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Pentamycin – a new option for the treatment of infections  
with *Trichomonas vaginalis*?

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## Abstract (deutsch)

Der einzellige, humanpathogene Parasit *Trichomonas vaginalis* ist der Erreger der Trichomonose, die mit rund 170 Millionen Neuinfektionen pro Jahr die weltweit häufigste nicht-virale Geschlechtskrankheit darstellt. Die Trichomonose ist zwar nicht primär lebensbedrohlich, ist aber eine sehr unangenehme Infektion und kann mitunter auch schwer verlaufen. Chronische Infektionen gelten als Risikofaktor für verschiedene Krebserkrankungen und begünstigen eine HIV-Infektion. Im Falle einer Schwangerschaft führen sie meist zu einer Frühgeburt und niedrigem Geburtsgewicht. Zur Behandlung der Trichomonose wird seit nunmehr 50 Jahren Metronidazol eingesetzt. Dieses zu den Nitroimidazolen zählende Antibiotikum wird oral verabreicht und im Allgemeinen auch gut vertragen, hat jedoch zum Teil schwere Nebenwirkungen und ist für Schwangere wegen seiner Plazentagängigkeit ungeeignet. Außerdem führt die wachsende Zahl an Metronidazol-resistenten Trichomonaden-Stämmen immer wieder zu Therapieversagen. Bisher steht allerdings kein gutes Alternativpräparat zur Behandlung der Trichomonose zur Verfügung.

Pentamycin, ein Antimykotikum, das zu den Polyenen zählt, wurde bislang hauptsächlich gegen Infektionen mit *Candida*-Pilzen angewendet, erwies sich allerdings in Vorversuchen auch als wirksam gegen Trichomonaden.

Das Ziel der vorliegenden Arbeit war die genaue Untersuchung der Wirksamkeit von Pentamycin gegen *T. vaginalis*. Hierzu wurden sowohl die inkubationszeit- und dosisabhängige Effektivität von Pentamycin gegen vier unterschiedlich Metronidazol-sensitive Stämme als auch die Zusammensetzung des Proteoms vor und nach der

Behandlung untersucht. Außerdem wurde über einen Zeitraum von sechs Monaten die Ausbildung von Resistenzen überwacht.

Es konnte gezeigt werden, dass Pentamycin außerordentlich effektiv gegen *T. vaginalis* ist. Eine hundertprozentige Abtötung der Trichomonaden wurde bereits mit einer Konzentration von 15 µg/ml und einer Behandlungsdauer von 1h erreicht. Alle vier *Trichomonas*-Stämme zeigten eine annähernd gleiche Sensitivität. Die mittels SDS-PAGE analysierten Proteinprofile sprechen dafür, dass die Wirkung von Pentamycin auf einer Schädigung der Zellmembran beruht, die in weiterer Folge zu einer totalen Lyse der Trichomonaden führt. Die vier untersuchten *T. vaginalis*-Stämme haben auch nach sechsmonatiger Dauerbehandlung mit niedrigen Dosen von Pentamycin keine Resistenzen ausgebildet, es konnte allerdings ein reversibler Gewöhnungseffekt beobachtet werden. Ebenso konnte eine Kreuzresistenz mit dem verwandten Antibiotikum Amphotericin B, das vor allem gegen Leishmanien angewendet wird, ausgeschlossen werden; allerdings war ein synergistischer Effekt bei gleichzeitiger Anwendung beider Wirkstoffe feststellbar.

Die Ergebnisse dieser Studie sprechen für eine hohe Wirksamkeit von Pentamycin gegenüber *T. vaginalis*, besonders vielversprechend ist das gute Ansprechen von Metronidazol-resistenten Stämmen auf die Behandlung.

## Abstract (english)

The protozoan parasite *Trichomonas 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) worldwide. Although trichomonosis is not a primarily lethal disease, the clinical picture can include severe urogenital inflammations. Chronic infections have been associated with cervical/prostate cancer and a predisposition of HIV infections. In case of pregnancy, chronic infections can also lead to preterm delivery and low birth weight. For more than 50 years, metronidazole, a nitroimidazole antibiotic, has been in use for the treatment of trichomonosis. It is applied orally and although it is mostly compliant, it can have serious side effects. It is also not applicable for pregnant women due to its ability to pass the placenta. Furthermore, an increasing number of emerging metronidazole-resistant *T. vaginalis* strains has lead to more treatment failures in the last few years. To this day, however, there is no effective alternative drug against trichomonosis available.

Pentamycin is a polyene antimycotic and has been in use in the treatment of candidiasis, in preliminary studies it also turned out to be effective against trichomonads.

The aim of this study was to evaluate the efficacy of pentamycin against *T. vaginalis* and the ability to develop resistances in vitro. For these purposes, the dose-effect relationship between pentamycin and four differently metronidazole-sensitive *T. vaginalis* strains was investigated. Moreover, the protein composition before and after the treatment was compared. To induce resistance, strains were treated with sublethal concentrations of pentamycin within a time of six months.

## ABSTRACTS

It could be shown that pentamycin is highly effective against *T. vaginalis*. A 100% eradication of trichomonads was reached with a concentration of 15 µg/ml and an incubation time of 1h. All four differently metronidazole-sensitive strains showed almost the same sensitivity to pentamycin. The comparison of the protein profiles of untreated and treated cells analysed by SDS-PAGE showed that the mode of action of pentamycin is based on an interaction and subsequent damage of the cell membrane which consequently leads to total lysis and death of the cell. After six months of long-term treatment, no establishment of resistance but a partial, reversible adaptation to low doses was recordable. Moreover, *T. vaginalis* did not express cross resistance to amphotericin B – a pentamycin related polyene antibiotic that is mostly applied against leishmaniosis – but both drugs acted synergistically when administered simultaneously.

The results of this study confirm the high efficacy of pentamycin against *T. vaginalis*, and particularly the susceptibility of metronidazole-resistant strains to pentamycin is promising.

# 1 Introduction

This diploma thesis deals with the unicellular human parasite *Trichomonas vaginalis*, the disease caused by it, the treatment with metronidazole, and, which is the main part of the thesis, the investigation of the effect of the polyene drug pentamycin on *T. vaginalis*.

## 1.1 Trichomonads

### 1.1.1 Parasitism and protozoa

The term “parasitology” is defined as the work with parasites, parasitism and parasitoses. The word “parasite” has its origin in Greek (“para” for besides, next to; “sitos” for eating). Originally it described people who participated in sacrificial feasts by tasting the slaughtered animals to avoid food poisoning. This was an honourable occupation and did not have any negative meaning yet. The term later received negative connotation when rich people invited such parasites not only to eat with them but also to entertain them, whereupon the parasites became dependent on their hosts. So the understanding of a parasite became established as someone who lives at the expense of someone else (HIEPE, 2006).

In the biological-medical context a parasite is defined as an organism that lives obligatorily or temporarily in or on a foreign, usually larger organism (host), harming it as a consequence of depriving life energy for its benefit but without killing it. In the narrower context it defines exclusively organisms that belong to the arthropods,

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helminths, or protozoans, of these only the arthropods representing a true phylum (ASPÖCK & WALOCHNIK, 2002; DEPLAZES & ECKERT, 2005).

The term “protozoa” has been introduced by the German biologist Georg August GOLDFUSS in 1818 from the Greek (“proto” for first; “zoa” for animals) and was considered as a term for a discrete and monophyletic systematic group for a long time, although it comprises a variety of organisms that are not really related to each other. Today the term is used as a collective for unicellular, heterotrophic eukaryotes without a cell wall and is only based on morphological characteristics (HEMPHILL & GOTTSTEIN, 2006).

### 1.1.2 Research history

The first isolation of trichomonads, which were classified as *Trichomonas tenax* by Clifford DOBELL later in 1939, was achieved in the year 1773 by the Danish scientist Otho Fridericus MÜLLER. 63 years later, in 1836, Alfred DONNÉ succeeded in the first isolation of *T. vaginalis*. He named it *Trichomonas* as he believed to recognize properties of the two protozoa *Tricodes* and *Monas*, and *vaginalis* because he had found it in a woman suffering from vaginitis. Nevertheless, he mentioned that the organism can occur in the genital secretion of both women and men. Around the turn of the 19<sup>th</sup> to the 20<sup>th</sup> century, Ernst HAECKEL published the classic “Kunstformen der Natur”, in which he precisely described the morphology of *Trichomonas* and painted it on page 13, figure 4 (Fig.1). He named it *Trichomonas intestinalis* because he observed mainly *Trichomonas* species living in the intestines of vertebrates (ACKERS, 2001; WALOCHNIK & ASPÖCK, 2002).

## INTRODUCTION

In the 1880s, Otto BÜTSCHLI (1848-1920), a German zoologist who is honoured as the “architect of protozoology”, published the standard work “Protozoa” which has been released in three editions and stands out as a very precise description of families and genera of trichomonads. Moreover, BÜTSCHLI tried to understand the evolution of eukaryotic cells and to relate the different groups. So he created the term “undulipodia” which is not correct regarding to systematics but should clarify the common origin of evolution between pseudopodia and flagellata (BÜTSCHLI, 1880–1889).

From the date of DONNÉ’S discovery, *T. vaginalis* was not considered as a human pathogenic organism until 1916, when HÖHNE proved it to be the causative agent in some cases of vaginitis (HÖHNE, 1916). As recently as in 1978, this concept became universally accepted by the work of HONIGBERG (HONIGBERG, 1978).

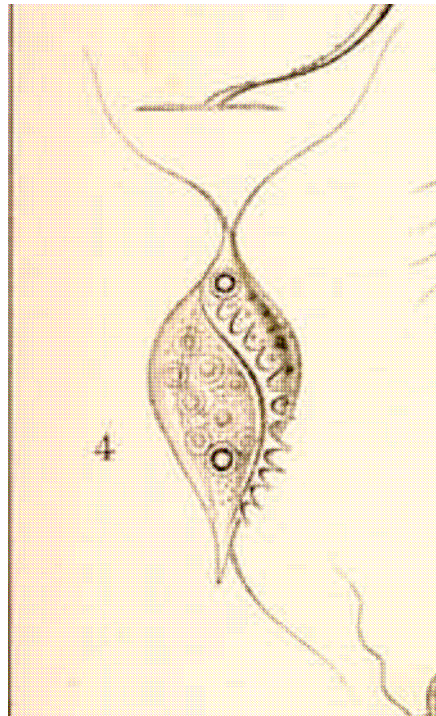
The invention of the electronic microscope around 1940 revolutionized the field of biological sciences and allowed the elucidation of small cellular compartments due to its higher resolution compared to light microscopy. The anatomy of eukaryotic flagella was discovered and described as well as the existence of the hydrogenosomes, organella for anaerobic metabolism and characteristic for trichomonads (WALOCHNIK & ASPÖCK, 2002).

Before the discovery of metronidazole, the human venereal disease caused by *Trichomonas vaginalis* was treated with natural remedies like garlic, cranberries and other red berries in naturopathy – today some authors still believe in that way of treatment – (HEMERKA, 2008) and typically with substances like acetarsol, trichomycin and potassium iodide in orthodox medicine, which may have relieved the symptoms but did not cure the disease as they are unable to take effect in the genitourinary



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region (DUREL et al., 1960). In 1959, the 5-nitro-imidazole drug metronidazole was introduced for treatment of trichomonosis and it has been the standard drug until now, although metronidazole-resistant trichomonads were already discovered and described in 1979 (COSAR & JULOU, 1959; KULDA, 1999).



**Fig.1:** *Trichomonas intestinalis* from Ernst HAECKEL, *Kunstformen der Natur*;  
page 13, fig. 4: Die Geißelthierchen.

### 1.1.3 Systematics and evolution

Ernst HAECKEL, the man who made Darwin's theory of evolution popular, did not only describe the morphological properties of trichomonads very precisely, he also established a phylogenetic system in which he classified the trichomonads as members of the phylum Protozoa, the order of the Infusoria and the class of the Flagellata. The taxon Flagellata had already been established in 1853 by COHN for

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unicellular organisms (including also bacteria) with one or more flagella. Later, Otto BÜTSCHLI added the criterion that these organisms have flagella only in the main stage of their life cycle. So it was possible to distinguish between “real flagellates” and “unreal flagellates” like gametes (WALOCHNIK & ASPÖCK, 2002).

This system was later replaced by a more precise but still morphology-based one that divided the eukaryotes into the Protozoa and the Metazoa, the Protozoa including six groups: the Flagellata, the Rhizopodia, the Sporozoa, the Microspora, the Myxozoa and the Ciliata. Amongst six other orders, the order Trichomonidida belonged to the class of the Flagellates (STORCH & WELSCH, 1997).

This widely used classification system was more or less kept up until 2005. Currently, the most widely accepted classification scheme is the one by ADL et al., who classified the kingdom of the eukaryotes into six supergroups: The Amoebozoa (Tubulinea, Flabellinaea, Stereomyxida, Acanthamoebidae, Entamoebida, Mastigamoebida, Pelomyxa and Eumycetozoa), the Opisthokonta (Fungi, Mesomycetozoa, Chonomonada, and Metazoa, including all multi-cellular animals), the Rhizaria (Cercozoa, Haplosporidia, Foraminifera, Gromia and Radiolaria), the Archaeplastida (Glaucophyta, Rhodophyceae and Chloroplastida, including the foliage plants), the Chromalveolata (Cryptophyceae, Haptophyta, Stramenopyles and Alveolata) and the Excavata (Fornicata, *Malawimonas*, Parabasala, Praexostylata, Jakobida, Heterolobosea and Euglenozoa) (Tab.1). In this system, *Trichomonas vaginalis* is one of four species of the genus *Trichomonas* in the family Trichomonadida and the order Trichomonadidae. Further families are Calonymphidae, Cochlosomatidae, Devescovichidae and Monocercomonadidae,

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which all belong to the group of the Parabasalia within the Excavata supergroup (ADL et al., 2005).


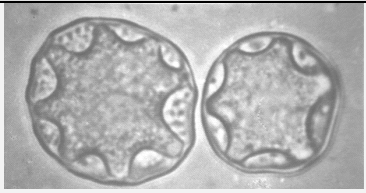
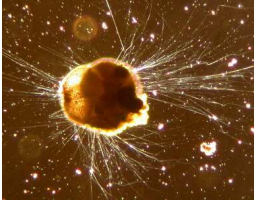
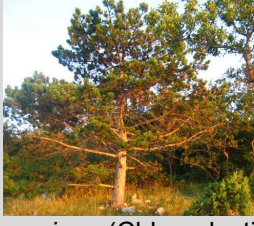
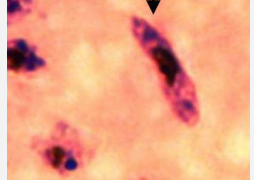
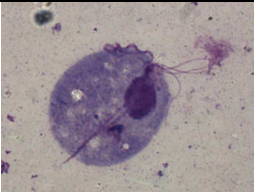
A characteristic of the Excavata is their cytostome, a sort of mouth. Additionally their subgroup – the Parabasalids – is characterised by an anaerobic metabolism, lack of mitochondria, amoeboid-like cell surface and complex microtubule structures. They can exist only in a community with animals, whether by symbiosis, commensalism, or parasitism, like *T. vaginalis* (CAVALIER-SMITH, 2002).

As *Trichomonas* – as well as diplomonads, microsporidians and *Entamoeba* – do not possess mitochondria, they have special organella, the so-called hydrogenosomes, for their anaerobic metabolism. Initially, it was thought that eukaryotes without mitochondria are primitive and had already developed before endosymbiotic bacteria led to the evolution of mitochondria. In the 1980s, CAVALIER-SMITH created a new term for this group, the Archezoa, including diplomonads, microsporidians and trichomonads, but not *Entamoeba* due to their higher evolutionary level. This has led to a theory of secondary loss of mitochondria (CAVALIER-SMITH, 1987; TOVAR et al., 1999).

Later, in 1998, CAVALIER-SMITH published “A revised six-kingdom system of life”, in which he divided the kingdom Archezoa into two phyla, the Metamonada, including *Giardia* and *Chylomastix*, and the Trichozoa. The Trichozoa were divided further into the Anaeromonada, including *Trimastix*, and the Parabasala, including the families Trichomonadida and Trichonympha (CAVALIER-SMITH, 1998).

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**Tab. 1:** Systematic table of the eukaryotes, modified after ADL et al., 2005.

EUKARYA		
Opisthokonta	Fungi Mesomycetozoa Chonomonada Metazoa	 <p><i>Asterias rubens</i> (Metazoa) (Photo: Original)</p>
Amoebozoa	Flabellinea Stereomyxida Acanthamoebidae Entamoebida Mastigamoebida Pelomyxa Eumycetozoa	 <p><i>Acanthamoeba astronyxis</i> (Acanthamoebidae) (Photo from PAGE, 1991)</p>
Rhizaria	Cercozoa Haplosporidia Foraminifera Gromia Radiolaria	 <p><i>Ammonia tepida</i> (Foraminifera) (Photo: From English Wikipedia. Original description: Live <i>Ammonia tepida</i> benthic foraminiferan collected from San Francisco Bay. Phase-contrast photomicrograph by Scott Fay, UC Berkeley, 2005)</p>
Archaeplastida	Glaucophyta Rhodophyceae Chloroplastida	 <p><i>Pinus pinea</i> (Chloroplastida) (Photo: Original)</p>
Chrom-alveolata	Chryptophyceae Haptophyta Stramenopyles Alveolata	 <p><i>Plasmodium falciparum</i> (Alveolata) (Photo from Astelbauer, 2010)</p>
Excavata	Formicata Malawimonas Parabasala Praexostylata Jakobida Heterolobosea Euglenozoa	 <p><i>Trichomonas vaginalis</i> (Parabasala) (Photo: M. Syrowatka, Giemsa stained)</p>

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However, more recent molecularbiological studies have shown that trichomonads as well as the other members of the Archezoa primarily did have mitochondria but either lost them by adaptation to their anaerobic mode of life, as in diplomonads and microsporidians, or were converted to hydrogenosomes, as in trichomonads (EMBLEY et al., 2003). Also, genes that seem to have a mitochondrial origin were found in their nuclei. These genes code for adenylate kinase, heat-shock proteins 10, 60, and 70 kDa, triosephosphate isomerase, and the valyl-tRNA synthetase (EMBLEY & HIRT, 1998). Because organisms with mitochondria possess genes that were transferred from mitochondria into the nucleus during the evolution ("numts"), this concedes the case that the mitochondrial genes of trichomonads have their origin in true mitochondria (LOPEZ et al., 1994). Nevertheless, trichomonads seem to have diverged from the eukaryotic tree of life very early. This theory is supported by many molecular biological studies and 18S rRNA analyses that have also shown that the Trichomonadida are a monophyletic lineage and represent a deep branch of the eukaryotic evolutionary tree (GUNDERSON et al., 1995). Apparently, the lack of mitochondria does not mean an ancestral role in the tree of life implicitly, but may be a result of adaptations to environment. Data from phylogenetical analyses of fungi and ciliates possessing hydrogenosomes and lacking mitochondria have also proved that these organelle must have evolved from mitochondria and also many times amongst the eukaryotes (EMBLEY et al., 2003).

Further molecular biological and phylogenetical studies have confirmed that the family of the Trichomonadidae represents a monophyletic group (DELGADO-VISCOGLIOSI et al., 2000). Even all investigated strains within the species *Trichomonas vaginalis* are monophyletic although there are some differences in their metabolism. Nevertheless, or even for this reason, there is a positive correlation

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between the degree of relationship and the susceptibility to metronidazole. On the other hand, the relationship does not correlate with virulence or geographic origin (HAMPL et al., 2001).

18S rRNA analyses indicate that the radiation of the recent trichomonadae occurred rather recently in the history of Earth, maybe in connection with the radiation of their animal hosts which are mostly mammals and birds (GUNDERSON et al., 1995).

A recent study assumes that even dinosaurs may have had trichomonads, which could have harmed them severely. In 2009, scientists found out that lesions in the jaw bones of a *Tyrannosaurus rex* had been caused by trichomonads. These infections might have led to starvation as the dinosaur could not move its jaws anymore. Interestingly, these trichomonads are closely related to recent *Trichomonas gallinae* that are specialised on birds and can harm them profoundly in a similar way. This does not only show that trichomonads have existed before their current hosts but also supports the relationship between dinosaurs and birds on the level of their parasites (WOLFF et al., 2009).

