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Trichomonas vaginalis –

Efficacy of Pentamycin and Studies on the Mode of Action

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1 Introduction

1.1 *Trichomonas vaginalis*

1.1.1 History

The invention and fast improvement of microscopes in the end of the 16th, 17th and 18th century opened new possibilities for the detection of and research on microorganisms. This is associated with famous names like Janssen, van Leeuwenhoek and Hook, which paved the way with their new optical constructions for further research on bacteria and also protists.

In 1773, Otho Fridericus Müller was the first, who isolated a trichomonad, which was much later identified by Dobel as *Trichomonas tenax*, a commensal of the human oral cavity. In 1836, Alfred François Donné first described *T. vaginalis* and named it, following an advice from Félix Dujardin, *Trichomonas*, because of its similarity to the genera “Tricodes” and “Monas” (Powell, 1936; Thorburn, 1974; Walochnik, 2002). Approximately at that time Hausmann recognized that trichomonads could be found only in some women with unusual genital conditions, but never in genitally healthy women (Schmid and Kamnicker, 1926). The German protologist Christian Gottfried Ehrenberg established the full name, giving credit to Donné for his discovery. The first staining experiments on *Trichomonas* were performed by Hoffman and Weigert with carmine and fuchsin (Thorburn, 1974).

In 1916, Ottomar Hoehne described the clinical picture in women and he also introduced the term “trichomoniasis” or “Trichomonadenkolpitis” (Jirovec and Petru, 1968; Harp and Chowdhury, 2011). About 14 years later the first report of *T. vaginalis* infection in men was given by Capek (Lanceley and Mcentegart, 1953; Liston and Lees, 1940). In 1940, Trussell and Plass obtained the first axenic culture in a medium of blood agar, sugars, milk and brain broth and they also demonstrated trichomonal infection by transplants (Hesseltine et al., 1942). In another study, male volunteers were infected with *Trichomonas* to proof that infection in man was also possible (Lanceley and Mcentegart, 1953). Even the famous German biologist Ernst Haeckel described trichomonads (*T. intestinalis*) in

his ground breaking work “Kunstformen der Natur” namely as a spindle-shaped parasite of the vertebrate intestine (Walochnik, 2002).

In the 1960s and 70s of the last century several biochemical tests were established and microscopic examinations performed to investigate growth characteristics and behavior of *T. vaginalis*. Later, research mainly focused on immunology and pathogenesis of this parasite. In 2007, the *T. vaginalis* genome sequencing project revealed the whole genome and thereby paved the way for numerous new findings (Harp and Chowdhury, 2011).

1.1.2 Classification

Already the ancient Greek philosophers attempted a classification of the living things, particularly Aristoteles from Thracia (384-322 B.C.) concerning the animals (Historia Naturalium) (Lennox, 1980) and Theophrastos from Eresos (371-282 B.C.) concerning the plants (Historia plantarum) (Schaffner, 1934; Thanos, 1994).

In the beginning of the 18th century, Georg A. Goldfuss was the first who used the term “Protozoa” (Greek: proto= first/early zoa=animals) for a group including Infusoria, Lithozoa, Phytozoa and Medusinae. Carl Theodor von Siebold introduced the invertebrate phylum protozoa within the kingdom Animalia, including the Infusoria and Rhizopoda (Scamardella, 1999). Carl Linnaeus divided the living things into three kingdoms (Animalia, Plantae and Minerals) and in the middle of the 19th century, amongst others, John Hogg (1860) and also Richard Owen claimed an additional kingdom. Owen arranged Amorphozoa, Foraminifera or Rhizopods, Polycystinae, Diatomacea, Desmidiaceae, Gregarinae and most of the Polygastra of Ehrenberg in this kingdom. Hogg, also classifying multi- and unicellular organisms in the kingdom, claimed to rename the group “Primigenal Kingdom” and coined the term “Protoctista” containing Protozoa and Protophyta. In 1866, Ernst Haeckel introduced the term “Protista” (“the first of all, primordial”) for a third kingdom besides animals and plants in “Generelle Morphologie der Organismen” (General morphology of organisms) and he also excluded the (kingdom) minerals from the living things. The kingdom Protista contained the phyla Diatomaceae, Flagellata, Myxomycetes, Noctilucae, Protoplasta, Rhizopoda, Spongiae and the Monera (bacteria and some slime molds). It thus included

unicellular and multicellular, non-nucleated and nucleated organisms, Prokaryota and Eukaryota. Haeckel also proposed to integrate the Infusoria into the animal kingdom until Otto Butschli (1873) showed their unicellularity (Imam, 2009; Scamardella, 1999).

In 1998, Cavalier-Smith reduced the number from eight to six kingdoms and divided the eukaryotic domain into five kingdoms (Cavalier-Smith, 1998). The infrakingdom Excavata within the kingdom eukaryotic Protozoa contains the Parabasalia (Cavalier-Smith, 2003; Hampl et al., 2009). The common characteristics of this superclass are the absence of classical mitochondria, a parabasal apparatus (a Golgi complex with parabasal fibres), a microtubular axostyle–pelta complex, nuclear division by cryptopleuromitosis and most of these flagellates lacking the ability to form cysts (Noda et al., 2009; Cepicka et al., 2010). Within this group, the class Trichomonadea is characterized by a single karyomastigont with 2-6 flagella and by the absence of a comb-like structure and an infrakinetosomal body. This class contains the order Trichomonadida with four families including the Trichomonadidae. This family within the parabasalia is characterized by a unique B-type costa, five flagella and an undulating membrane (Cepicka et al., 2010). Besides the genera *Pentatrichomonas*, *Tetratrichomonas*, *Pseudotrichomonas* and *Cochlosoma* the family contains also the genus *Trichomonas*. It is one of the most important in this family concerning its medical and veterinary relevance. The genus comprises the well-known human parasites *T. vaginalis*, penetrating the human urogenital tract and *T. tenax*, a commensal of the oral cavity. *T. hominis* is a commensal of the human gut, but it is also referred to as *Pentatrichomonas hominis*. It potentially causes diarrhoea in human as well as in dogs and cats (Compaore et al., 2013; Nielsen et al., 2012). Trichomonal species are also known from other animals, e.g. *T. equibuccalis* (horse), *T. anatis* (duck), *T. gallinae* (birds), *T. pavirovi* (cattles), *T. buttreyi* (swines), *T. microti* (rodents), *T. canistoimae* (dogs), *T. felistome* (cats), *T. macacovaginae* (monkey) and *T. phasianii* (pheasants) (Alderete and Chang, 2011; Pennycott, 1996).

The phylum and all its subordinated ranks are subjected to regular changes due to reordering processes and upcoming new phylogenetic models and phylogenetic

programs (Cepicka et al., 2010; Adl et al., 2007; Cavalier-Smith, 2012) . A revised and comprehensive classification is given in Tab. 1.

Tab. 1 New classification scheme of *Trichomonas* after Cavalier-Smith (Cavalier-Smith, 2012).

<u>Phylum Loukozoa</u> Cavalier-Smith, 1999 em.
<u>Subphylum Metamonada</u> Grassé, 1952 em. Cavalier-Smith, 2003
<u>Infraphylum Trichozoa</u> Cavalier-Smith, 1997 em. 2003 stat. n.n
<u>Superclass Parabasalia</u> Honigberg, 1973 stat. n. Cavalier-Smith, 2003
<u>Class Trichomonadea</u> Kirby, 1947 stat. n. Margulis, 1974 em
<u>Subclass Eotrichomonadida</u> subcl. n.
<u>Order Trichomonadida</u> Kirby, 1947
<u>Suborder Trichomonadina</u> subord. n.
<u>Family Trichomonadidae</u> Chalmers and Pekkoloa, 1918 sensu Hampl
e.g. <i>Trichomonas</i> , <i>Pentatrichomonas</i> , <i>Cochlosoma</i>

1.1.3 Morphology

T. vaginalis is a unicellular protist with only a trophozoite and no known cyst form in its life cycle. The parasite's morphology depends on environmental conditions, in particular pH, temperature, iron availability as well as the presence of human vaginal epithelial cells (VEC) (Benchimol, 2004) and other parameters (De Jesus, et al. 2007; Lecker, et al. 1991).

In axenic culture, *T. vaginalis* seems to be more pear shaped and oval with 7 to 23 µm in length and 5 to 15 µm in width, but when attached to VEC it appears in a rather amoeboid form (Arroyo et al., 1993; Lal et al., 2006). Under adverse environmental conditions the parasite rounds up and internalizes the flagella. This reaction is similar to that of other Parabasalia living in the urogenital tract e.g. *Tritrichomonas foetus* (Pereira-Neves et al., 2003). These degenerative forms are also supposed to be pseudocystic forms with no real cyst wall and high levels of immobility (Petrin et al., 1998; Afzan and Suresh, 2012). According to another theory, there are also differences between pseudocysts and trophozoites in flagella location, polymerization status of axostyla and costa, and an divergent mitotic process (Pereira-Neves et al., 2003).

In culture under normal conditions *T. vaginalis* has five flagella, four at the front and the fifth as part of the undulating membrane, which is supported by the costa (Gerbod et al., 2000) (Fig. 1). Besides this B-type costa another

trichomonadidal-specific structure is the axostyle, a microtubule formed rod originating at the pelta and ending up at the posterior end in a sharp tip. The axostyle is supposed to play an important role in the attachment to VEC. Axostyle and pelta are built up of a line of connected microtubules, which are also coupled with the v-shaped parabasal bodies, the analogues to the Golgi apparatus, and attached to other structures like the basal bodies via parabasale, striated fibres (Mowbrey and Dacks, 2009; Benchimol, 2004; Benchimol et al., 2001; Brugerolle and Viscogliosi, 1994; Amos and Grimston, 1968).

In the cell plasma there are different granules with varying functions, for example glycogen granules, chromatic granules (hydrogenosomes) and paracostal granules (Kim et al., 2006; Lindmark and Muller, 1973; Honigberg and King, 1964; Benchimol et al., 1996). Hydrogenosomes replace the function of the absent mitochondria in *Trichomonas* and have an essential role in energy metabolism and drug resistance (Benchimol, 2009). In the anterior part there is the nucleus enveloped in a porous nuclear membrane with rough endoplasmic reticulum (Benchimol, 2004).

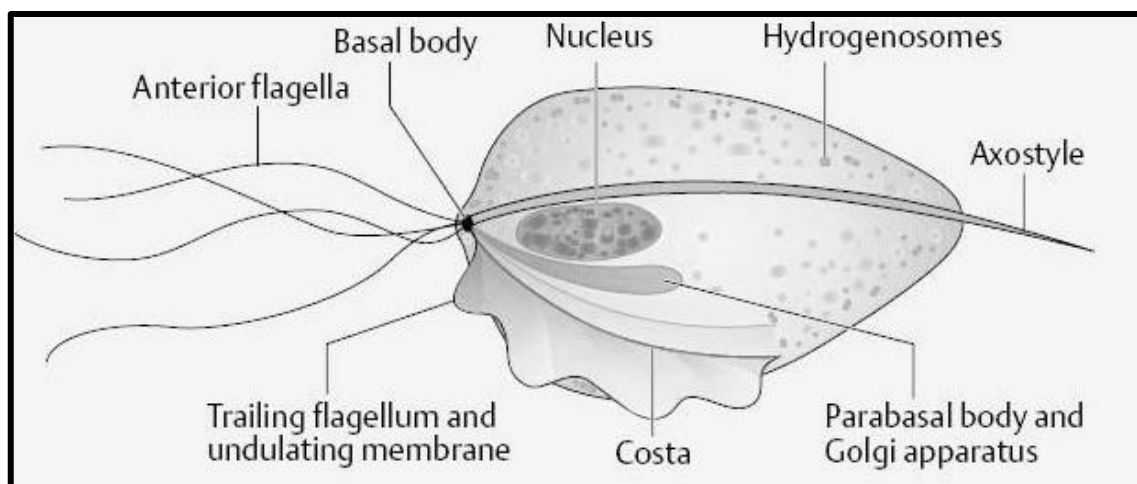


Fig. 1 Morphology of *Trichomonas vaginalis* (<http://www.hakeem-sy.com/main/node/31747>).

1.1.4 Metabolism

Although *T. vaginalis* is a primitive eukaryote and has several eukaryotic characteristics, the parasite also has various metabolic similarities to anaerobic bacteria for example a carbohydrate metabolism, which performs fermentation under anaerobic and aerobic conditions.

During glycolysis, glucose is converted into phosphoenolpyruvate in the cytoplasm and via the Emden-Meyerhof-Parnas pathway further into pyruvate. There are several interesting enzymes concerning their origin and function along this pathway. In the trichomonal genera there is an unusual hexokinase, which is closely related to those of proteobacterial/cyanobacterial lineages (Wu et al., 2001; Henze et al., 2001). *T. vaginalis* also has a pyrophosphate-dependent phosphofructokinase (PFP), a pyruvate-phosphate dikinase (PPDK) instead of a phosphofructase (PFK) and the enzyme pyruvate:ferredoxin oxidoreductase used in oxidative pyruvate decarboxylation (PFOR) instead of the mitochondrial pyruvate dehydrogenase (Benchimol, 2009).

Energy is generated by substrate-level-phosphorylation, but in *Trichomonas* there is also another pathway to generate ATP via an arginine dihydrolase (ADH) pathway by removing nitrogen from arginine similar to the vertebrate urea cycle (Morada et al., 2010; Morada et al., 2011; Linstead and Cranshaw, 1983) (Fig. 2). ATP is also produced during phosphate level phosphorylation by conversion of succinyl-CoA into succinate by the enzyme succinyl-CoA synthase (SCS, succinate thiokinase) as back reaction in acetate:succinate CoA transferase (ASCT)-mediated acetate production in the hydrogenosomes (Bringaud et al., 2010; Tielens et al., 2010; van Grinsven et al., 2008; Steinbuchel and Muller, 1986).

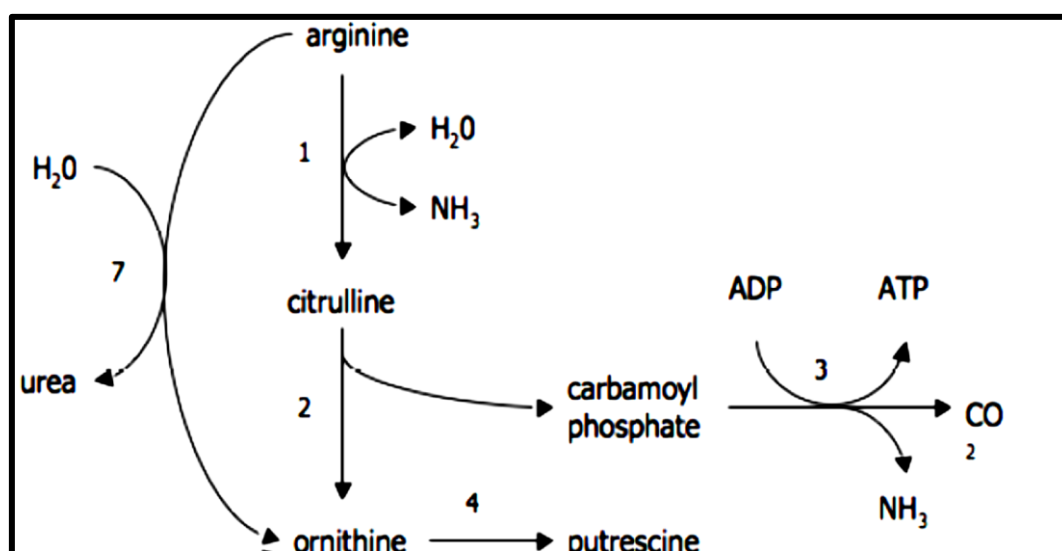


Fig. 2 Arginine dehydrogenase pathway. Arginine is converted to ornithine by arginine deiminase (1), and ornithine carbamoyltransferase (2) or by the enzyme arginase (7). ATP is generated by carbamate kinase (3) (Carlton et al., 2007a).

The major lipids in *T. vaginalis* are cholesterol, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin. *Trichomonas* cannot synthesise fatty acids *de novo*, but the medium-provided unesterified fatty acids are used for phosphoglyceride and sphingolipid biosynthesis although they are not used in cholesteryl ester or triacylglycerol biosynthesis. Cholesterol is not synthesised *de novo*, but can be incorporated from the medium and cannot be esterified or modified otherwise (Beach et al., 1990).

Many studies have shown the importance of the presence and abundance of sterols in the membrane, especially concerning the susceptibility to drugs (Marechal et al., 2011). Further information is provided in chapter 1.2.

In general, there are two common precursors of isoprenoids and therefore also of sterol, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are both generated by two separate pathways (Lichtenthaler, 2000) (Fig. 3). Isoprenoids are generated via the non-mevalonate pathway, also called 1-deoxy-D-xylulose-5-phosphate/ 2-C-methyl-D-erythritol-4-phosphate (DOXP/MEP) or glyceraldehyde phosphate (GAP)/ pyruvate pathway (Rohmer et al., 1993), which is known from Gram-negative bacteria (Grawert et al., 2011; Rohmer et al., 1996; Lherbet et al., 2006; Putra et al., 1998; Zhou and White, 1991), several Gram-positive bacteria, (Duvold et al., 1997), plants (Paseshnikchenko, 1998; Knoss et al., 1997; Schwarz, 1994; Chen et al., 2009; Matsuda et al., 2005; Hampel et al., 2006), algae (Disch et al., 1998) and cyanobacteria (Proteau, 1998; Disch et al., 1998; Okada and Hase, 2005).

On the other hand there is an acetate/ mevalonic acid (Ac/MVA) pathway, a mevalonate (MEV) dependent pathway with predicted enzymes in most plants (Hampel et al., 2006), many Gram-positive (Begley et al., 2008; Grawert et al., 2011) and Gram-negative bacteria and with a few alterations in almost all archaeans, in insects (Belles et al., 2005; Blomquist et al., 2010) and in mammals (Kovacs et al., 2007; Hogenboom et al., 2003).

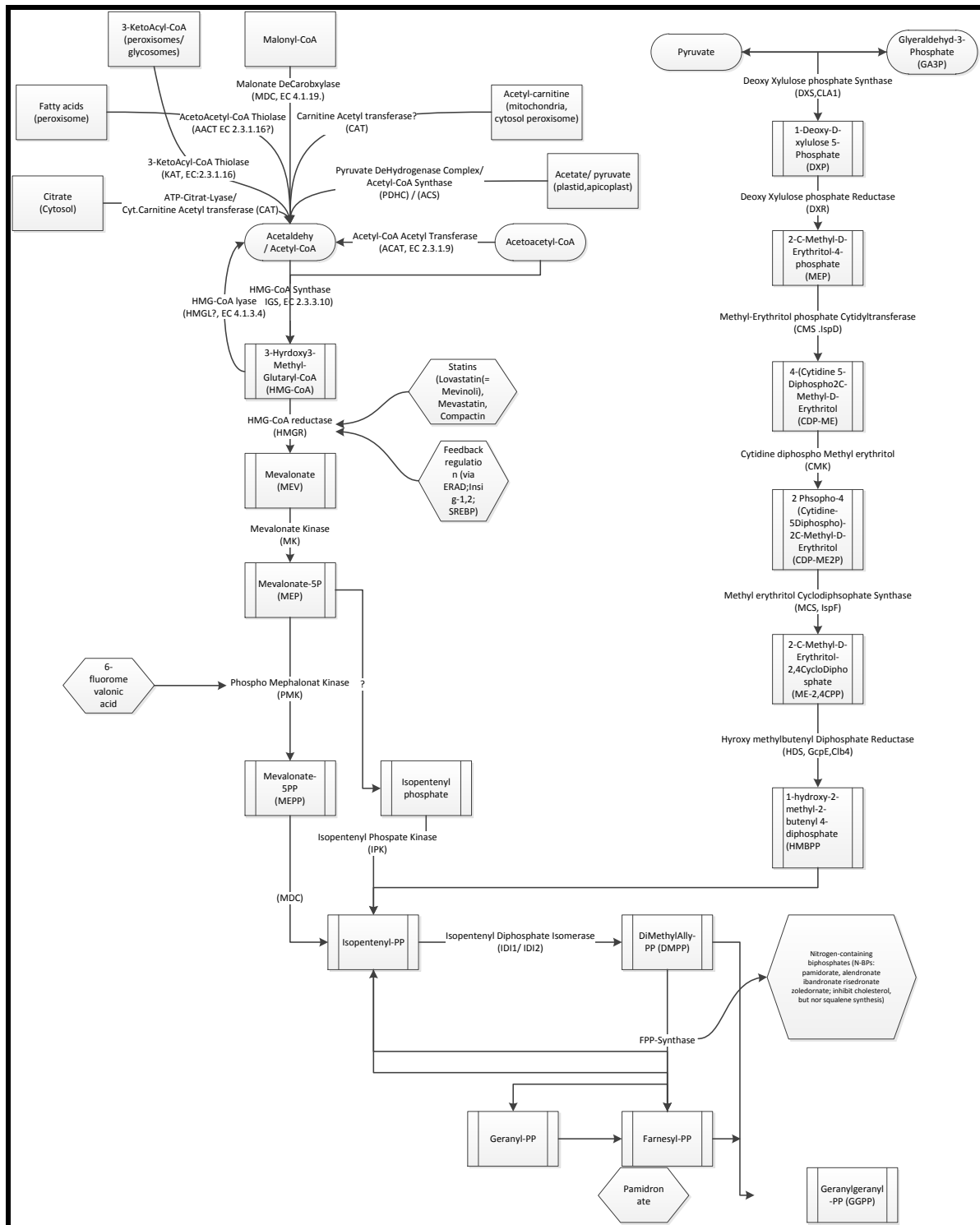


Fig. 3 Schematic mevalonate and non-mevalonate pathways (modified from KEGG) with enzymes, products (in squares) and inhibitors (in hexagons).

Simplified, the mevalonate pathway is used for IPP/DMAPP synthesis in eukaryotes and archaeans and the non-mevalonate pathway in bacteria and phototrophic eukaryotes with exceptions on both sides (Lombard and Moreira, 2011; Rohmer, 1999; Miziorko, 2011).

1.1.5 Genetics

First approaches to the genetic characterisation of *T. vaginalis* were performed in the early 1980s by isoenzym assays and monoclonal antibody panels. Around the millennium techniques like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and chromosomal DNA hybridisation were used to receive further information. In the last few years also microsatellite typing and multilocus sequencing typing (MLST) were used for trichomonal genotyping (Meade and Carlton, 2013).

The genome size of *T. vaginalis* was first estimated with 160 megabases (Mb) including more than 60,000 protein-coding genes (Carlton et al., 2007b), but based on new data the genome size may be up to 175 Mb. However the number of protein-coding genes was reduced to 45,000. In spite of this large number of genes, only 65 genes contain introns in their sequences, each having a canonical 5' splice site and a highly conserved 12 nt motif. The highly conserved small nuclear RNAs (snRNAs), namely U1, U2, U3, U4, U5 and U6 for the splicosomal process are all present in *T. vaginalis*. RNA interference (RNAi) activity via microRNA (miRNA) and other regulatory small RNAs are also expected (Smith and Johnson 2011).

The main RNA polymerases (RNAP) in eukaryotes are RNAP I, II and III, which synthesize ribosomal RNA (rRNA), messenger RNA (mRNA), small nuclear RNA (snRNA) and transfer RNA (tRNA). In general, eukaryotic transcription of protein-coding genes is mediated by α -amanitin sensitive RNAP II, which is assembled as pre-initiation complex at specific DNA-sequences, e.g. core promoter elements, for example Inr-elements (Smith and Johnson, 2011). The trichomonal transcription is unique concerning an amanitin-resistant RNAP II and there is only one common core promoter element described in *Trichomonas*, the highly conserved, DNA initiator (Inr) element. The trichomonal Inr element is equivalent to the metazoan one in function and structure, but it is not recognized by the TATA box-binding protein-associated factor1 (TAF1). Instead, it is recognized by a novel trichomonal specific 39-kDa transcription factor, the initiator binding protein 39 (IBP39). Also, two novel eukaryotic core promotor elements were found in *T. vaginalis*: motif 3 and the M3 binding protein (M3BP) (Smith et al., 2011).

In trichomonads, which vary in shape and growth behaviour under altered iron concentrations, iron-dependent mechanisms are particularly essential for cytoadherence, cytotoxicity and resistance to the complement system. In many organisms transcription can be regulated via cytoplasmic iron regulatory proteins (IRP) and their binding to iron-responsive elements (IRE) in the mRNA sequence. IRP/IRE-like proteins (tvcp12) have also already been detected in *Trichomonas* (Torres-Romero and Arroyo, 2009).

1.1.6 Medical relevance

T. vaginalis is the causative agent of trichomonosis, which is with more than 170 million new cases each year the most prevalent non-viral sexually transmitted disease (STD) (Gerbase et al., 1998).

T. vaginalis infects the squamous epithelium in the human urogenital tract with an incubation period of 4 to 28 days. The disease caused by *T. vaginalis* includes the following symptoms in women: yellowish-green (42%) frothy (10%) discharge, foul odour of vaginal discharge (50%), oedema or erythema (22-37%), pruritus, dysuria, dyspareunia, the characteristic “strawberry” cervix-punctate hemorrhagic lesions (50%), vaginal itching, urinary tract irritation, soreness, lower abdominal pain and elevated pH (>5.0). Besides vaginitis, there are other common manifestations like infection of Bartholin’s gland, adnexitis as well as infertility, low birth weight, cervical erosion, and increased HIV transmission. One of the main problems in trichomonal treatment is the missing contraception and the omitted treatment of the asymptomatic carrier state of most men and many women (Krashin et al., 2010; Schwebke and Hook III, 2003; Soper, 2004; Petrin et al., 1998; Cudmore and Garber, 2009).

Men are usually asymptomatic carriers, possible clinical symptoms are urethral discharge, urethritis or gonococcal urethritis, irritation, non-gonococcal urethritis (NGU) or prostatitis, epididymitis, reduced sperm function and infertility (Petrin et al., 1998; Harp and Chowdhury, 2011; Cudmore and Garber, 2009).

One of the first diagnostic methods was the wet mount preparation by Donne (1936), which is based on a vaginal discharge sample examined under the

microscope for the presence of *T. vaginalis*. However, this has sensitivity of only 35-80% depending on the experience of the microscopist and a detection minimum of 10^4 cells/ml. Broth cultivation in Diamond's medium has a higher sensitivity and only 300-500 cells/ml are required for cultivation, but a disadvantage is the culture period of 2-7 days (Petrin et al., 1998). Other cell culture techniques used for diagnostics with detection limits down to three cells/ml are rather expensive. Generally, culture is not recommended for rapid diagnosis (Tab. 2). Several staining techniques can be used to gain a better performance, e.g. acridine orange, Leishman, periodic acid-Schiff, Fontana, Papanicolaou staining, Eosin and Giemsa staining (Sood and Kapil, 2008; Walochnik, 2002).

Tab. 2: *T. vaginalis* - diagnostic tests (Harpl, Chowdhury 2011).

Methods		Sensitivity	Specificity
Old diagnostic tests (Microscopic evaluation)	1. Papanicolaou (Pap) smear	50%	90%
	2. Staining techniques	30–60%	less specific
	3. Wet mount	50-60%	>90%
	4. The agar plate technique	>90%	>90%
New diagnostic tests	1. Broth culture (Gold standard)	~85–95%	>95–100%
	2. Odor test	–	poor specificity
	3. XenoStrip-Tv technology	~66%	100%
Newest diagnostic tests	1. Affirm VPIII test	<90% (false +ve from dead organisms)	99%
	2. Rapid antigen test	83–90%	99–100%
	3. Nucleic acid amplification test (NAAT)	96–98% (vaginal swab), 88% (urine specimen)	>98%
	4. TV Polymerase chain reaction (PCR)-based test	64–89%	97–100%

1.1.7 Treatment

Since the 1960ies, metronidazole, a 5-nitroimidazole, has been the drug of choice used in therapy of *T. vaginalis* vaginitis, but emerging resistances with various mechanisms, are pushing the search for alternative drugs (Lofmark et al., 2005; Leitsch et al., 2009). In addition to the side effects like anorexia and nausea the drug is not recommended to be used during pregnancy. It also has mutagenic effects in bacteria and a known carcinogenic potential in rodents (Nyirjesy, 1999; Upcroft and Upcroft, 2001). Another disadvantage is the cross-resistance with other nitroimidazoles (Upcroft and Upcroft, 2001). The mode of action under anaerobic conditions is supposed to rely on the reduction of nitro groups of metronidazole in the hydrogenosomes by pyruvate-ferredoxin oxidoreductase and the resulting nitro radicals are supposed to damage DNA strands and therefore mediate cell death (Nyirjesy, 1999). Recent studies support a new theory for its efficacy by metronidazole adducts formation (e.g.: thioredoxin reductase) via thioredoxin reductase (Leitsch et al., 2009). Additionally, there is also a difference between clinically and laboratory-induced metronidazole resistance (Wright et al., 2010c).

1.2 Antimicrobial agents

1.2.1 History

Already in the ancient civilisations of Egypt, Mesopotamia, India and China treatments with mould compounds were in use (Forrest, 1982; Wainwright, 1989; Bassett et al., 1980). In the 17th century, the British King's herbarian John Parkinson mentioned the effect of moulds on infections (Wainwright, 1989).

At the end of the 19th century, John Burdon Sanderson, William Roberts and particularly John Tyndall (1876) observed the inhibiting effect of fungi on bacterial growth (Campos et al., 2007). Robert Koch, "the founder of bacteriology" , influenced the work of Paul Ehrlich, who already expected the correlation between chemical structure and function of antimicrobial compounds (e.g. Atoxyl) and also synthesised the first man-made antibiotic arsphenamine (1907), which was used for the chemotherapy of syphilis (Salvarsan® 1909) after further experiments in mice in cooperation with Sahachiro Hata (Bosch and Rosich, 2008).

