range used depends on the antimicrobial drug and on the microorganism tested. Result can be reported as quantitative MIC value (in μg/ml) and/or as the classification of the microorganism into categories: susceptible, intermediate, or resistant, based on the interpretive standards (Jorgensen *et al.*, 1999; NCCLS, 2002b). Thus, at the same time MIC value enables us to quantify the dose of the antimicrobial drug required for the effective therapy (Schlegelova and Rysanek, 1999).

According to the MIC interpretive standards, recommended by NCCLS (2000a,b), microorganisms other than streptococci are considered to be resistant to tetracycline if MIC ≥ 16 μg/ml, intermediate in case of MIC = 8 μg/ml and susceptible if MIC ≤ 4 μg/ml. For streptococci, strains exhibiting MIC values ≥ 8 μg/ml are considered as resistant, those with MIC values = 4 μg/ml intermediate and strains showing MIC values ≤ 2 μg/ml as susceptible.

The E-test (AB Biodisk, Solna, Sweden) is another method for the quantitative determination of antimicrobial susceptibility. It uses plastic-coated strips with a predefined gradient of an antimicrobial agent, which are applied onto the agar medium inoculated with a microorganism (Baker *et al.*, 1991; Jorgensen *et al.*, 1999). The MIC is directly read from the scale of the strip, where the boundary of the suppressed

growth of the microorganism intersects the strip. Several strips containing different antimicrobial agents can be placed onto the surface of one large plate (Jorgensen *et al.*, 1999).

6.2 Disk diffusion method

Disk diffusion test results in qualitative information about the susceptibility of the microorganism (Schlegelova and Rysanek, 1999). For this test, commercially prepared paper disks impregnated with a defined amount of antibacterial agent are used. The amount for each antimicrobial agent in the disk is standardized (NCCLS, 2000b). This method is based on the diffusion of the drug from the disk and the creation of the concentration gradient in the agar medium surrounding the disk. In the area where the concentration of the drug is inhibitory, no growth is observed. Disks are applied onto the surface of the agar medium inoculated with a microorganism and after the incubation the diameter of the zone with suppressed growth is measured (Bauer *et al.*, 1966; NCCLS, 2000b).

According to the interpretive standards, recommended by NCCLS (2000a; 2002b), microorganisms other than streptococci are considered as resistant to tetracycline if the diameter of the zone of growth inhibition is ≤ 14 mm, as intermediate if zone diameter is between 15 and 18 mm, and as susceptible if the zone diameter is ≥ 19 mm when using a disk charged with 30 μg of tetracycline. Streptococci exhibiting a zone diameter ≤ 18 mm are considered as resistant, those with a zone diameter from 19 to 22 mm as intermediate, and those showing a zone diameter ≥ 23 mm as susceptible.

6.3 Genetic methods for the detection of antimicrobial resistance genes

Genetic methods may confirm the presence of specific genes conferring tetracycline resistance, however, the presence of genes alone does not necessarily mean resistance of the microorganism, as it is possible (although unlikely) that resistance genes may not be expressed. Genetic methods can be fast and it is possible to use them directly on clinical specimens (Tenover and Rasheed, 1999). The most used methods is PCR (polymerase chain reaction) with specific primers for specific resistance genes; although DNA hybridization, using specific labeled molecular probes is another method for detecting resistance genes. Multiplex PCR using several pairs of primers for several different resistance genes in a single reaction may allow the detection of more than one resistance genes at a time (Warsa *et al.*, 1996; Ng *et al.*, 2001).

7. Localization of tetracycline resistance determinants in the genome and their transfer

Tetracycline resistance determinants are localized either on the chromosome or on conjugative or non-conjugative plasmids. Many *tet* genes are associated with non-conjugative or conjugative transposons and these in turn can be located on plasmids or on the chromosome (Roberts, 1994). The mobile nature of many of the tetracycline resistance genes can in part explain their wide distribution among many bacterial species (Roberts, 1996).

7.1 Active efflux determinants of gram-negative bacteria

Efflux genes of gram-negative bacteria are widely distributed and usually associated with large plasmids which belong to different incompatibility groups (Mendez *et al.*, 1980; Jones *et al.*, 1992b; Roberts, 1996). Tetracycline resistance genes are frequently part of transposons, which are able to change their location within the cell, and achieve increased mobility by inserting into conjugative plasmids, e.g. Tn*10*-located *tet*(B) (Coleman *et al.*, 1983; Sherburne *et al.*, 2000) found on the conjugative plasmids of *Actinobacillus* (Roe *et al.*, 1995) or *Aeromonas* (Rhodes *et al.*, 2000), Tn*1721*-located *tet*(A) (Allmeier *et al.*, 1992) and a Tn*1721*-analogous transposon integrated into the 47-kb conjugative plasmid pGFT1 in *Salmonella enterica* (Frech and Schwarz, 1998).