There are still some incertainties regarding the role of *Trichomonas* in the tree of life. The early branching of *Trichomonas* and also *Giardia* in 18S rRNA trees can also be explained by the “long branch attraction”, which says that long branches always form a group, neglecting the phylogenetic relationship. So these two groups could also have developed simultaneously with all other eukaryotes. Moreover, analyses of RBP1 as well as EF-1 $\alpha$  and EF-2 could not confirm an early diverging of *Trichomonas* and *Giardia*. The “hydrogen hypothesis”, a theory that supports a sister group relationship between *Trichomonas* and *Giardia*, assumes that proteobacteria became incorporated first by an archaeobacterium, evolving to mitochondria later, or

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to hydrogenosomes in anaerobians, respectively. A relationship between *Trichomonas* and *Giardia* would also support the theory of modified mitochondria (*Trichomonas*) and secondary loss of mitochondria (*Giardia*) (EMBLEY & HIRT, 1998).

### 1.1.3 The Trichomonadea

Tab. 2 shows the phylogenetic position of *T. vaginalis*. Basing on recent ultrastructural and molecular phylogenetic studies, the parabasalids consist of six main lineages at the rank of a class: Hypotrichomonadea, Trichomonadea, Tritrichomonadea, Cristamonadea, Trichononymphea, and Spirotrichonymphea. The presence of a single mastigont with 2–6 flagella and the absence of a comb-like structure as well as an infrakinetosomal body are characteristic for the Trichomonadea. The class of the Trichomonadea is divided into two orders which are monophyletic: Trichomonadida, containing the family Trichomonadidae (with a costa), and Honigbergiellida, containing the families Honigbergiellidae, Hexamastigidae, and Tricercomitidae (without a costa).

The order Trichomonadida is further subdivided into the family Trichomonadidae which splits into two groups: The *Trichomonas* group and the *Pentatrichomonas* group. Within the *Trichomonas* group, four genera branch: *Trichomonas*, *Trichomonoides*, *Tetratrichomonas*, and *Pentatrichomonas*. Representatives of *Trichomonas* colonize the urogenital tract, intestine, and oral cavity of birds and mammals and comprise some true pathogens (CEPICKA et al., 2010):

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**Tab. 2:** Classification of *Trichomonas vaginalis* (after CEPCICKA et al., 2010).

Domain	Eukarya
Kingdom	Excavata
Phylum	Parabasalia
Class	Trichomonadea
Order	Trichomonadidae
Family	Trichomonadida
Genus	<i>Trichomonas</i>
Species	<i>Trichomonas vaginalis</i>

*Trichomonas gallinae* parasitizes birds, especially pigeons, and can lead to severe decimations in the populations. The trichomonads colonize the mucosa of the pharynx, the oesophagus and the struma, causing yellow accretions that disable ingestion and lead to starvation (ROBINSON et al., 2010).

*Trichomonas tenax*, sometimes also referred to as *T. buccalis* or *T. elongata*, colonizes the oral cavity, especially the enamel and gingival, and feeds on the bacterial flora. Cases of a *T. tenax* infestation of workers in Eastern Europe and the Former Soviet Union are linked to periodontal disease (ACKERS, 2001). It is also reported that *T. tenax* can habitate the nasopharyngeal region and can cause pulmonic trichomonosis, when the patients had a pulmonic disease (STRATAKIS et al., 1999).

*T. vaginalis* is the only representative of the genus *Trichomonas* which is pathogenic for humans, and therefore the most relevant for human medical research. The genus



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*Tetratrichomonas* is the most diversified one of the *Trichomonas* group, with representatives living in vertebrates, molluscs, and leeches, and includes also one newly described species – *T. undula* sp. nov. –, which is the only known free-living species of the order Trichomonadida. The genus *Trichomonoides* contains only one member, *T. trypanoides*, which has been found in termites. Further species colonising *Homo sapiens* are *Pentatrichomonas hominis* (formerly referred as *Trichomonas hominis*) of the genus *Pentatrichomonas*, which is a non-pathogenic commensal of the ileum although it was reported to be associated with cases of diarrhea, and *Dientamoeba fragilis* of the genus *Dientamoeba*, family Dientamoebidae, order Tritrichomonadida, class Tritrichomonadea (thus not belonging to the Trichomonadea, although formerly it had been classified as member of the family Monocercomonadidae, order Trichomonadida after CAVALIER-SMITH, 1998), which is assumed to evoke gastrointestinal symptoms, however, of low pathogenic potential (ACKERS, 2001; CEPICKA et al., 2010).

All other members of the Trichomonadea are associated with animals and of different pathogenic potential.

### 1.2 *Trichomonas vaginalis*

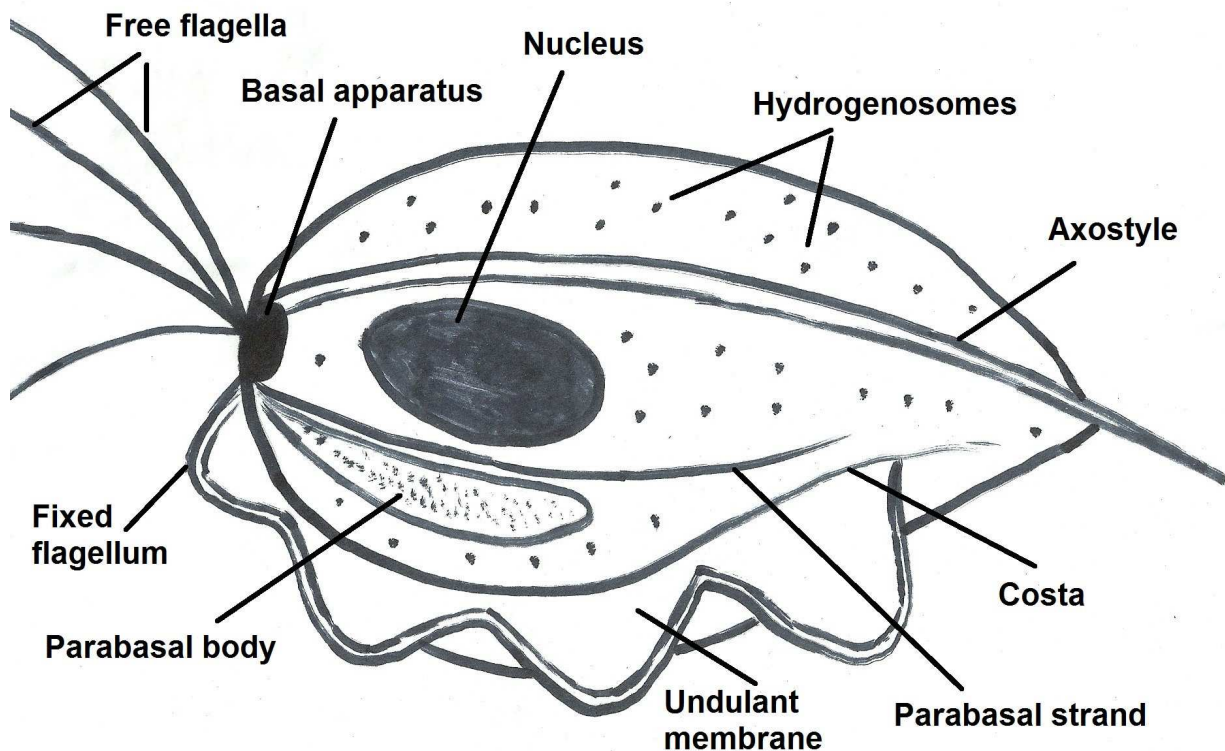
#### 1.2.1 Morphology

*T. vaginalis* is approximately 10 µm (5-15 µm) wide and 15 µm (8-25 µm) long. It appears as pear-shaped and oval, however, it also shows an amorphous-amoeboid morphology especially when attached to epithelial cells of the host (ARROYO et al., 1993). It is also known that the parasite can appear as an over-dimensioned sphere, with or without flagella, and often with multiple nuclei. The reason for this phenomenon is not yet completely understood, but it may be a reaction to physical discomfort or a cellular developmental stage (ABONYI, 1995).

The cell has four anterior flagella which are fixed on the apical pole. The undulant membrane runs along one side for about two-thirds of the length and is confined by the costa on the proximal side, which functions as a balancer, and by the fifth flagella on the distal side, which generates the wave-like motion of the membrane. The end of the flagellum is fixed in the pellicula. By flapping the free flagella, the parasite generates a hectic, staggering, and characteristic movement. The cell consists of plasma, nucleus, cytoskeleton, parabasal bodies, basal apparatus, axostyle, and hydrogenosomes. There are also granula for glycogen-storage, phagosomes and organelles, similar to lysosomes. The axostyle, a microtubular rod, divides the cell into two longitudinal halves and runs from the anterior to the posterior pole of the cell, where it breaks out as a sharp pinnacle. The purpose of this structure may be cell stabilization and for docking onto vaginal epithelial cells. Around the nucleus, the axostyle forms a collar-like pelta, which makes a cavity surrounding the nucleus and two parabasal bodies. The parabasal bodies consist of a modified Golgi apparatus

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and two filamentous elements containing dictyosomes and fibrillae (Fig. 2). The cytoplasm contains glycogen granules, large vacuoles as well as free and membrane-bound ribosomes. Furthermore, *T. vaginalis* has no mitochondria, but hydrogenosomes which are analogous organelle for an anaerobic metabolism. Mainly they are arranged alongside the costa and the axostyle (THOMASON & GELBART, 1989; ACKERS, 2001).



**Fig. 2:** Schematic view of *Trichomonas vaginalis* (Original).

### 1.2.2 Distribution

*T. vaginalis* is distributed worldwide, occurs in every climate zone with no seasonal variability and was found in every racial group and socio-economical strata, with differences in incidence, though. In Central Europe, it is one of the three most frequent pathogenic protozoa, besides *Toxoplasma gondii* and *Giardia lamblia*. It cannot form cysts, hence, it is dependent on environmental factors like temperature and humidity for its survival. The only way for transmission is direct contact. Other ways of transmission as the common use of towels are only theoretically possible because the parasite can exist outside the human body only for a short time. Swimming pool water as a transmitter can be excluded (ACKERS, 2001).

As a microaerophilic anaerobian with a pH optimum of 5.4–6.0, which correlates with the pH of the human vaginal milieu, the parasite is adapted for living in the genitourinary tract. There it lives on the epithelial layer of the anterior fornix vaginae, skene glands and urethra in women, and in urethra, prostate and occasionally under the foreskin in men. Results from *in vitro* studies have indicated a pH optimum of 6.0–6.3 and a decrease of motility below 4.5 (DIAMOND, 1986). At 0°C, the organism can survive for up to 5 days, however, at 60°C and above, it is killed within 4 minutes (THOMASON & GELBART, 1989). Crucially, *T. vaginalis* is very sensitive to dehydration and can survive in bath waters with its different osmotic value from 30 minutes to 3 hours and in chlorinated swimming pool waters (44 mg/l) only for a few seconds to minutes. In supply water, the survival span can reach up to 24 hours. As mentioned before, *Homo sapiens* is the only natural host of *T. vaginalis*, however, experimentally it can infect and breed it in the urogenital tracts as well as in the peritoneum of mice (ACKERS, 2001; WALOCHNIK & ASPÖCK, 2002).

### 1.2.3 Life cycle

*T. vaginalis* is an extracellular parasite and does only exist in the trophozoite stage. Vectors or cysts for transmission are not needed as it is directly transmitted from human to human during sexual intercourse. The trophozoite feeds by pinocytosis, a specification of phagocytosis, and ingests bacteria, yeasts, and erythrocytes, whereas liquids and solved nutrients are taken off by pseudopodia-like extensions (THOMASON & GELBART, 1989).

*T. vaginalis* reproduces by longitudinal binary fission every four to six hours, but in contrast to other eukaryotes the nucleus membrane does not disappear during this event. The nucleus divides by kryptopleuromitosis in which the spindle is located outside the nucleus. After disaggregation of the axostyle, the flagella double and the mitosis starts by forming two attractophores on both sides of the nucleus that become the poles of mitosis. From there, microtubules reach into the nucleus and attach on the centromeres. The extranuclear spindle between the attractophores, called paradesmose, elongates and the cell divides when nuclei and flagella migrate to separate poles. Subsequently, daughter cells produce the missing organella. Round forms – as mentioned in 1.2.1 – are assumed to divide by amitotic budding. Some species of the Trichomonadida are also able to form pseudocysts that are not protective but allow a faeco-oral transmission. Hence, it is assumed that *T. vaginalis* has lost its ability to form cysts as a result of its obligatory parasitic lifestyle and the direct way of transmission (PETRIN et al., 1998).

Interestingly, *T. vaginalis* also seems to have a meiotic recombination machinery as 27 of 29 resembling meiotic genes were found as ortholog genes. Due to the presence of 21 of these genes also in *Giardia intestinalis*, the meiosis machinery

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seems to be present in a common ancestor of both organisms and, hence, occurred early in the evolution. This theory is supported by the high genetic variation between *T. vaginalis* strains (MALIK et al., 2008).

### 1.2.4 Metabolism

The energy metabolism of *T. vaginalis* is, along with those of *Giardia* and *Enteromonas*, more similar to anaerobic bacteria than to eukaryotes. Trichomonads lack mitochondria and peroxisomes, thus they can oxidize glucose only partially and cannot catabolise  $H_2O_2$ . Moreover, their parasitic habitus also seems to result from their disability to synthesize nucleic acids, so that they have to incorporate purines and pyrimidines from their host cells. Adenine and guanine salvage is mediated by nucleoside phosphorylases and kinases, whereas thymine, cytidine and uracil are metabolized by phosphoribosyltransferases and nucleoside kinases. Nucleosides are transferred through the cell membrane by active carrier transport and are converted into nucleotides by specific kinases (PETRIN et al., 1998).

De novo lipid biosynthesis is confined to the phospholipid phosphatidylethanolamine, a component of the cell membrane. Other lipids, including cholesterol, are most likely acquired from exogenous factors because of the absence of their enzyme-coding genes and degradation of the metabolic pathways (CARLTON et al., 2007).

Amino acids are consumed in higher amounts when the main energy source, carbohydrates, is not available or limited (PETRIN et al., 1998). Carbohydrate metabolism takes place in the cytoplasm, where glucose is converted to phosphoenolpyruvate and pyruvate via glycolysis (Emden-Meyerhoff-Parnas

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pathway), and subsequently in the hydrogenosomes, where pyruvate is oxidatively decarboxylated to acetyl-coA by the enzyme PFOR. In the respiratory chain, acetyl-coA is converted to acetate, coenzyme A, and ATP. Under physiological stress, *T. vaginalis* decreases the activity of hydrogenosomal enzymes and changes to cytosolic conversion of pyruvate to lactate by enhanced activity of the enzyme LDH. The catabolic end products of *T. vaginalis* are acetate, lactate, malate, glycerol, CO<sub>2</sub>, and H<sub>2</sub> (KIM et al., 2006; LEITSCH et al., 2010; PETRIN et al., 1998).

Hydrogenosomes are organelles with a size of 0.5–1 µm and represent an analogue to mitochondria in anaerobic organisms. Contrary to them, they lack cristae, cytochromes, and also own DNA, (except in the ciliate *Nyctotherus ovalis*) – which corroborates the theory of a common ancestor of hydrogenosomes and mitochondria (the hypotheses on the origin of hydrogenosomes range from modified or degenerated mitochondria to the endosymbiosis of anaerobic bacteria in eukaryotic cells). The main function of the hydrogenosomes is the generation of energy by substrate-level phosphorylation of acetyl-coA to ATP (HACKSTEIN et al., 2001; PETRIN et al., 1998).

The ATP-generating pump works identically as those in mitochondria, however, it loses its function in the presence of oxygen as it is catalyzed by the strictly anaerobic enzyme hydrogenase. Due to the production of hydrogen and the consecutive conversion to methane by methanogenic bacteria, anaerobic protozoa with hydrogenosomes also contribute to the greenhouse effect significantly. PFOR is also supposed to play a role in the resistance mechanism against metronidazole (MÜLLER, 1986; MÜLLER, 1990; KULDA, 1999).

### 1.2.5 Genetics

The karyotype of the Trichomonadida depends on the species and varies from three to twelve chromosomes. *T. vaginalis* contains six chromosomes, comprising of three maxichromosomes, two intermediate chromosomes, and one minichromosome. The same number of chromosomes was found in 15 isolates of *T. vaginalis* from different geographic regions, which supports the theory of a highly conserved karyotype within the genus *Trichomonas* (LEKHER & ALDERTE, 1999).

In 2007, the “*T. vaginalis* Genome Project” was completed by CARLTON et al. (2007), and the draft genome sequence was published. The whole size of the genome was found to be 160 megabases (Mb) in size, which is about tenfolds larger than originally estimated by LEKHER & ALDERTE (1999). About 60.000 protein-coding genes were identified, which is more than in *Homo sapiens* with “only” 30–40.000 coding genes. Moreover, more than two thirds of these genes contain repeats and transposable elements. It is assumed that this enlargement of the genome occurred by lateral gene transfer from bacteria, viruses, and phagocytosis from host proteins as well as by the parasite’s relocation from the digestive into the urogenital tract. An enlargement of the genome might correlate with an enlargement of the cell (*T. vaginalis* cells are distinctly larger than those of other Trichomonadida) – probably a benefit at colonizing new habitats. Numerous of the identified genes are responsible for specific metabolic pathways and pathomechanism. About 800 genes stand by for the process of attachment on host cells, about 400 are responsible for the degradome (protein-degrading peptidases) (CARLTON et al., 2007).



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A recent study has shown that in case of the transmembrane adenylyl cyclase genes (TMAC genes), a high percentage are pseudogenes (46%) and the high number of gene copies result from a recent duplication of a small ancestral gene family.

Especially mavericks – giant transposable elements – are abundant and constitute 30% of the total genome and 50% of the putative protein-coding genes (PRITHAM et al., 2006; Cui et al., 2010). Introns and non-coding elements can be no explanation for the large-scale genome amplification as they are rare and short. In *T. vaginalis*, only 65 introns have been identified. Additionally, the size of the genome appears not to be correlated to pathogenicity although the largest genomes were found in the pathogenic species *T. vaginalis* and *T. foetus* (177 Mb). However, a similar genome size was found in the non-pathogenic species *T. augusta*, a harmless commensal in reptile intestines, (165 Mb) and *T. tenax* (133 Mb), whereas the smallest genome was found in the bird-pathogen *T. gallinarum* (86 Mb) (ZUBÁČOVÁ et al., 2008).

Arguably, a high polymorphism between microsatellite markers of a population of common laboratory strains indicates that *T. vaginalis* is a genetically diverse organism, however, sequencing of 5.8S rDNA and the flanking ITS regions of *T. vaginalis* isolates from sex workers on the Philippines showed low genetic polymorphism between the strains (RIVERA et al., 2009; CONRAD et al., 2010).

The identification of genes for the biosynthesis of common cytosolic FeS proteins gives a distinct support of the hypothesis that hydrogenosomes may have their origin in mitochondria. Furthermore, hydrogenosomes may also be able to metabolize amino acids. The existence of an RNA interference pathway might be a possible target for developing drugs manipulating the parasite's gene expression (CARLTON et al., 2007).

### 1.3 Trichomonosis

#### 1.3.1 Epidemiology

*T. vaginalis* is the causative agent of trichomonosis and therefore of high medical and socio-economical relevance. Trichomonosis is the most frequent non-viral sexually transmitted disease, with 174 million cases globally and an incidence of 2–3 million new infections annually (WHO, 2001; NIAID, 2003). Prevalence varies from region to region and ranges from 3% in the USA (ALLSWORTH et al., 2009), over 10–20% in Europe and the Republic of Korea (ASPÖCK, 1994; RYU & MIN, 2006) up to 31% in Mozambique (MENÉNDEZ et al., 2010) and 40% in Papua New Guinea (VALLELY et al., 2010). From Mongolia, a prevalence of 67% was reported (SCHWEBKE et al., 1998). In Vienna, Austria, the prevalence of *T. vaginalis* in prostitutes was found to be approximately 5% (STARY et al., 1991).

Studies from the USA have shown that trichomonosis is 12 times more prevalent among black women, besides it correlates also positively with young age, high frequency of changing sexual partners (especially with older partners), high poverty level, other genital infections (especially *Neisseria gonorrhoeae*, *Chlamydia*, herpes, warts), and low educational and hygienic level (ALLSWORTH et al., 2009; KRASHIN et al., 2010). A much higher prevalence – from 8.5% to 47% – was also found in female US jail and prison inmates (SUTCLIFFE et al., 2010). Moreover, in Greece *T. vaginalis* was more frequently detected in immigrants (8%) than in native women (3%) (PIPERAKI et al., 2010).