The first definition of antibiotics was given by Selman Waksman in 1942, but even earlier in 1889 the concept of “antibiosis” was formulated by Vuillemin (Waksman, 1947). In 1928, the Scottish biologist Alexander Fleming recognized that a compound from the ascomycetous fungus *Penicillium notatum*, formerly known as *Penicillium rubrum* (Campos et al., 2007), killed *Staphylococcus aureus*. In World War II and after on this substance was called penicillin and became a commonly used antibiotic (Demain and Sanchez, 2009). With penicillin the “golden era” of antimicrobial research started (Kumazawa and Yagisawa, 2002; Wright, 2007).

Although the first disease caused by fungi was recorded in 1665 it took nearly 200 years until the first real antifungal compound was discovered, namely nystatin (1949) (Fromtling, 1988). From the 60ies of the last century to the millennium no important new classes of drugs were discovered, which resulted in an invention gap in drug research (Fischbach and Walsh, 2009).

1.2.2 Classification

Antimicrobial agents include all agents, which inhibit growth or infectiousness of microorganisms. Antibiotics can be classified by their manufacturing process in natural, semisynthetic and synthetic drugs or by their chemical structure in aminoglycosides (e.g. gentamycin), tetracyclines (e.g. tetracycline), β -lactams (e.g. penicillin), glycopeptides (e.g. vancomycin), fluoroquinolones (e.g. ciprofloxacin), rifamycins (e.g. rifamycin), lipopeptides (e.g. daptomycin), macrolides (e.g. erythromycin), streptogramins (e.g. pristnamycin), phenicols (e.g. chloramphenicol) and more. Antibiotics can also be divided into groups by their mode of action. The mechanism can include inhibition of RNA, DNA, protein or cell wall synthesis as well as influence on the cell membrane integrity or interference of various metabolic pathways (Périchon and Courvalin, 2009; Kohanski et al., 2010; Depardieu et al., 2007).

In addition to the antibiotics, also antimycotic drugs are used in antiprotozoal therapy. Classes of antifungal drugs are azoles, allylamines and thiocarbamates, polyene macrolides, fluorinated pyrimidines (5-FC), griseofulvin, oxaboroles and (echino) candins (Van Minnebruggen et al., 2010). The main targets for antifungal drugs are the cell wall and membrane, especially sterols and their biosynthesis,

but also protein and DNA/RNA synthesis as well as microtubule aggregation (Odds et al., 2003; Ostrosky-Zeichner et al., 2010).

1.2.3 Resistance mechanisms

To date, there is already at least one resistance mechanism for almost every class of drug. The emergence and development of these resistances dependent on several factors, like distribution, mechanism of drug, transfer abilities and others. An important source of resistance mechanisms is given by the antibiotic-producing organism itself or other organisms in their environment, that have to protect themselves from these metabolic products. In addition to the natural antibiotic resistome there is also a clinical and in the broader sense an anthropogenic impact on the evolution and dissemination of antibiotics and resistances in hospitals, agriculture, aquaculture (e.g. wastewater treatment) and associated environments (Allen et al., 2010; Davies and Davies, 2010).

Resistances against antibiotics and antifungals are growing global issues with various mechanisms and reasons for their development and prevalence. In general, two major types of resistances can be distinguished. On the one hand, the natural (intrinsic) resistance, which is present in all members of one species or genus and is inherent due to absence, inaccessibility or low affinity of the target for the drug (e.g. multidrug efflux systems in *Pseudomonas aeruginosa*). On the other hand there is the acquired resistance, which is present in originally susceptible strains and results from mutations of the target or horizontal gene transfer of resistance mechanisms (Normark and Normark, 2002; P  richon and Courvalin, 2009; Toprak et al., 2012). This transfer can be mediated via transformation, conjugation and transposition by plasmids, phages, transposons and further mechanism (Wright, 2007).

One of the main concerns is the fast emergence of new resistances compared to the long time period of drug development (Tonkens, 2005; Clatworthy et al., 2007; Schmieder and Edwards, 2012). The implementation from drug discovery to drug approval is a time-consuming and cost-intensive process with considerations in political, ethic and juristic perspectives. It takes about 10 years on average from investigation to regular approval compared to the average of eight years for

resistance development against several classes of antibiotics (Fig. 4) (Schmieder and Edwards, 2012).

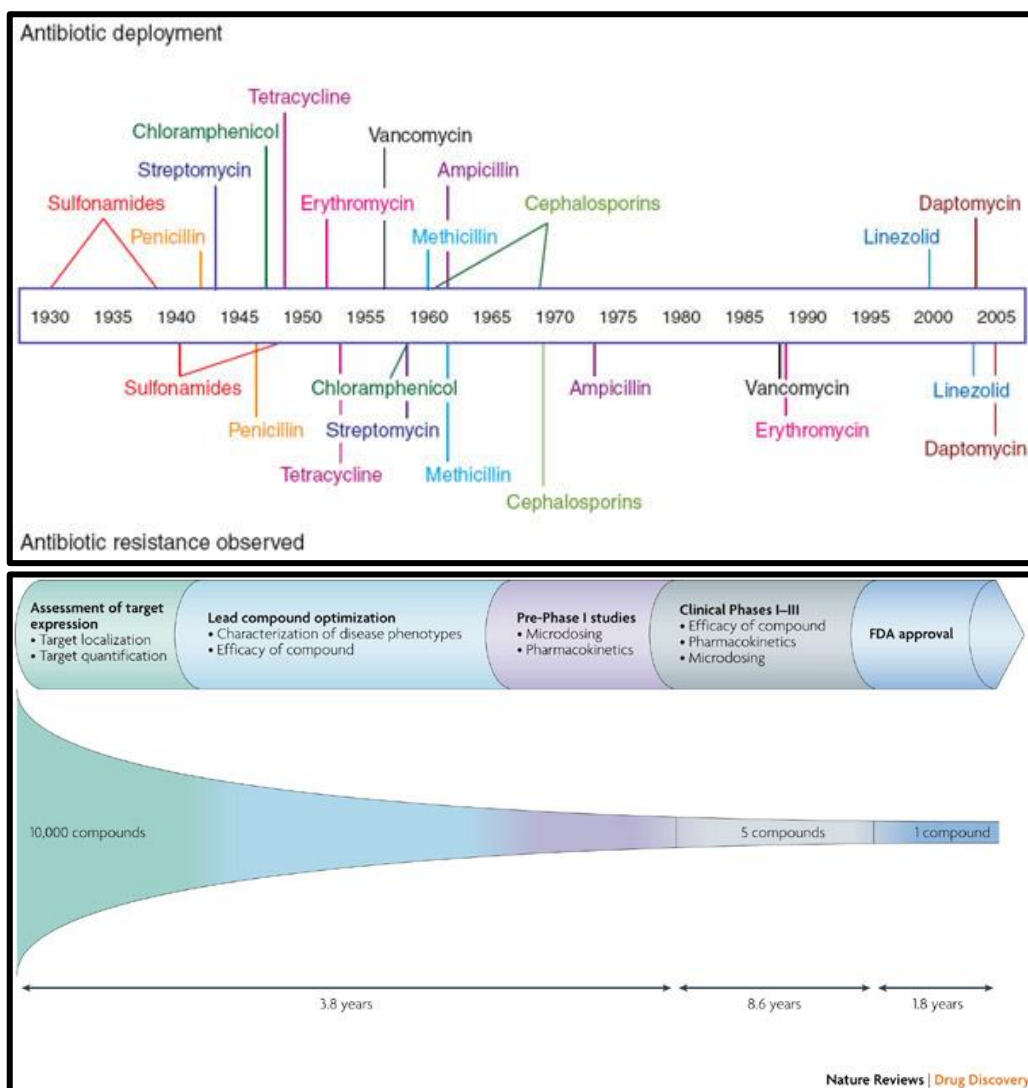


Fig. 4 Timeline for antibiotic introductions and emergence of resistances (Clatworthy et al., 2007) and the timeline for a single drug development (Willmann et al., 2008).

The emergence of several multi-drug-resistant (MDR) organisms also known as superbugs (e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and extended spectrum β -lactamases (ESBL) bacteria) demand the scientific attention for resistance development and their medical impact (Backman et al., 2011; Alekshun and Levy, 2007). Beside the development of resistance also the dissemination and ecological consequences, especially for humans, are major tasks in the current drug research projects (Schmieder and Edwards, 2012).

The control and reduction of resistance development will be an on-going project with different solutions proposed by international committees and organisations (e.g. WHO, CDC, ECDC, ISDA) (Jarlier et al., 2012). One of the main objectives is the strict control of antibiotic use to prevent abuse and incorrect medication in therapy as well as the prevention from spreading of these resistances. Amongst others, Goossens (2009) has shown in his work the positive correlation between drug consumption and emerging resistances in several countries. Further, new strategies are needed to control and reduce multidrug resistances. Therefore, new drugs and targets have to be investigated, even if successional resistance might be inevitable (Davies and Davies, 2010; Leung et al., 2011). An alternative to finding new drug targets is given by chemical modification of already known drugs to obtain drugs with improved antimicrobial activity and better safety profiles. Further, there is no need for new classes of drugs, if new effective compounds can be found within the old classes (Bush, 2012). High fitness costs for acquiring resistance could also be an important point in the consideration for new drug targets as well as a low compensatory rate (Andersson and Hughes, 2010; Handel et al., 2006). Research on the mechanisms and circumstances for evolution of resistances and the conceivably affected organisms will be an imperative necessity. A possible contribution to the identification of future resistances and mechanisms may be given by investigation of the evolution of already known resistance genes by the applications of metagenomic analysis. Further, metagenomic approaches can also be used for the identification of possible novel resistance genes. (Schmieder and Edwards, 2012).

1.3 Metronidazole and metronidazole resistance

Metronidazole, a 5-nitroimidazole drug, is one of the most commonly used drugs. It is effective against various intestinal parasites, e.g. *Entamoeba histolytica*, *Giardia intestinalis*, *T. vaginalis*, as well as against pathogenic bacteria (*Clostridium difficile*, *Helicobacter pylori*, *Gardnerella vaginalis* and *Bacteroides fragilis*) (Adagu et al., 2002a). For the treatment of trichomonosis it has been the drug of choice for more than 45 years (Löfmark et al., 2010).

In vitro and *in vivo* resistances to metronidazole are known from several *T. vaginalis* strains. Wrigth et al. (2010) induced microaerobic resistance in

BRIS/92/STD/L/B7268 (B7268) and BRIS/92/STD/L/F1623 (F1623) within 50 days by incremental increased drug concentrations as it has been described also by Brown et al. (Wright et al., 2010c; Wright et al., 2010b; Brown et al., 1999). Kulda et al. (1993) induced anaerobic resistance in the strains TV 10-02 and MRP by gradually increasing drug pressure for 12 to 21 months, but they could not develop fully developed resistance, which means stable resistance after cryopreservation and cultivation without drug pressure (Kulda et al., 1993). Subcultures (MR-3, MR-5, MR-30, MR-100) of the strain TV 10-02 were grown in concentrations up to 100 µg/ ml MTZ in TYM medium for 50 days under anaerobic and aerobic conditions with less resistance development in anaerobic grown *Trichomonas* (MLC: 200 µg/ ml) compared to the aerobic set up (Minimum lethal concentration (MLC): 4 µg/ ml) (Rasoloson et al., 2002). Brown et al. (Brown et al., 1999) were able to introduce resistance in the strains BRIS/92/STD/L/B7708 (B7708) and F1623 within 400 days (Brown et al., 1999). Tachezy et al. (Tachezy et al., 1993) tried to induce resistance in the strains TV 10-02 and MRP-2MT under aerobic and anaerobic conditions by cultivation with low concentrations of MTZ (2-3 µg/ ml) for 50 days (Tachezy et al., 1993). High MLCs (216-261 µg/ ml) were obvious only under aerobic conditions, anaerobic MLC: 4.2-6.3 µg/ ml for TV 10-02, MRP-2MT: aerobic MLC: 88.2 µg/ ml, anaerobic: 6.3 µg/ ml. Wright et al. (2010) displayed the results from Brown et al. (Brown et al., 1999) for the development of resistance in F1623-M (Fig. 5). It took more than a year to get stable MTZ-resistant strains (>400 µM).

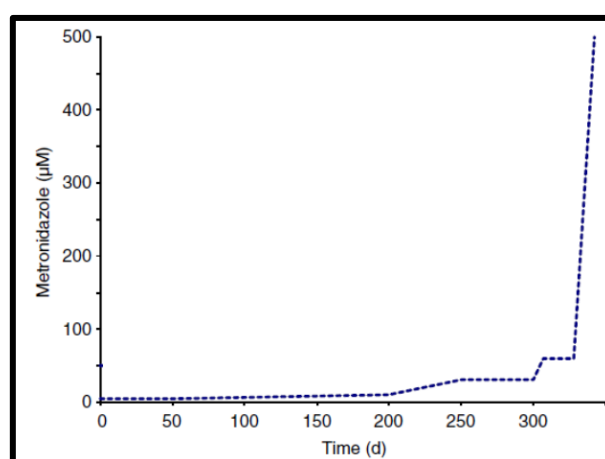


Fig. 5 In vitro development of metronidazole.
Modified (Wright et al., 2010a).

Adagu et al. (2002) and Müller et al. (1988) showed a geometrical mean of aerobic MLCs of 24.1 mg/L in susceptible and 195.5 mg/L in resistant trichomonal isolates compared to anaerobic MLC of 1.6 µg/ml in susceptible and 5.05 µg/ml in resistant strains (Müller et al., 1988; Adagu et al., 2002b). All these experiments reflect the problem of a universal set up for resistance measurement and controlled resistance development. The varieties of available strains as well as genetic variations within the strains are current problems in the comparability of the susceptibility of strains (Tab. 3). Adagu et al. (2002a) mentioned the problem of various modes of presentation for the susceptibility of strains e.g. by using minimal lethal concentration (MLC), minimal inhibitory concentration (MIC) or inhibitory concentration IC50.

Tab. 3 Examples for aerobic and anaerobic metronidazole resistance in *T. vaginalis*.

Strain	An- /aerobic	Resistance	Source
TV 10-02 (MTZ susceptible)	ae	200 µg/ml (~1.2mM)	(Rasoloson et al., 2002)
TV 10-02 (MTZ susceptible)	an	4 µg/ml	(Rasoloson et al., 2002)
TV 10-02 (derivate MR-30,-50,- 100)	an	1120-1425 µg/ml (6.5-8.3 mM)	(Rasoloson et al., 2002)
TV 10-02 (derivate MR100)	an	100µg/ml (grow at)	(Rasoloson et al., 2002)
TV 10-02	an	550-1.600µg/ml	(Kulda et al., 1993)
MRP-2	an	550-1.600µg/ml	(Kulda et al., 1993)
BRIS/92/STD/L/F1623		400µM	(Brown et al., 1999)
BRIS/92/STD/L/B7708		400µM	(Brown et al., 1999)
TV 10-02	an	4.2-6.3 µg/ml	(Tachezy et al., 1993)
TV 10-02	ae	216-261.5 µg/ml	(Tachezy et al., 1993)
MRP-2MT	ae	88.2 µg/ml (50-100µg/ml)	(Tachezy et al., 1993)
	an	6.3 µg/ml	(Tachezy, et al. 1993)
BRIS/92/STD/L/B7268 (patient isolate)	an	25 mM	(Dunne et al., 2003)
BRIS/92/STD/L/B7268	ae	> 200 mM	(Dunne et al., 2003)
BRIS/92/BSHC/11147	an	6.3 mM	(Dunne et al., 2003)
BRIS/92/BSHC/11147	ae	50 mM	(Dunne et al., 2003)
BRIS/92/STD/L/F1623	an	3.2 mM	(Dunne et al., 2003)
BRIS/92/STD/L/F1623	ae	25 mM	(Dunne et al., 2003)
BRIS/92/STD/L/F1623-M1	an	>100 mM	(Dunne et al., 2003)

1.4 Polyene drugs

1.4.1 Classification of polyenes

Streptomyces is one of the most important genera within the actinomycetes, producing various antibiotics such as the aminoglycosides, the tetracyclines, the polyene macrolides, the rifamycins, chloramphenicol and ivermectin (Raja and Prabakaran, 2011; Watve et al., 2001). The importance of these compounds is underlined by the fact, that 45% of 22,500 known biologically active compounds are from actinomycetic origin (Demain and Sanchez, 2009). There are two main groups of macrolides, on the one hand the erythromycin group, which is active against gram-positive bacteria by inhibiting the peptidyltransferase center and the ribosomal translocation. On the other hand, the polyene group, which is highly effective against fungi by altering the cell permeability (Hamilton-Miller, 1973).

The chemical structure of the macrolide class is a macrocyclic ring formed by lactonization, which contains 20-40 carbon atoms and additional polyene groups with several conjugated double bonds (Fjaervik and Zotchev, 2005; Hamilton-Miller, 1973). The classification of polyene antibiotics relies on the number of double bounds in the macrolide ring. The differences within this drug class can be seen in the ultraviolet absorption spectra, which vary between all classes, from triene up to heptaene compounds. The group of interest in this study has five double bounds (pentanes) and is subdivided into a normal group (methylpentanes) and a lacton-conjugated group, which contains pentamycin and the filipin complex (Hamilton-Miller, 1973).

1.4.2 Amphotericin B

One of the best investigated polyene drugs is Amphotericin B (AmB). This polyene macrolide is closely related to pentamycin and binds ergosterol with higher affinity than cholesterol, probably because of the structural differences. The higher ratio of ergosterol:phospholipids in fungi than mammals plays also an important role in its activity (Odds et al., 2003). The binding for both, sterol and ergosterol, is further the reason for the severe toxic side effects in mammals, including high fever, chills, hypotension, anorexia, dyspnoea and anaphylactic and cardiogenic shocks (Odds et al., 2003; Walochnik and Astelbauer, 2011). AmB is supposed to bind to sterol and induce channel formation with increased potassium efflux if bound to

ergosterol compared to cholesterol (Solovieva et al., 2011). Recent studies claim that the antibacterial effect does not only rely on the channel forming capability of AmB. The binding of ergosterol itself is proposed to be a main factor for AmB efficacy (Gray et al., 2012). Further, AmB is also involved in lipid peroxidation by concomitant-free radical formation from its autoxidation (Kovacic and Cooksy, 2012).

1.4.3 Pentamycin

In 1958, pentamycin was first mentioned in the literature (Umezawa et al., 1958). There are several types of pentamycin based on stereochemical differences (12 asymmetric centres), but all substances are produced by *Streptomyces* spp. Pentamycin produced by *S. pentaticus* (Oishi 1989) is called “fungichromin”, which can also be extracted from *S. padanus*, (Shih et al., 2003), *S. cellulose* (Noguchi et al., 1988) or *S. griseus* (Pandey et al., 1982). “Lagosin” is produced by *S. cinnamomeus*, *S. roseoluteus* or *S. cellulosa* (Hamilton-Miller, 1973; Pozsgay et al., 1976). Other types are “cogomycin” (Pozsgay et al., 1976) or “moldcidin B” (Hamilton-Miller 1973) (Tab. 4). The main problems in isolation and stability of pentamycin are auto-oxidation, byproducts and sensitivity to degradative conditions, as temperature, atmospheric oxygen, polycalent metals and light exposure. Therefore, the maximum level of purification has long been 75%. With crystallization of methanol-solven pentamycin in the 1980ies 94% purity and recently >95% purity could be obtained (Reuter et al., 2009). This first drug formulation was available as Pentacin®, but the product had to be withdrawn from the market for reasons for safety and purity (Reuter et al., 2009).

Tab. 4 Physicochemical comparison of fungichromin, lagosin and cogomycin (Pandey et al, 1982).

Property	Fungichromin (NSC-277813)		Lagosin	Cogomycin
Producing organism	<i>Streptomyces cellulosa</i> ^{1,2)}	<i>S. griseus</i> (FCRC-21) ⁷⁾	<i>S. roseoluteus</i> ^{3,4)}	<i>S. fradiae</i> ^{5,6)}
Nature	Light yellow crystals	Light yellow crystals	Light yellow crystals	Light yellow crystals
Molecular formula	C ₃₅ H ₅₅ O ₁₂	C ₃₅ H ₅₅ O ₁₂	C ₃₅ H ₅₅ O ₁₂	C ₃₅ H ₅₅ O ₁₂
Molecular weight	670	670	670	670
Melting point (°C)	205 ~ 210	220 ~ 225	230 ~ 240	190 ~ 210
[α] _D	−176 ± 4° (MeOH)	−227.7° (DMF)	−160 ± 4° (MeOH) −279 ± 3° (DMF)	−157° (MeOH)
IR (cm ^{−1})	3268, 1709, 1639, 1138, 1010, 846	3440, 1725, 1635, 1135, 1005, 850	3310, 1710, 1635, 1136, 1010, 852	1730, 1005
UV λ _{max} (E _{1cm} ^{1%})	357(1460), 339(1550), 323(960), 310(sh)	357(1231), 338(1250), 322(786), 308(sh)	356(1480), 338(1490), 322(930), 308(420)	357(1270), 339(1300), 322(731), 309(326)

In the current study pentamycin (C₃₅H₆₀O₁₃) from *Streptomyces pentaticus* was used. Pentamycin has the typical macrolide cyclic ring (lactone ring) with a polyene group with five conjugated double bonds and an UV absorption spectrum with specific bands at 850 cm^{−1} (Hamilton-Miller, 1973; Martin and McDaniel, 1977; Reuter et al., 2009) (Fig. 6).

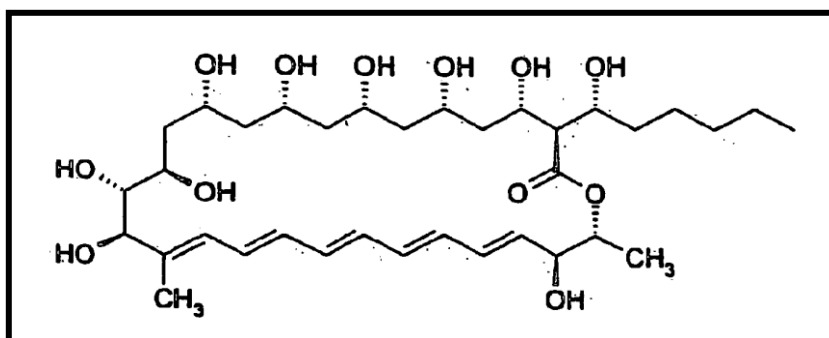


Fig. 6 Chemical structure of pentamycin (Reuter, et al. 2009).

Pentamycin is effective in anti-fungal therapy (e.g. in *Candida* species) and has anti-bacterial activity *in vitro* (Balmer, 2009). Pentamycin for vaginal application is available under the brand name Pruri-Ex® in Switzerland and as FemiFect® in other countries. The vaginal tablets are used for the treatment of vaginitis, whether caused by fungi (*Candida albicans*) or other pathogens. One of the advantages of pentamycin is the fact that it does not penetrate into the circulation due to its high molecular weight and bi-polar molecular structure. Another advantage is its effectivity against azole-resistant strains of *Candida albicans* (Balmer, 2009). Furthermore, pentamycin is well tolerated and the compliance is usually very

good. No adverse events have been observed so far (Balmer, 2009; Reuter et al., 2009). Also, the prescription of only one drug for mixed infections and the treatment period of only a few days is a reason for the high treatment acceptance and good treatment success (Balmer, 2009). Because preliminary testings were promising, recently a clinical phase 2 trial has been initiated by Lumavita for the treatment of trichomonosis with pentamycin.

1.5 Mode of action of polyene drugs

The mode of action of drugs is often bound to the gene expression of distinct genes, e.g. enzymes or other proteins. Therefore gathering information on the pathway and control mechanism of the target gene/enzyme plays an important role in understanding the mode of action.

1.5.1 KEGG metabolic pathway

One of these databases is the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>). It is one of the biggest database for pathways and it is linked to other databases and libraries, e.g. NCBI, PubMed, Pfam via the DBGET and LIGAND networks (Ogata et al., 1999; Ogata et al., 1998). To date, it contains genome information of more than 171 eukaryotes (17 draft), 124 archeans and 1777 bacteria. Several updates and additional tools have been added and upgraded KEGG to an innovative database with information on genomic (KEGG GENOME, KEGG GENES), chemical (KEGG COMPOUND, KEGG REACTION, KEGG ENZYME), and disease-related data (KEGG DISEASE). Information on organism-specific pathways as well as data on drug influence (KEGG DRUG) and systematic issues (KEGG BRITE, KEGG PATHWAY) have been included (Ogata et al., 1998; Kanehisa and Goto, 2000; Kanehisa et al., 2006; Kanehisa et al., 2010; Kanehisa et al., 2012). The connections within the KEGG database and the links to other databases are displayed in Fig. 7.

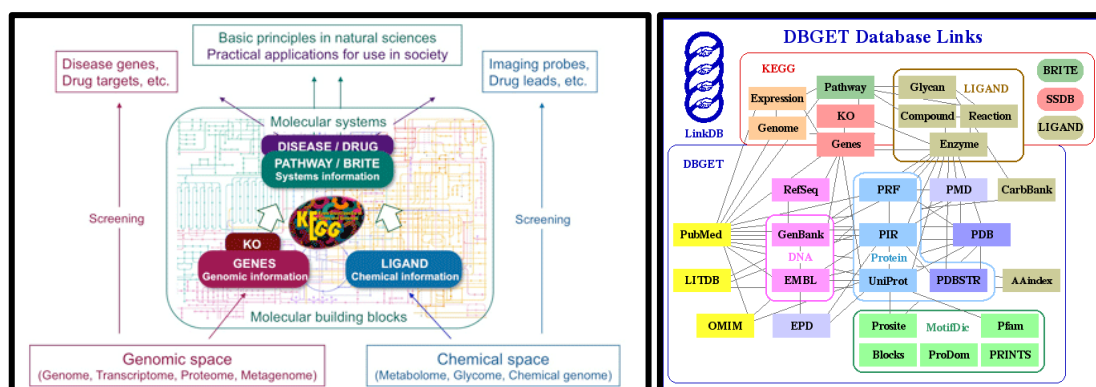


Fig. 7 The functional diagram of the different programs and applications within KEGG and the links to other databases (<http://www.genome.jp/kegg/kegg1a.html>).

Other pathway databases are MetaCyc (Karp et al., 2000; Karp et al., 2002), ExPASy (Gasteiger et al., 2003) and BioCyc or AnEnPi (Alves-Ferreira et al., 2009; Otto et al., 2008).

1.5.2 Lipidomics

At the beginning of the 21st century a new “omics” field emerged, called lipidomics. It deals with the lipid composition and metabolism of biological systems. There have been amazing advances and innovative methods invented in the analytical characterisation and quantification of lipids, particularly using mass spectrometry (Brouwers, 2011; Wenk, 2005; Han and Gross, 2005; Milne et al., 2006). The lipid composition of the cell membrane has an important influence on the efficacy of drugs and resistances against drugs, because of its function in membrane structure, signal transduction (second messenger: diacylglycerol), bioenergetics potential (energy source: triglycerides) etc. (Marechal et al., 2011; Gross and Han, 2011).

1.5.3 Current state of knowledge

Pentamycin is more closely related to filipin (pentaene) than to amphotericin B (Hamilton-Miller, 1973; Van Leeuwen et al., 2009). In contrast to amphotericin and nystatin the pentane filipin cannot induce pore formation in membranes. This is due to reduced molecule length and the absence of certain charged carboxyl groups. Instead of inducing pore formation, it is able to affect the membrane by inducing its fragmentation and leaking (Van Leeuwen et al., 2009; Gimpl and Gehrig-Burger, 2007). The efficacy of filipin is dependent on the type and

availability of sterols in the membrane, which should also be valid for pentamycin (Kleinschmidt and Chough, 1972).

1.6 Aims

The central aim of the study was to evaluate the efficacy of pentamycin on *Trichomonas vaginalis*. The idea was to assess the effectiveness of pentamycin against MTZ-resistant strains and therefore its potency as a potential alternative drug. This was achieved by testing the efficacy of pentamycin against strains with varying metronidazole (MTZ) susceptibilities, including one MTZ-sensitive (ATCC 30001), two normally MTZ-sensitive (TV2, ATCC 30236) and one MTZ-resistant (ATCC 50138) strain.

The second major aim was to investigate whether pentamycin is as prone to the development of resistance as MTZ. Therefore, a low-dose long-term treatment plan for permanent cultures of *T. vaginalis* was set up and the efficacy of pentamycin against these sub-strains grown under continuous drug-stress was evaluated at various time points within a one-year schedule.

Finally, it was aimed to set the basis for the elucidation of the mode of action of pentamycin against *T. vaginalis*. To achieve this, an *in silico* screening for potential targets of pentamycin was performed in the genome of the reference strain of *T. vaginalis*. Moreover, important enzymes along the eukaryotic biosynthesis pathway of isoprenoids were searched for by PCR in different strains of *T. vaginalis*.

2 Materials & Methods

2.1 Strains & cultivation

2.1.1 *Trichomonas vaginalis*

In this study four strains with different metronidazole susceptibility, which varied from resistant to sensitive (Tab. 5), were tested. The IR78 (ATCC 50138), NHM (ATCC 30001) and ATCC 30236 strain are related to the American Type Culture Collection types. The TV2 was isolated from a patient and kindly provided by Professor A. Stary from the STD-Ambulatory in Vienna (Blaha et al., 2006).