Tn*10* and Tn*1721* are examples of non-conjugative bacterial transposons of Class 1 and Class 2, respectively. Class 1 transposons (composite transposons) are characterized by the presence of direct or inverted copies of insertion sequences (ISs) at the ends, that supplies the transposition functions, whereas the members of the Class 2 (complex transposons) transposons are flanked by inverted repeats (30–40 bp) and the transposition encoded in the middle (Schoffl *et al.*, 1981).

The Tn*10* element (9.1-kb) carries nine substantial open reading frames (ORFs), including the tetracycline resistance determinant *tet*(B), and is flanked by the inverted repeats of IS*10* (Chalmers *et al.*, 2000). IS*10*-Right is fully functional insertion element that encodes a transposase, which mobilize either the insertion sequence or the whole transposon. Tn*10* moves in the genome by the mechanism of conservative transposition (Sakai *et al.*, 1995). During this process, number of element copies is conserved; the transposon leaves the original replicon and moves to another. The level of the transposition is reduced by several mechanisms, e.g. by the IS*10*-encoded small antisense RNA hybridizing to the mRNA for the transposase; by the preferential *cis* action of the transposase namely on the hemi-methylated DNA, that limits the transposition to the very short period after the replication (Mahillon and Chandler, 1998).

The Tn*1721* transposon (11.1-kb) is flanked by terminal 38-bp inverted repeats, a 38-bp internal repeat separates the transposon into two parts, one of which contains the genes required for transposition and the other with the tetracycline resistance gene *tet*(A) (Schoffl *et al.*, 1981; Allmeier *et al.*, 1992). Contrary to Tn*10*, it transposes always as a unit and by the replicative mechanism resulting in the increased number of copies of the transposon.

Large plasmids of gram-negative bacteria often carry various antibiotic resistance determinants (in addition to heavy metal resistance determinants and/or toxin coding genes), and thus confer the multidrug-resistant phenotype. Having studied the conjugal transfer of these plasmids, the resistance genes were observed to be transmitted as a cluster. They were demonstrated to be localized on the integron (Tosini *et al.*, 1998).

Integrons are genetic elements capable of capturing and disseminating resistance genes and thus conferring the antibiotic resistance, especially in gram-negative bacteria. They carry a site-specific recombination system that recognizes and captures resistance genes assembled as mobile gene cassettes (Hall and Collis, 1995). Gene cassette is generally defined as a single gene or open reading frame coupled with a downstream 59-bp recombination site (Hall *et al.*, 1991). Integrons generally consist of the gene *intI* (coding for the integrase), the gene *sulI* (conferring the resistance to sulfonamides) and of the attachment site, *attI*, where the gene cassettes integrate. The intergase mediates the integration of a gene cassette by the site-specific recombination between the *attI* site of the integron and the 59-bp element of the gene cassette, called *attC* site. The fragment *intI-attI* is highly conserved in all integrons and is called 59-CS (Sabate and Prats, 2002). Gene cassettes can exist free in circular form, but do not contain functions for their mobility and replication (Collis and Hall, 1992). In addition, the genes within the cassettes are promoterless. They can be only transcribed being integrated in an integron, namely from a common promotor P_c (Collis and Hall, 1995). Integrons are not self-transposable, but they are often associated with conjugative plasmids (Tosini *et al.*, 1998) and transposons that mobilize them, e.g. transposons Tn*21* and Tn*7* (Sundstrom *et al.*, 1991; Liebert *et al.*, 1999).

Conjugation is thought to be the most common way of the spread of antibiotic resistance among bacteria. Conjugation can be performed via conjugative plasmids or conjugative transposons (Roberts, 2003). This process requires the cell-to-cell contact that reaches to the transfer of the genetic material from the donor through the mating channel to the recipient. The transfer apparatus ensuring the conjugation process is encoded by the *tra* gene complex of the self-transmissible plasmid or conjugative transposon of the donor cell (Bennet, 1995).