The maximum prevalence is between 20 and 45 years, which is of higher age than for the most sexually transmitted diseases. However, this correlates with the age of

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maximal sexual activity. High-risk groups are prostitutes and promiscuous individuals. Besides, an altered vaginal pH milieu, caused by hormonal changes like birth control pill or pregnancy, leads to a stronger incidence of infections with *T. vaginalis*. With the beginning of the menopause, the disease often heals spontaneously (PETRIN et al., 1998). Infected mothers can transmit trichomonads to their child during birth (perinatal mode of infection) with a risk of approximately 5%. In this case, the child can acquire an infection of trichomonads in the urogenital tract, however, the infection heals spontaneously a few weeks after birth and requires normally no treatment (WALOCHNIK & ASPÖCK, 2004). Trichomonads can even be detected in infants – in this case, it is mainly an evidence for sexual child abuse (LEWIN, 2007).

### 1.3.2 Clinical manifestations

The habitat of *T. vaginalis* is the human urogenital tract where it colonizes the mucosal epithel cells. Both women and men can be infected and act as a vector for transmission. In women, the disease occurs mainly between adolescence and menopause. Primary affected organs are vagina, cervix and – additionally – urethra in 75–90% of the cases, whereas bladder and uterus are not affected normally. In 50% of infected and symptomatic women, the incubation time is between 4 and 28 days (THOMASON & GELBART, 1989; PETRIN et al., 1998). 10–50% of infected women show no symptoms and also have a normal vaginal flora and pH. However, 50% of these women will develop symptoms within the next six months (PERAZZI et al., 2010).

In contrast to women, infections with *T. vaginalis* are asymptomatic in men in 90% of the cases. The parasite could be detected in 72% of male partners of infected

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women, of whom 78% were asymptomatic. These cases may lead to misdiagnosis which supports further distribution (SEÑA et al., 2007). The incubation time in symptomatic men is approximately 10 days, in which the trichomonads colonize the foreskin, urethra, and prostate gland. In about 10% of the cases, urethritis and prostatitis can occur. In women, the symptoms are more severe and include mostly considerable irritations with a fulminant vaginitis. The first symptoms are strong urgency, pain at urinating, and itching of the vagina. Typical symptoms are a sore and reddend vulva, an edematous and reddend vagina, “fluor vaginalis” (a foamy, malodorous, viridescent discharge), and a “strawberry-cervix” (punctual, hemorrhagic lesions in the vaginal cervix). Furthermore, the number of leukocytes in and the pH-value of the vagina can be increased dramatically, thus the vaginal flora is altered and susceptible for further infections. Dyspareunia and postcoital hemorrhage can also occur. When therapy fails, the acute stage fades into a chronic stage, in which the symptoms continue but in a far milder way, being ignored often. This is also an important factor for the transmission of the disease (PETRIN et al., 1998).

In many cases, the symptoms are unspecific and can be confounded with those of other venereal diseases. The “strawberry cervix” is observed in 2% of cases and “fluor vaginalis” in 12% of cases only (FOUTS & KRAUS, 1980).

Even though trichomonosis is not a lethal disease, an infection can impair health and life quality severely. Besides the above mentioned symptoms, it is also believed that an infection with *T. vaginalis* can cause complications during pregnancy including damage of the placenta, higher risk of premature birth and a low birth weight (COTCH et al., 1997). Furthermore, there seems to be a coherence of trichomonosis and cancer, especially when the infection has passed into the chronic stage. Steadily

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inflamed urogenital organs like ovaries and prostate gland have a higher risk to develop neoplasia and cancer (SUTCLIFFE et al., 2009). In rare cases, a fulminant infection may also lead to temporary sterility (EL-SHAZLY et al., 2001).

In 2008, an acquisition of trichomonads in neonatals during their passage through the birth canal and consequently evoked infections of the childrens' respiratory tract could be detected (CARTER & WHITHAUS, 2008).

Finally, it is reported that trichomonosis may increase the risk for co-infections, not only for fungi (*Candida*) and bacteria (*Mycoplasma*), but also for HIV as an infection with *T. vaginalis* leads to a weaker integrity of the epithelial cells, to a decreased innate immunity and an accumulation of HIV-affine cells like lymphocytes and macrophages (LAGA et al., 1993; SORVILLO et al., 2001; THURMAN & DONCEL, 2011).

### 1.3.3 Pathomechanism

*T. vaginalis* is an extracellular parasite and primary affine to erythrocytes and vaginal epithelial cells but also to immune cells and vaginal bacteria. Additionally, *T. vaginalis* internalizes also viruses like HIV and human papillomavirus and can act as a Trojan horse for bacteria such as *Mycoplasma* that survives and multiplies inside the trichomonad cell. Similarly, after being tranferred from one person to another, *T. vaginalis* can support the spread of HIV by this way (TAYLOR-ROBINSON, 1998; HIRT et al., 2007).

The mechanism of pathogenesis is complex – as well not fully understood yet – and includes cell-cell-adhesion, haemolysis, excretion of lytic factors, endocytosis of host

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cell content, and degradation of immunoglobulins and complement proteins (HIRT et al., 2007).

Adhesion to epithelial cells is an essential step in pathogenesis. *T. vaginalis* attaches with the side opposite to the undulant membrane, changing from its ovoid shape to an amoeboid form. This step depends on temperature, pH value, and time span of contact. A key protein of this action is alpha-actinin, an actin-binding protein that participates in the morphological transformation by the redistribution of actin. Hence, the parasite's shape becomes amoeboid-amorphous and capable for adhesion onto host cells. *In vitro* studies have shown that motile parasites form large aggregates and recruit other parasites. After a few hours, retraction, rounding, and detachment of the epithelial cells were observed (ALDERETE & GARZA, 1985; FIORI et al., 1999). Adhesion seems to be dependent on adhesion proteins (AP65, AP51, AP33, AP120, AP23) and certain components of the glycocalyx of the cell membrane, especially its main component, lipophosphoglycan (LPG). *In vitro* studies have shown that LPG defective mutants have less adherence and cytotoxicity for human ectocervical cells (BASTIDA-CORCUERA et al., 2005; ZHANG et al., 2010).

Regarding the adhesion proteins, it is reported that lectins, considered as important virulence factors also in other pathogens (f.e. *Entamoeba histolytica*), could be involved in the adhesion process via binding to sugar moieties from glycoconjugates of host cells. Furthermore, hydrogenosomal enzymes are claimed to have dual cellular localizations as surface proteins, where they function as adhesins (so called "moonlighting proteins"). Although these data are controversial, it could be proved that the adhesion protein AP120 is identical to the hydrogenosomal enzyme PFOR. In this context, an outstanding feature is the absence of glycosylphosphatidylinositol-

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anchored surface proteins which are characteristic for eukaryotes (MORENO-BRITO et al., 2005; HIRT et al., 2007).

Haemolysis is dependent on temperature, pH value,  $\text{Ca}^{2+}$ , effector concentration, and includes several steps. After adhesion, cysteine proteinases – transmembrane pore-forming proteins – induce lysis of host cells by pore formation and can also manipulate the host's immune system by degrading antibodies. For example, CP39, one of the best characterized cysteine proteinases, is able to degrade collagens I, III, IV, and V, human fibronectin, hemoglobin, and immunoglobulins A and G (HERNÁNDEZ-GUTIÉRREZ et al., 2004).

During this step of haemolysis, the parasite detaches from the cell via a specific cell-detaching factor (CDF) and the contents of the lysed cell are phagocytosed. Red blood cells are a means for acquiring lipids and iron, essential nutrients for the parasite. It was also found that spectrin, the main protein of the cytoskeleton of the membrane of red blood cells, is totally absent after exposure to *T. vaginalis*. This may be a strategy of the parasite to increase the sensitivity of host cells to pore-forming proteins (FIORI et al., 1999). A decrease of cysteine proteinase expression also correlates with a low virulence phenotype (DE JESUS et al., 2008).

Recently, it was found that not only cysteine proteinases may be involved in pathogenicity; the first putative serine proteinase (SUB1; subtilisin-like serine protease) was identified and characterized (HERNÁNDEZ-ROMANO et al., 2010). The emergence of inflammation may be contributed to the recruitment and accumulation of neutrophils by the release of chemokines by *T. vaginalis* like IL-8 and GRO- $\alpha$  (RYU & MIN, 2006).

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An additional important factor in regulating virulence is iron, as trichomonads exhibit less cysteine proteinase activity and therefore low cytotoxicity levels at highest iron concentrations and vice versa (ALVAREZ-SÁNCHEZ et al., 2007).

*T. vaginalis* has allocated a great many of gene clans and families and proteins for its pathomechanism. There are about 800 genes for the process of cell-cell-adhesion and about 23 different proteinase activities (LEÓN-SICAÍROS et al., 2004; CARLTON et al., 2007). Curiously, proteins like CDF can even act independently from direct cell contact. It could be shown that epithelial cells in a trichomonad-free filtrate containing CDF react by rounding and detaching from the culture flask (GARBER et al., 1989).

An important role in pathogenesis accords to the interactions of *T. vaginalis* with the vaginal flora. The population of *Lactobacillus acidophilus*, an important symbiont of the vaginal milieu, can be reduced or disappear following on a massive reproduction of trichomonads. Consequently, the pH value – normally at 4.5 – can be deranged as trichomonads prefer a pH >5 which is disadvantageous for vaginal bacteria. Further co-infections can also appear, especially with *Candida* and *Mycoplasma hominis* (PETRIN et al., 1998; VAN BELKUM et al., 2001).

### 1.3.4 Diagnostics

Due to the mentioned unspecific symptoms of trichomonosis, laboratory diagnosis is of major importance and focused on the direct detection of parasites in vaginal smears. If diagnosis is based only upon the classic clinical symptoms (“strawberry cervix”, frothy discharge), 88% of infections cannot be diagnosed, and 29% of uninfected women would be false positive in being infected (FOUTS & KRAUS, 1980).



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In men, trichomonads are more difficult to detect than in women, as they occur in lower numbers and may be reduced by micturition too. In women, the pH value – measured on Nitrazine paper – is elevated and can be as high as 6.0. Sample collection is performed with cotton-tip swabs from the anterior and lateral vaginal fornices, prostatic secretions, and semen. Mucosal cells from the urethra can be collected on a wire loop (THOMASON & GELBART, 1989; RAJEBALI, 2008).

Wet-mount examination by phase-contrast, dark-field or bright-field illumination is a simple and fast diagnostic method. The secretions are inoculated onto a drop of physiological saline on a slide and examined. However, this method is of low specificity and has a sensitivity rate from as low as 38% to as high as 92% as the secretions contain not only trichomonads but bacteria and leukocytes too. Trichomonads often appear as rounded forms that are similar to the shape of white blood cells. They can be clearly identified by their motility. However, they have a limited life span outside of the human body, which depends on the temperature and sample material. Using wet-mount examination it is only possible to detect living trichomonads in at most three-quarters of infected women and only in 1–20% of infected men (THOMASON & GELBART, 1989; PETRIN et al., 1998; ACKERS, 2001).

Culture in Kupferberg Medium or Modified Diamond Medium provides a higher reliability. However, although this technique is simple and requires only 300–500 trichomonads/ml of inoculum to obtain a growing culture, it has its limitation in its incubation period of 2 to 7 days. During this time, infected patients can further transmit the parasites. Methods like the InPouch system and the plastic envelope method combine the promptness of wet-mount examination with the high sensitivity (97%) of culture, improving the specificity up to 100% (PETRIN et al., 1998).

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Staining techniques such as Papanicolaou, acridine orange, Leishman, periodic acid-Schiff and also Giemsa and Eosin increase the sensitivity of direct microscopy. It allows distinguishing similar shaped trichomonads from white blood cells, although sometimes the typical morphological features are altered or lost during fixation and staining. Used solely, staining has a fairly low specificity and sensitivity due to a high number of false positive and false negative findings (THOMASON & GELBART, 1989; PETRIN et al., 1998; RAJEBALI, 2008).

Staining methods using monoclonal antibodies maintain a sensitivity (80.6%) and specificity (98.6%) similar to that of culture. Trichomonas Direct Enzyme Immunoassay and Fluorescent Direct Immunoassay (California Integrated Diagnostics, Benicia, CA) use peroxidise- and fluorescent-labeled cocktails of monoclonal antibodies, making structures like membrane, cytoplasm, nuclei, and flagella visible. The examination can be performed with an ordinary light microscope within 1 hour. As even nonmotile trichomonads can be detected and it approves both diagnosis and treatment at a single consultation, this method is suitable for most clinics and physicians (THOMASON & GELBART, 1989). New tests as the OSOM Trichomonas Rapid Test (Genzyme Diagnostics) and the Affirm VP III (Becton Dickinson) are promising with a sensitivity >83% and a specificity >97% (WENDEL & WORKOWSKI, 2007).

Serological methods such as agglutination, complement fixation, and enzyme-linked immunosorbent assay may be applied to detect antitrichomonal antibodies, however, they are not suitable as first time diagnostics because it is not possible to distinguish between current and past infections. Trichomonal antibodies may persist for a long time after healing of the infection. In general, the sensitivity is low in solely used

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serological methods: if the level of antibodies is too low or the serum humoral response can not be elicited, it will not be possible to perform a successful diagnosis (PETRIN et al., 1998).

With the establishment of molecular biological methods in most laboratories, PCR is the primary method of choice in diagnosis nowadays. By this technique it is possible to detect even low numbers of pathogens by repeating amplification of a specific part of the genome (SAIKI et al., 1988). Sensitivity and specificity of an established PCR for *T. vaginalis* were calculated as 97.8% and 97.4%, respectively, for genital samples and 80% and 99.6 %, respectively, for urine samples. Hence, the reliability is similar to that of culture and, in contrary, enormously time-saving (JORDAN et al., 2001; VATANSHENASSAN et al., 2010).

Due to the disadvantage of high costs, a combination of PCR and microscopic examination seems to be most reasonable (LAB TESTS ONLINE, 2007). Today, co-infections by *T. vaginalis* and other pathogens as *Mycoplasma hominis* and *Ureaplasma urealyticum* can be detected rapidly with a newly established multiplex PCR (DIAZ et al., 2010).

### 1.3.5 Therapy

The medical treatment of trichomonosis is performed mainly by chemotherapy, and in this case, metronidazole has been the drug of choice since it was discovered by scientists of the Pasteur Institute in 1959 (COSAR & JULOU 1959). It is applied orally mostly, but can also be applied as vaginal suppository. After uptake, the drug is absorbed by the mucosa, resorbed in the gut, catabolized in the liver and excreted by

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the kidneys with a bioavailability of 90% and higher. The dosage regimen is referred as 750 mg three times a day for 7 days or as a single dose of 2 g, with a success rate of 82% to 88% and even 95% when all sexual partners are treated simultaneously (NIX et al., 1995; PETRIN et al., 1998).

Metronidazole belongs to the chemical class of 5-nitroimidazoles and has a high tolerability in general, nevertheless, there are some side effects as headache, metallic taste, anorexia, nausea, parasthesia, allergic reactions, neuropathy, and malfunctions of the digestion can occur. Furthermore, in some cases neutropenia, hepatitis, and jaundice have been reported. Metronidazole must not applied when diseases of the central nervous system, blood, and liver are present, and also during the pregnancy/lactation period, especially in the first trimester, due to its putative teratogenic potential. Studies in mice have attributed a cancerogenic effect to metronidazole as it provokes lung tumours after long-term application of high doses. Alternative drugs for treatment, although of lower effectivity, are clotrimazole, a combination of tinidazole and paramomycin, carnidazole, ornidazole, secnidazole, flunidazole, and nimorazole. Amphotericin B, a broadband antibiotic generally used against infections with *Naegleria*, *Leishmania* and *Trypanosoma*, can also be applied against trichomonosis although it has severe nephrotoxic potential (PETRIN et al., 1998; ACKERS, 2001; REDIGUIERI et al., 2011).

Resistance to metronidazole is a serious problem and a reason for treatment failure. It is reported from laboratory strains since the 1960s and from patient isolates since the late 1970s (RASOLOSON et al., 2002). Collateral to the broad use of metronidazole against trichomonosis, resistance is increasing due to adaptation of the parasite to the drug's mode of action. Treatment failure as the exposure of trichomonads to

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sublethal drug concentrations may lead to resistance and, furthermore, cross resistances to other nitroimidazoles – to which most of the antitrichomonal drugs belong – occur more frequently. Resistance may be overcome with higher and increasing doses. An alternative treatment of metronidazole-resistant strains is possible with tinidazole, however, it is successful only in a limited number of cases (KULDA, 1999; NYIRJESI, 1999; WENDEL & WORKOWSKI, 2007). Hence, there is a requisition for new antitrichomonal agents with a similar effectivity of metronidazole but a different mode of action.

Finally, it is important that not only the infected patient gets treated but also all sexual partners to avoid reinfections. Sexual intercourse should not be practised during the duration of treatment (PETRIN et al., 1998).

### 1.3.6 Immunity and prophylaxis

A vaccine is not available against *T. vaginalis* to this date. A reason might be the complexity of the interactions between host and parasite, which is generally the main problem in the establishment of vaccines against parasites (LUCIUS & LOOS-FRANK, 2008). There is no protective immunity of *H. sapiens* against *T. vaginalis*, even after repeated infections. Cell-mediated immune response was found to be invoked as well as anti-trichomonal antibodies IgA, IgG, IgG subclass, and IgM were found to be present during and a few months after an infection but an acquired, protective immunity could not be confirmed to this date. An explanation may be the antigenic heterogeneity of the parasite. However, innate mucosal immunity exists. Interestingly, in mice – although *H. sapiens* is the only natural host for *T. vaginalis*, it is also

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possible to breed it in the peritoneum and urogenital tract of mice (see 1.2.2) – immunity could be achieved after subcutaneous infection of living trichomonads. Amazingly, a protection could not be achieved after vaginal infection. Thus, also the localization of the pathogen in the vagina may play a role in systemic antigen presentation and acquiring immunity (ABRAHAM et al., 1996; KAUR et al., 2008; THURMAN & DONCEL, 2011).

As mentioned before, an infection with *T. vaginalis* must not lead to symptoms obligatorily, and even if it does, spontaneous healing can occur, particularly in men. It is assumed that production of cervical IL-8 and vaginal  $\alpha$ -defensins, mediated by *T. vaginalis* itself, and the consecutive infiltration with neutrophiles in the local area play a role in infection healing and asymptomatic infections, as neutrophiles have potential in killing pathogens and are important mediators in inflammatory regulation (BRENIER-PINCHART et al., 2001; SIMHAN et al., 2007).

The parasite may also be able to be resistant against the complement system which is present in the menstruation blood but not in the mucosa. It was found that a high level of iron supports a resistance, whereas low iron levels lead to more killing of trichomonads by the complement. This knowledge and the fact that many virulence factors of *T. vaginalis* are iron-mediated may be an explanation why the symptoms are more serious during the menses (ALDERETE et al., 1995).

As in other venereal diseases, the best prophylaxis is the use of condoms and the avoidance of promiscuity. Educational campaigns, controllings of risk groups, and a treatment of all sexual partners of the patient amend the prophylactic methods. A reduction of the time span of infection and an efficient treatment are important to control the spreading of trichomonosis (WALOCHNIK & ASPÖCK, 2002).

### 1.4 Scientific background

#### 1.4.1 Pentamycin – a polyene antibiotic

Pentamycin was discovered and described in 1958. It has been investigated as a drug against fungal infections, like candidiasis, a common co-infection of trichomonosis (UMEZAWA et al., 1958; CHEN et al., 1978; BALMER, 2009).

Chemically it belongs to the polyenes, a subdivision of the macrolide class. Substances in this class are characterized by a macrocyclic ring of carbon atoms closed by lactonization. The polyene group has, in addition, a series of conjugated carbon-carbon double bonds. The number of these bonds allows to classify polyenes into dienes (two bonds), trienes (three bonds), tetraenes (four bonds), pentaenes (five bonds), hexaenes (six bonds) and heptaenes (seven bonds) (HAMILTON-MILLER, 1973) .

A feature allowing classification of polyenes is the highly characteristic ultraviolet absorption spectrum, leading to specifically emitted colors: Trienes are colorless, tetraenes are pale yellow, pentaenes are yellow, and heptaenes appear orange. Many polyenes are amphoteric, possessing one basic and one acidic group, and also many act as antibiotics, mainly antifungals, and are produced by several *Streptomyces* species. The most effective antifungals belong to the heptaenes, like amphotericin B and candicidin, but also trienes, tetraenes, pentaenes and hexaenes include such strong substances. As its name indicates, pentamycin belongs to the pentaenes (HAMILTON-MILLER, 1973).