Tab. 5 *T. vaginalis* strains included in this study and their susceptibility to metronidazole

Species	Strain	Metronidazole susceptibility
<i>Trichomonas vaginalis</i>	ATCC 30001	highly sensitive
	ATCC 30236	normal sensitive
	ATCC 50138	resistant
	TV2	normal sensitive

2.1.2 Medium preparation

One litre of modified TYM (= modified Diamond's medium) (Clark and Diamond, 2002) contained 20.0 g trypticase; 10.0 g yeast extract; 5.0 g maltose; 1.0 g L-cysteine hydrochloride; 0.8 g potassium phosphate monobasic; 0.8 g potassium phosphate, dibasic and 0.2 g ascorbic acid dissolved with 30 ml Diamond Vitamin Tween 80 Solution and 100 ml 10% heat-inactivated serum and 500 U/ml penicillin G sodium and finally filled up to one litre with distillate water. After pH adjustment with hydrochloric acid to a value of six, the suspension was sterilised by filtration through a vacuum filtration system with 0.2 µm pore size and stored at 4°C for further use. Fresh medium was prepared at least every week or more often according to the demand.

2.1.3 Cultivation

All strains were cultivated in 12.5 cm² tissue culture flasks (Falcon) at 37°C in 40 ml modified Diamond's TYM medium (Clark and Diamond, 2002) under semi-aerobic conditions. After 2 to 3 days of incubation, depending on cellular growth

and health status, 100 µl of the culture were transferred to a new culture flask filled with 40 ml pre-warmed medium. If higher cell densities were needed, the old medium in the flask was discarded and the flask was refilled with 40 ml of fresh medium.

2.1.4 Cryopreservation

For long term storage, half the volume of a flask with trichomonal culture in log phase growth was transferred to a 50 ml tube. After gentle manual shaking of the flask the remaining culture was also transferred into the tube. After centrifugation for 10 min at 700 g the supernatant was discarded and the pellet was resuspended in 1.8 ml cell freezing medium with 10% DMSO (Sigma, Vienna, Austria). The suspensions were transferred into cryotubes and initially stored at -80°C for several weeks and finally in liquid nitrogen.

2.1.5 Thawing

Thawing of cryopreserved samples was achieved by taking the cryotubes out of the freezers, warming them up for 2 min in a 37°C water bath and pouring the entire content into a culture flask filled with 38 ml pre-warmed TYM-medium. After incubation at 37°C and at least one sub-cultivation step, the respective strain was used for further purposes.

2.1.6 Giemsa staining

Approximately 100 µl of a cell suspension were spotted on a microscope slide with a pipette. With another slide the drop was smeared over the whole slide length. Afterwards the cell smear was dried at 37°C, the slides were set in a methanol containing Coplin jar for two min and subsequently dried again. Then the slides were incubated in another jar in an azure-eosin methyleneblue (Giemsa) solution mixed 1:10 with Weise's Buffer (pH 7.2), for 30 min. The slides were removed, cautiously rinsed with water and finally observed under the microscope and photographed (Fig. 13).

2.2 Microtiter Assays

The pentamycin susceptibility of *T. vaginalis* was evaluated in a 24-well plate system with different dilutions of pentamycin (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10 µg/

ml) including two positive and two negative controls. All assays were performed in a doublet of triplets together with another student (Mag. Markus Kranzler).

2.2.1 Preparation of drugs

A stock solution (10:1) was prepared by dissolving 10 mg of pentamycin with a purity of 94% (Ch.-B. C-017084-PRS-03; Lumavita, Basel, Switzerland) in 1 ml DMSO by several steps of up and down pipetting, inverting and gentle shaking. This stock solution (10 mg/ml) was drawn up with a syringe, the needle was removed and replaced by a 0.45 µm-filter unit and the solution was sterile-filtered into a fresh 1.5 ml-reaction tube. The working solution was prepared by diluting the stock solution in TYM-medium at a ratio of 1:10.

The same procedure was performed using a batch of pentamycin with a purity of 28% (Ch.-B., Batch 20; Lumavita, Basel, Switzerland). This batch had been already tested in a previous study (Report G40/07) and was used as a positive control. Metronidazole (MTZ) was included as a second control and it was also prepared initially as a 10 mg/ml stock solution with DMSO. Trophozoites cultured in TYM-medium without addition of any reactive agent were used as a negative control. Trophozoites cultivated in TYM-medium with respective DMSO concentrations were included as second control.

2.2.2 Tests

The 24-well microtiter plates were pre-warmed in the incubator at 37°C for a few minutes to prevent the cultures from temperature shock. In the meantime, the culture flasks were gently inverted to detach the trichomonads from the bottom of the flasks. The cell densities were determined by counting the cells with a Fuchs-Rosenthal haemocytometer under a light microscope. The appropriate cell number (1×10^6 cells/ml) was adjusted with pre-warmed TYM-medium. The culture was gently mixed, 2.5 ml were filled into each well and then the plates were incubated for 15 min to allow the trichomonads to recover from the transferring process.

The drugs were added to the cultures in the respective wells to obtain the following final concentrations: 0.0 (negative control), 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 µg/ml of pentamycin (purity 94%), 20 µg/ml pentamycin with 28% purity (positive

control), 50 µg/ ml metronidazole and 12,5 µl DMSO (Fig. 8). The prepared plate was set onto an orbital shaker (GFL, ~150/min) for a few minutes to receive a homogenous distribution of the drug and to avoid attachment of the cells. Afterwards the plate was incubated at 37°C.

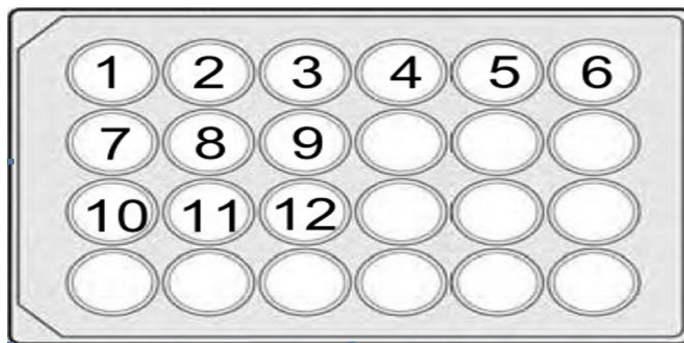


Fig. 8 24-well microtiter plate assay. The numbered wells contained 2.5 ml of *T. vaginalis* suspension in TYM medium with 0.0 (1), 0.5 (2), 1.0 (3), 2.0 (4), 3.0 (5), 4.0 (6), 5.0 (7), 10 (8), and 20 µg/ ml (9) of 94% pure pentamycin, 20 µg/ ml 28% pure pentamycin (10), 50 µg/ ml metronidazole (11) and DMSO (12).

2.2.3 Evaluation

After one, three and six hours the cell densities were measured by counting the cells in a Fuchs-Rosenthal haemocytometer. For each evaluation the inoculated cultures were softly shaken to detach the cells from the bottom of the well. Afterwards an aliquot of the respective suspension was pipetted between coverslip and counting chamber to fill the chamber by capillary force. The cells were counted under a light microscope in the following manner: cells were counted in four small squares along the diagonal of one large square, the number was multiplied with 4 to receive the relative number of cells within large square. The cell number of four large squares along the diagonal of the whole counting area were summed up and multiplied with 4 to get the calculated number of trichomonads for all 16 large squares, which equals the cell number per 3 µl. The calculated number was multiplied with 1000 (3 ml) and divided by 3 to obtain the number of cells per ml.

2.2.4 Trypan blue assay

Trypan blue (Sigma, Vienna, Austria) staining is an inexpensive and fast method to visually separate vital cells from fading ones (Strober, 2001). Trypan blue, a diazo-naphthalene sulfonate, stains cells without intact cell membrane. The trypan blue solution (0.4%, sterile-filtered) was mixed with the respective suspension in a

1:1 ratio and left on the bench for 5 min. Afterwards, the solution was pipetted into a haemocytometer and the relative number of blue-stained cells (Fig. 9) was determined under the light microscope. For photographical imaging under the microscope, the cell culture was shortly centrifuged in a 50 ml- falcon tube and washed with phosphate buffered saline (PBS, 8 g NaCl, 0.2 g KCl, 1.78 g Na_2HPO_4 , 0.27 g KH_2PO_4 , H_2O per liter; pH 7.4) to get rid of the debris and the medium.



Fig. 9: Dead cell of *T. vaginalis* strain ATCC50138 stained with trypan blue (orig.; 1000 x magnification).

2.2.5 Effective and inhibitory concentrations

The number of dead cells in proportion to the living cells is the basic information for calculating the effective concentrations (EC). EC_{50ies} and EC_{90ies} represent the required concentrations to kill 50% and 90% of the cells. The half maximal inhibitory concentration (IC), the IC₅₀, is based on the determined concentration to immobilize 50% of the cells. The EC_{50ies}, EC_{90ies}, IC_{50ies} and IC_{90ies} were calculated by linear regression analysis for all drugs analysed. Statistical calculations were performed with the computer programs SPSS 16.0 and PASW 18.0.

2.3 Long-term treatment

For evaluation of a potential adaption and loss of susceptibility, respectively trichomonal cultures were grown in modified TYM-medium with sub-lethal concentrations of pentamycin. During the first four month of long-term treatment, increasing concentrations of pentamycin (0.5 µg/ ml steps) were added to the cultures every two to three days.

After four months of cultivation, the method was modified by adding lower concentrations to new cultures, less than 0.2 µg/ ml once or twice per day. Depending on the trichomonal growth behavior and tolerance level, pentamycin concentrations were increased slowly, adapted to the fitness of the respective trichomonal strain. If the cell number was rapidly decreasing the treatment was halted to keep the culture alive. After addition of the drug the flasks were fastened on a rocking platform for a few minutes and then re-incubated at 37°C. At least every two weeks, a culture in log phase from every long-term treated culture was prepared for cryopreservation as a backup for all cultures.

2.4 Molecular Biology

2.4.1 Primer design

The first step in the primer design was to search for similar sequences in other eukaryotes. KOALA (KEGG Orthology and Links Annotation) is using the sequence similarity database (SSDB) in KEGG to identify orthologous sequences. This tool was used to search for orthologous sequences for PMVK (Tab. 6) and HMGS (Tab. 7). The search results for some of the most relevant organism for this work are listed down below: hsa = *Homo sapiens*; ath = *Arabidopsis thaliana*; cdu/cal = *Candida dubliniensis*/ *C. albicans*; gla = *Giardia lamblia*; tva = *Trichomonas vaginalis*; ngr = *Naegleria gruberi*; acan = *Acanthamoeba castellanii*; lma = *Leishmania major*; lif = *Leishmania infantum*; lbz = *Leishmania braziliensis*

Tab. 6 KOALA excerpt for PMVK ([E2.7.4.2](#)): GRP (E.=eukaryote, Pla=plant, Fun=fungi, Pro=protists), ORG (organism acronym), KEGG ID (identity document in KEGG), KO (KEGG Ortholog).

GRP	ORG	KEGG ID	KO (KOALA)	KO (GENES)	Orth (GENES)
E.Pla	ath	> ath:AT1G31910(450)	K00938 *	K00938	E2.7.4.2
E.Fun	cdu	> cdu:CD36_41760(433)	K00938 *	K00938	E2.7.4.2
E.Fun	cdu	> cdu:CD36_41760(433)	K00938 *	K00938	E2.7.4.2
E.Fun	cal	> cal:CaO19.4606(432)	K00938 *	K00938	E2.7.4.2
E.Fun	cal	> cal:CaO19.12076(432)	K00938 *	K00938	E2.7.4.2
E.Pro	tbr	> tbr:Tb09.160.3690(471)	K00938 *	K00938	E2.7.4.2
E.Pro	tcr	> tcr:507913.20(476)	K00938 *	K00938	E2.7.4.2
E.Pro	tcr	> tcr:508277.140(476)	K00938 *	K00938	E2.7.4.2
E.Pro	lma	> lma:LMJF_15_1460(459)	K00938 *	K00938	E2.7.4.2
E.Pro	lif	> lif:LINJ_15_1510(458)	K00938 *	K00938	E2.7.4.2
E.Pro	lbz	> lbz:LBRM_15_1450(458)	K00938 *	K00938	E2.7.4.2
E.Pro	gla	> gla:GL50803_16552(358)	K00938 *	K00938	E2.7.4.2
E.Pro	tva	> tva:TVAG_128700(363)	K00938 *	K00938	E2.7.4.2

Tab. 7 KOALA search for orthologs for HMGS ([E2.3.3.10](#)): GRP (E.=eukaryote, Pla=plant, Fun=fungi, Pro=protists), ORG (organism acronym), KEGG ID (identity document in KEGG), KO (KEGG Ortholog) Orth (ortholog) .

GRP	ORG	KEGG ID	KO (KOALA)	KO (GENES)	Orth (GENES)
E.Ani	hsa	> hsa:3157(520)	K01641 *	K01641	E2.3.3.10
E.Ani	hsa	> hsa:3158(466)	K01641 *	K01641	E2.3.3.10
E.Pla	ath	> ath:AT4G11820(461)	K01641 *	K01641	E2.3.3.10
E.Fun	cdu	> cdu:CD36_34550(449)	K01641 *	K01641	E2.3.3.10
E.Fun	cal	> cal:CaO19.7312(451)	K01641 *	K01641	E2.3.3.10
E.Pro	gla	> gla:GL50803_13962(489)	K01641 *	K01641	E2.3.3.10
E.Pro	tva	> tva:TVAG_438940(433)	K01641 *	K01641	E2.3.3.10
E.Pro	ngr	> ngr:NAEGRDRAFT_77778(495)	K01641 *	K01641	E2.3.3.10
E.Pro	acan	> acan:ACA1_048170(513)	K01641 *	K01641	E2.3.3.10

Universal PCR primers were designed for sequences of enzymes along the biosynthesis pathway of isoprenoids. For this, two enzymes of the mevalonate pathway, the phosphomevalonate kinase, which is still listed as hypothetical protein (PMVK, [XP_001324679.1](#), [XP_001330015.1](#)) and the hydroxymethylglutaryl-CoA synthase family protein (HMGS, [XP_001302712.1](#), [XP_001313745.1](#)) were chosen. The presence of conserved sequences of these enzymes in trichomonal strains was determined by PCR. The primers are assumed to bind homologous sequences of PMVK and HMGS in the majority of other eukaryotic cells.

Information on the chosen enzymes was obtained by the online database KEGG (Kyoto Encyclopedia of Genes and Genomes). The terpenoid backbone biosynthesis pathway offered by KEGG includes the mevalonate and also the non-mevalonate pathway. Via the operating line within this program the *T. vaginalis* (green) specific enzymes in the reference pathway can be shown (Fig. 10).

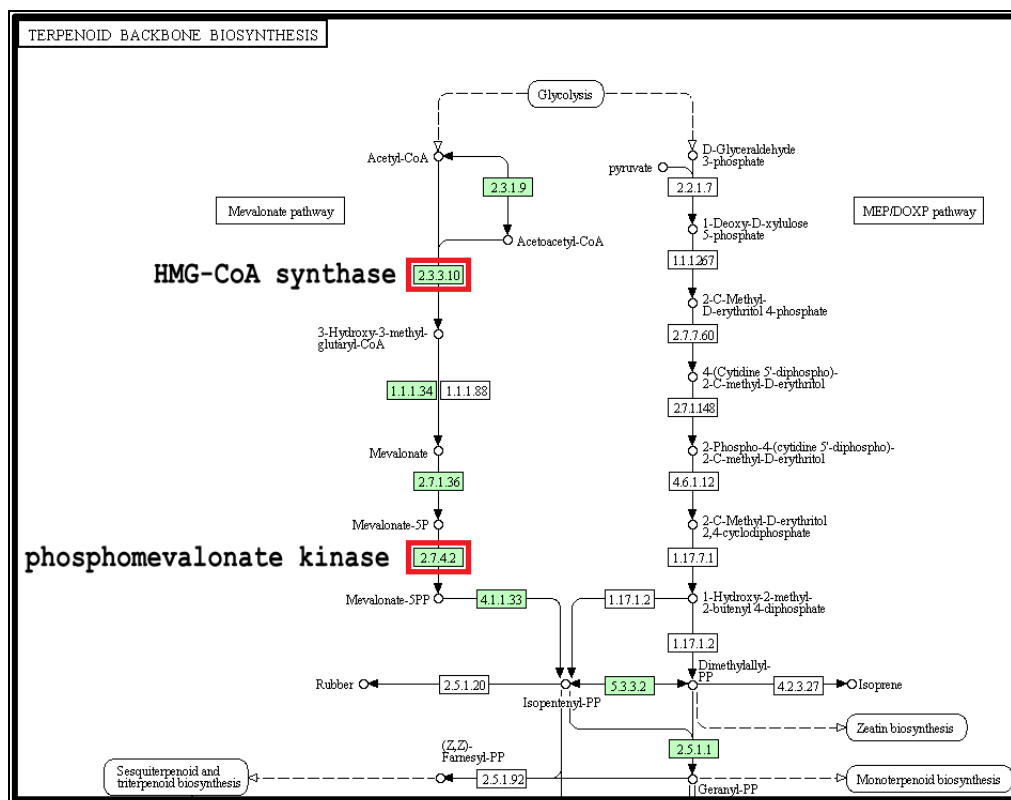


Fig. 10 Terpenoid backbone biosynthesis pathway offered by KEGG (modified). The enzymes of interest are red framed and highlighted: phosphomevalonate kinase (PMVK, hypothetical) ([EC:2.7.4.2]) and hydroxymethylglutaryl-CoA (HMG-CoA) synthase ([EC:2.3.3.10]). The supposed enzymes in the trichomonal synthesis pathway are highlighted in green by the program.

The link-connection to the NCBI files for these enzymes from the linkage number in the pathway was used to obtain the FASTA-files for the protein sequences. The orthologous and homologous sequences of the enzymes were shown in KEGG genomics and listed by KOALA (KEGG Orthology and Links Annotation) (Kanehisa et al., 2010). The sequences for HMGS and PMVK from other eukaryotes were also investigated by searching nucleotide and protein sequences for these enzymes in KEGG and in the following part also in NCBI via Blast.

For primer design the protein sequences of these enzymes were blasted to other organisms to search for the conserved universal sequence segments. Protein sequences instead of nucleotide sequences were chosen to be blasted, because of the more conserved regions in protein sequences and also because of the presence of silent mutations or wobble bases in nucleotide sequence (Crick, 1966; Rogalski et al., 2008; Riddihough, 2008).

Similar protein sequences for HMGS and PMVK to trichomonal ones, namely the *T. vaginalis* hypothetical protein sequence ([XP_001324679.1](#)) (Fig. 11) and for the hydroxymethylglutaryl-CoA synthase (HMGS) protein sequence ([XP_001302712.1](#)) (Fig. 12) were searched via BlastP in other eukaryotes. The position and the amino acid sequence of the conserved regions of the protein sequence was revealed and compared to other eukaryotic sequences.

>ref|NP_001149345.1| **UG** phosphomevalonate kinase [Zea mays]
Length=512

GENE ID: 100282968 LOC100282968 | phosphomevalonate kinase [Zea mays]
(10 or fewer PubMed links)

Score = 69.7 bits (169), Expect = 1e-14, Method: Compositional matrix adjust.
Identities = 76/201 (27%), Positives = 114/201 (41%), Gaps = 60/201 (24%)

Query	134	GKTGLGSSSATIIISIMSLMKI-----SQIIEEITPEQDQLFQLASVANSIAOK	184
		KTGLGSSST A S++ +L+ Q + + + DL+ +A A+ EC	
Sbjct	184	AKTGLGSSAAMTTSVVAALLHVLGAVNLSCFGQSSGDNASGRELDLVHTIAQSAHCLAQ	243
Query	185	KIGSGFDLSCAIWCPQIFRR--PESSLISTENIG-----KDWGNVHTKFLHP	229
		KIGSGFDL S A++G Q + R PE L S + IG + W + + +F LP	
Sbjct	244	KIGSGFDVSAAYYGSQRVRFSP-ILSSAQIGGTVLPDVVSDVLTQRWDHENKQFSLP	302
Query	230	DNMRLVLIKSGE-GSSTADLVBSFNH-RSDP-KDTPLYEELIKANTL-----	274
		M L L + G GSST +V S RSDP K + +L AN+	
Sbjct	303	PLMTLLLGEPGTGSSSTPSMVGSVKRWLKSDEPKSRDTSKLAIANSTLENQLRIINGLS	362
Query	275	-----ACDALQSGDIEQI-----KIKFAELRKIQARITDEWK	306
		+C L G ++ + E+R +	
Sbjct	363	ENHHEAVESMVRSCSHLTYGKAEVATNQHQELIIRSLAARDACLEIRLHMREMGIAAG	422
Query	307	VPILPMEIRELISKFEKIPNYITAIPPGAGGYDAIAVITG 347	
		VPI P L+ + + A PGAGG+DA+ + G	
Sbjct	423	VPIDPSQTRLLDATMMEGVLLAGVPGAGGDAVFSVVLG 463	

>ref|NP_001049577.1| **UGM** Os03g0253100 [Oryza sativa Japonica Group]
Length=512

Fig. 11 Alignment of the *T. vaginalis* hypothetical protein against other eukaryotic (taxid:2759) protein sequences (e.g. *Zea mays*). The conserved sequences are framed red and are also present in other eukaryotes.

hydroxymethylglutaryl-CoA synthase [Arthroderma gypseum CBS 118893]
Sequence ID: ref|XP_003172654.1| Length: 445 Number of Matches: 1
See 1 more title(s)

Range 1: 1 to 441 GenPept Graphics

Score	Expect	Method	Identities	Positives	Gaps
353 bits(905)	1e-114	Compositional matrix adjust.	193/453(43%)	268/453(59%)	38/453(8%)

Query	3	VPQNIKAIQVNI PRSYVTQDDLEETEISILGEESRTKIKGFTKGLGQTALSFCOTDHE	62
		+ Q+GIKAI++ P YV Q +LE + LG S GK+T GLGQT +SFC D E	
Sbjct	1	MAQDVGIKAIETIYFPSRYVAQSELE----TYLGASS----GKYTIGLGQTKMSFCDDRE	51
Query	63	DTASIAMSAVQRLMDTYGYKPTDFGRLEIGSETAIDRSKSIKSFVMOLFENHSIMGVND	122
		DT S+A++ V L+ Y P GRLE+G+ET +D+SKS KS +MOLF EN I GVD	
Sbjct	52	DTYSIALTTVSLLRKYSIDPNTIGRLEVGTETMLDKSKSKSVLMOLFGENSDIEGVDT	111
Query	123	TNACYGCTAFLNLSLAWIESSQWDGRLALVVCADIAVYDDRAARPTGGCGAVAMVLGPDA	182
		NACYGCT A N++ W+ESS WDGR A+VV ADIA+YD AARPTGG G VAM++GP+A	
Sbjct	112	YNACYGCTNALFNAINWVSSAWDGRDAIVVAADIALYDKLAARPTGGAGCVAMLIGPNA	171
Query	183	PIVFE--KGSLTNYFTHSFDFWKPNPANHPKVDGSLSLTYECLENCYMQFRTRHP--	238
		P+VFE +GS + H +DF+K + + +P VDG S Y + L+Ncy ++ + +	
Sbjct	172	PLVFEPTRGs---FMQHVYDFYKADFKSEYPIVDGQFSNKCYLKALDNCYKRYQAKKAAR	228
Query	239	---GTT----LDKFDYVVFHTPFQNHKKCTGRNLFDQHODNNDP-----EIIKTRS	284
		G T LD FDY+ FHTP + K RL + D D +P E+ +	
Sbjct	229	LVNGVTGNIPLDSFDYLAFHTPNCKLVAKSYARLLYNDYLDADPNKPNVFKDIPAEVKEIDY	288
Query	285	EHQTVSQQSHDA-----AQIFKDKVQPASLMSAMCGNGYATASYMALSSLIFN---RDLT	336
		E ++ A + F ++VQP + + MCGN YTAS+Y L SL+ N ++L	
Sbjct	289	ESSLTDRKVEKAFMALTKRFSERVQPLMAATMCGNSYTASVYFGLISLLSNVQSKELM	348
Query	337	GKRLMMFSYSGGAASSMYVLNVGDIKFMKDALNLEERLANRVORKVSDYDHDIKLADQL	396
		GKR++ FSYGS ASSM+ L V G+ M LNL +RL R YD +L ++	
Sbjct	349	GKRVVLFYSGGLASSMFLRVKNTDEMVSKLNLQRLDARKAESPEFYDEMCRLEKA	408
Query	397	YRTAPYEPSCSLDLIESGAWYLEKIDDNWRRYI 429	
		Y+ Y P +++ + G +YL +D+ +RR Y	
Sbjct	409	YQQRNYTPQGNITLAPGTYYLVNVDETFRRTY 441	

Fig. 12 Alignment of the *T. vaginalis* hypothetical protein against other eukaryotic (taxid:2759) protein sequences (e.g. *Arthroderma gypseum*). The conserved sequences are red framed and are also present in other eukaryotes.

For alignment three conserved nucleotide sequence segments of each enzyme were chosen. The competitive nucleotide sequences were aligned with sequences from NCBI by ClustalX to proof the presence of this enzyme sequence in other eukaryotes (Tab. 8).

Tab. 8 Primer sites within the HMGS and PMVK sequences.

enzyme	protein sequence	nucleotide sequence
HMGS1	nacyggt	aatgcctgttatggcgggaacc
HMGS1	aarptgg	gctgccagaccaacaggagga
HMGS1	fsygsaa	ttagctacggtagtggcagca
PMVK	ktglgss	aaaacaggtcttgatcaagc
PMVK	aqkkigsgfd	gcacaaaagaagattggatcaggattcgac
PMVK	pgaggydaia	cctg gtgcaggtggctacgatgctatagca

The GC-contents, lengths and melting temperatures (T_m) of the potential primer pairs were calculated with OligoCalc (Kibbe, 2007). The primers were designed by optimisation of the lengths (17-25 bp) and the guanine-cytosine contents (40 to 60%). The primers were designed to not contain thymine/adenine and less than three guanine/cytosine. Further, the primers were reversed, complemented and blasted against each other and also to the other primer pair to prevent primer dimerization. The optimized primer sequences were then megablasted to *T. vaginalis* (taxid:5722) and other eukaryotes (taxid:2759). Further, the primer sequences for each enzyme were aligned to genomic and mRNA sequence of HMGS ([XM_001302711.1](#); [gi|123405960:c21644-20343](#)) and PMVK ([XM_001324644.1](#); [gi|123486451 c12508-11417](#)) to visualize their precise binding site and also to prove the absence of introns in these genes.

2.4.2 DNA Isolation

DNA was isolated from *T. vaginalis* cultures in log phase growth. To obtain these, 100 µl of culture were added to flask with already pre-warmed TYM-medium. On the following day the cell density was measured with a haemocytometer, diluted to 5 x 10⁶/ml and harvested by centrifugation at 1200 g for 11 min. The supernatant was discarded and the pellet with the mainly disrupted cell was resolved in 200 µl PBS.

DNA isolation was performed according to the QIAamp protocol for cultured cells. Cell lysis was achieved by adding 20 µl of QIAGEN protease (proteinase K) and 200 µl of lysis buffer (Buffer AL) to the resuspended cells and vortexing the suspension briefly. After 10 min in the thermoblock (56°C), the samples were briefly centrifuged and 200 µl ethanol (96%) were added. The solution was pipetted onto a QIAamp Spin Column fitted in a 2 ml-tube and the DNA was bound onto the silica membrane by centrifugation at 6,000 x g for 1 min. The filtrate was discarded and the column was washed with 500 µl washing buffer (Buffer AW1) and centrifuged (6,000x g, 1 min). The discharge was poured out, 500 µl of the second washing buffer (AW2) was added, centrifuged (20,000x g, 3 min) and the flow-through was discarded. The column with the bound DNA was placed into a fresh 1.5 ml-reaction tube, 200 µl of elution buffer (buffer AE) was added and the DNA was eluted by spinning at 6,000 x g for 1 min and finally stored at -20°C.

2.4.3 Control PCRs

In order to control DNA quality two control PCRs were performed, both well-established PCRs for routine diagnostics (Tab. 9). The first primer pair BUTB9/2 (Madico et al., 1998; Crucitti et al., 2003) targets a 112 bp long fragment of a conserved region of the β -tubulin sequence (GenBank: btub1([L05468](#); btub2 ([L05469](#)); btub3 ([L05470](#))), which is specific for *Trichomonas*. The other primer pair TVK3/7 (Kengne et al., 1994) binds a trichomonal specific repeat sequence, which is 261 bp long (Pillay et al., 2004).

Tab. 9 Primers for β -tubulin and specific repeat sequence.

primers for β -tubulin (btub)			
Name	Sequence (5'-3')	Tm	Length of the amplicon
BTUB 9	CATTGATAACGAAGCTCTTTACGAT	52.8	112
BTUB 2	GCATGTTGTGCCGGACATAACCAT	57.4	
primers for the <i>Trichomonas</i> -specific repeat sequence (tvk)			
TVK3	ATTGTCGAACATTGGTCTTACCCTC	56.0	261
TVK7	TCTGTGCCGTCTTCAAGTATGC	54.8	

For both primer pairs a 50 µl-reaction (5 µl DNA buffer, 5 µl MgCl₂, 11 µl ddH₂O, 1 µl dNTP, 5 µl primer1, 5 µl primer2, 0,25 µl polymerase, x dH₂O) with 1, 3 and 5 µl of trichomonal DNA with an additional PCR control (without DNA) was performed with the following PCR setup (Gastb54 program): pre-denaturation (95°C, 7 min), 35 cycles with denaturation (95°C, 1min), primer annealing (54°C, 2 min) and elongation (72°C, 3 min) and final elongation (72°C, 7 min).