The gene *tet*(H) was originally found on the plasmid pVM111 of an avian strain of *Pasteurella multocida* (Hansen *et al*., 1993) but subsequently, it was also localized on the chromosome (Hansen *et al.*, 1996). In 1998, Kehrenberg and coworkers discovered a transposon-like element, Tn*5706*, carrying a copy of *tet*(H) flanked by the insertion sequences IS*1596* and IS*1597*. This 4.3-kb element was localized on the 6.8-kb plasmid pPMT1 of *Pasteurella multocida* (Kehrenberg *et al.*, 1998) and its truncated copy on the plasmid pPAT1 of *P. multocida* and *P. aerogenes* (Kehrenberg and Schwarz, 2000). In the isolates of *Pasteurella* spp. and *Mannheimia* spp., the *tet*(H) gene was identified to be carried by a small plasmid, designed pMHT1, as well as by the chromosome but only chromosome located *tet*(H) was part of the complete element Tn*5706* (Kehrenberg *et al.*, 2001). Recently, new bacterial genera, *Moraxella* spp. and *Acinetobacter* spp., were dicovered to carry the *tet*(H) gene as part of Tn*5706* element. Although, in *Moraxella* spp. isolates, the gene was flanked by a smaller insertion element, designed IS*1599* (Miranda *et al.*, 2003).

The gene *tet*(E) differs from the others because it is associated with large plasmids which are neither mobile nor conjugative. That may explain its lim-

ited distribution among prokaryotes (DePaola and Roberts, 1995). The gene *tet*(E) has been also found in the chromosomal DNA (Lee *et al.*, 1993).

Genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(Y), *tet*(30) and *tet*(31) are exclusively found in gram-negative genera (Roberts, 1996).

Other *tet* genes have been identified in gram-negative bacteria: *tet*(Y) in *Echerichia coli*; *tet*(30) in *Agrobacterium* spp.; *tet*(31) in *Aeromonas* spp. (Levy *et al.*, 1999).

7.2 Active efflux determinants of gram-positive bacteria

Tetracycline resistance genes involved in active efflux and found in gram-positive bacteria include *tet*(K), *tet*(L), *tetA*(P), *tet*(V), *tet*(Z), *tet*(33), *tcr*3 and *otr*(B). These genes are often associated with small mobilizable plasmids.

The 4.45-kb plasmid pT181 from *Staphylococcus aureus* is considered the prototype *tet*(K) plasmid. It is generally found in number about 20 per cell and belongs to the incompatibility group inc3 (Khan and Novick, 1983). Plasmids of pT181-family are closely similar in size and structure and can be differentiated by restriction mapping (Schwarz and Noble, 1994). pT181-like plasmids have also been detected either integrated in the large plasmids or in the chromosome. They were always flanked by directly repeated insertion sequences of the type IS*257* (Needham *et al.*, 1994; Werckenthin *et al.*, 1996), a small (0,79 kb) mobile genetic element originally detected in *S. aureus* (Rouch and Skurray, 1989).

The gene *tet*(L) was found to be commonly located on small *Bacillus* plasmids. Plasmids carrying *tet*(L) are more variable in sizes and occasionally contain additional resistance genes (Schwarz and Noble, 1994). Chromosomal *tet*(L) gene of *Bacillus subtilis* is the exception (Stasinopoulos *et al.*, 1998). In 1992, Schwarz *et al.* (1992) described the *tet*(L)-carrying plasmid pSTE1 in *Staphylococcus hyicus*. In 1996, *tet*(L) was found to be carried by naturally occurring plasmid pSTS7 of *Staphylococcus epidermidis* (Schwarz *et al.*, 1996). The gene *tet*(L) is the second most prevalent tetracycline resistance gene in streptococci and enterococci (Poyart-Salmeron *et al.*, 1992).

Small *tet*(K) or *tet*(L)-carrying plasmids are not self-transmissible but can be transmitted into the recipient cells by the conjugation by means of conjugative plasmids or conjugative transposons co-resident in the same cell (Naglich and Andrews, 1988). These provide their conjugative apparatus and mobilize small plasmids either in *trans* or in *cis*. In case of *trans* mobilization, plasmid contains the *mob* locus, coding for the Mob protein, a relaxase, that recognizes the origin of transfer (*oriT*) on the plasmid where it nicks the plasmid DNA to a single strand and thus initiates the transfer. This variant of mobilization, called donation, results in the copies of only the mobilized plasmid in the transconjugant (Projan and Archer, 1989). In the *cis* case, the plasmid is transferred as a cointegrate with a conjugative element (it results from the integration of the conjugative transposon into the plasmid or of the small plasmid into the conjugative plasmid) which provides all the transfer functions and carries along the small plasmid when it transfers its own DNA during the conjugation (Needham *et al.*, 1994; Salyers *et al.*, 1995b). This model of mobilization, called conduction, results in transconjugants carrying copies of both elements (Projan and Archer, 1989). The transfer of the genetic material into the recipient occurs in the form of ssDNA (single-stranded DNA) where the complementary strand is synthesized (Roy, 1999).