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All polyenes interact with sterols in the cell membrane that are ergosterol in fungi and cholesterol in protozoa and higher eukaryotes, where they alter the permeability of the membrane, lead to  $K^+$  leakage, inhibition of glycolysis and finally to cell lysis. Differences in the mode of action have been noted in the ion selectivity of membrane pores produced, the relative preference for cholesterol- or ergosterol-containing membranes and in progressive or “all-or-none” modes (KOTLER-BRAJTBURG et al., 1979). In the 1960s, it was believed that polyenes are toxic for fungi and some other eukaryotes but not for bacteria due to their different membrane composition and lack of sterols. However, it was found out later that some polyenes like amphotericin B and nystatin are toxic for bacteria like *Acholeplasma laidlawii* too (DE KRUIJFF et al., 1974). Regarding the effectivity of the polyenes, it seems that their toxicity is only outweighed by the lack of resistance occurring to them (HAMILTON-MILLER, 1973).

However, only a few polyenes have found their way into clinical treatment, the most known among them is amphotericin B. It is applied against all human pathogenic fungi and some protozoa like *Leishmania*, *Trypanosoma*, *Naegleria*, and also *T. vaginalis*. Especially against the severe diseases visceral leishmaniosis and primary amoebic meningoencephalitis it is used as drug of last resort. Its disadvantages are its side effects, which can lead to severe and permanent kidney damage in the worst case. According to its clinical use, it is one of the most toxic polyenes for mammals too: Experiments have shown a  $LD_{50}$  in mice of 280 mg/kg after intraperitoneal application and >8,000 mg/kg after oral application. In comparison, pentamycin has shown a  $LD_{50}$  of 33.3 mg/kg after intraperitoneal application and 1,624 mg/kg after oral application (CARTER, 1969; OLLER, 1969; HAMILTON-MILLER, 1973; HAIDO & BARRETO-BERGTER, 1989; PAILA et al., 2010).



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Pentamycin ( $C_{35}H_{60}O_{13}$ ), produced by the actinomycete *Streptomyces pentaticus*, has been in laboratory use against fungal infections since then, in particular against candidiasis, since the 1960s but has also been described as an enhancer of the potent antitumor agent bleomycin (NAKASHIMA et al., 1974; ZYGMUNT, 1966). As recently as in 2009, it has been described as a successful drug in clinical use against infections with *Candida albicans*: Submitted as a vaginal tablet, it does not penetrate into the circulation due to its high molecular weight (670.85 g/mol) and bi-polar structure, which contributes to its tolerability. Because candidiasis is a common co-infection of trichomonosis, it was noticed that the efficacy is also significant in case of trichomonosis (BALMER, 2009).

The clinical importance of a new and effective drug in treating trichomonosis is getting distinctively due to the increasing resistance of *T. vaginalis* against metronidazole on which several theories exist meanwhile about the mechanism of resistance (MÜLLER, 1986; HRDY et al., 2005; LEITSCH et al., 2009).

### 1.4.2 The field of proteomic research

The term “proteome” is a cross between “protein” and “genome” and means the entirety of proteins in a cell or an organism, as transcriptome means the entirety of mRNA and genome the entirety of genes. Proteomics is the investigation of the proteome and can give an insight into the actual genomic activity of the cell. Because the genome is only the blueprint of its transcripts, the status of a cell or organism is much better represented by its expressed proteins.

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In contrary to the genome (aside from epigenetic regulation mechanisms), the proteome shows a highly dynamic activity as the span of expressed proteins differ from cell to cell and from time to time. Some proteins persist only for a few seconds whereas others can outlast for years. Also, it is not possible to conclude the number and functions of proteins from the genes because post-translational modifications of mRNAs (poly adenylation, capping, splicing, exon shuffling,...) and also of proteins (glycosylation, acetylation, phosphorylation, methylation,...) can lead to various functional proteins that are expressed by one single gene – or vice versa, as several genes may contribute to the transcript of one single protein. Finally, the activity of proteins also depends on their primary, secondary, tertiary, and quartary structure as well as new functions can emerge when proteins assemble to-gether to a complex (KNIPPERS, 2001) .

To simplify this complex research field, the aims of proteomics can be abstracted into two chapters (KNIPPERS, 2001; MURKEN et al., 2006):

- first, the identification and characterization of proteins, performed by 2D SDS-PAGE (two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis) and subsequent mass spectroscopy
- secondly, the investigation of the function of proteins, including the localization of specific proteins in the cell, performed by enzymatic tests and genetic methods like knockout-mutants, reverse genetics, mutant phenotypes, and other methods

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Alongside with the successful identification of the draft genome sequence of *T. vaginalis*, the research of its proteome has improved too in the last years. A study of 2009 by HUANG et al. has published a proteome reference map of the parasite by using 2D SDS-PAGE and MALDI-TOF mass spectroscopy. It was observed that the majority of the expressed proteins is settled in the acidic pH milieu, maybe due to the adaptation to the vaginal milieu. The identified proteins were classified into energy production and carbohydrate metabolism, cytoskeleton, defense and stress-related responses, nucleotide metabolism, pathogenesis and other. The most abundant group was responsible for energy metabolism (24.7% of total) that is located in both cytosol and hydrogenosomes. However, cytoskeletal proteins and cysteine proteinases, that participate both in the pathogenesis process as well as antioxidant proteins like thioredoxin reductase, accounted a considerable amount for the *T. vaginalis* proteome too (HUANG et al., 2009).

Furthermore, a study of 2007 has shown that trichomonads, cultivated in an iron-depleted medium, display a different protein profile than those grown in iron-rich medium. Iron is an important nutrient, and a lack causes alterations in the morphology and the metabolism, forcing the organism to change to alternative metabolic pathways. Enzymes involved in energy metabolism, proteolysis and hydrogenosomal iron-sulfur proteins were down-regulated or suppressed, whereas actin and lactate dehydrogenase were up-regulated in iron-lacking trichomonads (DE JESUS et al., 2007). This is a good example for the potential and amenities of proteomics in displaying the status of an organism, depending on the environmental conditions, which will also play a role in this study.

### 1.5 Aims of the study

The overall aim of this study was to investigate the mode of action of pentamycin against *T. vaginalis* via microscopic and proteomic methods. Four differently metronidazole-sensitive strains of *T. vaginalis* were used: ATCC 50138 (metronidazole-resistant), TV2 and ATCC 30236 (both normally metronidazole-sensitive), and ATCC 30001 (highly metronidazole-sensitive). In a preliminary study, pentamycin of less purity (28%) had been shown to be generally effective against all these strains.

First, it was aimed to investigate the effectivity under resistancy stress via establishing a long-term drug treatment with sublethal concentrations for six months.

Second, it was aimed to establish a dose-effect-relationship of trichomonads exposed to pentamycin and to look whether differently metronidazole-sensitive strains show different sensitivities to the treatment with pentamycin.

Third, it was aimed to investigate the mechanism of action with a focus on the effects on the protein level. Protein composition pre and post treatment was compared. It should be evaluated whether treatment alters the protein composition and if trichomonad cells respond to treatment and in which way. Additionally, trichomonads in long-term treatment were analysed by this way and compared at different stages of resistance by the protein profile to monitor their stage of resistance and to look for a correlation between resistance development and altering protein profile.

In a parallel study the mode of action of pentamycin on the DNA level was investigated. Here, the genetic regulation pre- and post-treatment was monitored by

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the establishment of cDNA libraries and comparing corresponding RNA profiles.

(Michael Syrowatka, in preparation).

## 2 Material and Methods

### 2.1 Strains

Four strains of *Trichomonas vaginalis*, differently susceptible to metronidazole, were used in this study: ATCC 30001 (highly susceptible), ATCC 30326, TV2 (both normally susceptible) and ATCC 50138 (resistant). First, all experiments were performed with two strains, ATCC 30001 and TV2. For the last six months, also the other two strains were included in the study.

### 2.2 Cultivation of *Trichomonas vaginalis*

*Trichomonas vaginalis* was cultured axenically at 37°C in prewarmed modified TYM medium (CLARK & DIAMOND, 2002) containing 10% heat-inactivated horse-serum, 3% Diamond Vitamin Tween 80 Solution (40x), and 500 U/ml penicillin in 42 ml culture flasks under microaerobic conditions (filled up to 40 ml). Medium was changed every 2–3 days, depending on growth of the trichomonads.

For standard incubation, 100 µl were transferred into 40 ml medium and incubated again for 2–3 days at 37°C. When it was necessary to obtain accrued cells within a shorter time, the culture was discarded and filled up again with fresh medium to 40 ml. For growth reduction and inhibition of reproduction, the cultures were incubated at 30°C or at room temperature. When growth was poor or cells were impaired/inhibited after thawing, respectively, medium was discarded and filled up with fresh medium. When the cells have recreated a few days later, the normal medium changing procedure was applied as described above. TYM medium was made freshly once a

week and stored in the fridge at 4°C. Before use, it was prewarmed at 37°C in a waterbath.

For cryopreservation, cells were centrifugated at 700 g, afterwards the pellets were suspended in 1.8 ml Cell Freezing Medium-DMSO, pipetted into Cryopreservation Tubes and stored at -85°C. For storage longer than two weeks, the tubes were transferred into a Liquid Nitrogen Deep Freezer.

For a side experiment dealing with the influence of temperature on the susceptibility to pentamycin (see 3.1), the cultures were split and incubated at 20°C, 26°C and 37°C after treating with 1, 2, and 3 µg/ml pentamycin, respectively.

Additionally, 0.025% ammonium ferric (III) citrate was added to TYM medium and when it was obvious that the iron-dependent enzyme PFOR was depleted due to lack of iron, as described in 3.5.4 and 4.3.3. To incubate the parasites with this supplement, 2.5 ml of a stock solution (10 mg/ml) were added to 1 litre of TYM medium.

### **2.3 Preparation of drugs**

For each of the applied drugs (pentamycin, amphotericin B, metronidazole) a stock solution of 10 mg/ml, dissolved in DMSO, was prepared. DMSO is the solvent of choice because it alleviates the penetration of the drug through the cell membrane as well as it has excellent solvent properties. In small dose rates it may stimulate the cell growth, however, in amounts over 10% it has an inhibiting and toxic effect on cells (JACOB, 1985). Working solutions were made by diluting the stock solution 1:10 in TYM medium.

## 2.4 Treatment of trichomonads

Pentamycin in DMSO as well as the other solutions of drugs were pipetted directly into the liquid cell culture. The flask was inverted slightly for several times or shaken mildly on the rocker to allow a constant reallocation of the drug solvent. It was worked with various doses, depending on the experiment and the application.

For the blank test, 1 µg/ml of pentamycin was added to a cell culture, fully adapted with trichomonads (see 3.1, fig. 4). For the experiments dealing with the correlations between cell density and applied doses, various concentrations (0.5, 1, 2, 3, 5, 10, 20 and 50 µg/ml) were added and incubated for 1h (see 3.2, fig. 10).

To prove the obviously directly related correlation of various doses of pentamycin to the total cell number, an assay using an empiric scale was performed. This scale was consulted to evaluate the vitality of the cells, whereby “5” means optimal vitality, “4” means vital cells with impaired motility, “3” means cells that are impaired in their vitality and motility moderately, “2” means cells that are impaired severely including lysed cells, “1” means only a few living cells are remaining and “0” means minimal vitality or EC<sub>100</sub>. Four cell densities were used ( $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  cells/ml), treated with pentamycin in various doses, and observed under the microscope for their vitality in periodic time intervals.

The first assay was performed with one  $10^6$  cells/ml, treated with 0.5, 1, 1.5, 2, and 2.5 µg/ml of pentamycin (Fig. 11). Then, a lower cell number ( $10^5$  cells/ml) and another strain were used (Fig. 12). The following assays were performed with two strains, four cell densities ( $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  cells/ml) and three concentrations of pentamycin (1, 2.5, and 5 µg/ml). The cultures were inspected after five minutes,



subsequently in an interval of 15 minutes and after one hour in an interval of 30 minutes (Fig. 13–15). Preparations for the microtiter assays were performed as described in 2.10.

## **2.5 Staining with Giemsa stain**

A slide was coated with a drop of cell culture and dried for one hour at 37°C. Giemsa azur-eosin-methylenblue solution of 1:9 dilution was prepared after Weise (pH 7.2). Briefly, 5 ml Giemsa and 45 ml phosphate buffer solution were mixed, the slides were fixed in methanol for two minutes, dried and stained in the solution for 30 minutes. After staining, the slides were dried and photographed under a phase-contrast microscope.

## **2.6 Staining with Trypan Blue stain**

100 µl of Trypan blue solution (0.4%) were mixed with 100 µl of cell suspension and incubated for approximately 5 minutes. The solution was observed under a microscope in a Fuchs-Rosenthal hemocytometer. Blue cells were assigned as dead, unstained cells were assigned as alive or inhibited when immotile.

## **2.7 Counting cells with the Fuchs-Rosenthal Hemacytometer**

This counting chamber has 4x4 large squares with a side length of each 1 mm. Each large square has 16 small squares which are the ones to count, so there are a total of 256 countable squares. 4 large squares were determined by counting the cell

number of 4 small squares in a diagonal way and multiply it with four which makes the cell number of 3  $\mu$ l. To get the cell number of 1 ml this value had to be multiplied with 333.3.

For determination of the inhibitory concentration of pentamycin, all immotile cells were counted and correlated to the total cell number. For determination of the effective concentration, only the dead – blue stained – cells were counted and correlated to the total cell number.

## 2.8 1D SDS-PAGE

The 1D SDS-PAGE (LAEMMLI, 1970) is a method to separate previously denatured proteins by their molecular mass and to make them visible by protein staining methods, mostly by Coomassie or silver staining. In this study, this method was used to compare protein profiles between pentamycin treated cultures of *T. vaginalis* and untreated cultures, and was performed in 5% stacking gels and 12% resolving gels (SAMBROOK et al., 2001) with the Bio-Rad system.

Cell cultures were treated with various doses of pentamycin and incubated for two hours. The time of incubation was set to one hour. For the first experiments, (see 3.4.1, fig. 28–33) low doses of 0.5 and 1  $\mu$ g/ml were chosen because the trichomonads should show discomfort in their morphology and motility, but should also be still vital. Higher doses like 2, 7, and 10  $\mu$ g/ml were chosen to show the severity of the effect on protein diminishment (see 3.4.1, fig. 34–36). In this case, a majority of the cells were dead at the time of analysis.

### 2.8.1 Sample preparation

Cells were grown to a cell density of around  $1 \times 10^6$  cells/ml, so that the culture medium was adnated densely with vital trichomonads. The cells were harvested by centrifugation at 700 g for 5 minutes. Pellets were washed in 1xPBS by the same centrifugation procedure, followed by another washing step with dH<sub>2</sub>O to remove residual serum proteins from the culture medium and salts from PBS. The supernatant was discarded and 400 µl of dH<sub>2</sub>O were added to the pellets, mixed and transferred into a 1.5 ml Eppendorf tube. The rest of the volume was filled up with 12.5% TCA in 90% acetone (-20°C) to precipitate the proteins. The time of precipitation was set from 1h to over night at -20°C.

Precipitated proteins were centrifuged at 13.300 g for 1 minute at 4°C, and washed twice with 90% ice-cold acetone at 13.300 g and 4°C for 5 minutes. These washing steps were essential to remove disturbing TCA. After washing, the pellets were air dried for at least 10 minutes until the residual acetone was gone and resuspended in an appropriate amount of lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 1% BioLyte; stored at -80°C). Small pellets were suspended in 100 to 300 µl, large pellets in 500 to 1000 µl, intermediate pellets in a range from 150 to 300 µl. Resolubilization was performed on a mild shaker for at least 2 hours or over night. Generally, temperature must not rise above 30°C, otherwise urea will lead to carbamylation of proteins. The neutral chaotropic agent urea denatures proteins, avoids hydrophobic interactions and provides their solubility, which can be increased by thiourea. The non-ionic detergens CHAPS avoids formation of aggregates and dithiothreitol reduces disulfide bonds of proteins. After resolubilization, a step of at least 1h of centrifugation at 13.300 g was performed to remove residual nucleic acids or debris.

Before loading onto the gel, 10  $\mu$ l of SDS-sample-buffer (20% (v/v) glycerol, 4% (w/v) SDS, 125 mM Tris-HCl pH 6.8, 0.02% (w/v) bromophenol blue, 2%  $\beta$ -mercaptoethanol) were added to 10  $\mu$ l of sample, which leads to denaturation of proteins after 5 minutes at 95°C. SDS degrades the native protein into its secondary, or even, primary structure,  $\beta$ -mercaptoethanol breaks up the disulfide bonds, whereas bromophenol blue works as a marker for the following electrophoresis (LEITSCH et al, 2005).

### 2.8.2 Gel preparation

Complementary glass plates of 1 mm and 0.75 mm thickness, respectively, were assembled into a clamp. The gels were prepared in 50 ml centrifugation tubes. 10 ml of a 12% resolving gel were made as following: 3.3 ml dH<sub>2</sub>O, 2.5 ml 4x lower Tris buffer pH 8.8 (containing 20% (w/v) SDS), 4.2 ml acrylamide (30%, w/v)/bisacrylamide (0.8%, w/v), 50  $\mu$ l 10% (w/v) APS, 5  $\mu$ l TEMED.

10% APS (w/v) was prepared freshly prior to each set up and used for maximum a week. After adding APS and TEMED, the liquid was mixed by inverting, and poured into the gap between the two glass plates rapidly due to the properties of APS as initiator of the polymerization of acrylamide to polyacrylamide, and TEMED as catalyzator of the reaction. When the plates were filled about 4/5 with the resolving gel, a layer of isopropanol was pipetted onto the surface to get it smooth, according to the process of polymerisation. The time of polymerisation was around from 15 to 30 minutes.

5 ml of a 5% stacking gel were made as following: 3 ml dH<sub>2</sub>O, 1.25 ml upper Tris buffer pH 6.8 (containing 20% (w/v) SDS), 800 µl acrylamide (30%, w/v)/bisacrylamide (0.8%, w/v), 40 µl 10% (w/v) APS, 5 µl TEMED.

### **2.8.3 Electrophoresis**

After polymerization of the resolving gel, isopropanol was poured off and the stacking gel was pipetted into the residual space above the resolving gel. Appropriate combs of 1 mm thickness were put into the stacking gel and removed when the gel has polymerized. Subsequently, the glass plates with the gel were put into the special clamp holder of the BioRad Mini-Protean system, fixed into the tray and filled up with 1x running buffer (25 mM Tris-HCl, 200 mM glycine, 0.1 % SDS (w/v)). 10 µl of the samples and 10 µl of Prestained Protein Marker MW (PeqLab) were loaded into each slot and electrophoresis was started. The proteins migrated at low voltage and amperage (22 mA, 80 V) for the first 20 minutes through the macropore stacking gel to focus them on a line, then the voltage was turned up to 150 V for the rest of the time. The migration of the proteins through the resolving gel lasted from 2 to 3 hours.

### **2.8.4 Staining with Coomassie stain**

After finishing, the gels were stained with Coomassie Brilliant Blue R-250 solution (45% dH<sub>2</sub>O, 10% acetic acid (v/v), 45% methanol (v/v), 0.8% Coomassie Brilliant Blue R-250 (w/v)) at least for 1 hour and destained with destaining solution (70% dH<sub>2</sub>O, 10% acetic acid (v/v), 20% methanol (v/v)) as long as necessary. After destaining, the gels were laid into water to remove residual methanol. When finished, the gels were shrinkwrapped and photographed.

### **2.8.5 Protein diminishment assay**

This was applied to look for proteins that had migrated from inside the cell into the extracellular medium and to compare the protein profiles of cells and supernatant, respectively. The cultures were centrifuged at 700 g for harvesting, subsequently the cell pellets were resuspended in 1 ml of PBS and incubated with 0, 2, 5, and 10 µg/ml pentamycin, respectively, for 30 minutes under mild shaking. Subsequently, the suspension was centrifuged for 30 seconds at 3.000 g, the supernatant was taken off until 200 µl from the bottom and centrifugated again. The proteins of the supernatant as well as the cell pellet fraction were precipitated and analysed by 1D SDS-PAGE as described previously. The experiment was repeated two times with each strain.

## **2.9 2D SDS-PAGE**

The two-dimensional SDS polyacrylamide gelelectrophoresis was also performed with the BioRad System after GÖRG et al. (2007), with modifications. This technique allows the visualisation of proteins after their separation by their isoelectric point in the first dimension (“horizontal separation”) and by their molecular mass in the second dimension (“vertical separation”). Mainly, it was performed to compare the protein profiles of wildtypes and those being in long-term treatment with pentamycin, assuming to detect differences that might concern metabolic pathways correlated with resistance establishment. Wildtypes and partial resistant trichomonads were analysed for a time span of 4 months, covering different time stages of long-term treatment (see 3.5.4, fig. 50–54).