2.4.4 Amplification of HMGS and PMVK

PCRs were run as shown in Tab. 10. The only difference was the annealing temperature.

Tab. 10 PCR set up for HMGS and PMVK.

PCR step	degrees	time	
pre denaturation	95°C	7 min	
denaturation	95°C	1 min	35 cycles
annealing	52°C (HMGS) / 54°C (PMVK)	2 min	
elongation	72°C	3 min	
final elongation	72°C	7 min	

2.4.5 Gel electrophoresis

A 2% agarose gel (1g agarose + 50 ml TAE buffer) was prepared with adding 4 µl ethidium bromide (EtBr) after cooling to approximately 50°C. 18 µl of the PCR products with 2 µl of loading buffer were pipetted into each slot and 25 µl of a Step ladder marker (Sigma-Aldrich, Vienna, Austria) was loaded into a separate slot. The gel was run in 1xTBE buffer for 30 min at 70 amp and afterwards the gel was photographed under UV light (3.2.3). For the set up with the PMVK and HMGS primers 27 µl of PCR products with 3 µl of loading buffer were pipetted into each slot.

2.4.6 DNA extraction from gel

The amplified bands were cut out with a sharp scalpel and transferred into 1.5 ml reaction tubes. The extracted bands were cleaned up with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Each fragment was weighted, 3 volumes of QG buffer were added to 1 volume of gel and the suspensions were incubated in the thermoblock at 50°C for 10 min with short breaks of vortexing every three minutes. One gel volume of isopropanol was added and the solution was transferred to the Qiaquick column. The DNA was bound on the QIAamp silica membrane by two washing (0.5 ml Buffer QG, 0.75 ml Buffer PE) and centrifugation (17,900 g, 1 min) steps. The bound DNA was eluted into a 1.5 reaction tube by adding 50 µl EB buffer.

2.4.7 Sequencing

The cleaned up PCR products from the gel were used for sequencing-PCR and run in a 10 µl setup. The PCR mix was composed of 2 µl Big Dye mix (BigDye Terminator, dNTPs, ddNTPs, Polymerase; Applied Biosystems, Vienna, Austria), 1 µl Sequencing buffer, 2 µl of the respective PCR-product, 3 µl distilled water and 2 µl of forward or reverse primer for each primer, respectively (Tab. 11).

Tab. 11 Sequencing PCR set up.

PCR step	degree	time	
pre denaturation	96°C	30 sec	
denaturation	96°C	10 sec	40 cycles
annealing	50°C	5 sec	
elongation	60°C	4 min	
hold	4°C	~	

The sequencing-PCR products were spun down and 1 µl of sodium acetate (NaAc) and 40 µl of ethanol (100%) was added to each product, mixed and left on ice for 20 min for precipitation. Afterwards, the tubes were centrifuged for 30 min at 12,000 g at 4°C. The supernatant was discarded, 90 µl ethanol (70%) were added to the pellet and the solution was centrifuged again for 10 min, 12,000 g at

4°C. The supernatant was discarded again and the pellet was air dried for five minutes. Afterwards, 20 µl of sequencing reagent (Hi-Di formamide, AB, Vienna, Austria) was added to each pellet. The tubes were incubated for 5 min at room temperature and then for 5 min at 95°C in the thermoblock. After brief vortexing, the reaction was stored on ice for another 10 min. The tube caps were cut off and the tubes were set into the ABI PRISM 310 Genetic Analyser (PE Applied Biosystems, Langen, Germany), a single-capillary sequencer for automatic sequencing.

The forward and the reverse sequences for each enzyme were edited with BioEdit 7.1.3 (Hall, 1999; Hall, 2004). A consensus sequence was obtained by multiple alignment of all sequences. The two obtained sequence fragments for each enzyme (PMVK1-2, PMVK2-3; HMGS1-2, HMGS2-3) were aligned and afterwards compared to reference sequences from GenBank.

3 Results

3.1 Effect of treatment

3.1.1 Morphology

Pentamycin treatment induced morphological changes in trichomonads, which varied in correlation with the drug concentration and the cell number. The normal, untreated *T. vaginalis* cells were pear-shaped or oval and about 10 x 15 µm in size. In Giemsa staining, *T. vaginalis* trophozoites show a blue to violet colour (Fig. 13).

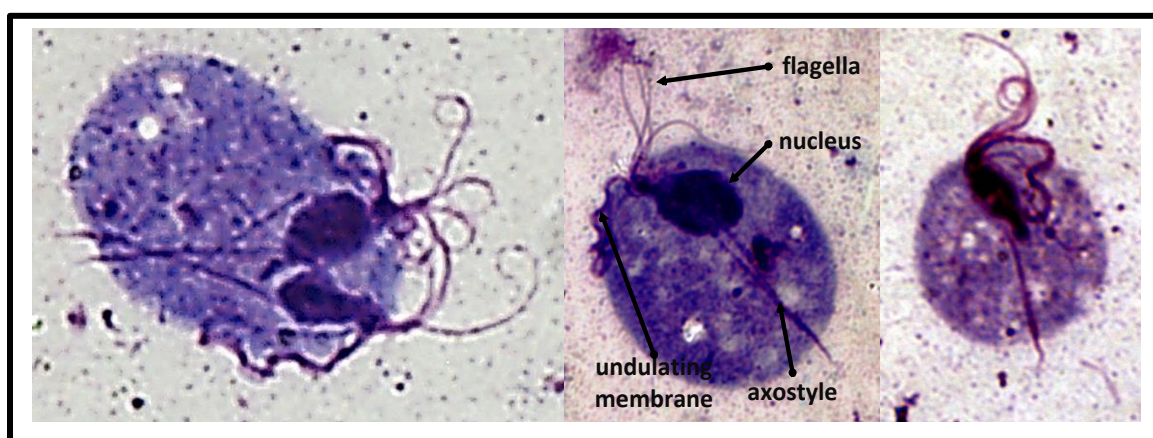


Fig. 13 Giemsa staining of *T. vaginalis* ATCC 30236 (orig.). The stained nucleus is visible at the anterior end, also the four flagella, the undulating membrane and the posterior axostyle are clearly visible.

After several hours in TYM-medium, local clusters of trophozoites formed monolayer-like patches on the culture flask surface. The attached cells appeared in a more amoeboid-like shape, but they were able to switch to an unattached stage, freely moving via the flagella apparatus. The trichomonal ability to change their shape is well-known. At low levels (<0.2 µg/ ml) of pentamycin treatment and under other induced stress situations (e.g. temperature shift) the cells internalised the flagella and started to round up, but they did not lyse. At higher levels of pentamycin treatment (>2 µg/ ml), immediate severe damage of the cell structures and also lysis were observed in all trichomonal strains. The increased lysis and damage effects were correlated with increased drug concentrations and incubation times. Cultures treated with pentamycin concentrations over 5.0 µg/ ml revealed higher levels of intracellular damage as well as dissolving of the cell structures even after only one hour of treatment. When incubated at these higher levels of

pentamycin for more than 6h, most of the cells were lysed and partly fragmented, whereas control cells had no remarkable morphological changes. In contrast, morphological changes caused by incubation with metronidazole (MTZ) included internalization of the flagella and cells becoming concentric spheres, but the cells did not lyse. Moreover, the morphological appearance of cells incubated with 50 $\mu\text{g}/\text{ml}$ metronidazole depended on the strain. The cells of the MTZ-sensitive strain (ATCC 30001) started to round up after incubation with MTZ, even after only one hour of incubation. After 6h of treatment with MTZ nearly all cells of the sensitive strain were spherical with internalized flagella (Fig. 14C)

However, no remarkable morphological changes could be detected within the MTZ-resistant strain (ATCC 50138) after one hour of MTZ treatment, even after six hours of treatment only few of the cells showed any effect (Fig. 15). Cell cultures incubated with DMSO appeared normal shaped without any notable difference to untreated cells (Fig.14)

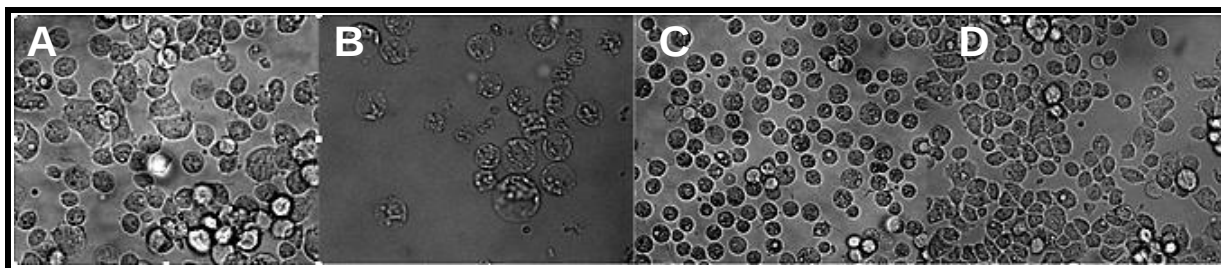


Fig. 14 Morphology of *T. vaginalis* (ATCC 30001) untreated (A) and after 6h of treatment with 3 $\mu\text{g}/\text{ml}$ pentamycin (B), 50 $\mu\text{g}/\text{ml}$ MTZ (C) and DMSO (D). The untreated cells and the cultures with DMSO were normal-shaped. The pentamycin-treated cells were lysed and the MTZ-treated ones were rounded up. 400x magnification (orig.)

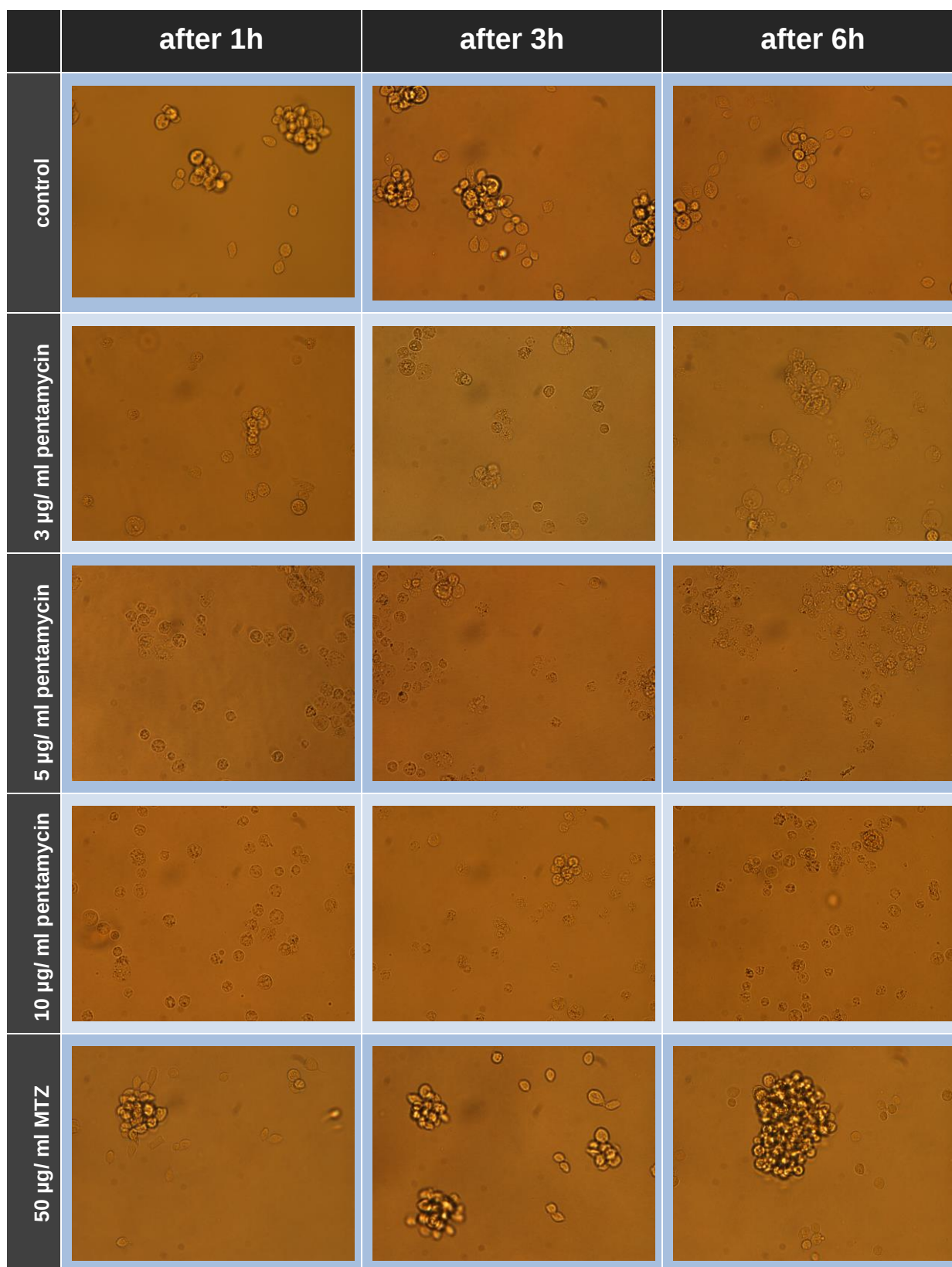


Fig. 15 Morphology of *T. vaginalis* (ATCC 50138) after incubation with varying concentrations of pentamycin after 1, 3 and 6 h. An increase in concentration or time had a negative effect on the viability of the cells; 400x magnification (orig).

Another interesting finding was the accumulation of cells within a few minutes, which could be observed particularly in the ATCC 50138 strain (Fig. 16) in normal grown cultures as well as in long-term treated cells. Even when the cells were separated by softly vortexing or up and down pipetting and were then single-spread, vital cells immediately started to accumulate again. After three minutes, accumulations of trichomonads of up to 30 cells were visible, but still many single cells were observed (approximately 50%, Fig. 16B). Seven minutes later (after 10 min) most of the trichomonads were accumulated in large agglomerates of up to approximately 100 cells (Fig. 16C). The cell number of these large accumulations could be only estimated, because of density and agility of the cells.

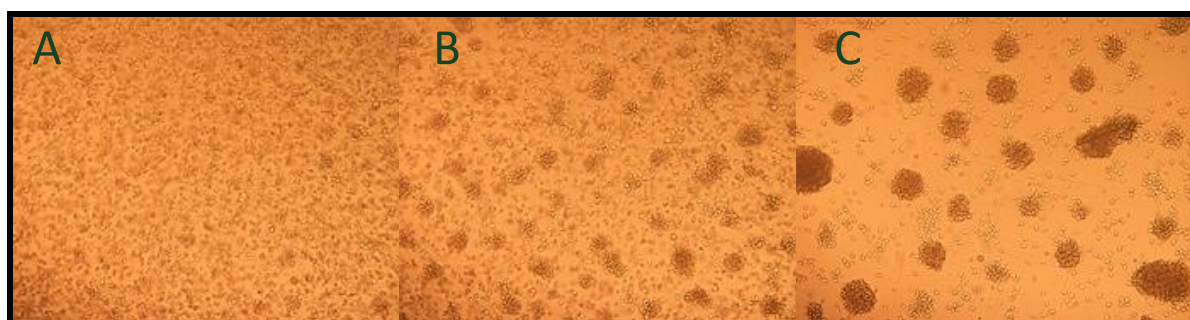


Fig. 16 Accumulating effect in a *T. vaginalis* culture (ATCC 50138) over time. Photos captured immediately after pipetting (A), after 3 min (B) and after 10 min (C). 100x magnification (orig).

Morphology of long-term treated cells

The long-term treated cells, which were grown under continuous drug stress, had aberrant morphological characteristics. In these cells, more immobility was observed, compared to the controls. The cells grown under permanent drug stress were also more rounded-up and often had bizarre shapes and abnormal flagella constructions as well as other morphological abnormalities (Fig. 17).

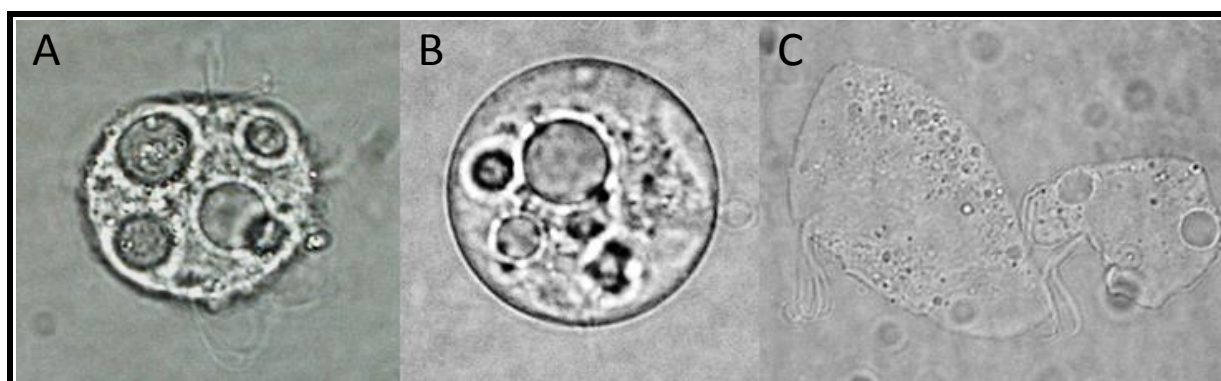


Fig. 17 *T. vaginalis* (ATCC 501238R) after 1 year of low-dose pentamycin treatment. 1000x magnification (orig).

3.1.2 Motility and Viability

In all strains investigated the viability and motility decreased with increasing pentamycin concentrations on one hand and with longer incubation times on the other hand.

Highly metronidazole-sensitive strain (ATCC 30001)

The susceptibility of the metronidazole-sensitive strain (ATCC 30001) after incubation for one, three and six hours with different dilutions of pentamycin (94% and 28% purity, respectively) and MTZ is illustrated in Fig. 18 and Fig. 19.

After 6h of incubation, at a concentration of 5 µg/ ml pentamycin all cells were immobilized, but interestingly, this was not the case after 1h or 3h. Nevertheless, this is a very low concentration, when comparing it to the batch of pentamycin with less purity (28%). Here, 20 µg/ ml only immobilized 97.65% of the cells after one hour, and 100% after 6h. MTZ-treatment (50 µg/ ml) did not show high efficacy after one hour, with 72.2% still mobile cells compared to 78.54% mobile cells in the untreated well. When treatment was extended to 6h only 15% were still mobile. This is equal to approximately 2 µg/ ml of pentamycin with 14% mobile cells. Altogether, when treatment with pentamycin was extended from 1h to 6h, the IC₅₀ies for pentamycin decreased by 30% to values around 0.64 µg/ ml and the IC₉₀ies decreased to 2 µg/ ml (Tab.12).

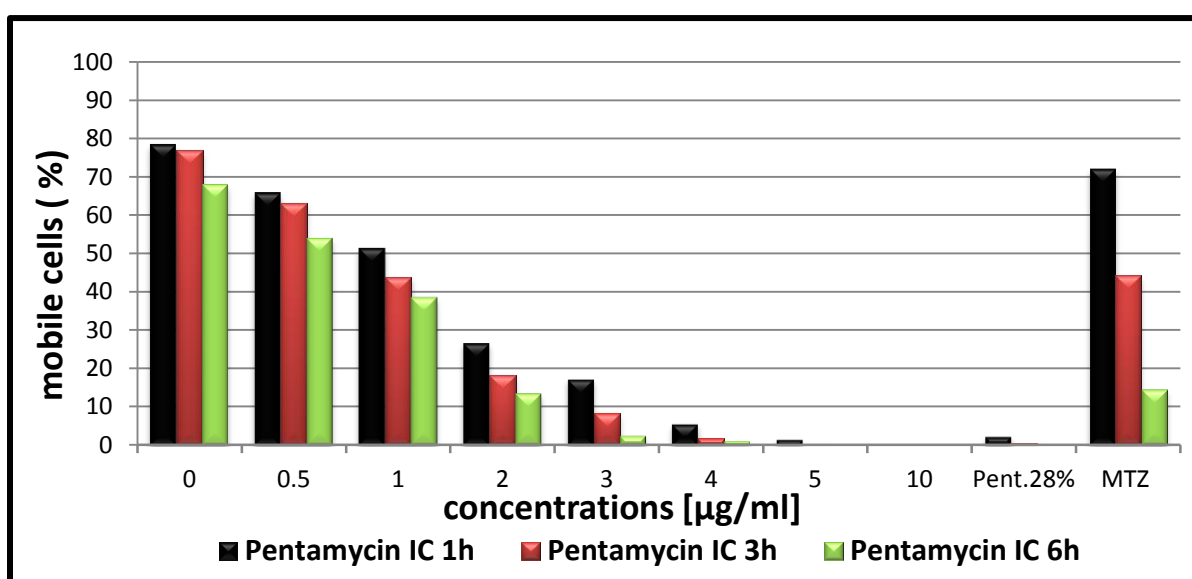


Fig. 18 Motility of *T. vaginalis* strain ATCC 30001 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

The viability after pentamycin treatment is displayed in Fig. 19 and obviously a dose as low as 10 µg/ ml pentamycin (94%) killed all cells at any time. Concerning the duration of treatment, EC₅₀ies ranged from 2.210 µg/ ml (after 1h) to 1.280 µg/ ml (after 6h) and the EC₉₀ies from 6.514 µg/ ml to 3.621 µg/ ml (Table 12). The efficacy of 20 µg/ ml pentamycin with 28% purity (1.18% viable cells) was rather similar to the efficacy of 5 µg/ ml pentamycin with 94% purity (0.71% viable cells). The effectiveness of MTZ (44.56% viable cells) was comparable to pentamycin treatment with 1 or 2 µg/ ml (36.5%).

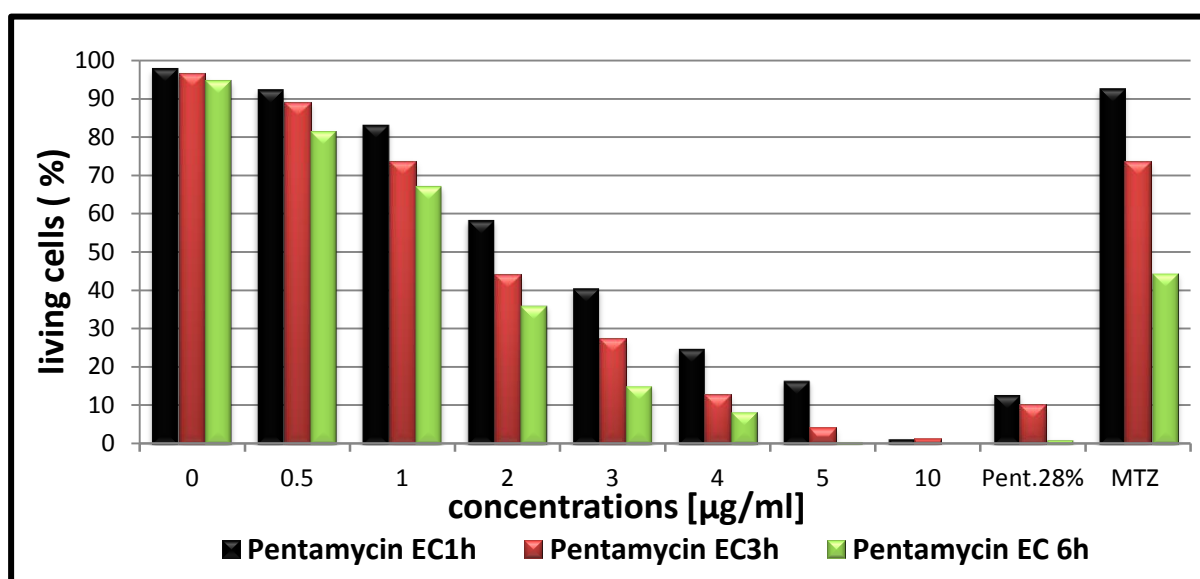


Fig. 19 Viability of *T. vaginalis* strain ATCC 30001 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% with (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

Normally metronidazole-sensitive strain (ATCC 30236)

The motility and viability of the MTZ-sensitive strain (ATCC30236) after incubation for one, three and six hours with different dilutions of pentamycin (94% and 28% purity) and MTZ are displayed in Fig. 20 and Fig. 21.

Concerning the effects after 6h of treatment, with pentamycin there were no motile or living cells left. The mobility rates of cells treated with the pentamycin with 28% purity were comparable with a concentration between 2 to 3 µg/ ml of pentamycin with 94% purity. After six hours MTZ-treatment (50 µg/ ml) approximately half of the cells were immobile (51.28%), which was also observed with 0.5 µg/ ml pentamycin (48.16%). The IC₅₀ies for pentamycin in the ATCC 30236 strain decreased by approximately 40% over time (0.533 µg/ ml).

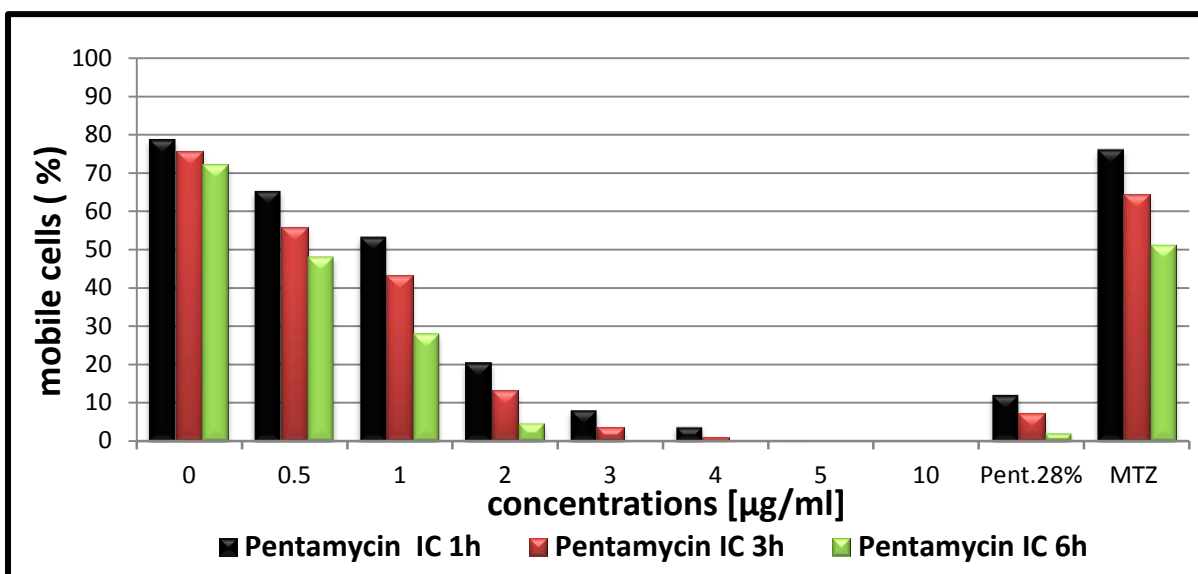


Fig. 20 Motility of *T. vaginalis* strain ATCC 30236 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% with (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

Concerning after six hours of treatment, 5 µg/ ml pentamycin killed all cells. Treatment with 20 µg/ ml pentamycin with less purity only 6.79% of the cells survived, which would be adequate to treatment with 2 µg/ ml (16.73% living cells) or rather 3 µg/ ml (4.26% living cells) of pentamycin with 94% purity. The efficacy of MTZ-treatment was comparable to the efficacy of 0.5 µg/ ml pentamycin with 94% purity, which both killed 70% of the cells. The EC₅₀ies decreased by approximately 50% as well as the EC₉₀ies (Tab. 12).

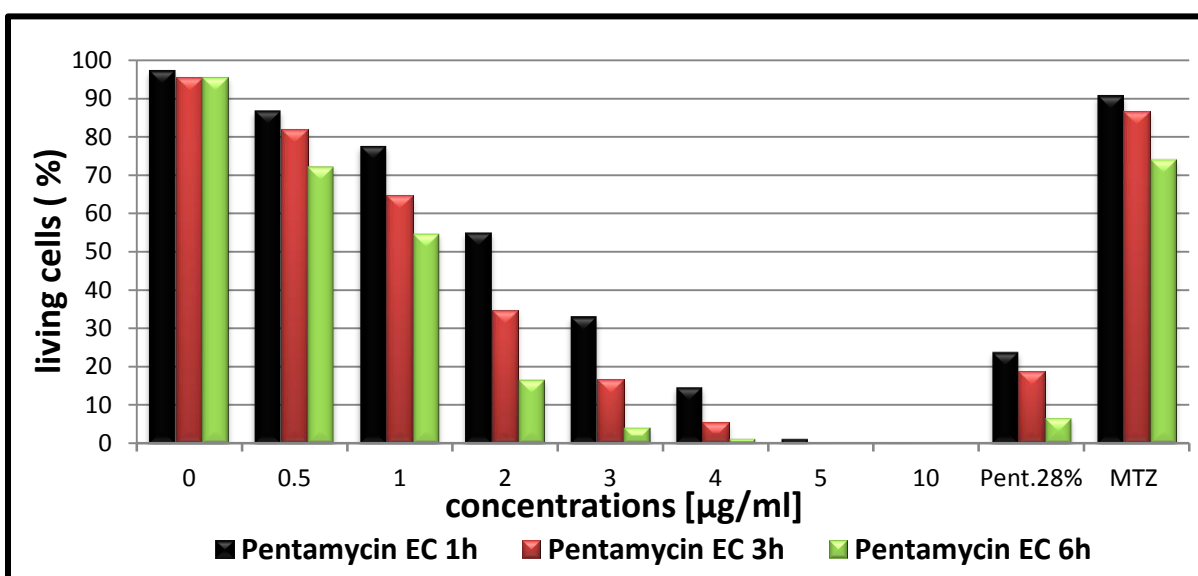


Fig. 21 Viability of *T. vaginalis* strain ATCC 30236 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% with (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

Normal metronidazole-sensitive strain (TV2)

The observed motility and viability after one, three and six hours of incubation for the strain isolated from a patient (TV2) is illustrated in Fig. 22 and Fig. 23.