The gene *tet*(Z) has been discovered on the 19-kb plasmid pAG1 of *Corynebacterium glutamicum*. Sequence analysis has revealed a homology to gram-negative efflux determinants and the highest level of amino acid similarity to Tet(A) has been observed (Tauch *et al.*, 2000).

The gene *tet*(33) was found on the 27.8-kb plasmid pTET3 from *Corynebacterium glutamicum* where it was flanked by identical copies of the widespread insertion sequence IS*6100* (Tauch *et al.*, 2002). The amino acid sequence of the encoded protein Tet(33) has shown a homology to the group 1 of tetracycline efflux systems, with the highest similarity to Tet(Z), also found in the same species.

7.3 Ribosomal protection determinants

Eight *tet* genes, *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *tetB*(P), *tet*; and one of *otr* genes, *otr*(A), code for the ribosomal protection mechanism of tetracycline resistance. They are generally associated with conjugative transposons, which have preference for the chromosome (Roberts, 1997). They are considered to be of gram-positive origin, but nowadays, they are often found in a variety of gram-negative species (Roberts, 1996).

The gene *tet*(M) is the most widely distributed tetracycline resistance gene in gram-positive bacteria (Roberts, 1996). It was first identified in *Streptococcus* spp. (Burdett, 1986), and subsequently, it has been described in a large number of gram-positive and gram-negative bacteria, mycoplasmas and ureoplasmas, as well (Roberts, 1994). The *tet*(M) gene is frequently associated with conjugative transposons of the Tn*916*-Tn*1545* family (Clewell *et al.*, 1995; Roberts, 1996) which often carry additional antibiotic resistance genes. According to the study of Schmitz *et al.* (2001), *tet*(M) is the most prevalent single tetracycline resistance determinant in MRSA (methicillin-resistant *Staphylococcus aureus*). The majority of *tet*(M)-positive *S. aureus* isolates also carry *tet*(K) and so MRSA isolates are typically of *tet*(M) or *tet*(K,M) genotype (Bismuth *et al.*, 1990).

The *tet*(Q) gene comes from *Bacteroides* spp., but it can be expressed in both gram-positive and gram-negative species (Nikolich *et al.*, 1992). It is also associated with conjugative transposons. *Bacteroides* conjugative transposons are large (> 60 kb) elements which often carry erythromycin resistance gene *erm*(F) in addition to *tet*(Q) (Li *et al.*, 1995a; Chung *et al.*, 1999a,b).

Conjugative transposons, e.g. conjugative transposons from *Bacteroides* spp. or conjugative transposons of the Tn*916*–Tn*1545* family, are mobile elements, which excise themselves to form covalently closed circular intermediates (excision seems to be Rec-dependent). After that, they can reintegrate into the genome of the same cell (intracellular transposition) or transfer by the conjugation to new recipient cells and integrate into their genomes (intercellular transposition) (Salyers *et al.*, 1995a). Conjugative transposons were first described in gram-positive cocci (Franke and Clewell, 1981). Due to their broad host range, they can transfer to the variety of gram-positive and gram-negative bacteria and thus spread various resistance determinants. They have been shown to co-transfer mobilizable plasmids as well as unlinked genomic DNA, e.g. segments of 10–12 kb in *Bacteroides* spp., called NBUs (non-replicating *Bacteroides* units) (Shoemaker *et al.*, 1993), namely within species as well as across species (Roberts, 1996).

Transfer frequencies of Tn*916*–Tn*1545* family as well as *Bacteroides* conjugative transposons can be significantly enhanced *in vitro* and *in vivo* by subinhibitory concentrations of tetracycline in the culture medium (Doucet-Populaire *et al.*, 1991).

The *tet*(O) gene is mobile only when located on conjugative plasmids and normally it is not associated with conjugative transposons (Roberts *et al.*, 1991). It was originally described in *Campylobacter jejuni* (Taylor *et al.*, 1987) and *Campylobacter coli* (Sougakoff *et al.*, 1987), but was also found in gram-positive bacteria such as streptococci, where both plasmid and chromosomal locations were described (Brown and Roberts, 1991).