Because the results led to the assumption that addition of pentamycin shortly before may affect the protein profile by its mode of action (see 3.4), which might be a false positive conclusion for the results in this study, a final experiment with 4 approaches was performed. It was attached importance to eliminate the proteolysis factor by not treating the R-strains (term used for adapted strains) up from 2 days before harvesting. The study design was defined as comparing untreated cells with one-time treated cells (2.5 µg/ml, 2 hours incubation) as well as comparing R-strains, untreated before harvesting, with R-strains, immediately treated before harvesting (2.5 µg/ml, 2 hours incubation) (Fig. 55). So it should have been possible to monitor and distinguish proteolysis effects from such ones that have developed from a possible resistance mechanism.

Due to the varying running times of the gels, caused by various technical problems, it was not possible to analyse the 2D SDS-PAGE protein spots via the Melanie software package. Instead, the spots were compared and identified manually with the support of David Leitsch.

### **2.9.1 Sample preparation**

Cell harvesting, protein precipitation and resolubilization were identical as described in 2.8.1. A necessary step before rehydration was measuring the protein concentration. For silver staining, 50–100 µg protein was required, for Coomassie staining 500–1000 µg. Protein concentrations were measured by Bradford-Assay: 800 µl dH<sub>2</sub>O + 3 µl sample + 200 µl Bradford solution were pipetted into a cuvette (for the blank control 3 µl of lysis buffer instead of sample). Extinction was measured photometric at a wavelength of 595 nm and divided by the calibration factor 0.14,

which results in the concentration per volume. Optimal concentration was between 5–10 mg/ml protein. The respective sample volume was filled up to 400 µl with lysis buffer and spiked with a tiny drop of bromophenol blue to make it visible as pale blue.

### **2.9.2 First dimension – Isoelectric focussing**

The first dimension was performed with the BioRad Protean IEF cell and 17 mm strips with immobilized pH gradient (IPG), which contain a series of chemically well defined acrylamide derivates (BJELLQVIST et al., 1982). In the current study, buffers with pK values between pK 3 and 10 were used. Prior to IEF, the IPG strips had to be rehydrated to their original thickness of 0.5 mm with a rehydration buffer that is identical to the lysis buffer. During the IEF, differently charged proteins migrate through the electrical field until they reach their specific isoelectrical point (IEP) where they become neutral and stop to migrate.

First, a sample volume of 400 µl was loaded into each slot, subsequently the IPG strips were added with the gel side downwards, and coated with 2.5 ml of mineral oil to avoid volatilization.

The programme was set as following and run overnight: Rehydration 50 mV for 12 hours, 150 V for 1h (rapid slope), 300 V for 1h (rapid slope), 2000 V for 1h (linear slope), 5000 V for 2h (linear slope) and 10.000 V for 6h (rapid slope). The last step was shortened to 3h when working with low sample loads (e.g. for subsequent silver staining). When finished, voltage dropped down to 500 V.



### 2.9.3 Gel preparation

For the following steps, resolution buffer and equilibration buffer were prepared. The resolution buffer contained 1.5 M Tris-Base, 0.4% SDS and was filled up with dH<sub>2</sub>O to 500 ml and adjusted to pH 8.6 with 20% HCl. The equilibration buffer contained 6M urea, 30% glycerol, 2% SDS, 16.7 ml resolution buffer and was filled up with dH<sub>2</sub>O to 500 ml. Two 12.5% resolving gels were prepared in 100 ml as following: 30 ml dH<sub>2</sub>O, 5 ml glycerol, 25 ml resolution buffer, 40 ml acrylamide (30%,w/v)/bisacrylamide (0.8%,w/v), 300 µl 10% (w/v) APS and 40 µl TEMED. The gel was casted between the glass plates as described above. The time of polymerisation was about 2h.

### 2.9.4 Equilibration

Equilibration consisted of two steps: Reduction of cysteine groups with dithiothreitol (DTT) and alkylation of reduced cysteines with iodacetamide (IAA). This is necessary to obtain clear spots. The IPG strips were placed into an equilibration tray, washed with dH<sub>2</sub>O, and incubated in 10 ml 1% DTT equilibration buffer for 15 min and in 10 ml 4% IAA equilibration buffer for 15 min under mild shaking.

### 2.9.5 Second dimension – Electrophoresis

First, a 0.8% agarose solution in resolution buffer was prepared, boiled in the microwave, spiked with a drop of bromophenol blue and cooled down in a waterbath to 37°C. The IPG strips were washed with dH<sub>2</sub>O and put onto the top of the resolving gel. Then, a layer of cooled down agarose was added and after a few minutes of polymerization, the two gel plates were put into the 2D SDS-PAGE tank, filled with 2l of running buffer. The tank was connected with the power supply and put into the

fridge where it ran overnight with an initial amperage of 15-20 mA. The voltage increased by progressing running time, which was around 15 hours.

### **2.9.6 Staining with Coomassie stain**

This step was identical to the one described in 2.8.4.

### **2.9.7 Staining with Silver stain**

This step followed the protocol after BLUM et al. (1987): Briefly, gels were fixed for 1h in 40% (v/v) ethanol and 10% (v/v) acetic acid. Acetic acid was removed with 30% ethanol for 2x20 minutes, ethanol was removed with dH<sub>2</sub>O for 1x30 minutes or 2x5 minutes. A solution of 0.02% (w/w) sodium-thiosulfate was added for 20 seconds of sensitizing and removed with dH<sub>2</sub>O for 3x20 seconds. Subsequently, the gels were incubated with a silver nitrate solution (2 g of silver nitrate + 200 µl of 37% formaldehyde in one litre) for ten minutes before washing with dH<sub>2</sub>O for 3x20 seconds. The developing solution contained sodium carbonate (30 g of sodium carbonate + 500 µl of 37% formaldehyde in one litre) and was carried out as long as necessary for the reduction of silver. Finally, a stop solution with 0.5% glycine was added and incubated for ten minutes. When finished, the gels were shrinkwrapped, scanned, and photographed.

## 2.10 Microtiter assays

To evaluate the doses of pentamycin, necessary to kill or to inhibit a certain percentage of cells, microtiter assays were performed in 24-well plates with 2.5 ml volume per well. First, cell cultures at a countable density of approximately  $1 \times 10^6$  cells/ml were pipetted into the wells and incubated at 37°C for about 15 minutes to give them recovery from previous pipetting stress.

Subsequently, test series with eight dilutions of 94% (Ch.-B. C-017084-PRS-03) pentamycin (0.5, 1, 2, 3, 4, 5, 10, and 15 µg/ml) were run including two positive and two negative controls. EC and IC data were used as an effective concentration of 20 µg/ml pentamycin (28%) (Ch.-B. 020) and as a less effective concentration of 50 µg/ml for metronidazole, referring to “Comparative studies on the activity and mode of action of pentamycin on *Trichomonas vaginalis*, Report G40/07”.

These concentrations were chosen to have an visible effect on the cells without killing them entirely. As negative controls, cells in culture medium without any addition (blank sample) and with the highest respective concentration of DMSO used in the drug supplements, were applied.

For the experiments on cross resistance, the following dilutions of amphotericin B were used: 10, 50, 100, 150, 200, 300, 400, and 500 µg/ml. The wells were counted after one, three and six hours of treatment with a phase contrast microscope and a cell counter. Positive and negative controls were applied as described above.

All four strains were used and each strain was counted in parallel triplicates. IC<sub>50/90</sub>s (inhibitory concentrations with 50% and 90%, respectively, of the cells immotile and inhibited) and EC<sub>50/90</sub>s (effective concentrations with 50% and 90%, respectively, of

the cells dead) were calculated by SPSS with linear regression/probit analysis. The results were expressed as diagrams and arithmetic means with standard deviations. Furthermore, the standard error of arithmetic means as well as the level of significance (=error probability; significance  $p \leq 5\%$ , high significant  $p \leq 1\%$ , highest significant  $p \leq 0.1\%$ ) was calculated by SPSS analysis of variance with repeated measurements.

In a side experiment, the influence of the temperature on susceptibility was investigated. For this purpose, two strains (ATCC 30236 and ATCC 50138) were incubated with 1, 2, 3, and 4  $\mu\text{g/ml}$  of pentamycin at temperatures of 20°C, 26°C, and 37°C for 1h. Subsequently, the amount of vital cells was counted.

## **2.11 Long-term treatment**

In the first four months from June to September 2009, the strains were treated every 2–3 days with rising concentrations of 0.5  $\mu\text{g/ml}$  pentamycin dissolved in DMSO. Trichomonads responded to the treatment with 100% lethality at various stages. A few cells seemed not to be affected by the drug, as they were inside a cell cluster, and led to a proliferated cell culture again. When it was obvious that this method results in dead cells at higher doses – probably due to the relative large intervals of incubation time and concentration – the approach was changed: At least once, but at maximum three times a day, a sublethal amount of pentamycin between 0.25  $\mu\text{g/ml}$  and 3.0  $\mu\text{g/ml}$  was added to the cultures, depending on cell density and vitality of trichomonads. Consecutively, the dose was increased by 0.1  $\mu\text{g/ml}$ , respectively. At good vitality, the applied dose was increased by 0.2–0.3  $\mu\text{g/ml}$ .

After 4 and 5 months of continuous, daily addition of sublethal doses, respectively, strain ATCC 50138 (metronidazole-resistant) was applied to check the susceptibility to pentamycin in comparison to wildtypes. For this purpose, microtiter assays with Trypan blue stain, as described in 2.5 and 2.10, were performed (see results 3.5.2).

Cross resistance to amphotericin B was also investigated, due to the similar action mechanism of these polyenes (ANDREOLI & MONAHAN, 1968; HSUCHEN & FEINGOLD, 1973). For this purpose, microtiter assays as described in 2.4 and 2.10, with seven concentrations of amphotericin B (10, 50, 100, 150, 200, 300, and 400 µg/ml) were performed. Two strains (ATCC 30236, TV2) were counted after four months of resistance treatment (see results 3.5.3).

### **2.12 Lactate dehydrogenase enzyme activity assay**

Lactate dehydrogenase (LDH) reduces pyruvate to lactate and oxidizes  $\text{NAD}^+$  to  $\text{NADH}/\text{H}^+$ . By measuring the turnover rate of  $\text{NAD}^+$  in cells, the quantity of LDH can be determined .

The following assay (after LEITSCH et. al, 2010) was performed subsequently to the last 2D SDS-PAGE experiment, to follow the idea of an obviously altered expression of LDH in R-strains: After harvesting and centrifuging at 700 g for 5 min, the cells were washed twice with PBS and  $\text{dH}_2\text{O}$ . 800 µl SM-buffer (250 mM sucrose + 10 mM MOPS (3-N-morpholino-propanesulfonic acid)) was added to the pellets and mixed. The suspension was transferred into a slotter, pounded on ice for 30 times and poured into an Eppendorf tube. Then it was centrifuged at 20.000 g for ten minutes at 10°C. The supernatant was taken off and was measured for protein concentration

after Bradford as described in 2.9.1. The enzyme activity assay was prepared with 100 mM NADH-solution and reaction buffer (100 mM Tris-HCl, pH 7.5; 0.2 mM NADH; 1mM EDTA; 2mM pyruvate) and started with 5 µg protein equivalent, mixed with 1 ml of reaction buffer. The measurement was performed at 340 nm and 25°C by noticing the photometric value every 30 seconds which corresponds with the turnover rate of the substrate.

### **2.13 Cell permeability assay**

This assay was performed according to the study of PALACIOS AND SERRANO (1978) that demonstrated the effect of polyene antibiotics on the proton permeability of the cell. In this case, a changing pH of the extracellular environment after administration of low, sublethal drug doses may have its reason in the formation of tiny membrane pores that let pass through small ions like  $H^+$  and  $K^+$ . The described methods were simplified by disclaiming the addition of 2-deoxyglucose. Measurements were performed in 12-well microtiter plates, each containing 5 ml of suspension with 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 3 µg/ml, 5 µg/ml, 10 µg/ml of pentamycin, respectively. As negative control pure cell culture in TYM medium was used. pH values were measured before the addition of pentamycin and 30 minutes, 1, 3, and 6 hours, subsequently.

## **2.14 Nile Red stain and fluorescence microscopy**

Nile Red (9-diethylamino-5H-benzo [a] phenoxazine-5-one) can be used as a fluorescent dye in order to monitor properties of the cell membrane. For this study, a protocol by KIERNAN (2008) was adapted and applied as following: A stock solution was prepared by dissolving 0.5 mg Nile Red in 1 ml of acetone. To obtain a working solution, 0.05 ml of the stock solution was added to 50 ml of a 75:25 glycerol-water mixture. A drop this solution was put on a glass slide, mixed with a drop of cell suspension, and dried at 37°C. The stained cells were observed under a fluorescence microscope with a UV filter at 340 nm.

### 3 Results

#### 3.1 Morphology of pentamycin-treated trichomonads

After treatment, cells appeared impaired, showed diminished motility and exuded severe discomfort. Some cells also appeared as rounded and/or enlarged, and were even lysed or in the process of lysis. The cells appeared more harmed with increasing time of incubation. For a preliminary test, a dose of 10 µg/ml was added, and after 10 minutes a distinctive effect with a large amount of lysed cells was observable. For the following analyses, smaller doses were used. Fig. 3–4 show the result of the consecutive treatment with addition of 1 µg/ml pentamycin incubated for 90 minutes .

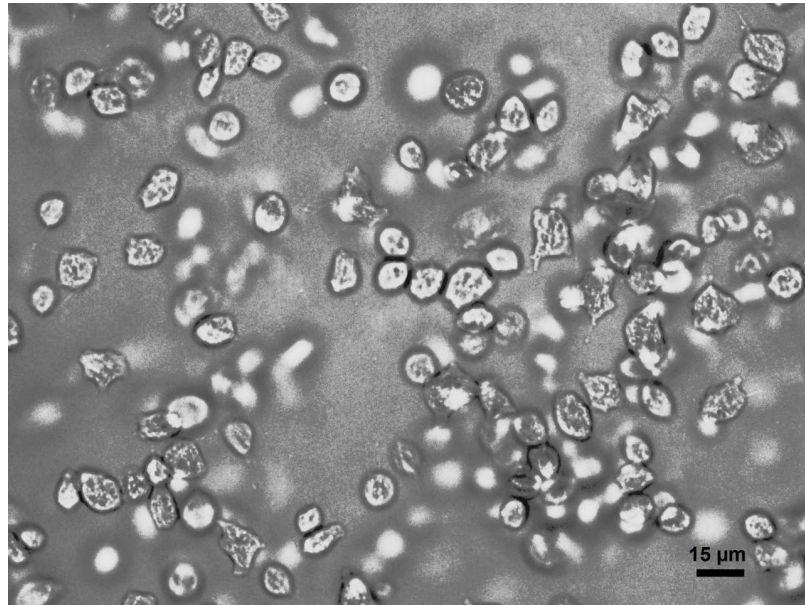
The comparison of treated and untreated cells indicated an increase of cell damage with higher doses (1, 3, 10 µg/ml), leading to lysis finally at 10 µg/ml. It was noticeable that the cells assembled in dense batches, and stained particles – probably flown out cell content – appeared in the environment of the cells. At 10 µg/ml, nearly all cells seemed to be completely lysed (Fig. 5–8, Giemsa stain).

A further observation, dealing on the susceptibility to pentamycin, was the influence of temperature. At a lower temperature of 26°C, trichomonads were less motile and vital but also seemed to be less susceptible to the drug. Especially at concentrations of 3 and 4 µg/ml, the difference was considerable between cells cultivated at 26°C and 37°C. Whereas there were 55% (3 µg/ml) and 35% of vital cells (4 µg/ml) at 26°C, there were 15% (3 µg/ml) and less than 5% (4 µg/ml) of vital cells observable at 37°C within the same incubation time (Fig. 9).



## RESULTS

This effect was reversible: When incubated at 37°C again, the trichomonads regained back their motility and also their susceptibility to pentamycin. After 3 hours, it could be observed that not only the motility has increased, but also the amount of lysed cells.