After 3h of pentamycin treatment with 10 $\mu\text{g}/\text{ml}$ no motile cells were left. The percentage of motile cells after 6h treatment with 20 $\text{mg}/\mu\text{l}$ pentamycin of less purity was close to the values for 4 $\mu\text{g}/\text{ml}$ pentamycin with 94% purity (5.14% motile cells). The number of motile MTZ-treated cells 2 $\mu\text{g}/\text{ml}$ pentamycin, with approximately 16% motile cells left after 6h treatment.

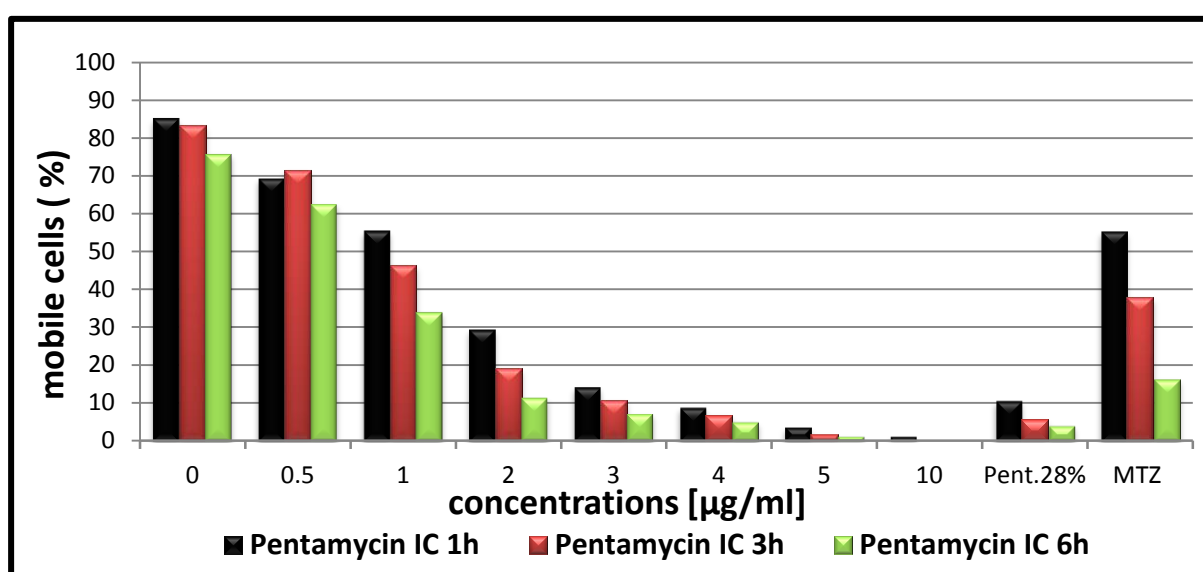


Fig. 22 Motility of *T. vaginalis* strain TV2 after 1, 3 and 6 hours of treatment with pentamycin (blue bars, 94% purity) at concentrations of 0.0 to 10.0 $\mu\text{g}/\text{ml}$, 20 $\mu\text{g}/\text{ml}$ pentamycin with a purity of 28% with (green) and 50 $\mu\text{g}/\text{ml}$ metronidazole (violet).

Concerning the viability, already 10 $\mu\text{g}/\text{ml}$ killed almost all cells after three hours of treatment. The efficacy of 20 $\mu\text{g}/\text{ml}$ pentamycin with less purity (28%) was similar to 4 $\mu\text{g}/\text{ml}$ of pentamycin with higher purity (94%), which killed approximately 90% of the cells. The viability after treatment with 50 $\mu\text{g}/\text{ml}$ MTZ was comparable to pentamycin concentration between 0.5 to 1.0 $\mu\text{g}/\text{ml}$. Also in this strain the EC50ies and the EC90ies were decreased over time by approximately 50%.

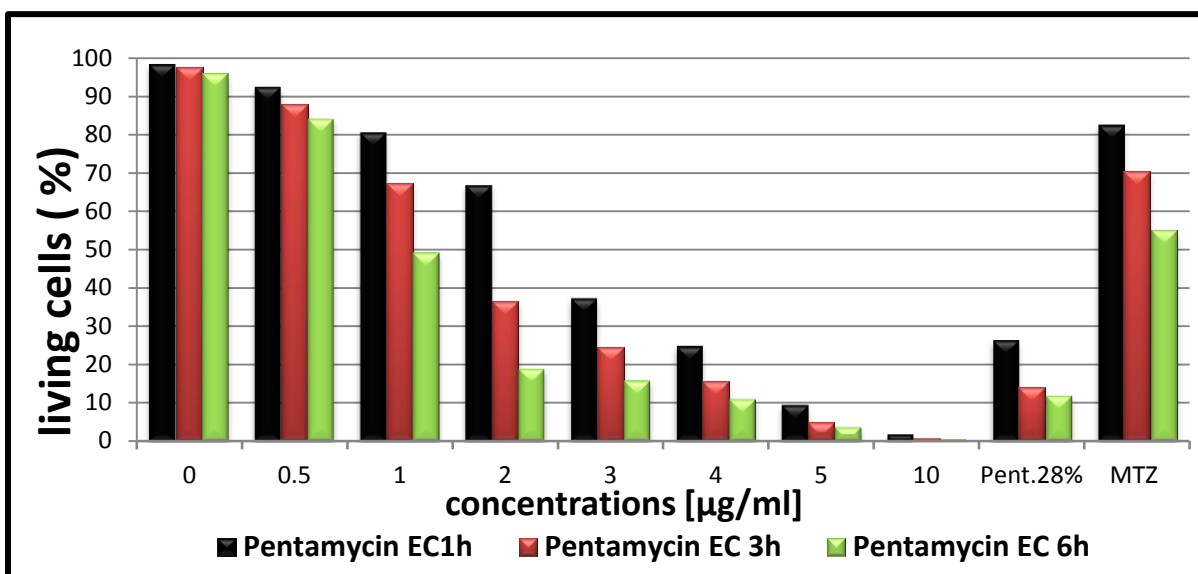


Fig. 23 Viability of *T. vaginalis* strain ATCC TV2 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% with (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

Metronidazole resistant strain (ATCC 50138)

The susceptibility of the MTZ-resistant strain (ATCC 50138) after incubation for one, three and six hours with different dilutions of pentamycin (94% and 28% purity) and MTZ is illustrated in Fig. 24 and Fig. 25.

A decrease of mobility of the cells could already be observed after one hour of treatment with 0.5 µg/ ml pentamycin (86.5% mobile cells) and was most obvious between 1 (73.58%) and 2 µg/ ml (36.05%). After 6h of treatment this effect was seen between 0.5 (76.56%) and 1 µg/ ml (27.27%). At that time point, all cells were immobile when treated with 5 µg/ ml. The efficacy of pentamycin with 28% purity was almost the same (10.56% mobile cells) as of 2 µg/ ml pentamycin with higher purity (11.92%). Whereas the efficacy of 50 µg/ ml MTZ (26.64% mobile cells) was more similar to the effectiveness of 1 µg/ ml pentamycin (27.27%). Altogether, the IC₅₀ies of pentamycin for the ATCC 50138 strain ranged from 1.35 µg/ ml (after 1h) to 0.766 µg/ ml (after 6h), the IC₉₀ies from 3.507 to 1.856 µg/ ml.

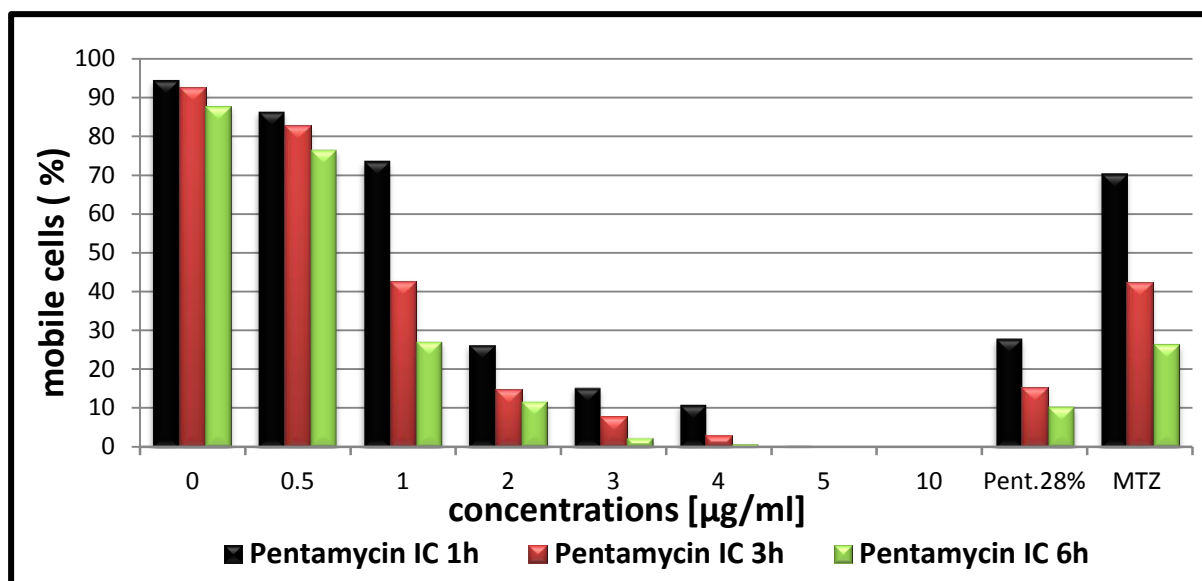


Fig. 24 Motility of *T. vaginalis* strain ATCC 50138 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% with (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

In this strain, the ECs were similar to the ICs. After 6h of pentamycin-treatment almost all cells were killed by 5 µg/ ml (0.07% living cells) and all cells at 10 µg/ ml pentamycin. At that time point, 18.41% of the cells were still alive when treated with 20 µg/ ml pentamycin with 28% purity, which is comparable to the efficacy of 2 µg/ ml pentamycin with 94% purity (22.03% living cells). The efficacy of 50 µg/ ml MTZ (65.77% living cells after 6 hours) is comparable to the one of 1 µg/ ml (46.39%) or 0.5 µg/ ml (89.64%) pentamycin. Altogether, over the entire treatment period, the EC50ies ranged from 2.62 to 1.06 µg/ ml and the EC90ies from 5.77 to 2.46 µg/ ml.

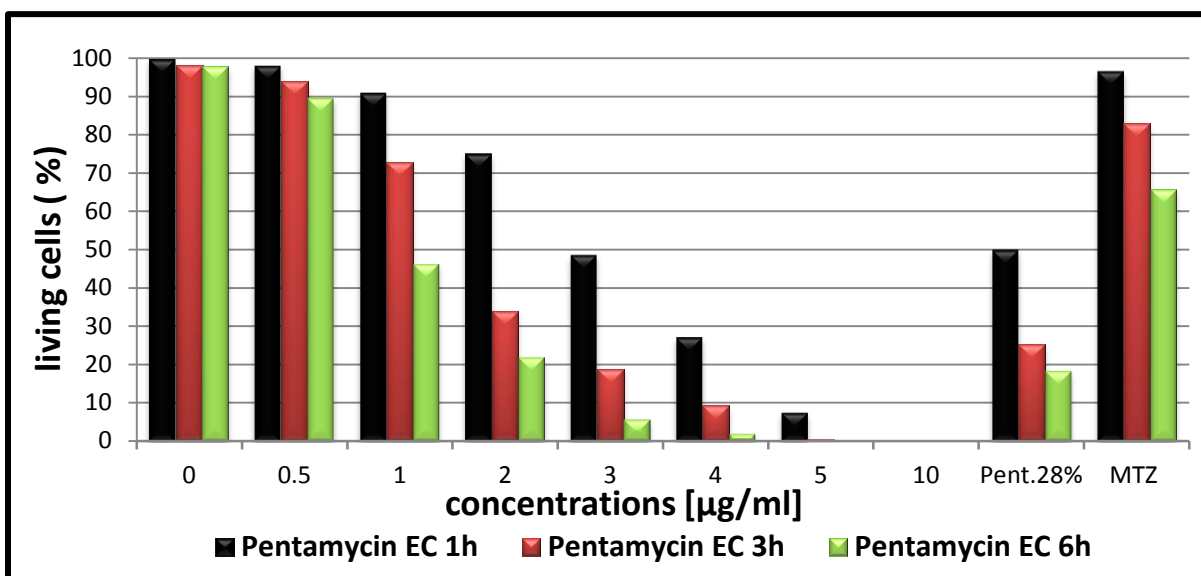


Fig. 25 Viability of *T. vaginalis* strain ATCC 50128 after 1, 3 and 6 hours of treatment with pentamycin (94% purity) at concentrations from 0 to 10.0 µg/ ml, 20 µg/ ml pentamycin with a purity of 28% with (Pent.28%) and 50 µg/ ml metronidazole (MTZ).

Tab.12 gives an overview of the ICs and ECs for all strains investigated. There were only minor differences between the strains. The highest IC value was evaluated for the ATCC 50138 strain (0.766 µg/ ml) and the lowest for the ATCC 30236 strain (0.533 µg/ ml). The EC values ranged from 0.913 µg/ ml for the ATCC 30236 strain to 1.28 µg/ ml for the ATCC 30001 strain.

Tab. 12 Summarized inhibitory (ICs) and effective (ECs) concentrations of pentamycin for all four strains tested after one, three and six hours of treatment.

Inhibitory (IC) and Effective Concentrations (EC)			
ATCC 50138	1h	3h	6h
IC 50	1.350	0.941	0.766
IC 90	3.507	2.378	1.856
EC 50	2.620	1.505	1.061
EC 90	5.770	3.568	2.459
ATCC 30236			
IC 50	0.869	0.680	0.533
IC 90	2.725	2.081	1.455
EC 50	1.744	1.248	0.913
EC 90	4.909	3.443	2.357
TV2			
IC 50	1.004	0.887	0.678
IC 90	3.778	2.904	2.362
EC 50	2.169	1.478	1.085
EC 90	6.205	4.481	3.467
ATCC 30001			
IC 50	0.922	0.774	0.639
IC 90	3.369	2.450	2.010
EC 50	2.210	1.620	1.280
EC 90	6.514	4.616	3.621

3.1.3 Long-term treatment (R-strains)

Generally, all strains were slightly less susceptible to pentamycin after long-term treatment. However, this effect was reversible when cells were again grown under normal conditions. Furthermore, after cryo-conservation of the R-strains their susceptibility to pentamycin was even enhanced.

Highly metronidazole-sensitive strain, long-term treated (ATCC 30001R)

The susceptibility of the long-term treated MTZ-sensitive strain after incubation for one, three and six hours with different dilutions of pentamycin is shown in Fig. 26, Fig. 27 and Fig. 28.

After 6h of treatment with 10 µg/ ml pentamycin all cells from the standard strain were killed, but at any time point a few cells of the long-term treated sub-strain were still alive at this concentration. The general decrease of the susceptibility to pentamycin is also shown by the increase of the EC₅₀ies from 1.62 µg/ ml in the standard strain to 3.36 µg/ ml in the R-strain after 12 months.

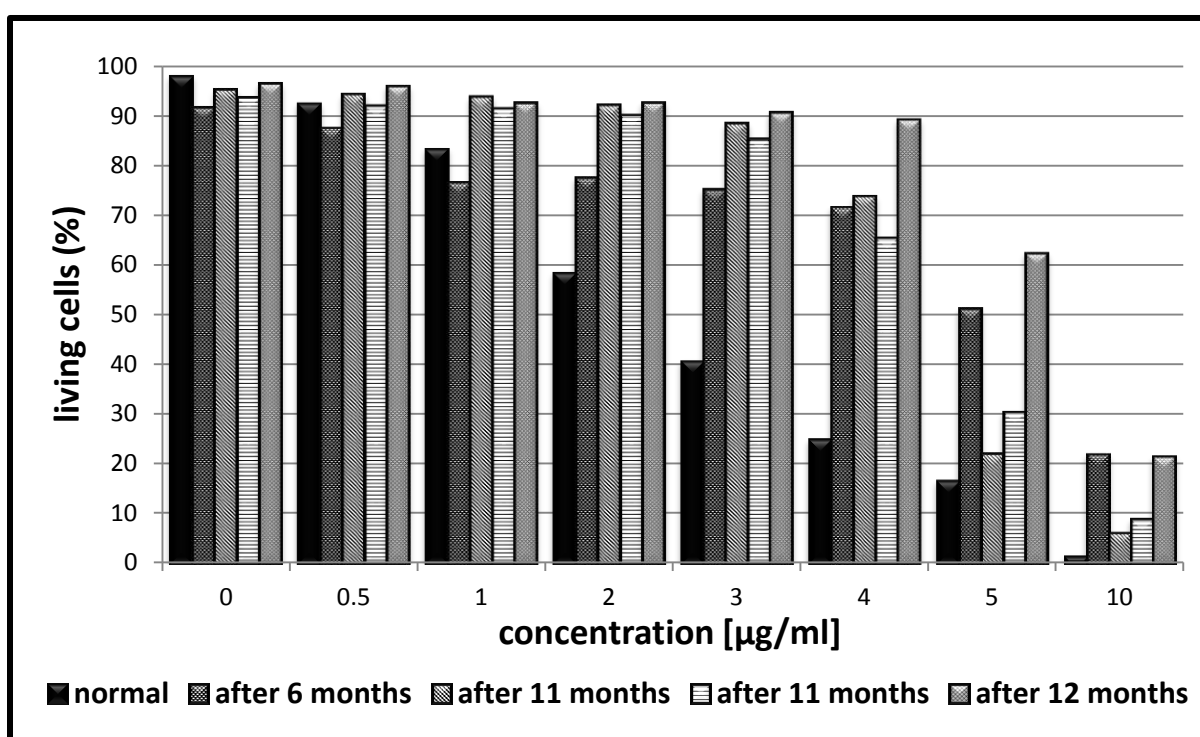


Fig. 26 Susceptibilities of the strains ATCC 30001 and ATCC 30001R to pentamycin over time with increasing concentrations of pentamycin after one hour of treatment.

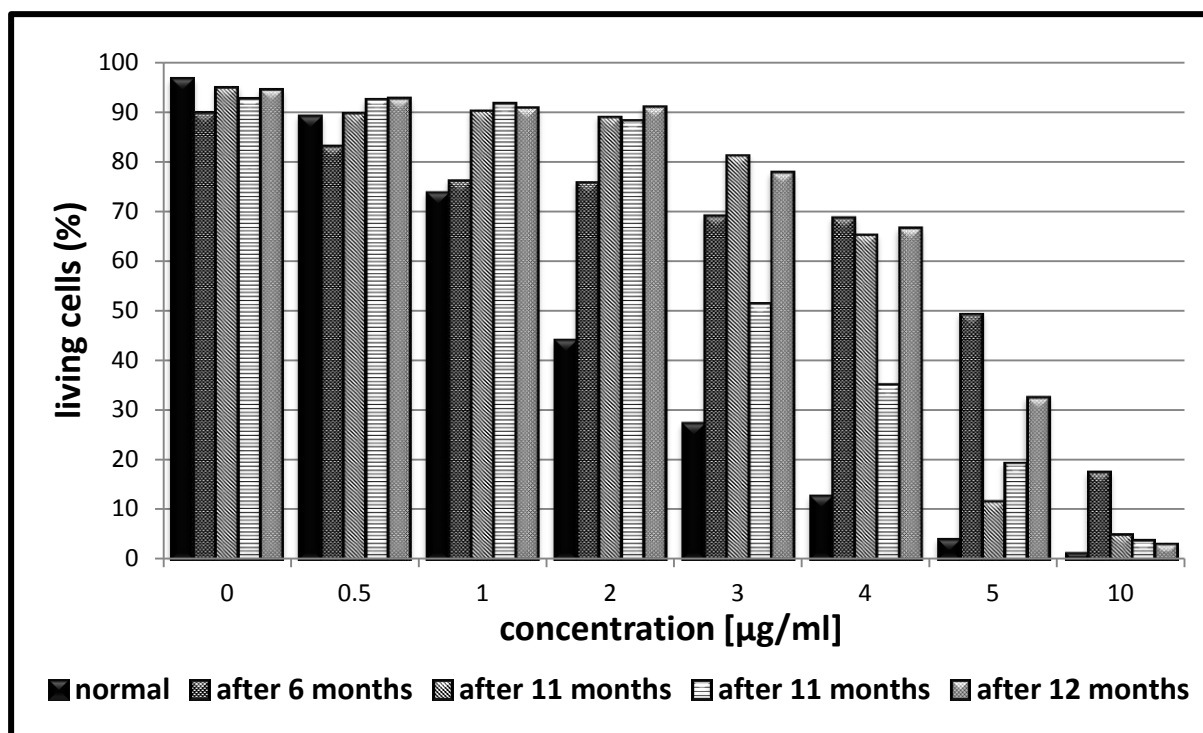


Fig. 27 Susceptibilities of the strains ATCC 30001 and ATCC 30001R to pentamycin over time, with increasing concentrations of pentamycin after three hours of treatment.

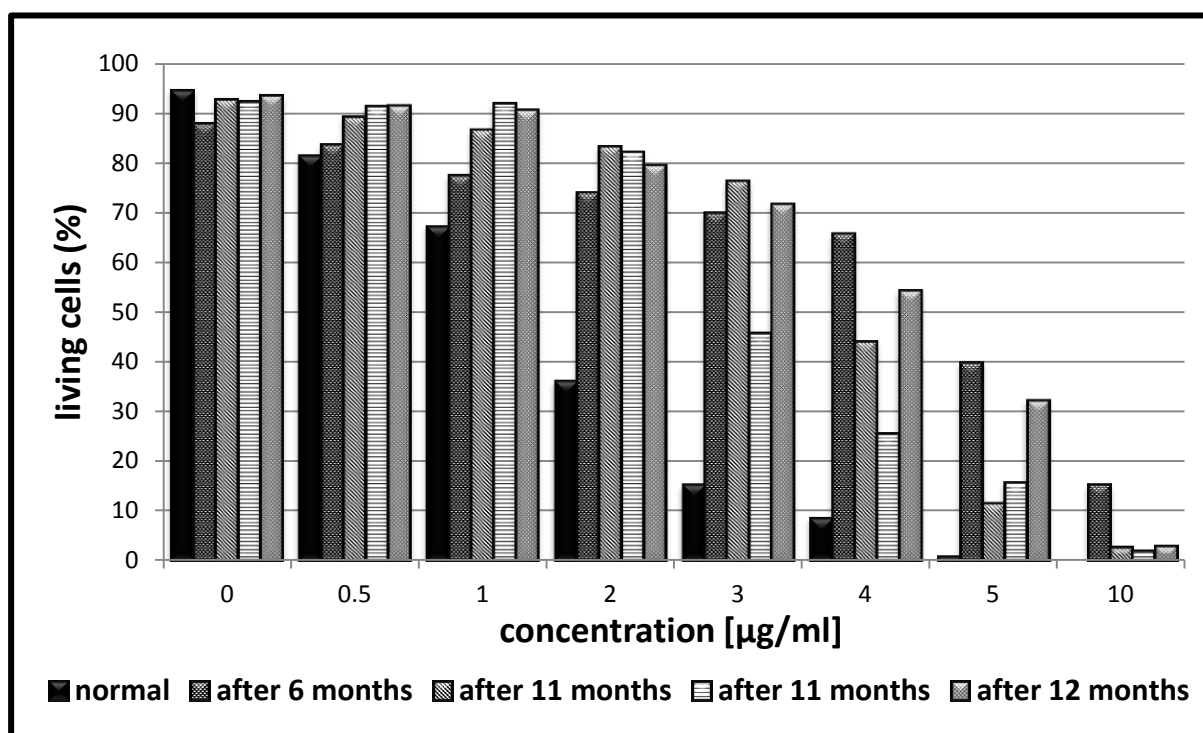


Fig. 28 Susceptibilities of the strains ATCC 30001 and ATCC 30001R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after six hours of treatment.

Normal metronidazole-sensitive strain, long-term treated (ATCC 30236R)

The susceptibility of the long-term treated strain ATCC 30236R is shown in Fig. 29, Fig. 30 and Fig. 31.

In this strain, after 5 month of low-dose long-term treatment the EC₅₀ies had increased to the approximately 3.3fold and after 11 month to the 2.54fold .

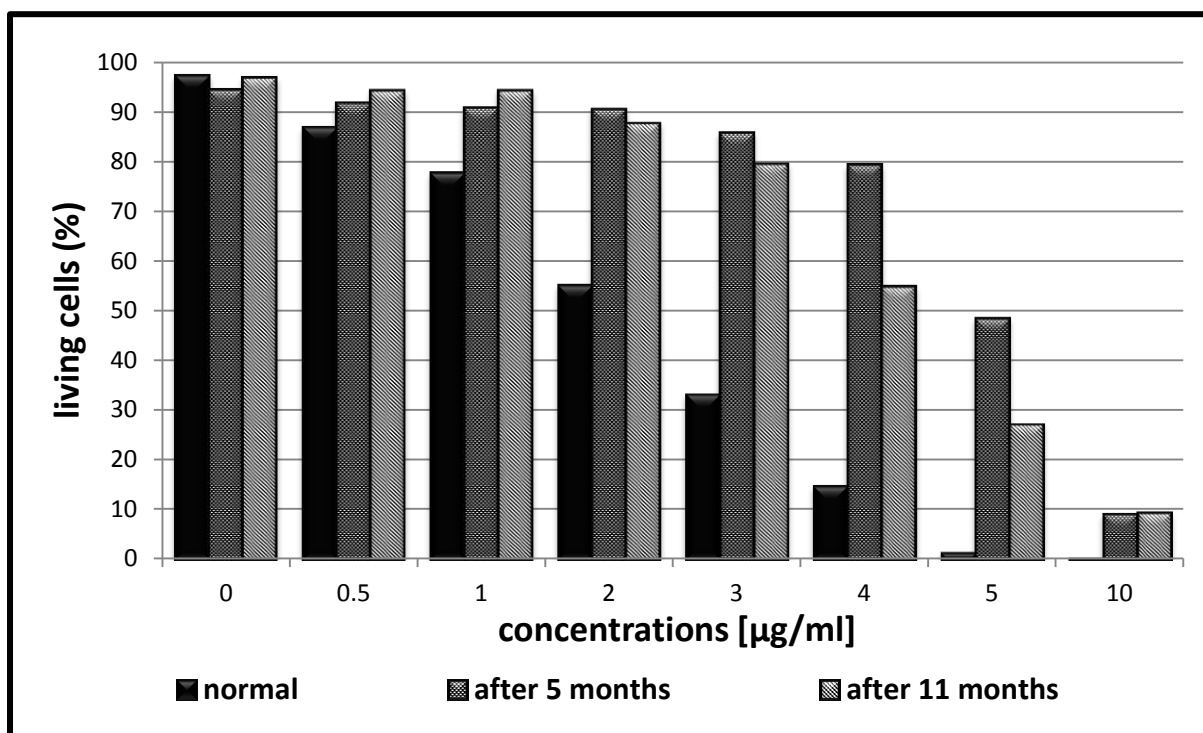


Fig. 29 Susceptibilities of the strains ATCC 30236 and ATCC 30236R to pentamycin over time with increasing concentrations of pentamycin after one hour of treatment.

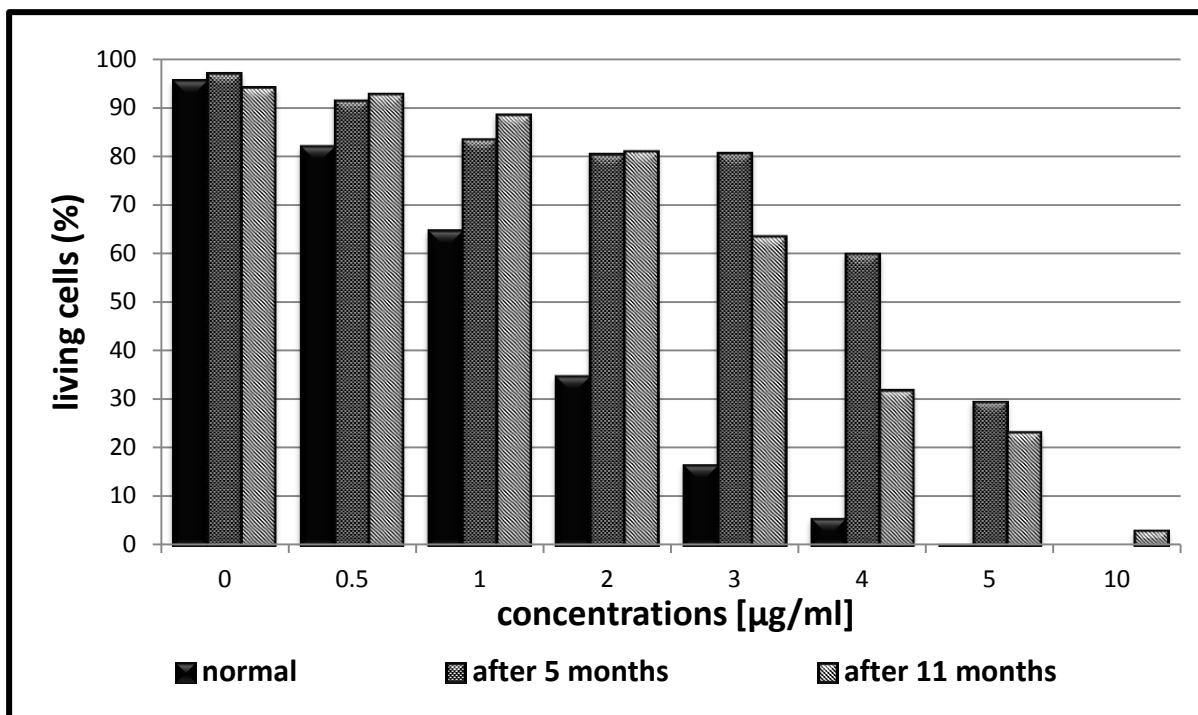


Fig. 30 Susceptibilities of the strains ATCC 30236 and ATCC 30236R to pentamycin over time with increasing concentrations of pentamycin after three hours of treatment.