The *tet*(P) gene is known from the 47-kb conjugative plasmid pCW3 of *Clostridium perfringens* (Sloan *et al.*, 1994). It consists of two genes overlapping by 17 bp: *tetA*(P), which codes for a putative 46-kDa efflux protein with 12 transmembrane domains, and *tetB*(P), coding for a putative 72.6-kDa ribosomal protection protein (Sloan *et al.*, 1994). The gene *tetA*(P) has been found alone without *tetB*(P) but not vice versa (Lyras and Rood, 1996).

The *tet*(S) gene is originally known from a *Listeria monocytogenes* plasmid (Charpentier *et al.*, 1993) but subsequently, it has been found on the chromosome of *Enterococcus faecalis* (Charpentier *et al.*, 1994; Francois *et al.*, 1997) and on the conjugative plasmid of *Lactococcus* spp. (Perreten, 1997).

The *tet*(T) gene has been discovered as a novel branching order in *Streptococcus pyogenes* A498 by Clermont and coworkers in 1997. It has been located on the chromosome and the Tet(T) protein has been found to be the most closely related to the protein Tet(Q) with 49% homology of the amino acid sequence (Clermont *et al.*, 1997).

The *tet*(W) gene has been originally identified in *Butyrivibrio fibrisolvens* isolate from the bovine rumen, subsequently found in the *Clostridium*-related human fecal anaerobe bacterium K10 and in human *Fusobacterium* and *Bifidobacterium* isolates and recently frequently isolated in bacteria from the human oral cavity (Scott *et al.*, 1997, 2000; Villedieu *et al.*, 2003). It was shown to be located on the conjugative transposon Tn*B1230* (Barbosa *et al.*, 1999).

The *otr*(A) gene was originally discovered on the chromosome of oxytetracycline producing *Streptomyces rimosus* (Ohnuki *et al.*, 1985; Doyle *et al.*, 1991). Subsequently, it has been found in *Mycobacterium* spp. and other *Streptomyces* spp. (Pang *et al.*, 1994).

7.4 Unknown resistance mechanism

The *tet*(U) gene was described to be located on a 1.9-kb plasmid of *Enterococcus faecium* and the

amino acid sequence of the encoded protein Tet(U) is unrelated to those of tetracycline efflux proteins or tetracycline ribosome protective proteins (Ridenhour *et al.*, 1996). The Tet(U) protein has no GTP-binding motif and appears to be very different in size and similarity to ribosomal protection proteins (Clermont *et al.*, 1997). It confers only low-level tetracycline resistance (Chopra and Roberts, 2001).

The *otr*(C) gene comes from *Streptomyces* spp., its nucleotide sequence has not been determined (Ohnuki *et al.*, 1985; Chopra and Roberts, 2001).

8. Conclusion

Long-term usage of tetracyclines in the therapy and also in the subtherapeutic doses for the prophylaxis or as growth promoters in animal feed exerting the permanent selective antimicrobial pressure on their bacterial flora is considered to be the greatest risk for the selection of resistance (Anonym, 1999). Since the appearance of the first tetracycline resistant bacteria, a wide variety of tetracycline resistance determinants have been discovered in various microorganisms. Despite the enormous amount of data published on tetracycline resistance during the last two decades, still not all mechanisms of tetracycline resistance are fully understood and further work is required to elucidate them. Horizontal transfer of resistance determinant is a dynamic process that occurs frequently in the nature and is stimulated by the antimicrobial selective pressure resulting in the maintenance of resistant strains in different bacterial populations. However, not entirely is still known what about the measure and the exact paths of this transfer, and where it exactly occurs. Another important question is the fate of tetracyclines in the environment and their role in the evolution and selecting tetracycline resistant environmental bacteria. Methods based on molecular biology have become the key instrument, which could possibly clarify this unknown.

Monitoring systems in the European countries and in the other countries over the world were established due to the necessity to know the actual situation about the use of antimicrobial agents and the incidence of resistant microorganisms. These data help to evaluate the potential risks for the human health and propose the solutions to the related problems. Nowadays, a general reduction of the application of antibiotics and their elimination as growth promoters in food animals are supposed to be useful for the reduction of the spread of the resistance even if not immediately. Tetracyclines, and antimicrobial agents generally, are widely used and irreplaceable for the therapeutic purposes in humans and animals and will be ever used. The main question and the problem is that if their use has ever been justified.

Since 1986 in Sweden, the ban on growth promoting agents was followed by the other countries in the European Union, and the general reduction of the amount of antimicrobials used in the animals has been observed during last two decades (IFAH, 2002).

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