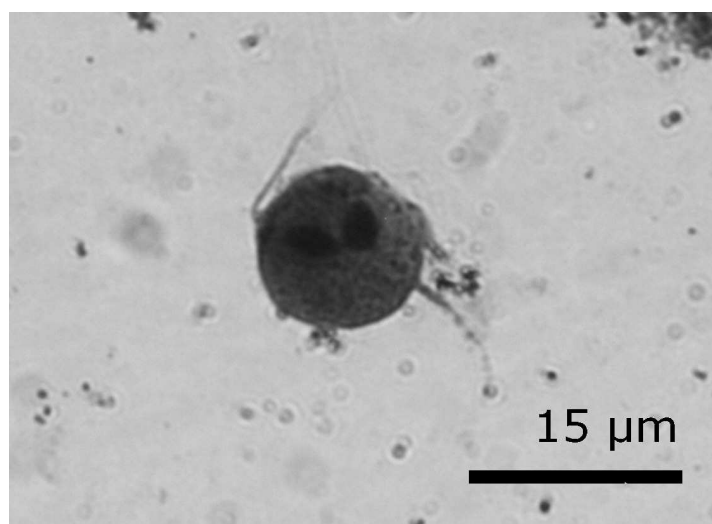


**Fig. 3:** Untreated, vital and motile trichomonads (40x magnified, ATCC 30001).

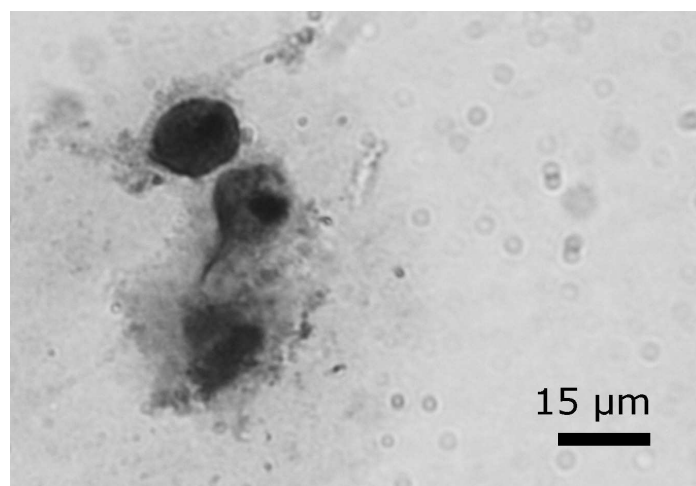


**Fig. 4:** Trichomonads, treated with 1 µg/ml, after 90 min of incubation (40x magnified).

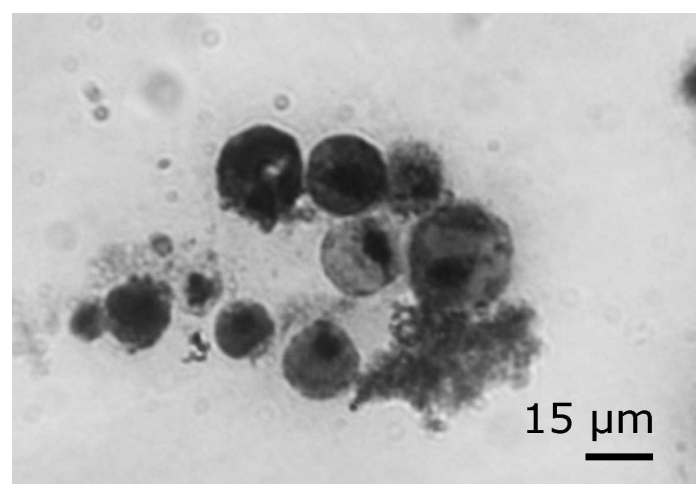
## RESULTS



**Fig. 5:** Untreated trophozoite (during mitosis, two nuclei), Giemsa stained (ATCC 30001).

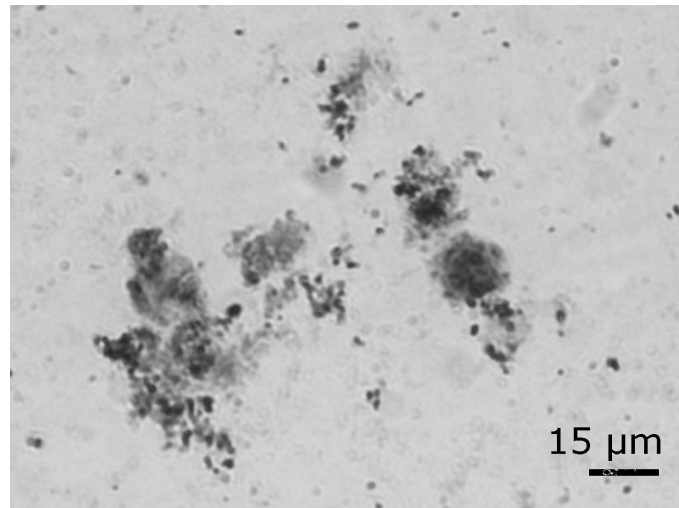


**Fig. 6:** Trophozoites treated with 1  $\mu\text{g/ml}$  pentamycin after 2h of incubation.

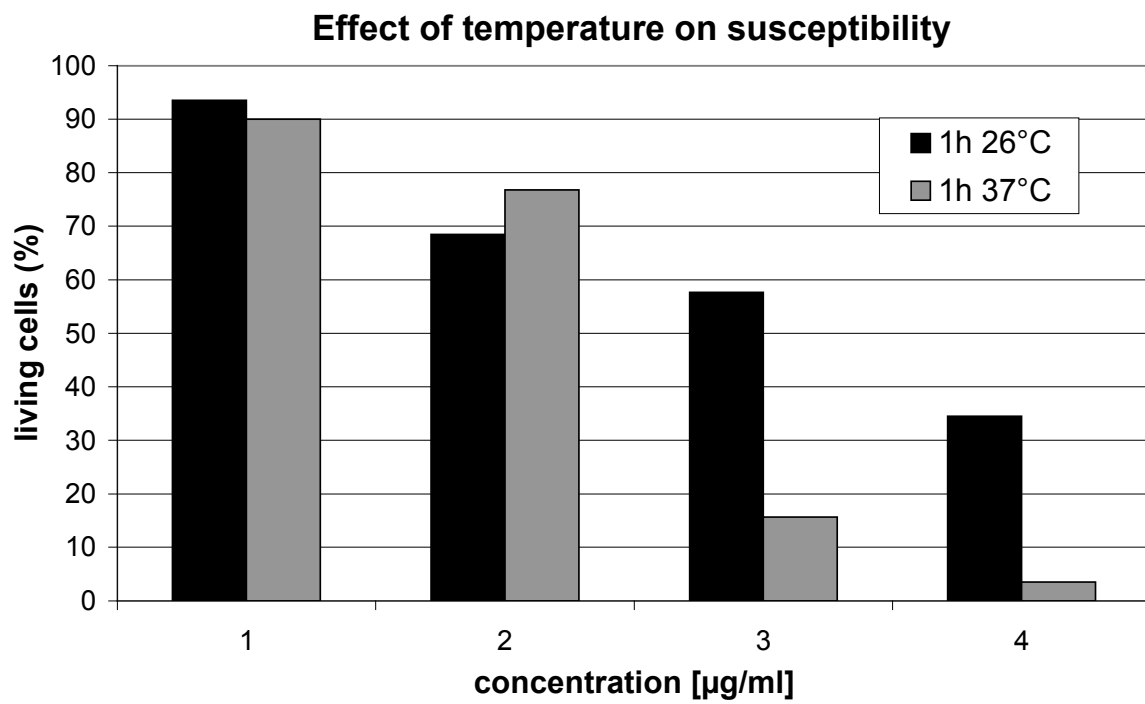


**Fig. 7:** Trophozoites treated with 3  $\mu\text{g/ml}$  pentamycin after 2h of incubation.

## RESULTS



**Fig. 8:** Trophozoites treated with 10 µg/ml pentamycin after 2h of incubation.



**Fig. 9:** ATCC 50138, cultivated at 26°C and 37°C, and incubated for 1h.

### 3.2 Correlation between cell density and dose rate

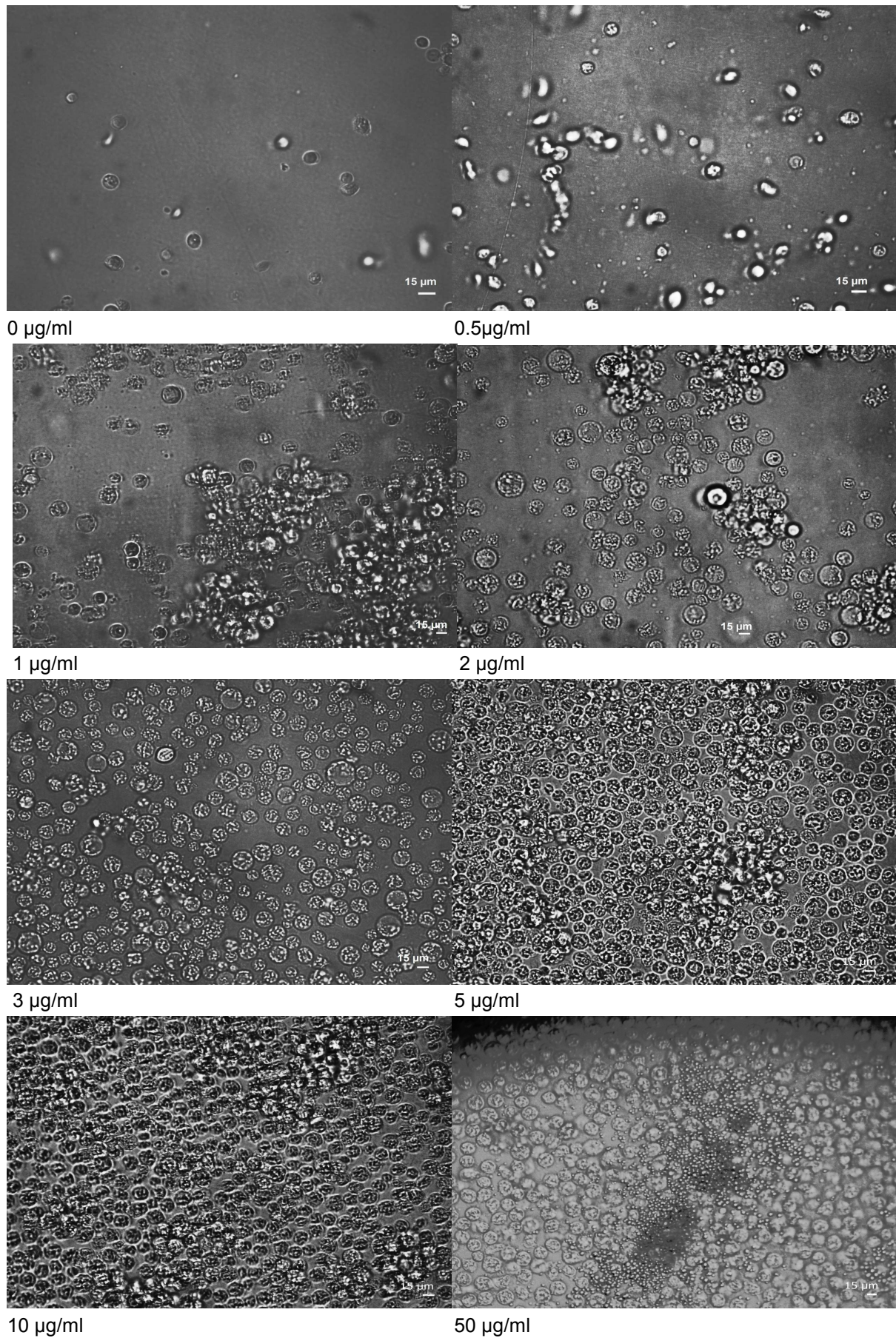
Cells treated with doses of 10 µg/ml and above were lysed in 100% of cases, whereas at lower doses a smaller amount of cells was affected. The cells that were still vital appeared impaired, round or amoeboid shaped, and with shrugging movements. 1h after treatment, nearly all cells were viable and motile at 0 and 0.5 µg/ml. From 1 to 3 µg/ml, the amount of round and lytic cells was increasing. At 5 µg/ml, only a few single cells were still motile but severely impaired. At 10 µg/ml, all cells were immotile and lysed. Interestingly, granula-like particles appeared around the lysed cells additionally, however, only after an overdose of 50 µg/ml (Fig. 10).

#### Blank test for the microtiter assays

An empiric scale was set up to mark the appearance of treated trichomonads: 5 (all cells are vital and motile), 4 (mostly vital with impaired motility of a few cells), 3 (vital but impaired with a few round forms and <50% lysed cells), 2 (severely impaired, round forms, >50% lysed cells), 1 (nearly all cells lysed with a few motile/viable cells), 0 (100% lysed cells). The estimation of the vitality after treatment with various doses, respectively, showed “4” after 0.5 µg/ml and “3” after 1 and 1.5 µg/ml. After 2 and 2.5 µg/ml it showed “1” after 10 min, and below “1” after 30 and 60 minutes (Fig. 11).

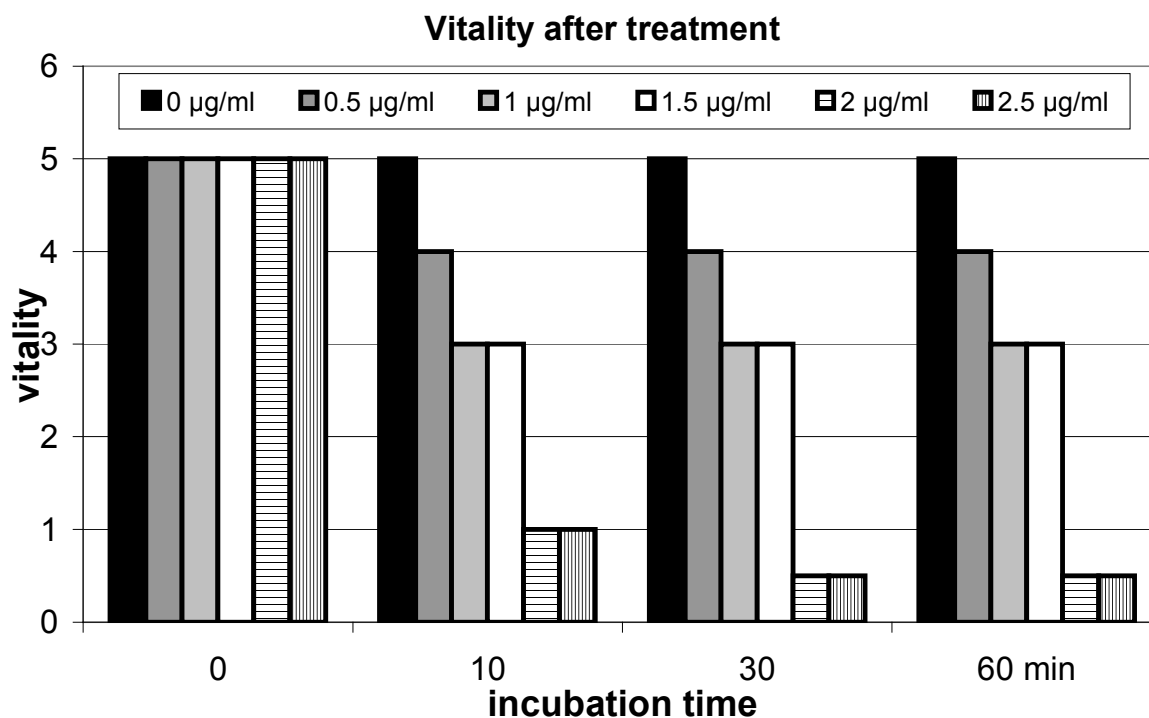


## RESULTS



**Fig. 10:** Process of cytolysis at 1h after treatment with pentamycin (94%). Note: while living cells were swimming around actively, dead cells sedimented on the bottom of the flasks.

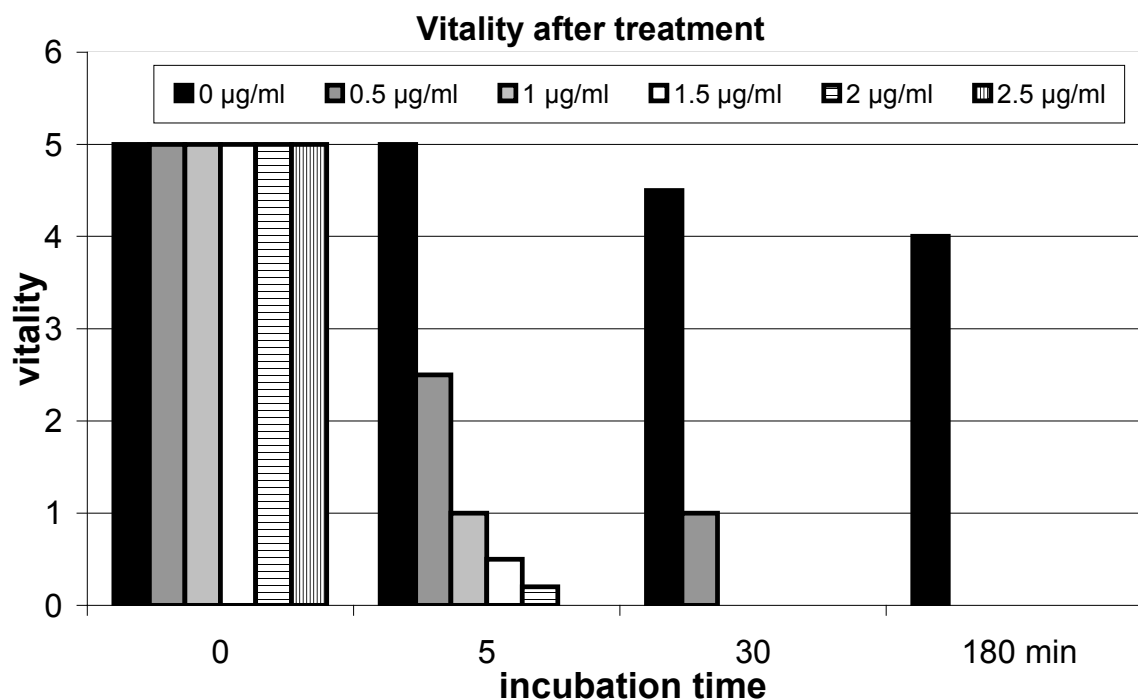
## RESULTS



**Fig. 11:** ATCC 30001 ( $10^6$  cells/ml), treated with various doses of pentamycin.

After treating  $10^5$  cells/ml, the condition of the cells was worse than previously: The estimation of the vitality showed “0” after treatment with 0.5 µg/ml and 3h of incubation. All higher doses than 0.5 µg/ml resulted in 100% dead cells after 30 minutes. Up from 1 µg/ml, it showed “1” and below after 5 min, at 0.5 µg/ml between “2” and “3” after 5 min and “1” after 30 min (Fig. 12). A stronger effect than in fig. 11 can be stated, most probably due to a smaller number of examined cells.

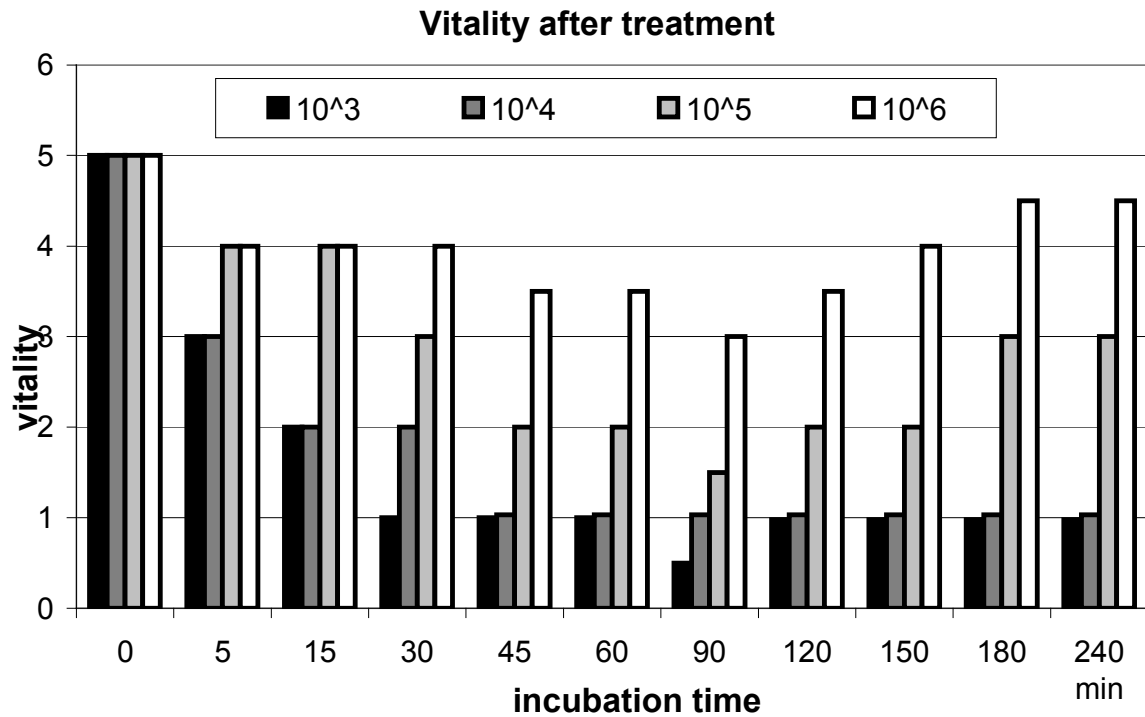
## RESULTS



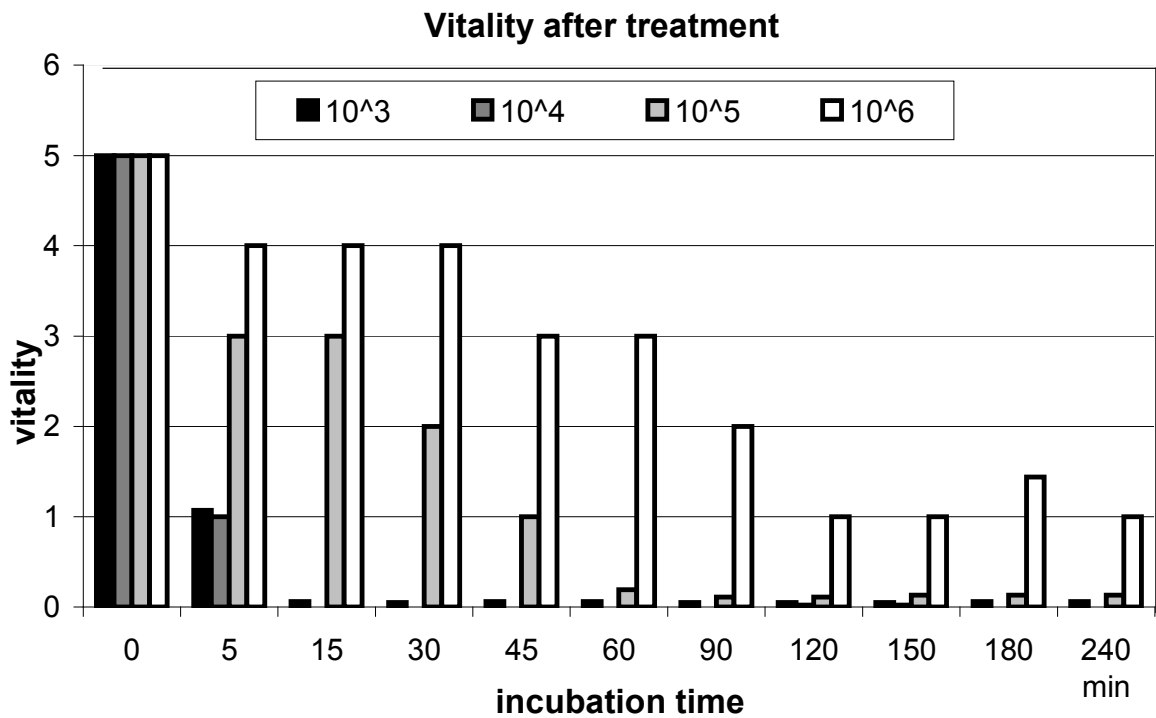
**Fig. 12:** Strain TV2 ( $10^5$  cells/ml), treated with various doses of pentamycin.

The following diagrams (Fig. 13–15) demonstrate that lower cell densities ( $10^3$  and  $10^4$  cells/ml) undergo a faster decline of their vitality than higher cell densities ( $10^5$  and  $10^6$  cells/ml) when exposed to pentamycin. The assay was performed with ATCC 30001 (highly metronidazole-sensitive) and TV2 (metronidazole-sensitive) (data of TV2 not shown), and both strains showed similar reactions to the treatment. They were severely affected and inhibited at 2.5 µg/ml after 15 min (Fig. 14), and at 5 µg/ml after 5 min (Fig. 15). A concentration of 1 µg/ml was obviously too low to be effective and led to more (low cell densities) or less (high cell densities) damage. The longer the incubation time and the higher the applied dose, the stronger the harming effect was. Interestingly, it was also observable that 90 minutes after treatment with 1 µg/ml, cells at a density of  $10^6$  cells/ml seemed to regenerate (Fig. 13). Altogether, there is a positive correlation between cell density and the dose rate of pentamycin.