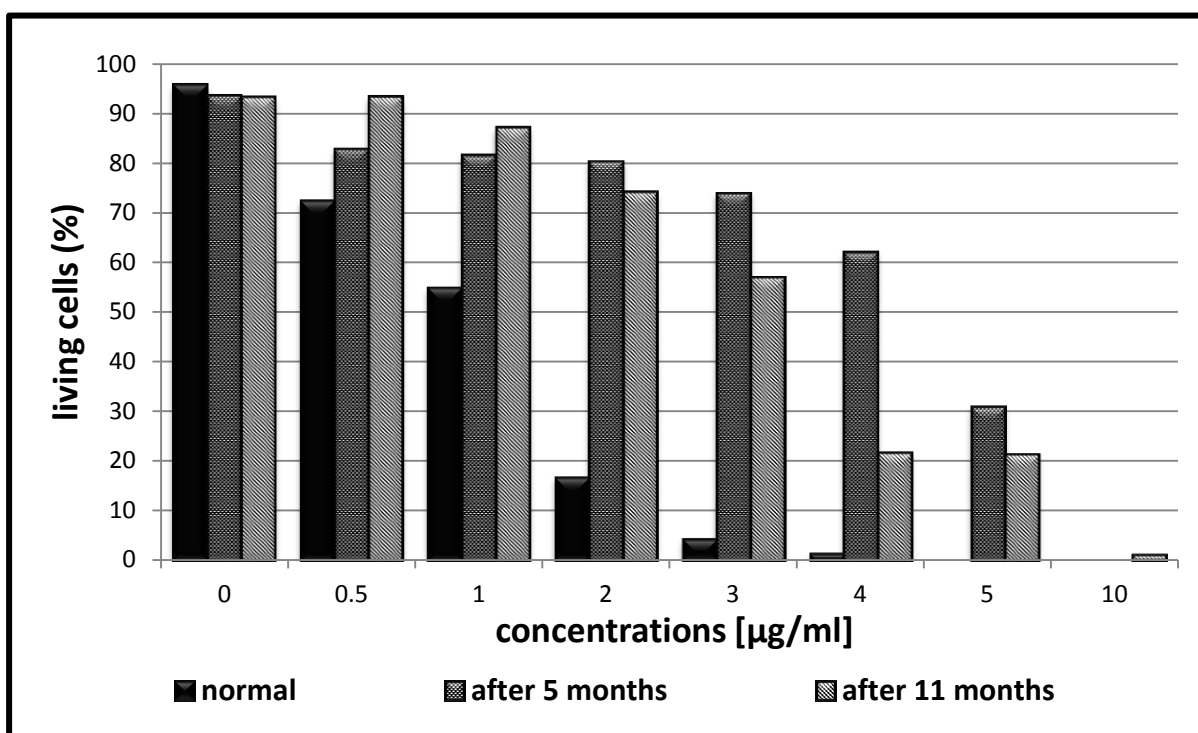


Fig. 31 Susceptibilities of the strains ATCC 30236 and ATCC 30236R to pentamycin over time with increasing concentrations of pentamycin after six hours of treatment.

Normal metronidazole-sensitive strain, long-term treated (TV2R)

The susceptibility of the long-term treated strain TV2R to pentamycin after one, three and six hours of treatment. is demonstrated in Fig. 32, Fig. 33 and Fig. 34.

After eleven months of long-term treatment the increase in tolerance to pentamycin compared to the normal strain was almost the same for all three time points, namely approximately the 3.16fold. Interestingly, after 12 months of long-term treatment, the susceptibility to pentamycin was higher than after 11 months (Fig. 32) . Generally, the differences in susceptibility to pentamycin between the R-strain and the normal strain were particularly significant at higher pentamycin concentrations.

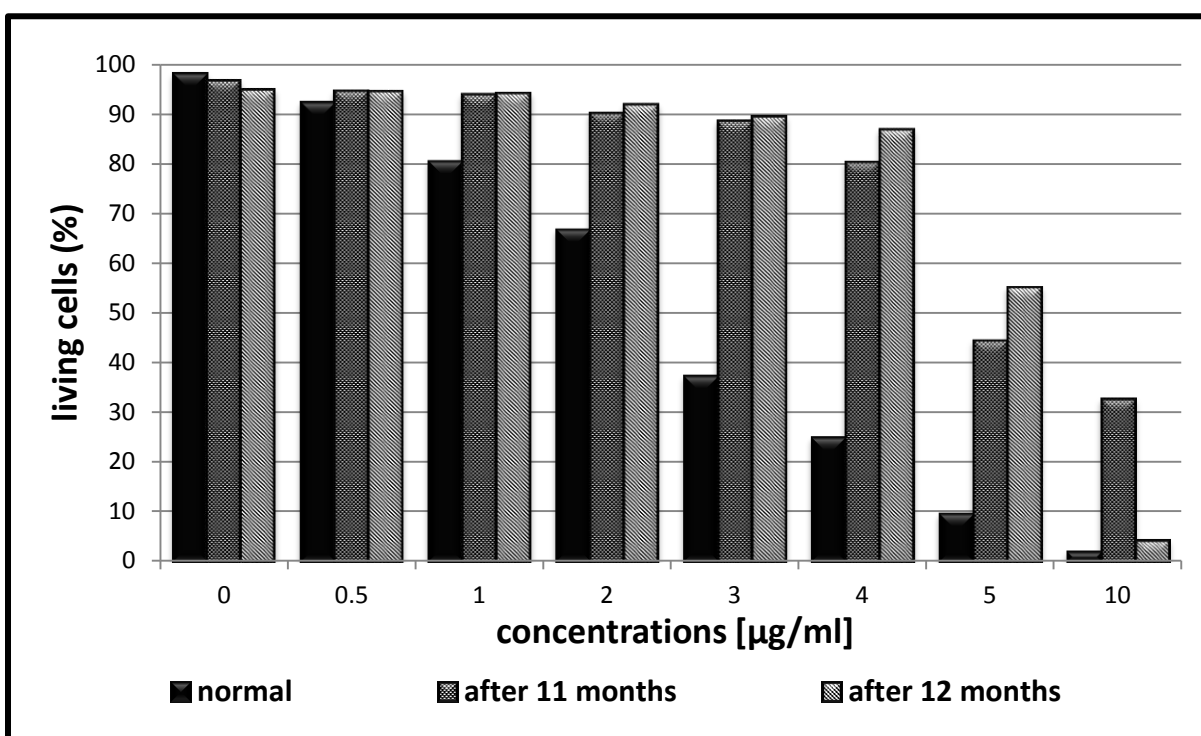


Fig. 32 Susceptibilities of the strains TV2 and TV2R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after one hour of treatment.

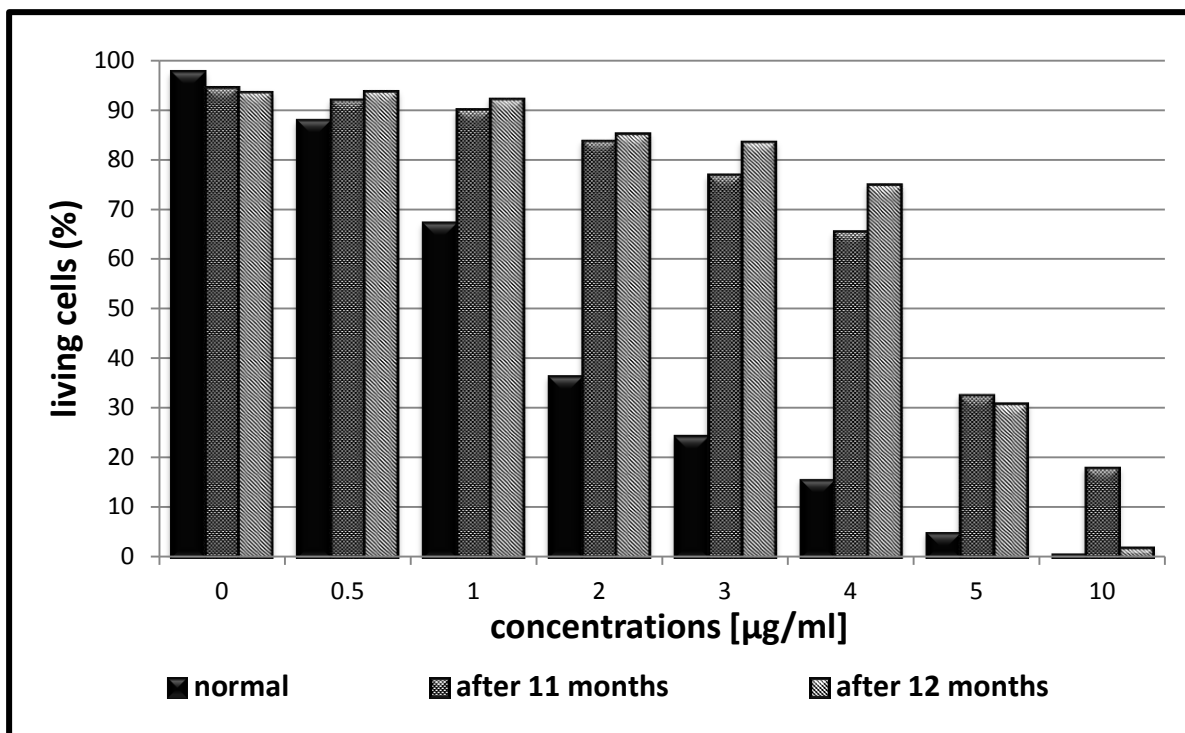


Fig. 33 Susceptibilities of the strains TV2 and TV2R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after three hours of treatment.

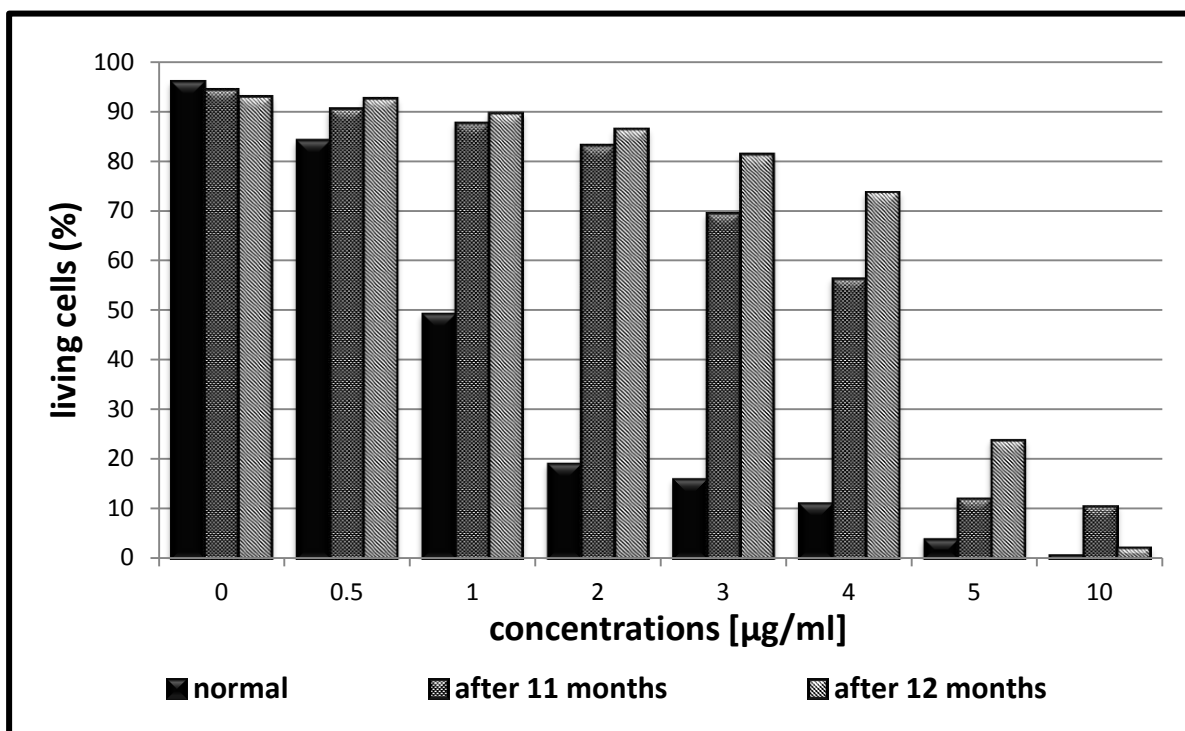


Fig. 34 Susceptibilities of the strains TV2 and TV2R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after six hours of treatment.

Metronidazole resistant strain, long-term treated (ATCC 50138)

The susceptibility to pentamycin for the long-term treated strain ATCC 50138R is illustrated in Fig. 35-Fig. 37 .

The effect of the long-term treatment was highest in the rapid effect of pentamycin. When the R-strain was compared to the normal strain after 1h of incubation with pentamycin the EC₅₀ies had increased to the 3fold, namely from 2.62 to 8.1 µg/ml after 11 months compared to only the 2.5fold after 12 months.

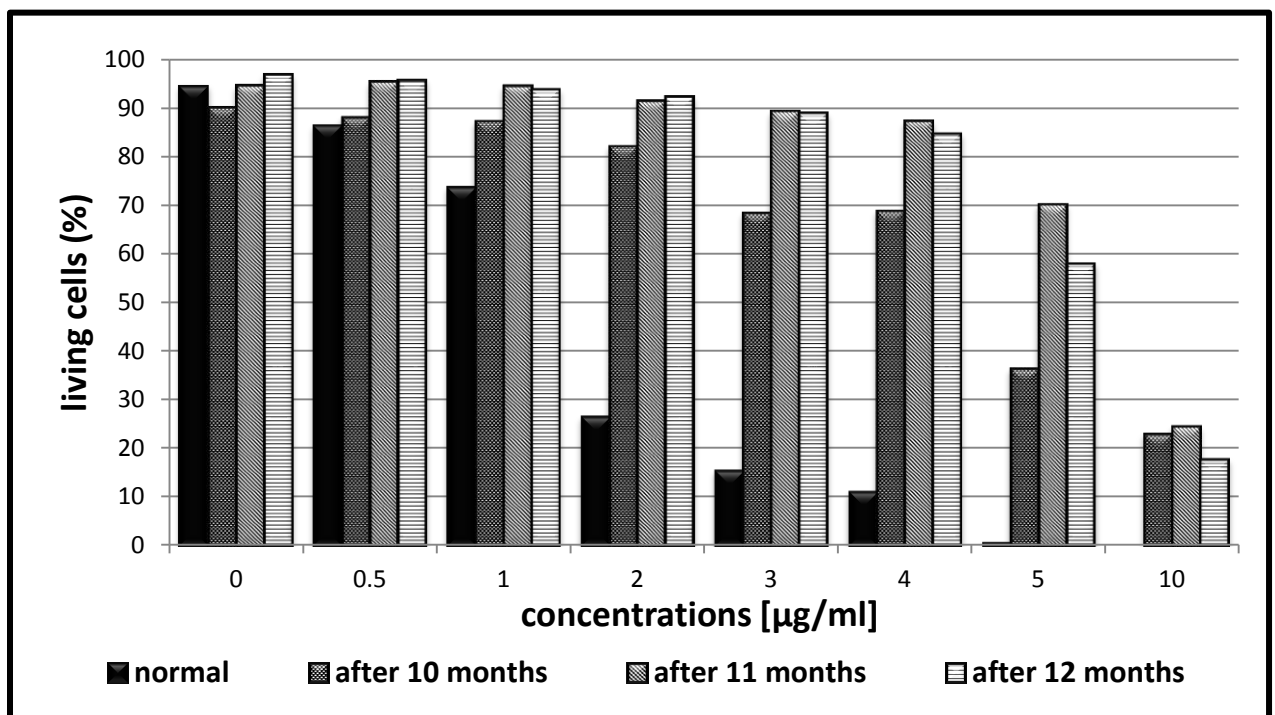


Fig. 35 Susceptibilities of the strains ATCC 50138 and ATCC 50138R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after one hour of treatment.

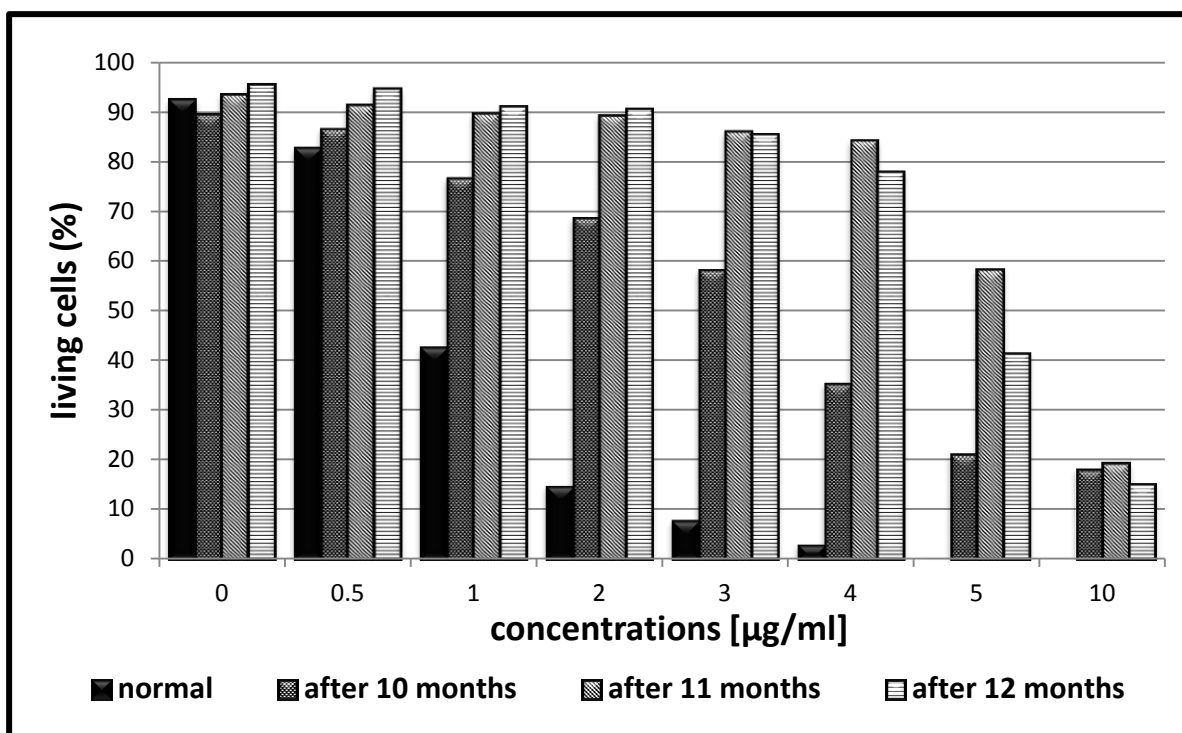


Fig. 36 Susceptibilities of the strains ATCC 50138 and ATCC 50138R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after three hours of treatment

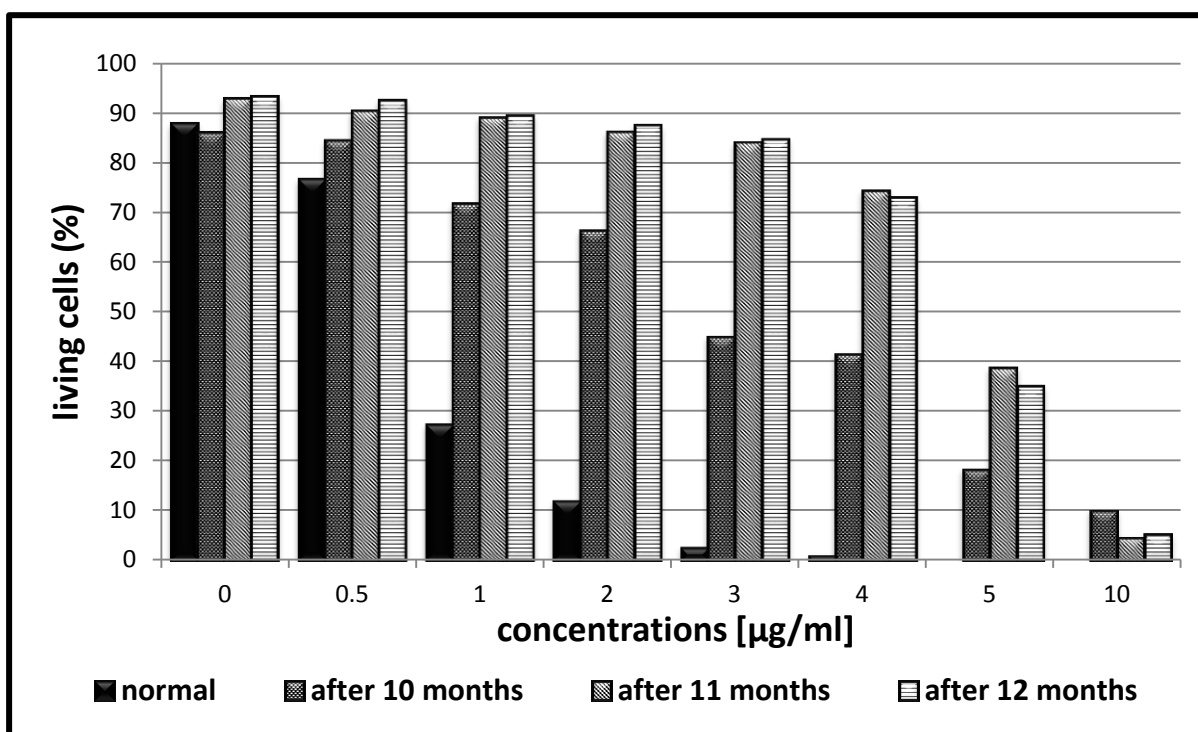


Fig. 37 Susceptibilities of the strains ATCC 50138 and ATCC 50138R to pentamycin over time. The viability is illustrated in correlation with increasing pentamycin concentrations after six hours of treatment.

Overview of effective concentrations (EC) for long-term treated strains

Tab. 13 summarizes the EC₅₀ies and EC₉₀ies of pentamycin for the long-term-treated strains, focussing on the effect of 12 months of long-term treatment. The tolerances to pentamycin of the long-term treated strains had increased to the 2.33-4.3fold concerning the EC₅₀ies and to the 2.49-6.52fold concerning the EC₉₀ies.

After one hour of treatment the strain ATCC 30001R showed the highest increase, however, this effect was reduced after six hours to the average level of the standard strains. In contrast, the ATCC 50138R strain showed an average decrease of susceptibility after one hour, but after three and six hours of treatment the increase in tolerance was significantly higher than in the other strains. Generally, an increase in tolerance could be observed in all four strains tested, but there were slight concerning the final tolerance levels and also the process of tolerance development (Tab. 13).

Tab. 13 Effective concentrations (ECs) of pentamycin for all four long-term treated strains tested (after 12 months of long-term treatment) after one, three and six hours of incubation. The ECs from normal strains (normal) are given for comparison.

Strain	EC ₅₀ (1h, µg/ ml)		EC ₅₀ (3h, µg/ ml)		EC ₅₀ (6h, µg/ ml)	
	normal	12 months	normal	12 months	normal	12 months
ATCC 30001	2.210	7.435	1.620	4.204	1.280	3.636
ATCC 30236	1.744	4.069	1.248	3.008	0.913	2.646
TV	2.169	5.712	1.478	4.337	1.085	4.094
ATCC 50138	2.620	6.578	1.505	5.437	1.061	4.563
	EC ₉₀ (1h, µg/ ml)		EC ₉₀ (3h, µg/ ml)		EC ₉₀ (6h, µg/ ml)	
	normal	12 months	normal	12 months	normal	12 months
ATCC 30001	6.514	28.799	4.616	13.303	3.621	12.392
ATCC 30236	4.909	12.199	3.443	9.093	2.357	7.581
TV	6.205	17.369	4.481	13.231	3.467	13.148
ATCC 50138	5.770	23.026	3.568	19.841	2.459	16.031

3.2 Molecular biology

3.2.1 Primer design

The selected conserved protein sequences for HMGS and PMVK were screened for primer binding sites and were blasted against the genome of the reference strain *T. vaginalis* G3. The primer sequences are given in tables 14 and 15.

Tab. 14 Primer sequences based on protein ([XP_001302712.1](#)) and DNA ([XM_001302711.1](#)) sequences of the reference strain of *T. vaginalis* (strain G3).

Protein sequence	Position	Primer designation	Primer sequence	Nucleotide position
NACYGGT	124	HMGS1fw	aatgcctgtta tggcggaacc	370
AARPTGG	163	HMGS2 fw /(rev)	gctgccagacc aacaggagga	490
FSYGSG(AA)	346	HMGS3rev	ttagctacggtag tggt(gcagca)	1027

Tab. 15 Hypothetical protein (PMVK): Primer sequences based on protein ([XP_001324679.1](#)) and DNA ([XM_001324644.1](#)) sequences of the reference strain of *T. vaginalis*.

Protein sequence	Position	Primer designation	Primer sequence	Nucleotide position
KTGLGSS	135	PMVK1fw	gtaaaacaggtcttggatcaa	403
AQKKIGSGF D	182	PMVK2 fw /(rev)	gaagattggatcaggattcga	543
PGAGGYDAI A	333	PMVK3rev	gctatagcatcgtagccacctgcacc	996

Altogether, eight primers, two primer pairs for each gene, were designed and evaluated for their suitability to amplify the genes for the hypothetical proteins PMVK and HMGS. The details of the designed primers are given in Tab. 16.

Tab. 16 Selected primers for PMVK and HMGS.

Primer	Sequence [5'-3']	Length [nt]	GC-content [%]	Melting temperature TM [°C]
PMVK1fw	GTA AAA CAG GTC TTG GAT CAA G	22	54	51.1
PMVK2rev.	GTC GAA TCC TGA TCC AAT CTT C	22	45	53.0
PMVK2fw	GAA GAT TGG ATC AGG ATT CGA	21	43	50.5
PMVK3rev.	GCT ATA GCA TCG TAG CCA CC	20	55	53.8
HMGS1fw.	GCC TGT TAT GGC GGA ACC	18	61	52.6
HMGS2rev.	GTC CTG TTG GTC TGG CAG	18	61	52.6
HMGS2fw	GCT GCC AGA CCA ACA GGA	18	61	52.6
HMGS3rev.	CTG CAC CAC TAC CGT AGC	18	61	52.6

The primer binding sites were evaluated by alignment of the primers to the corresponding DNA and mRNA sequences (an examples for such alignments are shown in Fig. 38 and Fig. 39). These alignments revealed the *in silico* absence of introns on the one hand and visualized the expected binding sides for the designed primers on the other hand.

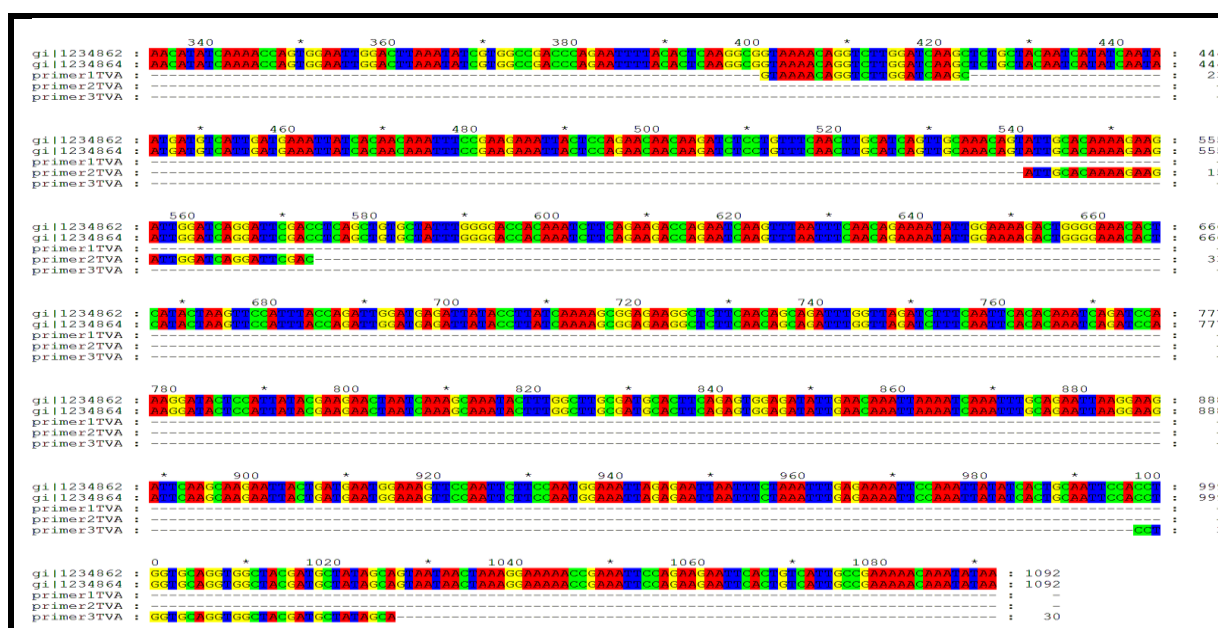


Fig. 38 ClustalX alignment of the primers designed to amplify a fragment of PMVK to the genomic (gi|1234862) and mRNA (gi|1234864) of the reference strain. Primer1TVA refers to PMVK1fw, primer2TVA to PMVK2rev./PMVK2fw and primer3TVA to PMVK3rev.