## RESULTS



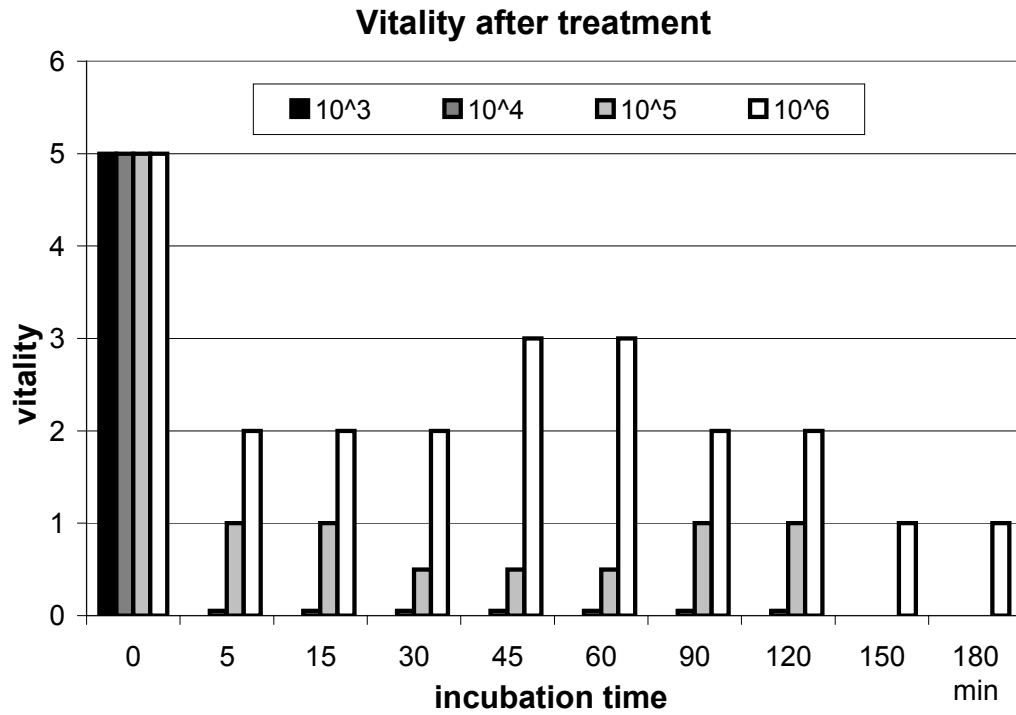
**Fig. 13:**  $10^3$  to  $10^6$  cells/ml (ATCC 30001) were treated with 1  $\mu$ g/ml and observed for their vitality in periodic time intervals.



**Fig. 14:**  $10^3$  to  $10^6$  cells/ml (ATCC 30001) were treated with 2.5  $\mu$ g/ml and observed for their vitality in periodic time intervals.



## RESULTS

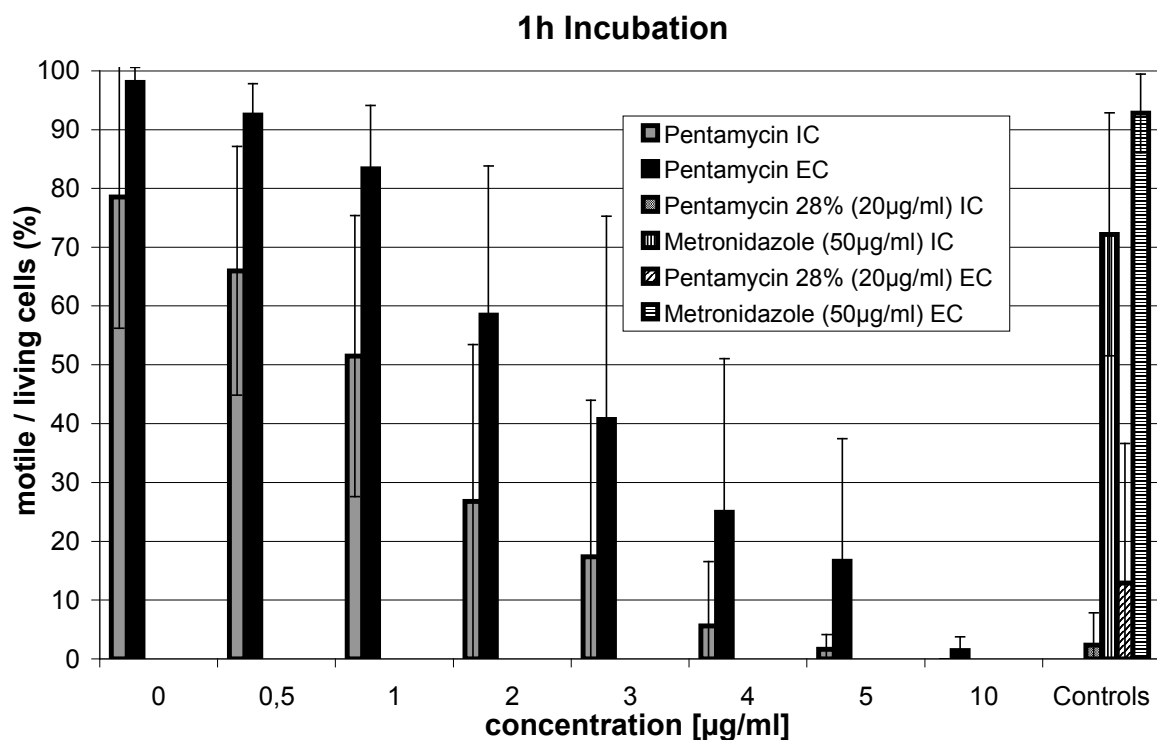


**Fig. 15:**  $10^3$  to  $10^6$  cells/ml (ATCC 30001) were treated with 15  $\mu$ g/ml and observed for their vitality in periodic time intervals.

### 3.3 Effective and inhibitory concentrations ( $EC_{50/90}$ , $IC_{50/90}$ ) of pentamycin on four *T. vaginalis* strains

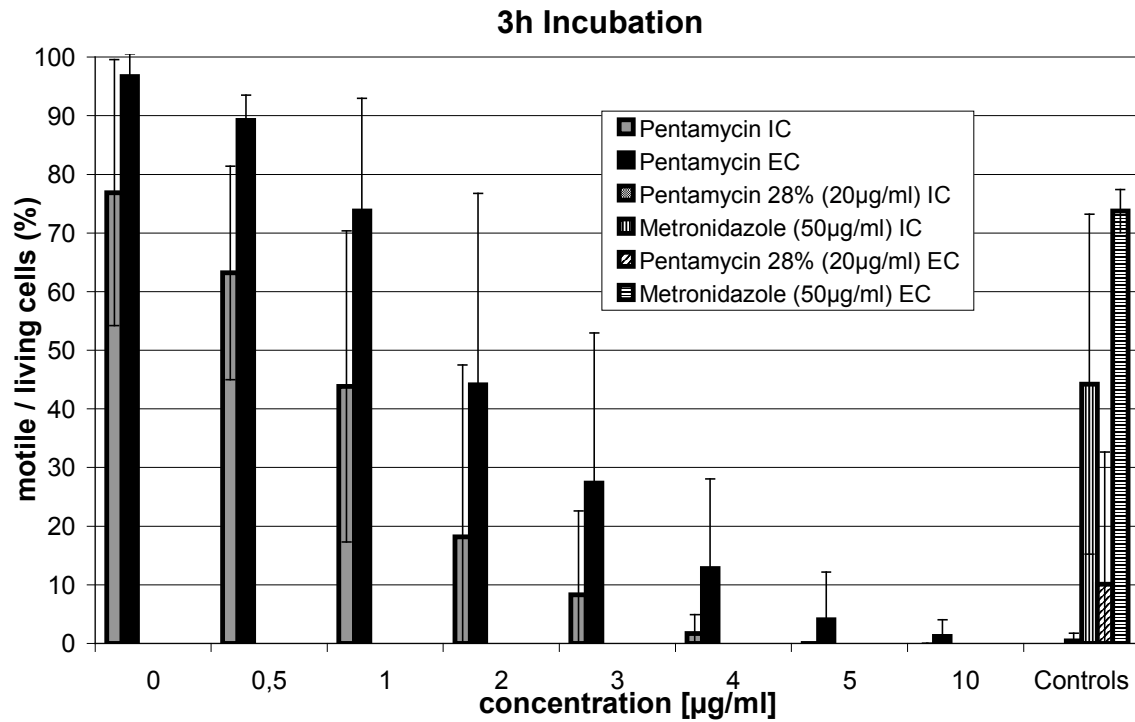
#### ATCC 30001 (highly metronidazole-sensitive strain)

Fig. 16–18 display the relative motility and vitality of this strain after treating with various doses of pentamycin and incubated for 1, 3, and 6 hours, respectively. It could be shown that the amount of motile and vital cells is decreasing with higher doses of pentamycin as well as with longer incubation time.

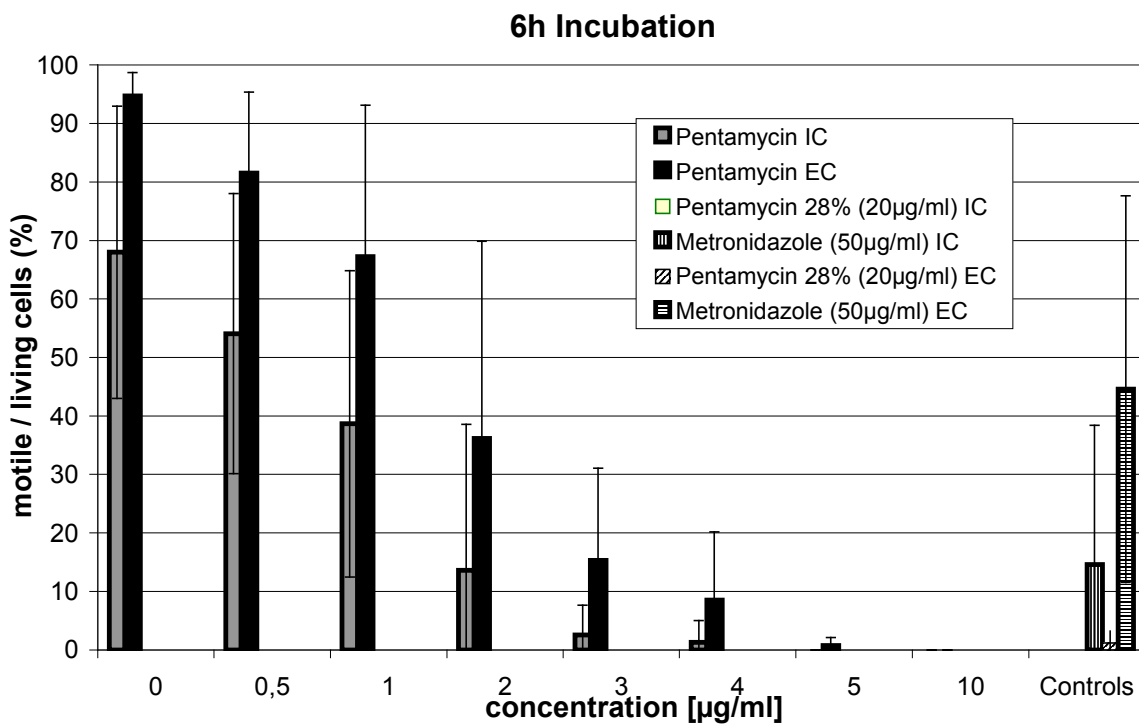


**Fig. 16:** Motility and vitality of strain ATCC 30001 after 1h of treatment with pentamycin.

## RESULTS



**Fig. 17:** Motility and vitality of strain ATCC 30001 after 3h of treatment with pentamycin.



**Fig. 18:** Motility and vitality of strain ATCC 30001 after 6h of treatment with pentamycin.

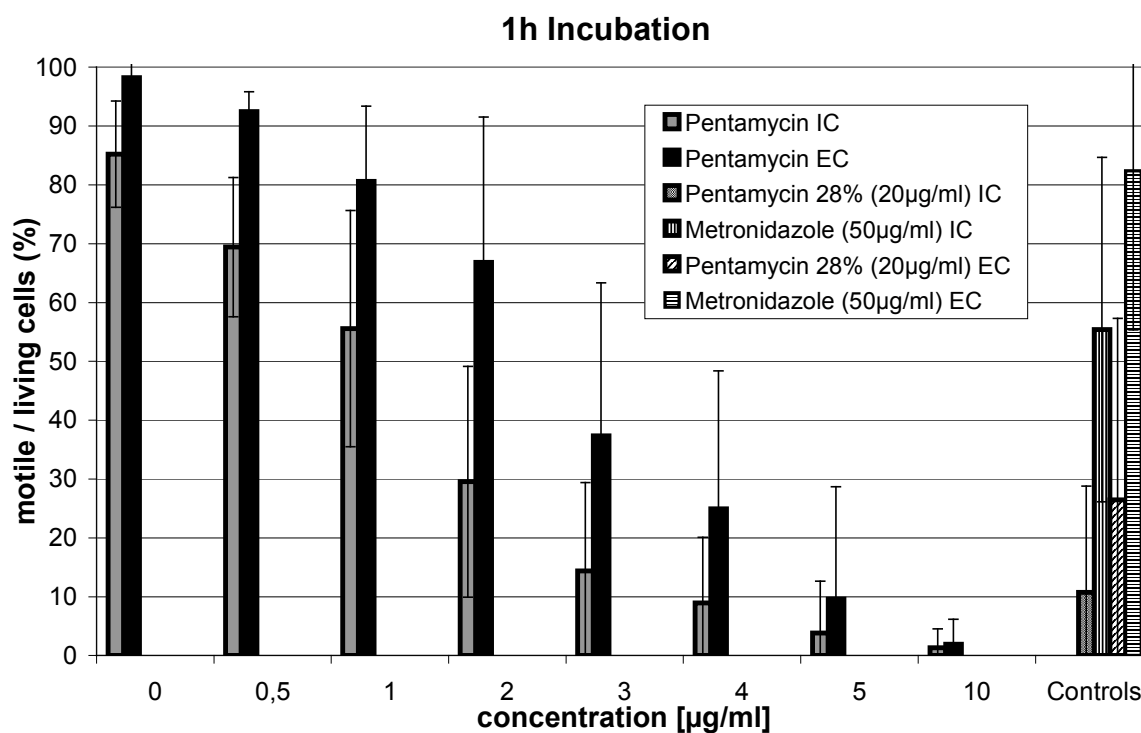
## RESULTS

**Tab 3:** EC and IC values ( $\mu\text{g/ml}$ ) of ATCC 30001 after 1h, 3h, and 6h.

ATCC 30001	1h	3h	6h
IC <sub>50</sub>	0,922	0,774	0,639
IC <sub>90</sub>	3,369	2,450	2,010
EC <sub>50</sub>	2,210	1,620	1,280
EC <sub>90</sub>	6,514	4,616	3,621

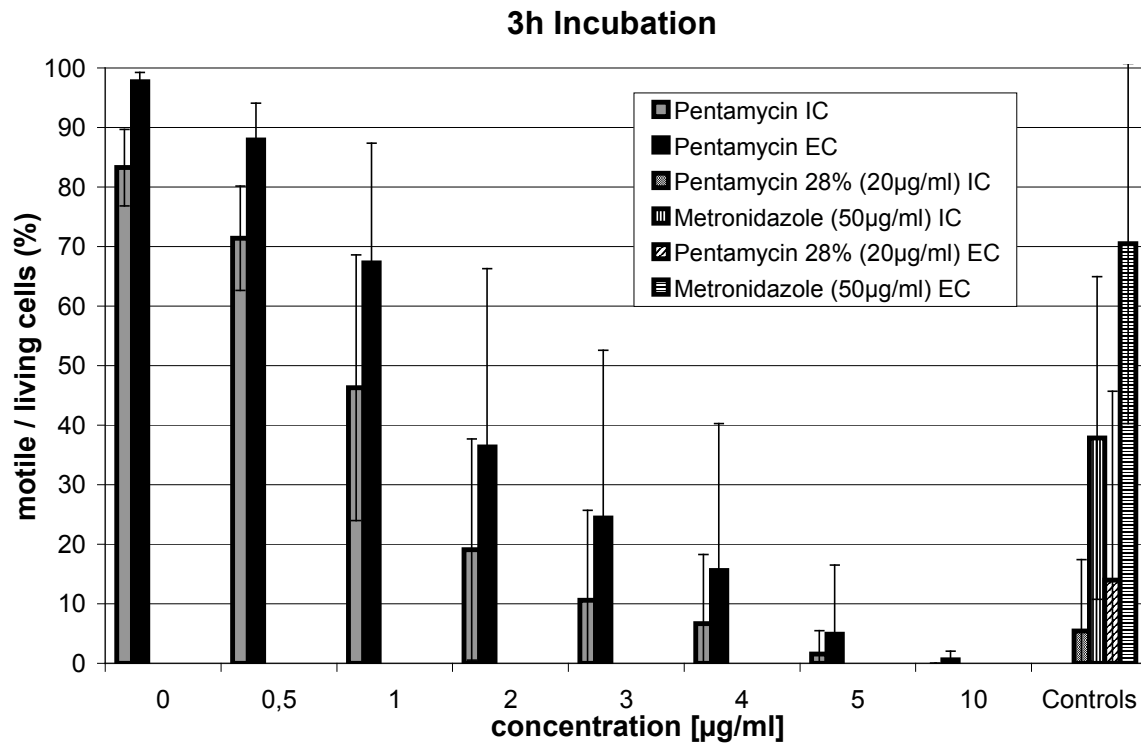
### **TV2 (normally metronidazole-sensitive strain)**

Also in this case, fig. 19–21 display the relative motility and vitality of this strain after treating with various doses of pentamycin and incubated for 1, 3, and 6 hours, respectively. It could be shown that the amount of motile and vital cells is decreasing with higher doses of pentamycin as well as with longer incubation time.