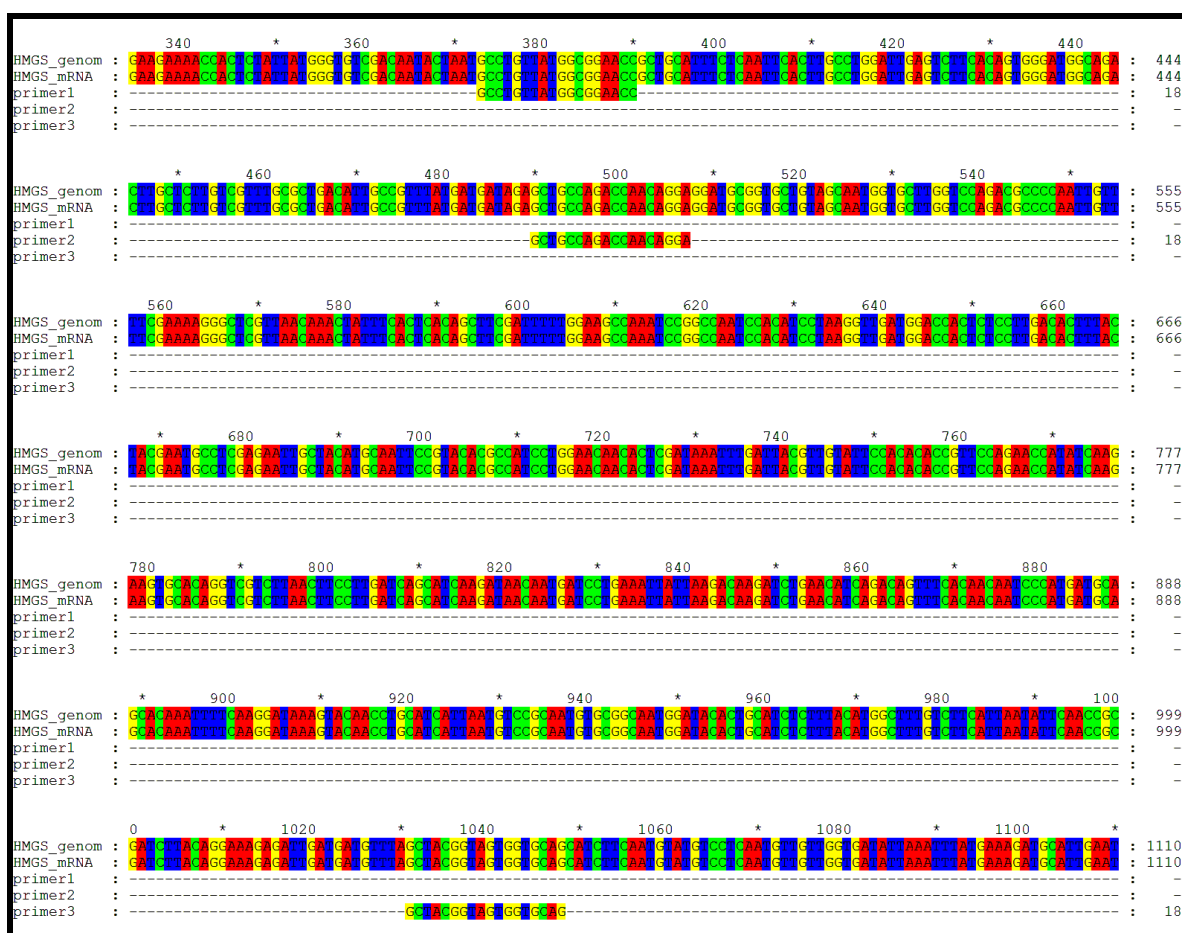


Fig. 39 ClustalX alignment of the primers designed to amplify a fragment of HMGS to the genomic (HMGS_genom; gi|123405960:c21644-20343) and mRNA (HMGS_mRNA; gi|123405955) of the reference strain. Primer1 refers to HMGS1fw, primer2 to HMGS2rev./HMGS2fw and primer3 to HMGS3rev.

3.2.2 Control PCR

As shown in Fig. 40, the extracted DNA was of good quality. The two control PCRs gave clear bands at the correct positions, namely at 100 bp for the BTUB-PCR and at approximately 250 bp for the TVK-PCR.

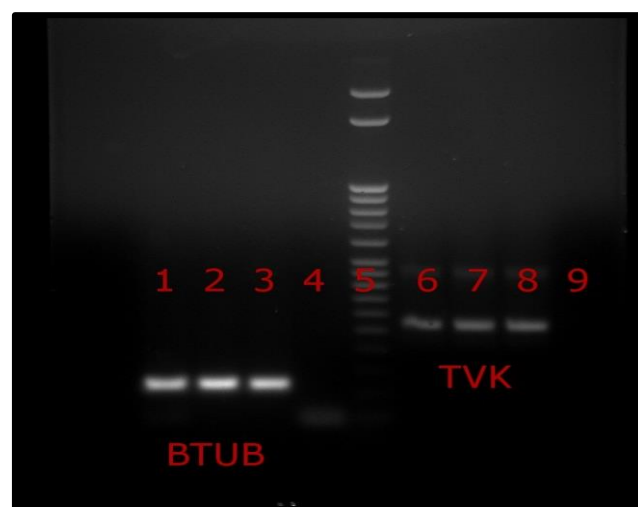


Fig. 40: Agarose Gel after DNA isolation & PCR: PCR for the β -tubulin gene (BTUB) (slots 1-3); and for TVK (6-9), step ladder marker (5); PCR controls (4,9)

3.2.3 PCR

As shown in Fig. 41 and Fig. 42 the amplification of PMVK and HMGS was successful. The PCR for PMVK gave bands at 100 bp (L1-L3) for the first primer pair (PMVK1fw/PMVK2rev) and at approximately 450 bp for the second one (PMVK2fw/PMVK3rev). Also the PCR for HMGS gave bands at 100 bp (L1-L3) (HMGS1fw/HMGS2rev) and at 500 bp for second primer pair (HMGS2fw/HMGS3rev). The smaller amplicon from the PMVK-PCR was present at 100 bp and the longer amplicon is present above the 500 bp line.



Fig. 41 PMVK-DNA bands on agarose gel from *T. vaginalis*: Primer PMVK1fw/PMVK2rev (line L1-L3) and PMVK2fw/PMVK3rev (L4-L6), the negative control are in line 7 (L7) and the DNA marker in line 9 (L9).

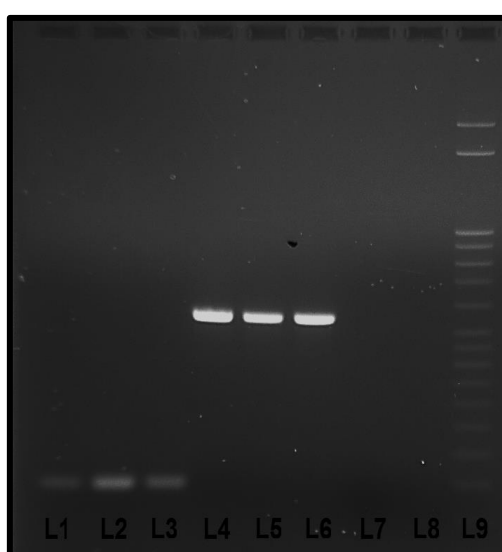


Fig. 42 HMVK-DNA bands on agarose gel from *T. vaginalis*: Primer HMVK1fw/HMVK2rev (line L1-L3) and HMVK2fw/HMVK3rev (L4-L6), the negative control are in line 7 (L7) and the DNA marker in line 9 (L9).

3.2.4 Sequencing

DNA sequencing of the PMVK PCR amplicons gave a fragment of 574 nucleotides (Fig. 43), which has a 99% similarity (573/574) to the respective gene of the reference strain *T. vaginalis* G3, as revealed by BLAST search against the *T. vaginalis* genome and subsequent alignment of the obtained sequence to the reference sequences.

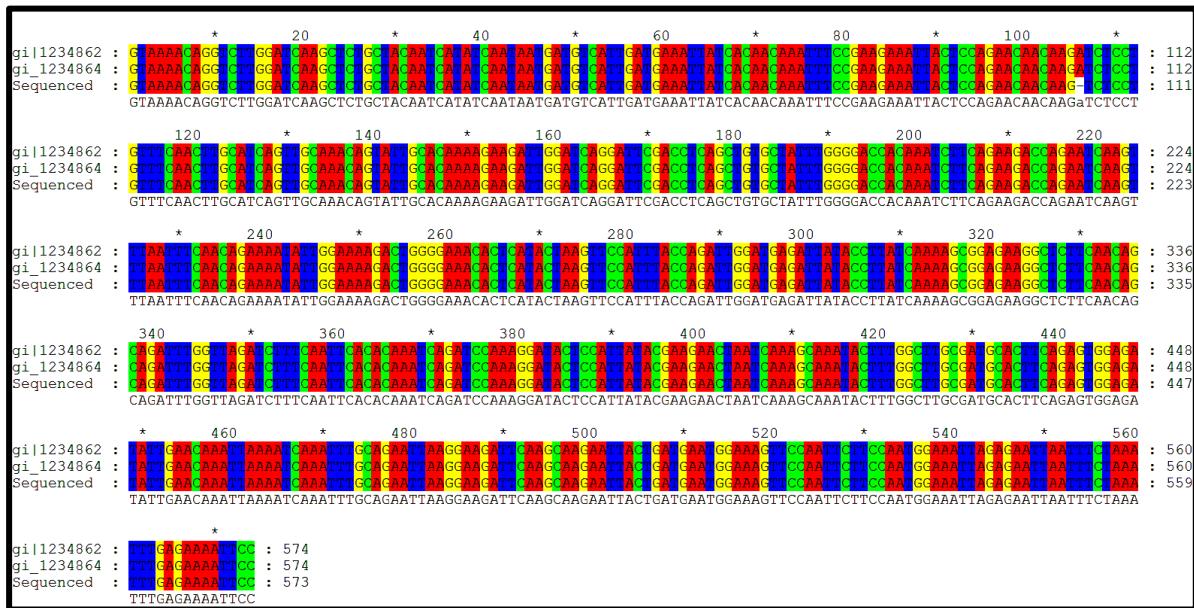


Fig. 43 ClustalX alignment of the obtained PMVK sequence to the genomic (gi|1234862) and mRNA (gi_1234864) sequences of the reference strain (*T. vaginalis* G3).

DNA sequencing of the HMGS PCR amplicons gave a fragment of 281 nucleotides (Fig. 44), which has a 98% similarity (277/281) to the respective gene of the reference strain *T. vaginalis* G3, as revealed by BLAST search against the *T. vaginalis* genome and subsequent alignment of the obtained sequence to the reference sequences.

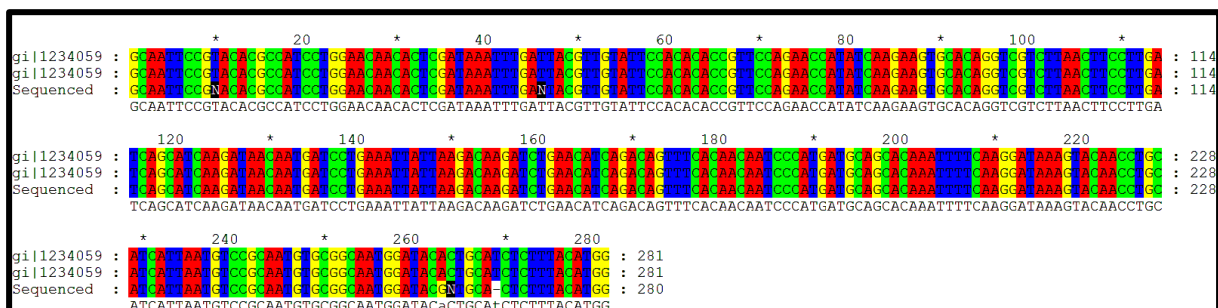


Fig. 44 ClustalX alignment of genomic (gi|1234059551; 1.line), mRNA (2.line) from the reference strain (*T. vaginalis* G3) and the HMGS sequence from the sequencer (Sequenced, 3.line).

4 Discussion

4.1 Pentamycin is highly active against *Trichomonas vaginalis*

4.1.1 Effect on morphology

Treatment with pentamycin lead to a rounding up of the cells and cell shape deformation, in normal-grown cells treated with low concentrations as well as in the long-term treated cells. Drug-induced stress is known to cause rounding up of cells. Especially in the highly MTZ-sensitive strain (ATCC 30001) the processes of rounding up and cell shape deformation were observed. These immobile and often enlarged round forms may be seen as pseudocysts or cysts. This controversially discussed trichomonal stage has been described as non-motile cells with internalized flagella and without cell walls also in other recent studies (Afzan and Suresh, 2012; Pereira-Neves et al., 2003). The round form seen in cultures in the current study look very similar to the “pseudocysts” found by Afzan and Suresh (Afzan and Suresh, 2012) as well as by Yusof and Kumar (Yusof and Kumar, 2012) (Fig. 45). From that point of view, pseudocysts were definitively present in our strains. On the other hand, the cell in Fig. 45 B is still flagellated and proliferating and therefore certainly not a degenerative form, while the round cell in Fig. 45C rather seems to be dead and cannot be regarded as pseudocystic.

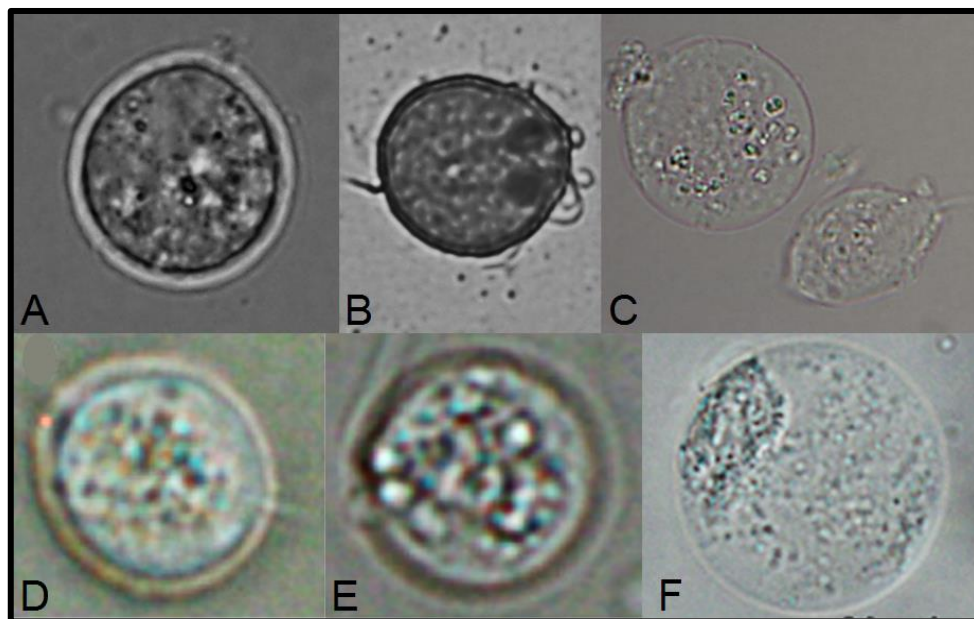


Fig. 45 Different strains with “pseudocystic” stages. Cells from long-term treated TV2 (A), from trypan blue-stained ATCC50138 (B) and ATCC 30001(C). Corresponding images of pseudocysts published by Yusof and Kumar (2012) (E) and by Afan and Suresh (2012 (D, F).

A rounding up of cells after pentamycin treatment was also observed in transformed rat fibroblastic cells (RFL-T) (Nakashima et al., 1974). They evaluated the synergistic effect of bleomycin (50 µg/ ml), a glycopeptic antibiotic, and pentamycin (1.5 µg/ ml) on RFL-T (1×10^5 cells/ ml). Treatment with 1.5 µg/ ml pentamycin as well as with 50 µg/ ml bleomycin did not result in alteration of cell morphology. Only when both compounds were added to the cultures the cells rounded up. The assumed low purity level of pentamycin at that time may be the reason for this observation. The pentamycin with 28% purity used in the current study might still be of better purity than the one used in 1974. Nevertheless also this did not kill all cells at that concentrations (Fig. 21). In addition to the presumably low purity of the pentamycin batches used in that previous study, also the low drug concentration (1.5 µg/ ml) might be a reason for the unaltered morphology of cells after treatment. Concentrations of 1 or 2 µg/ ml of pentamycin with 94% purity showed high potential to lyse cells. However, in that study RTL-T cells did not lyse, even after 18h of treatment with 1.5 µg/ ml pentamycin, which might be due the difference between trichomonal and RTL-T cells, but more likely because of the lower purity of pentamycin at that time.

Rounding up of cells under drug pressure has also been reported for other drugs. Miltefosine-treatment (hexadecylphosphocholine, HePC) also resulted in increased rounding up of cells as well as increased immobility, membrane blebbing and finally cell-lysis (Blaha et al., 2006). The fast lysis of cells after HePC-treatment (within 30 min) was also observed after pentamycin-treatment at corresponding concentrations. Also treatment with tinidazole (1µg/ ml for 1h), a nitroimidazole, leads to rounding up of *T. vaginalis* cells (Carosi et al., 1977). Treatment with albendazole also seems to alter the morphologic appearance of trichomonal membranes, which have been described to become thicker and less well defined (Oxberry et al., 1994). After 4h of treatment with 10 µg/ ml albendazole the trophozoites became swollen and showed cell coat invaginations. Treatment with low concentrations (<1 µg/ ml) of nitazoxanide, a 5-nitrothiazolyl derivate, also seemed to change the trichomonal shape from oval to round. At higher concentrations of nitazoxanide (>3 µg/ ml) membrane blebbing, cell swelling, empty cytoplasmic areas and alterations of vacuole distribution have been observed (CEDILLO-RIVERA et al., 2002). A swelling of the cells, even to

the double volume of untreated cells, was also seen in our cultures and also membrane deformations were observed, particularly in the long-term treated cells.

In general, *T. vaginalis* cells seems to round up and internalize flagella under unfavourable conditions (Petrin et al., 1998). Therefore, the morphology of *T. vaginalis* is also depended on environmental conditions including pH, oxygen saturation, carbohydrates and host cell conduct (Jesus et al., 2004).

4.1.2 Effective Concentrations (ECs)

In the current study, the 50% effective concentrations of pentamycin were around 1 µg/ ml, for all strains investigated. The concentrations of pentamycin, that completely inhibited cell mobility after 6h of incubation varied between 5 and 10 µg/ ml. All cells of all strains were killed at 20 µg/ ml pentamycin.

Efficacy of pentamycin against other organisms

Comparison of the efficacy of pentamycin against different organisms is difficult, not only because of the different investigation procedures, but also because of the different purities of the drug. In the following, the efficacy of pentamycin with high purity from the Swiss manufacturer (Lumivita AG, Basel) will be discussed separately from studies with pentamycin from other manufacturers.

Winnips et al. (2010) investigated the efficacy of pentamycin from the same Swiss manufacturer against 141 non-albicans *Candida* strains (*C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*) and 20 *Saccharomyces cerevisiae* strains. The evaluated MIC90ies after 48h of incubation with pentamycin for these yeasts were 1-4 µg/ ml and complete growth inhibition was observed at 4 µg/ ml pentamycin. These results are quite similar to the inhibitory concentrations of *T. vaginalis* in the current study. The use of pentamycin from the same manufacturer and of similar purity levels may be the reason for equal results for yeasts and trichomonads. Further, Astelbauer et al. (data not published yet) determined the efficacy of pentamycin against *Leishmania infantum* (MCAN/ES/89/IPZ 229/1/89), *Plasmodium falciparum* (clone 3D7, K1), *Trypanosoma cruzi*, *Giardia intestinalis* and *Entamoeba histolytica*. In all these strains pentamycin showed high efficacy at similar concentrations as in the current study. The EC50ies for *L. infantum* and *T.*

cruzi after 24h of pentamycin treatment were below 0.5 µg/ ml and therefore below the EC50ies for *T. vaginalis* strains (<1.3 µg/ ml). However altogether, in all these studies pentamycin from the same manufacturer showed high efficacy against parasites and fungi even at concentrations below 5 µg/ ml.

Xiong et al. (2012) evaluated the efficacy of fungichromin, an analogue of pentamycin, purified by silica gel column chromatography. The MIC50ies (0.21-3.45 µg/ ml) against several fungi (*Rhizoctonia solani*, *Helminthosporium sigmoideum* Cav, *Magnaporthe grisea*, *Penicillium notatum*, *Giberella zeae*, *Mucor rouxianus* and *Ustilagoidea virens*) were comparable to the results from the current study. Nevertheless, even though the values for the MIC50ies were quite similar, the MIC90ies varied between 3.88 µg/ ml (*P. notatum*) and 133.1 µg/ ml (*U. virens*) (Xiong et al., 2012). In the current study, the 90% effective concentrations did not exceed 10 µg/ ml and were comparable to the effective concentrations of fungichromin against e.g. *M. rouxianus* or *M. grisea*. Shih et al. (2003) used fungichromin (also purified by silica gel column chromatography) to determine the MIC90ies against *Rhizoctonia solani* Kühn, the anamorphic stage of *Thanatephorus curcumeris* Donk. The MIC (> 90% inhibition) against the *R. solani* (RST-04) was 72 µg/ ml and complete inhibition of the mycelial growth was observed at 80-120 µg/ ml. These values of growth inhibition are much higher than the values for pentamycin efficacy in the current study on *T. vaginalis* and underline the importance of the purity level and the manufacturer. In another study by Zygmunt (1966) the MICs for fungichrom against *Candida albicans* (NRRL Y477) were evaluated and the effective concentrations were far lower than in the current study on trichomonads. Interestingly in Zygmunt's study the ECs of fungichromin (MIC 80 µg/ ml) and pentamycin (MIC: 40 µg/ ml) differed from each other, although they generally have the same chemical structure. The reason for this variance might be the different drug manufacturers (fungichrom, Merck & Co; pentamycin, Nikken Chemicals Co). The phylogenetic distance between protozoa and fungi may be one of the reasons for the overall high divergence in the efficacy of pentamycin, but differences in purity and in methods used to evaluate the ECs are probably the main reasons.

The efficacy of pentamycin against *T. vaginalis* is genetically comparable to the efficacy of pentamycin from the same manufacturer against other protozoa and various fungal species. However, there have been only a few studies on the efficacy of pentamycin against eukaryotic cells and because of the various techniques used for susceptibility testing these results are difficult to compare to each other and to the results in the current study. Akiyama et al. (Akiyama et al., 1980) have determined the effect of pentamycin on Chinese hamster V79 cells and *Saccharomyces cerevisiae*. The efficacies were evaluated as the drug concentrations, which were sufficient for 50% inhibition of colony formation of the cells and are therefore not directly comparable with the results in the current study. Hidaka et al. (1978) also investigated the pentamycin-induced inhibition of colony formation of Chinese hamster cells, however, by the decrease of relative plating efficacies in correlation with drug concentrations (Hidaka et al., 1978). It is difficult to compare the results from these two different techniques, but the drug concentration sufficient for a 50% inhibition of colony formation in mammalian cells (1-2 µg/ ml) was similar to concentrations needed to inhibit the motility by 50% in trichomonads. Twentyman (Twentyman, 1976) did not observe killing of EMT6 mouse tumour cells after pentamycin treatment, but pentamycin boosted the efficacy of bleomycin *in vitro*. A comparison of the high efficacy of pentamycin used in the current study with even low concentrations (< 1 µg/ ml) sufficient to kill trichomonads, with the result of Twentyman leads to the conclusion, that firstly the biological difference between mammal tumour cells and protozoans is important, but again, also in this case probably the drug purity at that time was significantly lower than it is today. In Tab. 1 all studies mentioned in this part are summarized.

Tab. 17 Summary of studies on pentamycin (fungichromin) efficacy

Autor	Year	Drug	Species	MIC [µg/ ml]
Twentyman	1976	Pentamycin (gift from Prof. Umezawa)	EMT6 tumor cells	0
Hidaka et al.	1978	Pentamycin (Nikken Chemicals Co., Ltd.,Tokyo)	Chinese Hamster cells (V79)	5 ($>10^{-2}$ relative plating efficacy)
Akiyama et al.	1980	Pentamycin (Nikken Chemicals Co., Ltd.,Tokyo)	Chinese Hamster cells (V79)	1-2 (50 %colony forming blocked)
Zygmunt	1966	Pentamycin (Nikken Chemicals Co., Ltd., Tokyo)	<i>C. albicans</i>	40
		Fungichromin (Nikken Chemicals Co., Ltd., Tokyo)	<i>C. albicans</i>	80
Shih et al et al.	2003	Fungichromin (silica gel column chromatography)	<i>Rhizoctonia solani</i>	72
Winnips et al.	2010	Pentamycin (Lumavita Ag, Basel, Switzerland)	<i>C. glabrata</i> ; <i>C. parapsilosis</i> ; <i>C. tropicalis</i> ; <i>C. krusei</i> ; <i>S. cerevisiae</i>	MIC 90:1-4 MIC ≤ 4
Xiong et al.	2012	Fungichromin (silica gel column chromatography)	<i>Rhizoctonia solani</i> , <i>Helminthosporium sigmoideum</i> Cav, <i>Magnaporthe grisea</i> , <i>Penicillium notatum</i> , Plantain head blight fungus, <i>Gibberella zeae</i> , <i>Mucor rouxianus</i> , <i>Ustilaginoidea virens</i>	EC50: 1.47; 2.53; 3.45; 1.33; 3.17; 2.21; 2.11; 0.21
Astelbauer et al.	-	Pentamycin (Lumavita Ag, Basel, Switzerland)	<i>Leishmania infantum</i> ,; <i>Trypanosoma cruzi</i>	EC90: 1.41; 1.19
Syrowatka	-	Pentamycin (Lumavita Ag, Basel, Switzerland)	<i>Trichomonas vaginalis</i> (ATCC 30001, ATCC 30238, TV2 and ATCC 50138)	EC90: 1.46-2.36

Efficacy of pentamycin compared to other drugs

MTZ is the standard drug in trichomonosis treatment. In current study MTZ at the applied concentration (50 µg/ ml) showed hardly any effect on trichomonal cells after one hour of treatment. After 6h of treatment MTZ showed increased efficacy against *T. vaginalis*, but still lower efficacy than pentamycin.

The comparison of the efficacy of pentamycin to the efficacy of other drugs is difficult, because of different methods for activity measurement and different descriptions for the efficacy, like MLC, MIC, IC and EC (Adagu et al., 2002a). Additionally, the nitroimidazole activity in cells is dependent on the presence of oxygen and therefore the results from susceptibility testings of these drugs vary extremely (Conrad et al., 2013). For instance, the minimal lethal concentration for tinidazole, nimoreazole and MTZ for the strain ATCC 50138 (IR-78) has been determined under aerobic and anaerobic conditions by Meingassner and Thurner (1979). They showed a very low efficacy of MTZ in the presence of oxygen (MLC of 50-100 µg/ ml), but high efficacy under anaerobic conditions (MLC of 0.4-0.2 µg/ ml). This is discussed in more detail on page 84 will be discussed later in combination with theory on the mode of action of MTZ. Tinidazole has the double plasma half-life of MTZ and is used to treat MTZ-

resistant *T. vaginalis* infection. There is also a difference in the efficacy of tinidazole depending on the presence of oxygen and therefore between the aerobic MLCs (100-400 mg/l) and the anaerobic MLC (12.5-25 mg/l) (Saurina et al., 1998). Another drug tested for its activity against *T. vaginalis* is albendazole, a benzimidazole used commonly as an anti-helminthic drug. The IC₅₀ after 4h post treatment was 400 µM (= 102.4 µg/ ml) (Oxberry et al., 1994), which is a much higher concentration compared to the IC₅₀ies after 3h of pentamycin-treatment in trichomonads (0.8 µg/ ml). In a study by Fürnkranz et al. (2011) the *in vitro* activity of N-chlorotaurine (NCT), the N-chloro derivate of the amino acid taurine, against two trichomonal strains was evaluated. The EC₅₀ies of NCT against the strains ATCC 50138 (9.0 mM) and ATCC30001 (28.7 mM) after 1h of treatment were not as high as the EC₅₀ies of pentamycin (EC₅₀: 1.58 µM and 1.91 µM). Also the efficacy of curcumin, an extract from the Indian spice turmeric (*Curcuma longa*), has been evaluated in the same strains of *T. vaginalis* as used in the current study. The EC₅₀ies ranged from 183.30 µg/ ml (ATCC 30001) to 307.23 µg/ ml (ATCC 30236) after 6h and 73 µg/ ml (ATCC 30236) to 105 µg/ ml (ATCC 50138) after 24h of curcumin treatment (Wachter et al., data not published yet.). Even if curcumin has a high patient tolerance and has no known severe effects, the EC₅₀-concentrations are around 10² higher than the EC₅₀ies of pentamycin.

One of the few polyenes, which has been shown to be highly active against trichomonads in topical application is hamycin. It is used in India and is effective against MTZ-sensitive and resistant strains and 88% of the patients become cured. The MIC of 1 µg/ ml (0.87µM) (Gehrig and Efferth, 2009) is similar to the one of pentamycin, which can be explained by their similar chemical structure. Hamycin and pentamycin are both polyene macrolides, although hamycin has a mycosamine appendage and is closer related to AmB. AmB is also effective against *T. vaginalis*, but the IC₅₀ values are with 12.5 µg/ ml (13.5µM) higher than those of hamycin and pentamycin.

Altogether, the results of the current study underline the usefulness of pentamycin in the treatment of trichomonosis, because it has a high potential to kill trichomonads, even at very low concentrations.

4.2 Differences between strains

Although the susceptibilities to pentamycin of the four strains used in the current study were rather similar, there were differences in growth and behavior, like e.g. the increased aggregate formation of cells in strain ATCC 50138. Furthermore, aberrations from the arithmetic mean for the EC50, EC90, IC50s and IC90 were rather high, particularly after 6h of treatment. The EC50ies for the R-strains showed even up to 30% aberration from mean EC50.

Differences between *T. vaginalis* strains were also observed by F rnkranz et al. (F rnkranz et al., 2011). In that study, the MTZ-resistant strain (ATCC 50138) showed less susceptibility to *N*-chlorotaurine (NTC) than the MTZ-sensitive strain. Further, Blaha et al. (Blaha et al., 2006) observed a difference between the strains in their susceptibility to miltefosine. The lowest susceptible namely 10 μ M miltefosin, was detected in ATCC 50138, which was also the least susceptible strain in the current study (IC50: 0.766 μ g/ ml). This lower susceptibility may be due the high accumulation effect in this strain. Reduced target sites for drugs may be a consequence of these conglomerates and therefore a lower susceptibility (Fig. 16). The MTZ-resistant strain shows also increased resistance against other drugs, e.g. disulfiram (MIC ATCC 50138: 0.34 μ M), compared to the MTZ-sensitive strain (ATCC 30001: 0.11 μ M) (Bouma et al., 1998)

Further, differences between strains in the response to stress, which can be also induced by other stimuli than drugs, have been reported. For instance, trichomonads with high pathogenic potential show a 2- to 5- fold increased response to oxidative and heat stress compared to mild pathogenic strains (Davis-Hayman et al., 2000). *T. vaginalis* is also able to cope with temperature-induced stress, but also here differences in the capability to deal with this kind of stress have been reported between the strains. Trichomonads cultivated at lower temperatures than 37 C, namely 5 C and 22 C, even survived for a longer time period than the same strains cultivated at temperatures of 37 C. However, the relevant information from this study by Smith (Smith, 1983) is the difference in the viability between the strains under temperature-induced stress, e.g. from 10 tested strains only 4-7 strains could be cultivated at 22 C (for 6 days) and not more than 4 strains could be cultivated at 5 C (for 10 days). In the same study also a difference in heat tolerance was observed.

Under heat-induced stress situation (42°C) not more than 4 strains out of 10 strains could be cultivated for more than 4 days. These results support the hypothesis of a different susceptibility to stress between *T. vaginalis* strains. Usually temperature-induced stress, leads to an increased expression of chaperone proteins, which support the folding and unfolding process, correct conformation and prevention of protein aggregation of other proteins. The most prominent member of these chaperons is the heat-shock-protein HSP70, which is also present in *T. vaginalis* and is expressed in heat-shock-induced stress situations (Davis-Hayman et al., 2000). Meade et al. (2009) showed a genetic diversity of *T. vaginalis* clinical isolates by restriction fragment length polymorphism (RFLP) analysis of HSP70. In that study seven strains from the ATCC and 122 clinical isolates from different individuals in the USA were included, and they showed high levels of heterogeneity. Cornelius et al. (2010) demonstrated the genetic diversity in clinical and reference strains, and established a phylogenetic tree for Hsp70-RFLP patterns. Their analysis shows a wide distance in branching of two of the strains used in the current study (ATCC 30001 and ATCC 50138) (Cornelius et al., 2010). The authors further introduced a multilocus sequence typing (MLST) scheme by characterization of the internal sequence of seven house-keeping genes and analysis of 68 recent and historical isolates confirmed the high genetic diversity within *T. vaginalis* (Cornelius et al., 2012). A high genetic diversity within the entire genus *Trichomonas* was revealed by random amplified polymorphic DNA (RAPD) data of 42 strains from 10 species confirming the different branching of the sister clade C:1-NIH (ATCC30001)/JH31A (ATCC 30236) and IR78 (ATCC 50138) (Hampl et al., 2001). A study by Paschinger et al. (2012) analysed the *N*-linked oligosaccharides of four *T. vaginalis* strains by mass spectrometric (MS) and high-performance liquid chromatography (HPLC). They observed an interstrain difference in the glycome of *T. vaginalis* concerning the extent and type of pentosylation as well as the degree of modification with charged moieties and with *N*-acetyllactosamine. Leitsch et al. (2012) showed a difference in the protein activity (flavin reductase) and expression (alcohol dehydrogenase-1, ADH1) between *T. vaginalis* strains and this has an important influence on the intracellular activation of MTZ.

4.3 The target is primarily the membrane

Polyene macrolides are supposed to interact with sterols in the plasma membrane of cells. Based on comparison to the mode of action of other polyenes and of the effect of pentamycin on other microorganisms, and on what is known on the membrane composition of *T. vaginalis*, I hypothesize that also in *T. vaginalis* pentamycin targets primarily the membrane.

Before going into detail on the effect of polyene macrolides, considerations on the structure of various cell membranes and walls should be made. The differences in the effect of polyene macrolides on the membrane relies on their chemical structure as well as on the variations in membrane compositions. In contrast to protozoan/mammal cells, fungi have a cell wall, which consists of glycoproteins and polysaccharides. The major polysaccharides are glucans, particularly β -1,3-glucans are present in the cell wall scaffold. Most glycoproteins are modified by *O*-/*N*-linked oligosaccharides and vary between different fungi. Some of these proteins additionally have a glycosylphosphatidylinositol anchor (GPI) for their binding to the plasma membrane or the cell wall. GTP is also present in trypanosomes, yeasts and mammalian cells. In contrast to the cell wall of plants, which contains cellulose, the fungal cell wall also contains chitin (homopolymer, β -1,4-linked-acetylglucaosamine) in varying amounts, depending on the species (Bowman and Free, 2006).

The action of pentamycin and other macrolide polyenes does not affect the cell wall, but the underlying plasma membrane. The membrane is composed of a phospholipid double or bilyer. In the fungal membrane, the most prominent sterol is ergosterol, although if it is not present in all fungi. There are also other main sterols, e.g. cholesterol, 24-ethyl cholesterol, 24-methyl cholesterol and brassicasterol. Brassicasterol is the primary sterol in *Taphrina* and *Protomyces* and also in the fungal taxon Glomeromycota. It contains 24-ethyl cholesterol as its major sterol, which is also present in plants. Other species of this group of mycorrhizal fungi do not even contain ergosterol. In most of the early dividing fungal groups ergosterol is not found and cholesterol is no reported in the later diverging fungi (Weete et al., 2010). Although *T. vaginalis* cannot synthesise cholesterol *de novo*, it has been shown to incorporate cholesterol and cholesteryl ester into its membranes. Beach et al. (1990) evaluated the lipid composition of *T. vaginalis*, which had been cultivated in

Diamond's medium containing 12.1% cholesterol. The major component of the neutral lipids was cholesterol, representing 18.9% of the total lipids. The other major lipids were phospholipids like phosphatidylethanolamine (21.9% of total lipids), phosphatidylcholine (19.3%) and sphingomyelin (13.3%) (Beach et al., 1990). The presence and the high level of sterols in *T. vaginalis* is the prerequisite for the efficacy of polyene drugs against this parasite. Also the association of cholesterol with the phospholipids plays an important role for membrane function, which is particularly obvious seen in cholesterol/ sphingolipid-rich lipid rafts (Ohvo-Rekila et al., 2002; Marechal et al., 2011). The importance of the presence of sterols and other membrane components for drug resistance will be discussed later. However, the most important chemical structures of the sterols for interaction with polyene macrolides are the planar steroid ring, the hydrocarbon side chain at C-17 and the 3 β -hydroxyl group (Norman et al., 1972; Rog et al., 2009).

Only a few bacteria contain sterols, e.g. methylotrophic bacteria (4-methylsterol), cyanobacteria (4-desmethylsterol), *Nannocystis exedens* (mycobacterium; cholest-8(9)-en-3 β -ol), *Rhodococcus* spp., *Bacillus* spp., *Gemmata obscuriglobus* and *Methylococcus capsulatus* (Volkman, 2003; Desmond and Gribaldo, 2009). The sterol precursor isopentenyl diphosphate (IPP) is synthesized in bacteria via a biosynthesis pathway, that is different from the eukaryotic one. Within the synthesized squalene several ring structures are cyclized via squalene-hopane cyclase (SHC) to hopanoids, which are the alternative to eukaryotic sterols in bacteria. In eukaryotes, cyclization is mediated via squalene epoxide (squalene monooxygenase) and oxidosqualene cyclase (OSC) to either lanosterol which is then converted into ergosterol (in fungi) and cholesterol (vertebrates), or into cycloartenol and then metabolized to campesterol and stigmasterol (in plants) (Desmond and Gribaldo, 2009). The differences in the biosynthesis of sterols and the resulting sterol types have important implications on the susceptibility to drugs. For instance, Kleinschmidt and Chough (1972) have shown the different binding efficacies of filipin (pentane), a closely related to pentamycin, to various membrane-bound sterols. Due to the close chemical similarity of filipin and pentamycin, the varying efficacy in dependence of the sterol type should be also valid for pentamycin. Lagosin, the stereoisomer of fungichromin (=pentamycin) has also been proven to bind to cholesterol, but with less affinity than filipin (Bittman and Fischkoff, 1972).

The mode of action of amphotericin B (amB), as an example for another polyene, is dependent on the presence of sterols within the membrane, but also chemical substructures of the drug. The amB molecules are supposed to self-assemble into ion channels, which mediate membrane permeabilization. Gray et al. (2012) proclaim the importance of the mycosamin appendage at C19, which seems to be important for contact with ergosterol. Amphotericin without that appendage (AmdeB) cannot bind to ergosterol and induce ion channel formation, and AmB with a removed hydroxy group in C35 (C35deOAmB) can bind to ergosterol but does not induce membrane permeability (Gray et al., 2012). Most probably the absence of the mycosamine appendage in filipin explains its disability to form ion channels. Due to the high chemical similarity between filipin and pentamycin and the absence of the mycosamine appendix in pentamycin, a non-channel forming mode of action for pentamycin is assumed.

A special position concerning the presence and composition of the outer layer in organisms is upon the mollicutes, bacteria without cell walls. One of the well-studied species within this group is *Acholeplasma laidlawii* because this organism requires no sterol for growth (de Kruffy et al., 1972). The similarity between *A. laidlawii* and *T. vaginalis* is the incorporation of sterols, if provided in the growth medium. The ability to synthesise sterols *de novo* was also lost in nematodes, insects and marine vertebrates (Hannich et al., 2011). Hidka et al. (1978) already mentioned the dependence of AmB resistance on drug formulation as well as on the sterol level in the membrane.

The mode of action of metronidazole has still not been entirely elucidated and there are at least two different theories on how the drug in fact kills *T. vaginalis*. The common theory of the drug mechanism of 5-nitroimidazole drugs starts with the passive diffusion of the prodrug into the cytoplasm (in bacteria), hydrogenosomes (in trichomonads) or into other organelles in other protozoans. For activation of the drug, the 5-nitro group needs to be reduced by an electron donor like ferredoxin. The nitro radical production depends therefore on PFOR, which catalyses the pyruvate to acetate conversion and electron production. Also the presence of oxygen as electron acceptor, as well as malate conversion has an impact on the drug activation. The nitro-radical form binds to DNA and disrupts DNA synthesis or induces double- and

single-strand breaks (Kulda, 1999; Leitsch et al., 2009; Löfmark et al., 2010; Clark et al., 2010). Leitsch et al. showed an alternative mode of action with the flavin enzyme thioredoxin reductase (nitroreductase) as a major player, which reduces MTZ and protein adduct formation (Leitsch et al., 2009).

4.4 Resistance is not inducible *in vitro*

4.4.1 Pentamycin versus metronidazole

A comparison of the results from susceptibility testings in *Trichomonas* is complicated, because there are no established standard techniques. This is also valid for resistance tests and even more for long-term trials trying to induce resistance. The interpretation and comparison of the results from the current study with the data from the literature are difficult due to divergent experimental set ups and calculation methods as well as a lack of data on pentamycin resistant organisms (Adagu et al., 2002a).

In the current study, increasing pentamycin concentrations lead to a loss of motility as well as viability in the reference strains as well as in the long-term treated strains. Nevertheless long-term treatment caused diminished susceptibilities in all strains tested, particularly when long incubation times were chosen. In long-term treated strains the EC50ies had increased from the approximately 2 fold in strain ATCC 30236 to the ~ 4fold in strain ATCC 50138 after 6 hours. However, these diminished susceptibilities were not so obvious in the EC90ies and, more importantly, were not permanent – when these substrains were subcultivated in normal medium they were as susceptible (or partly even more susceptible) to pentamycin as the original strain. Thus, it can be concluded that, a true resistance (hereditary resistance) against pentamycin is not inducible *in vitro*, at least not within one year of permanent drug stress.

The resistance to pentamycin was not stable. Without the drug pressure and also after re-thawing frozen R-strains the susceptibility to pentamycin had increased again. Therefore, even after one year of cultivation under sub-lethal drug pressure, no stable resistance could be observed. Of course, this does not exclude that a true resistance against pentamycin can be induced by other methods.

Comparison to the literature concerning the induction of drug resistance in trichomonads reveals, that this process can take more than a year. Anaerobic MTZ-resistance (against up to 400 µg/ ml) in initially MTZ-sensitive trichomonal strains has been induced by permanent drug stress over 350 days or even longer (Brown et al., 1999; Wright et al., 2010a). Kulda et al. (1993) also induced MTZ-resistance by cultivation with increasing drug pressure under anaerobic conditions within 12 to 21 months. In another study trichomonads have shown an increased susceptibility to MTZ onto approximately the double of the initial value after 300 days of cultivation under drug pressure (Fig. 5) (Wright et al., 2010b).

However, the differences in the drug targets of MTZ make a comparison of these two drugs concerning their being prone to resistance development very difficult.

4.4.2 Possible resistance mechanisms in polyene drugs

Most probably – if resistance against pentamycin can develop in trichomonads – the mechanism is different to MTZ, against which resistance develops via hydrogenosome downsizing (Wright et al., 2010c) or by the down regulation of PFOR (Kulda et al., 1993), or flavin reductase and alcohol dehydrogenase (Leitsch et al., 2012). Resistance mechanisms to polyene macrolides are dependent on the presence or absence of sterols in the membrane. For instance, *Candida albicans* strains that are resistant to amphotericin show changes in the gene expression of genes for ergosterol biosynthesis (Barker et al., 2004). In *C. tropicalis* JEY162, the observed amphotericin B resistance (MIC >32µg/ ml) relies on the mutation of two ergosterol biosynthesis proteins (ERG11 and ERG3) (Eddouzi et al., 2013). The resistance to AmB in *Leishmania donovani* (Mbongo et al., 1998) is mediated via S-adenosyl-L-methionine:C-24-Δ-sterol methyltransferase, the replacement of ergosterol by cholesta-5,7,24-trien-3β-ol or by a ABC transport and a thiol metabolic inhibitor (Purkait et al., 2012). The importance of ergosterol absence in amphotericin resistance in *Leishmania* also emphasizes the crucial role of sterols in the efficacy of polyene macrolides. Other compounds, like 22,26-azasterol and 24(R,S),25-epiminolanosterol are affecting the sterol pathway by an inhibition of Delta-24(25)-sterol methyltransferase and result in membrane deformation and morphological changes (de Andrade Rosa et al., 2011).

Also members of other drug classes like miltefosine (hexadecylphosphocholine, HePC), depend on the presence of sterols for their effectivity. This was shown by sterol depletion in the membrane by methyl- β -cyclodextrin (MCD) and cholesterol oxidase (CH-OX) in *Leishmania donovani* (Saint-Pierre-Chazalet et al., 2009). HePC was further observed to effect the lipid composition in the membrane (Rakotomanga et al., 2007). This is supported by different sterol profiles of HePC resistant strains (Rakotomanga et al., 2005). The supposed correlation between miltefosine efficacy and sterol/ phospholipid biosynthesis is corroborated by the difference of the IC50 between epimastigotes (17.4 μ M) and trypomastigotes (29.5 μ M) in *Trypanosoma cruzi*, which differ in their sterol biosynthesis from each other (Menna-Barreto et al., 2009). The activity of this compound has also been tested in *T. vaginalis* and showed a cytotoxic effect at 8 μ M and this effect most probably also relies on the presence of sterol in the membrane (Blaha et al., 2006).

In the current study, the evolved lower susceptibilities to pentamycin in long-term treated strain varied between the strains tested, in the final result as well as in the process of development of diminished susceptibility. For instance, in the ATCC 30001 strain the maximum EC50 was seen after six months (4.198 μ g/ ml), while the final EC50 after 12 months (3.636 μ g/ ml) was surprisingly low. Further, the EC50ies within the total timeframe were below the first value. The second last EC50 value (EC50 after 11 month: 2.638 μ g/ ml) differed significantly from the last one (after 12 month: 3.636 μ g/ ml). This might have been caused by a spontaneous mutation in this substrain, but it also has to be taken into account that the deviations from the mean in these long-term drug pressure trials were rather high. In most studies on drug pressure the focus is not on the process of resistance development, but more on the mechanism itself and the final results. The process and progress of developing MTZ-resistance *in vitro* is known to be not continual. This is why in most studies different strains were treaded with different MTZ concentrations for different periods of time. In a study by Rasoloson (2002), strain MR-100 was cultivated for 4 days at 20 μ g/ ml and for 138 days at 30 μ g/ ml, but the MR-30 strain was cultivated for 112 days at 20 μ g/ ml MTZ.

The mechanism of action of polyene macrolides is also closely connected to the lipid composition of the target membranes. Therefore, the presence or absence of lipids and phospholipids determines the biochemical capabilities of this antibiotic group.

In *Saccharomyces cerevisiae* the alterations in the sterol metabolism are associated with polyene resistance (filipin, nystatin, pimaricin rimocidin and etruscomycin). Mutations in the genes *pol 1*, *2*, *3*, and *5* are supposed to be responsible for polyene resistance and reduced affinity to bind sterol (ergosterol and 24,(28) dehydro-ergosterol) (Molzahn and Woods, 1972). Also other fungi, e.g. *Aspergillus fennelliae*, with a lack of ergosterols show increased resistance to members of the polyene antibiotic group (amphotericin B, nystatin) (Kim and Kwon-Chung, 1974). Resistance to nystatin in *Neurospora crassa* is regulated by a mutation in *erg-3*, a C-5 sterol desaturase, and consequently reduced ergosterol production (Ellis et al., 1991). In *S. cerevisiae* the addition of cholesterol (+20 µg/ ml) results in increased MICs for filipin (from 1.0 to 17 µg/ ml) (Lampen et al., 1960).

4.5 Pentamycin as a candidate for treatment of *T. vaginalis* infections

Pentamycin does not only show high efficacy against *T. vaginalis* and has a low risk for resistance development – at least resistances do not develop under long-term low-dose drug stress, which is the main problem in the treatment of *T. vaginalis* infections with the standard drug metronidazole –, but it also has a good compliance and tolerability. Until now, no severe effects in connection with the application of pentamycin have been reported. This has been confirmed in a study with 40,000 patients, who had no adverse reaction after treatment with pentamycin. Even an overdose of pentamycin with the 10fold amount of the commonly used treatment-dose of 10 mg did not result in an increase of systemic or local adverse reactions (Tirri et al., 2010). In contrast, treatment of trichomonosis caused by MTZ-resistant *T. vaginalis* with very high doses of MTZ has severe toxic side-effects and still patients may develop a recalcitrant trichomonosis (Cudmore and Garber, 2009). Side effects of MTZ treatment include nausea, vomiting, headache, insomnia, dizziness, drowsiness, but also peripheral neuropathy, palpitation, confusion, eosinophilia and leukopenia (Cudmore and Garber, 2009). Further, a mutagenic potential in bacteria and carcinogenic potential in rodents have been reported, as well as association with

an increased risk of preterm delivery, low birth weight, infant mortality and predisposition to HIV (Upcroft and Upcroft, 2001).

Moreover, pentamycin is not only active against various fungi and protozoa, but it also has also proven bactericidal activity. Nevertheless it does not show any efficacy against lactobacilli, which is an additional advantage (Tirri et al., 2010). Lactobacilli are the most prevalent bacteria in the healthy vaginal microbiome and they inhibit the growth of pathogens. Also adhesion of *T. vaginalis* is negatively affected by the presence of lactobacilli (Valadkhani et al., 2013; Phukan et al., 2013).

Another advantage of topical application of pentamycin is its low ability to penetrate into the circulation because of its high molecular weight (670.85 g/mol) and bipolar molecular structure. In contrast to MTZ, which is given orally in high dosage to show sufficient efficacy at the site of infection pentamycin can be applied directly onto the vaginal mucosa. Vaginal application of MTZ shows only limited efficacy with elimination of the infection in only 20% of the patients (Muzny and Schwebke, 2013; Cudmore and Garber, 2009). Other drugs with topical and intravaginal application, e.g. paromomycin, an aminoglycoside effective in *E. histolytica*, *Cryptosporidium* spp., *Giardia intestinalis* and *Leishmania*, and also hamycin, another polyene drug, are partly effective against *T. vaginalis*, but have severe side effects (Cudmore and Garber, 2009; Singh and Jain, 2007a; Ali and Nozaki, 2007). Also clotrimazole has been used for topical treatment, but it was not as effective as MTZ (Singh and Jain, 2007b).

Generally, polyene drugs when applied systematically – but here reliable data are only available for Amphotericin B, include side effects like high fever, chills, hypotension, anorexia, dyspnoea, tachypnoea and, exceptionally, anaphylactic shock or cardiogenic shock as well as hepatotoxicity and nephrotoxicity. Although alternative formulations of this drug, e.g. liposomal amphotericin B (L-AmB), in a lipid complex (ABLC, amphotericin B lipid complex) or a colloidal dispersion of AmB (ABCD) are less toxic, but some of the side effects still remain (Astelbauer and Walochnik, 2011; Laniado-Laborín and Cabrales-Vargas, 2009; Safdar et al., 2010; Torrado et al., 2008).

Possible future developments

As in most other infectious diseases there have been approaches to develop a vaccine against infections with trichomonads, and although until now there is no vaccine on the market against *T. vaginalis*, there has been some success in developing vaccines against trichomonal infections in animals – nevertheless, of course, these are other species or even genera of trichomonads and also very different diseases. Vaccinations in heifers against the causing agents (*Tritrichomonas foetus*) of bovine trichomonosis are commercially available and are very efficacious in preventing infection and reducing foetal calves' mortality. In humans, the first studies on a possible vaccine were performed with heat-inactivated trichomonads in the 1960 (Cudmore and Garber, 2010). This year another study in mice testing a whole cell vaccination with the adjuvant Alhydrogel resulted in a decrease of infections after 7 days and an increased clearance within 28 days (Smith and Garber, 2013).

5 Literature

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6 Appendix

6.1 Abstracts

6.1.1 English

Trichomonas vaginalis is the causative agent of trichomonosis which is with more than 170 million new cases each year worldwide and 11 million in West Europe, the most prevalent non-viral sexually transmitted disease (STD). Increased HIV transmission and acquisition, cervical cancer low birth weight, abortion, impotence, infertility and other severe disease are also associated with *T. vaginalis* infection (el Seoud et al., 1998). Metronidazole has been the drug of choice since the 1960ies, but emerging resistances are pushing the search for alternative drugs.

In this study, the efficacy of the polyene macrolide drug pentamycin against *T. vaginalis* was evaluated using a microtiter plate system and four strains of *T. vaginalis* with different metronidazole resistances. Moreover, the susceptibility of trichomonads to pentamycin under resistance stress was revealed by establishing a long-term treatment with sub-lethal concentrations.

Trichomonads treated with pentamycin showed cell lysis or changes in shape and structure. The four strains with their varying metronidazole-susceptibilities, showed almost similar susceptibilities to pentamycin. The EC₅₀ies for all strains were below 1.28 µg/ ml, which is remarkably low compared to metronidazole. The EC₉₀ies of the four strains ranged from 2.36 to 3.62 µg/ ml and a 100% eradication of all *Trichomonas* strains tested was reached with a concentration of 20 µg/ ml pentamycin within 1h. Resistance against pentamycin could not be induced in any of the tested strains, even after 1 year of permanent sub-lethal treatment.

Altogether, this study established the efficacy of pentamycin against *Trichomonas vaginalis*, particularly the susceptibility of metronidazole-resistant strains makes pentamycin a promising drug. Moreover, as pentamycin has also been shown to be active against fungal vaginitis and co-infections with bacteria.

6.1.2 German

Trichomonas vaginalis ist ein humanparasitärer Protozoon und der Erreger der Trichomonosis, die mit 170 Millionen Neuinfektionen weltweit und mit 11 Millionen Neuinfektionen in West Europa pro Jahr, die häufigste nicht virale, sexuell übertragene Krankheit ist. In Zusammenhang mit dem Erreger stehen auch eine erhöhte HIV Transmission und Akquisition, Gebärmutterhalskrebs, ein verringertes Geburtsgewicht, Abort, Impotenz, Unfruchtbarkeit und weitere schwere Krankheiten. Seit den 1960igern wird Metronidazol als Medikament der Wahl verabreicht, aber das vermehrte Auftreten von Resistenzen forciert die Suche nach alternativen Medikamenten.

In dieser Studie wurde die Effektivität des neu patentierten, polyenen Makrolids Pentamycin an vier verschiedenen Metronidazol-resistenten Trichomonadenstämmen ermittelt. Des Weiteren wurden die Stämme auf deren Empfindlichkeit und Resistenzentwicklung während einer Langzeitbehandlung durch steigende, sub-lethale Pentamycinkonzentration untersucht.

Generell verursachte Pentamycin Zellyse und Veränderung der Zellform und Struktur. Trotz der unterschiedlichen Metronidazoleverträglichkeiten, zeigten alle vier Stämme in etwa die gleiche Pentamycinverträglichkeit. Die errechneten EC_{50} -Werte lagen bei allen Stämmen unter $1,28 \mu\text{g/ml}$ und sind wesentlich geringer als jene für Metronidazole. Die EC_{90} -Werte lagen zwischen $2,36$ und $3,62 \mu\text{g/ml}$ und eine 100%ige Abtötung der Zellen wurde bei Konzentration von $20 \mu\text{g/ml}$ innerhalb einer Stunde bereits erreicht. Selbst nach einem Jahr konnte in keiner der vier Kulturstämme, die unter sub-lethalen Pentamycinkonzentration gewachsen sind, eine dauerhafte Resistenz festgestellt werden, jedoch eine gewisse Verträglichkeit.

Abbreviations

µl	Microliter
µm	Micrometer
Ac/MVA	acetate/ mevalonic acid
ADH	arginine dihydrolase
ampB	Amphotericin B, AmB,
ASCT	acetate:succinate CoA transferase
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSA	Bovines-Serumalbumin
cDNA	complementary deoxyribonucleic acid
cDNA:	complementary DNA
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Didesoxinukleotid-Triphosphat
DOXP	1-deoxy-D-xylulose-5-phosphate/ 2-C-methyl-D-erythritol-4-phosphate
EC	effective concntration
EDTA	Ethylen-Diamin-Tetra-Azetat
EthBr	Ethidium-Bromid
GAP	glyceraldehyde phosphate
HePC	hexadecylphosphocholine
HIV	Human Immunodeficiency Virus
HMGS	HydroxyMethylGlutaryl Coenzyme A synthase
IC	inhibitory concentration
IPP	isopentenyl diphosphate
IRE	iron-responsive elements
IRP	regulatory proteins
kDA	Kilo-Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOALA	KEGG Orthology and Links Annotation
Mb	megabases
MDR	multi-drug-resistant
MEV	mevalonate
MIC	Minimum inhibitory concentraion

miRNA	mirco-RNA
MLC	Minimum lethal concentration
mRNA	messenger RNA
MRSA	methicillin- resistant Staphylococcus aureus
MTZ	Metronidazol
NaAc	sodium acetate
PCR	Polymerase-Chain-Reaction
Pent	pentamycin
PFK	phosphofructase
PFOR	pyruvate:ferredoxin oxidoreductase
PFP	pyrophosphate-dependent phosphofructokinase
PMVK	phosphomevalonate kinase
PPDK	pyruvate-phosphate dkinase
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
RNAP	RNA-polymerase
rRNA	ribosomal RNA
snRNA	small nuclear RNA
STD	sexually transmitted disease
tRNA	transfer RNA
U	Unit
VRE	vancomycin-resistant enterococci
WHO	World Health Organsation

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6.3 Curriculum vitae

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- 2012 "Parasitological expert discussion: Parasite induced changes: from the host to the ecosystem"
- 2011 **Good Clinical Practice (GCP)**, Medical University of Vienna
- 2011 45th Annual Conference of Austrian Society for Tropical Medicine and Parasitology,
Poster presentation: „The polyene macrolide drug pentamycin is highly effective against *Trichomonas vaginalis*"
- 2011 Retreat of the Center of Pathophysiology, Infectiology & Immunology
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- 2010 44th Annual Conference of Austrian Society for Tropical Medicine and Parasitology, Meerscheinschloss Graz, Austria
Oral presentation:
"Trichomonas vaginalis: Efficacy of Pentamycin and Possible Resistance"
Student Travel Grant
- 2010 Retreat of the Center of Pathophysiology, Infectiology & Immunology
Poster presentation, One-Slide presentation:
"Trichomonas vaginalis: Susceptibility to pentamycin & response to long-term treatment"
- 2010 „Reise und Reisemitbringsel- Schwerpunkt Südamerika"
Fortbildungsveranstaltung der ÖGTP
- 2009 43th Annual Conference of Austrian Society for Tropical Medicine and Parasitology, NHM Vienna, Austria

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