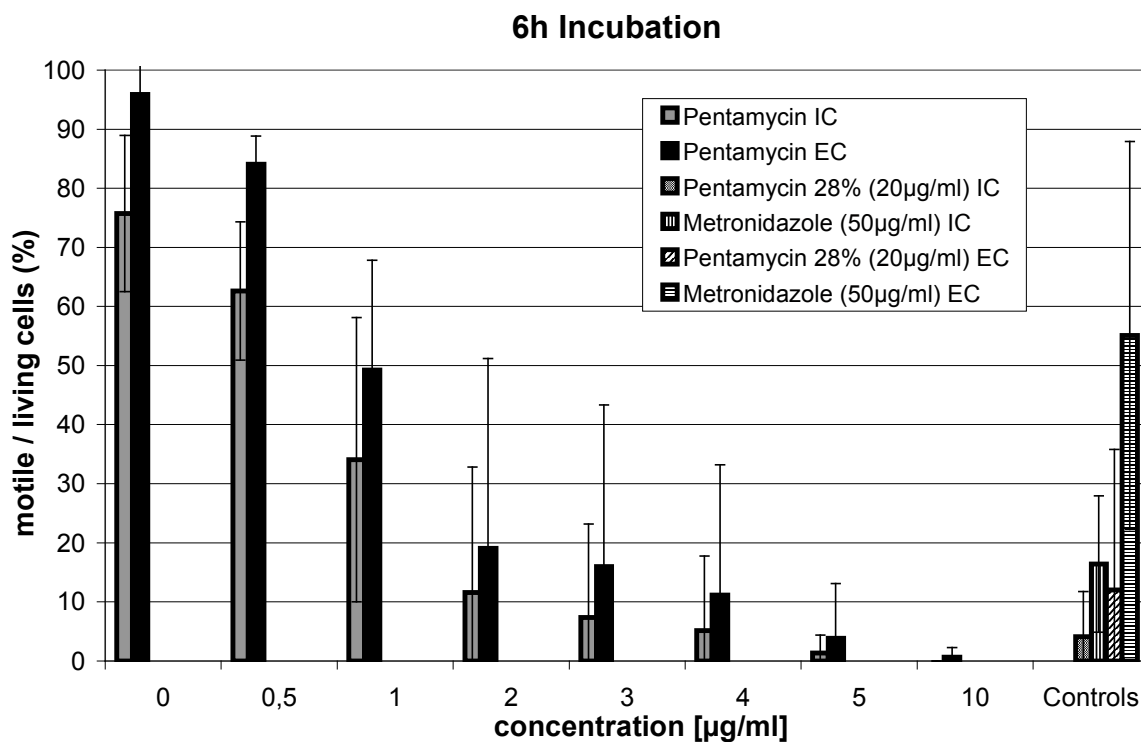


**Fig. 19:** Motility and vitality of strain TV2 after 1h of treatment with pentamycin.

## RESULTS



**Fig. 20:** Motility and vitality of strain TV2 after 3h of treatment with pentamycin.



**Fig. 21:** Motility and vitality of strain TV2 after 6h of treatment with pentamycin.

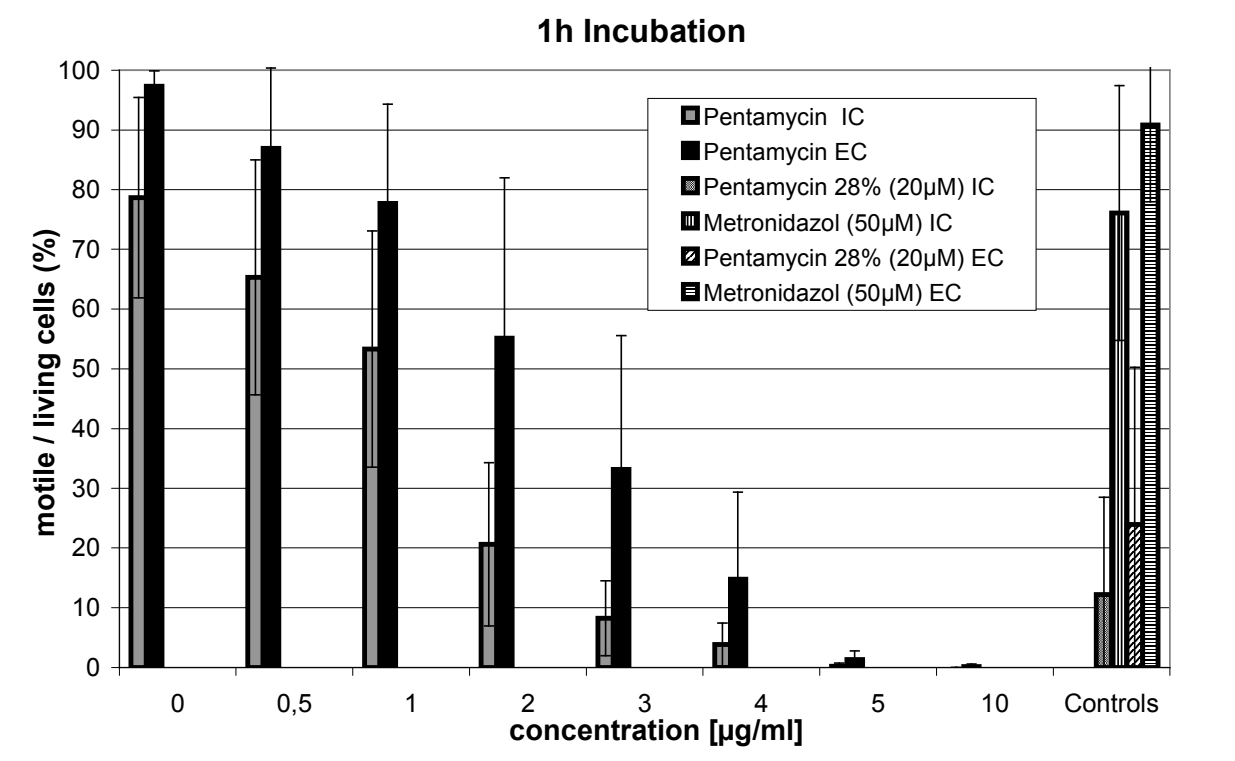
## RESULTS

**Tab 4:** EC and IC values ( $\mu\text{g/ml}$ ) of TV2 after 1h, 3h and 6h.

TV2	1h	3h	6h
IC <sub>50</sub>	1,004	0,887	0,678
IC <sub>90</sub>	3,778	2,904	2,362
EC <sub>50</sub>	2,169	1,478	1,085
EC <sub>90</sub>	6,205	4,481	3,467

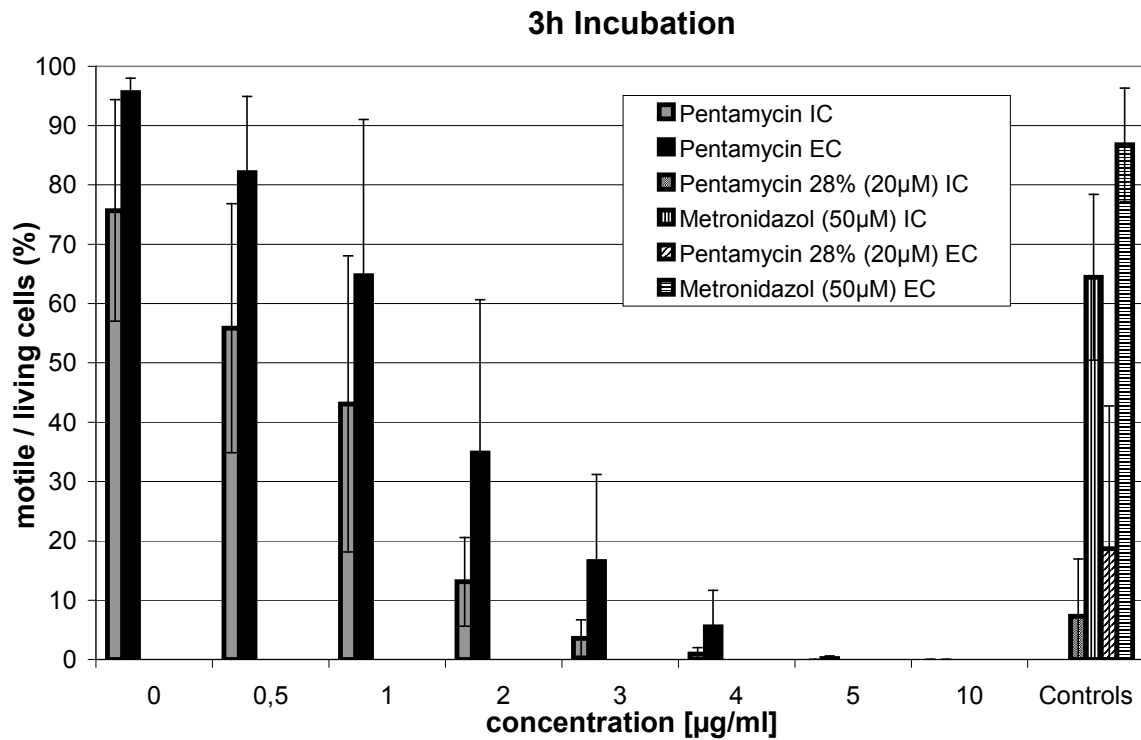
### **ATCC 30236 (normally metronidazole-sensitive strain)**

As before, fig. 22–24 display the relative motility and vitality of this strain, after treating with various doses of pentamycin and incubated for 1, 3, and 6 hours, respectively. It could be shown that the amount of motile and vital cells is decreasing with higher doses of pentamycin as well as with longer incubation time.

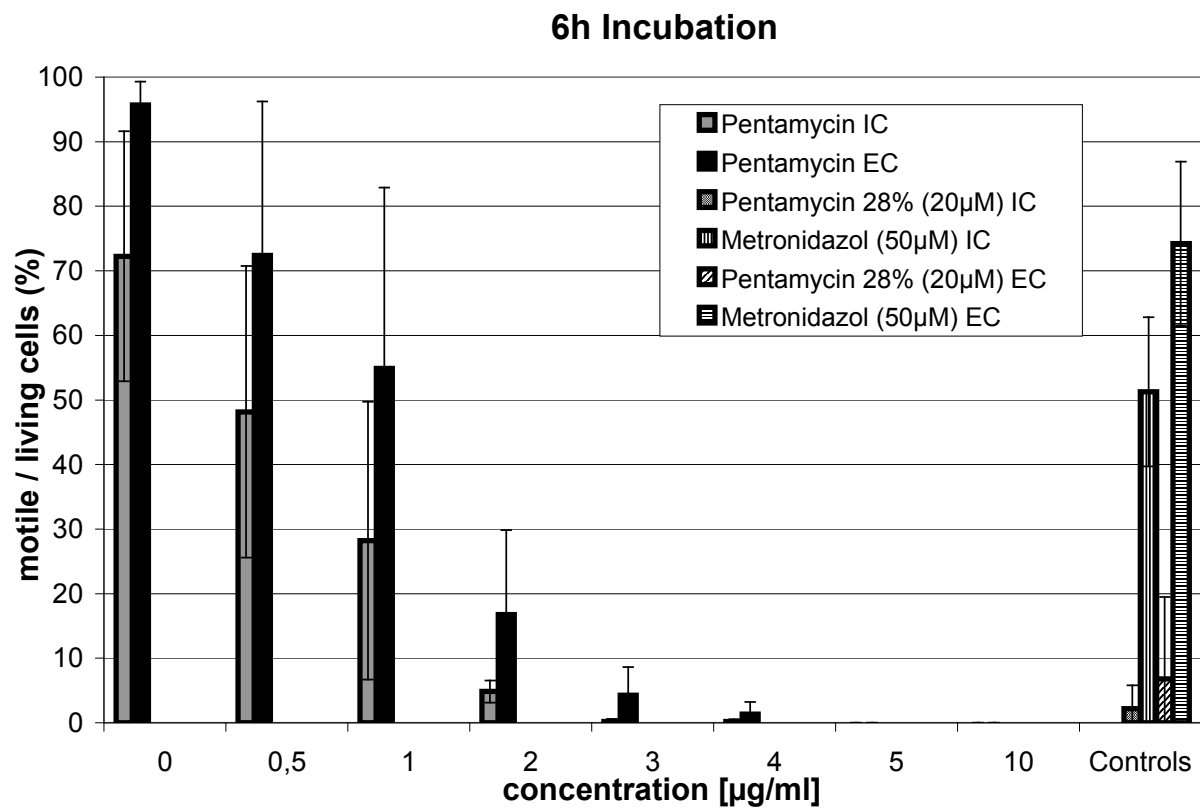


**Fig. 22:** Motility and vitality of strain ATCC 30236 after 1h of treatment with pentamycin.

## RESULTS



**Fig. 23:** Motility and vitality of strain ATCC 30236 after 3h of treatment with pentamycin.



**Fig. 24:** Motility and vitality of strain ATCC 30236 after 6h of treatment with pentamycin.

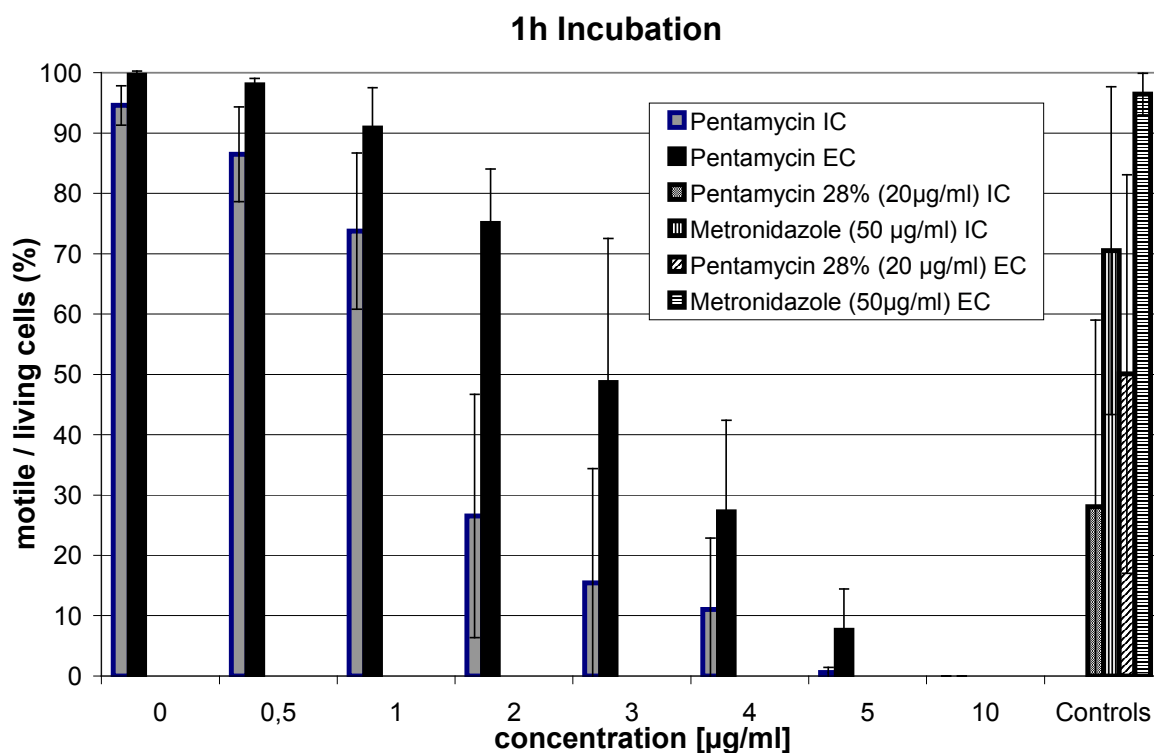
## RESULTS

**Tab. 5:** EC and IC values ( $\mu\text{g/ml}$ ) of ATCC 30236 after 1h, 3h and 6h.

ATCC 30236	1h	3h	6h
IC <sub>50</sub>	0,869	0,680	0,533
IC <sub>90</sub>	2,725	2,081	1,455
EC <sub>50</sub>	1,744	1,248	0,913
EC <sub>90</sub>	4,909	3,443	2,357

### **ATCC 50138 (metronidazole-resistant strain)**

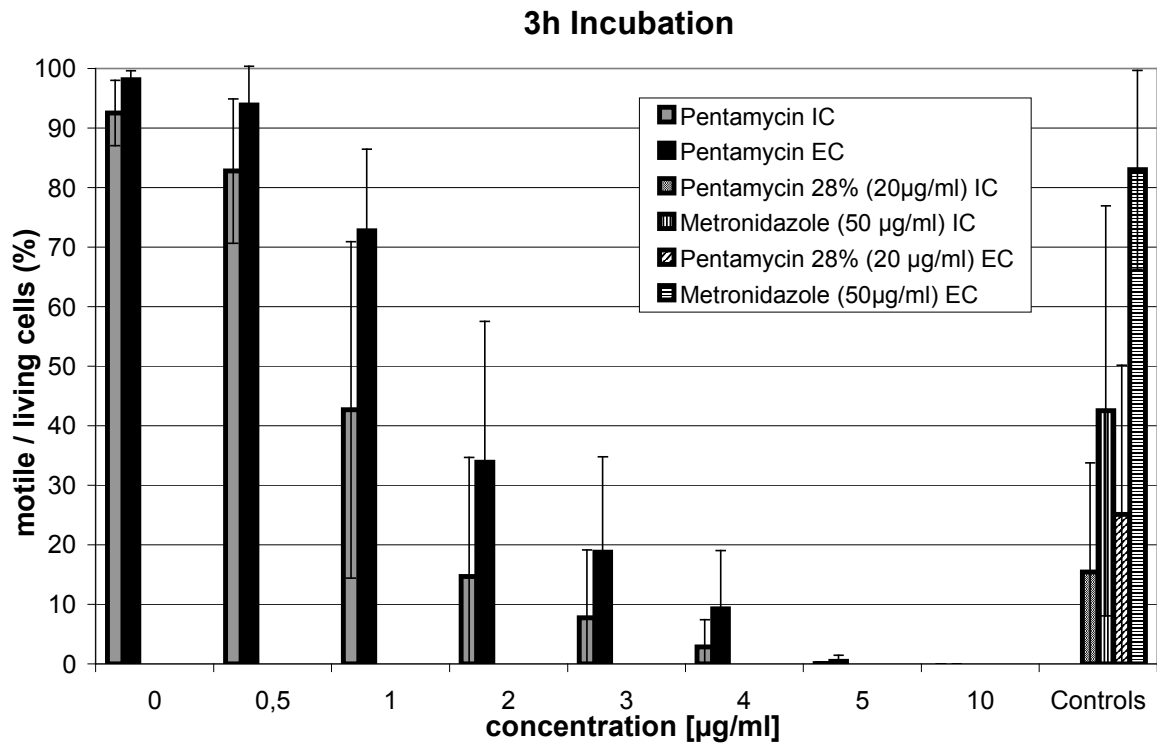
Here, fig. 25–27 display the relative motility and vitality of this strain, after treating with various concentrations of pentamycin and incubated for 1, 3, and 6 hours, respectively. It could be shown that the amount of motile and vital cells is decreasing with higher doses of pentamycin as well as with longer incubation time.



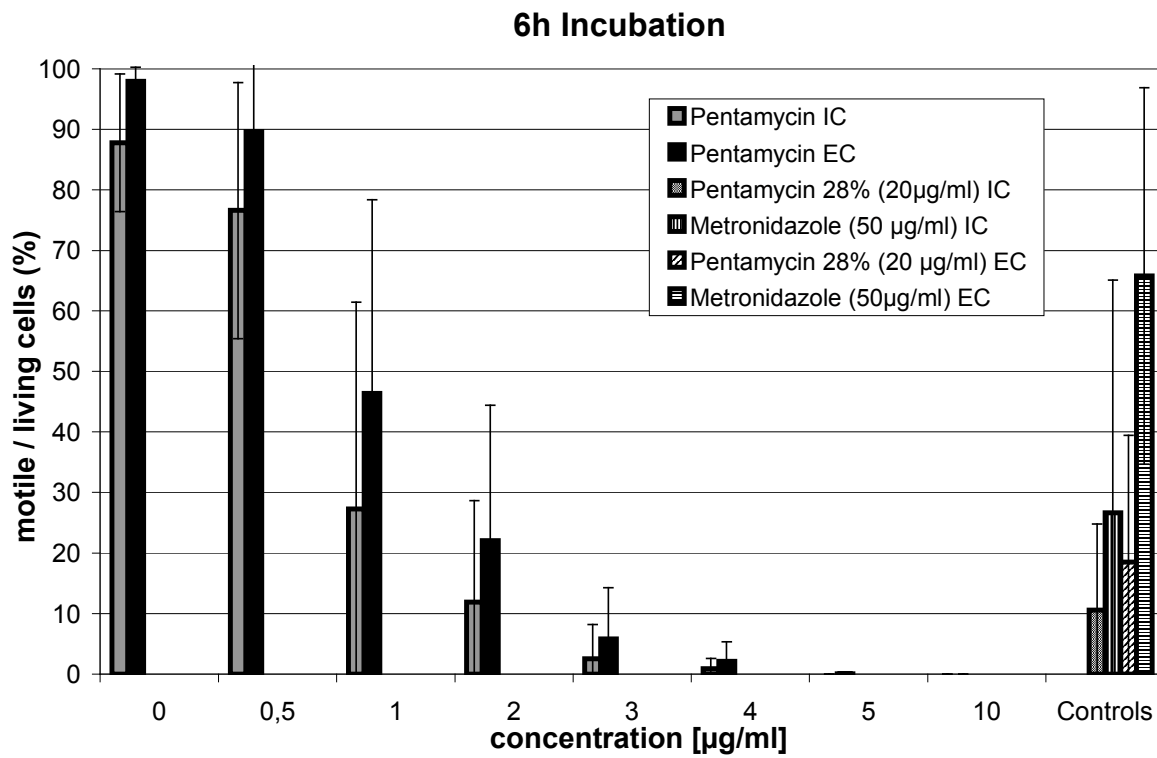
**Fig. 25:** Motility and vitality of strain ATCC 50138 after 1h of treatment with pentamycin.



## RESULTS



**Fig. 26:** Motility and vitality of strain ATCC 50138 after 3h of treatment with pentamycin.



**Fig. 27:** Motility and vitality of strain ATCC 50138 after 6h of treatment with pentamycin.

## RESULTS

**Tab. 6:** EC and IC values ( $\mu\text{g/ml}$ ) of ATCC 50138 after 1h, 3h and 6h.

ATCC 50138	1h	3h	6h
IC <sub>50</sub>	1,350	0,941	0,766
IC <sub>90</sub>	3,507	2,378	1,856
EC <sub>50</sub>	2,620	1,505	1,061
EC <sub>90</sub>	5,770	3,568	2,459

Due to the large number of data, IC<sub>50</sub>s, IC<sub>90</sub>s, EC<sub>50</sub>s, and EC<sub>90</sub>s of all strains were summed up and their arithmetic mean was calculated for a better illustration of these results. This should demonstrate the efficacy of pentamycin (94%) (Tab. 7) and the low dose required for inhibition and eradication of trichomonads.

In general, all strains showed almost the same susceptibility to pentamycin, though not significant but with a distinct tendency. The level of significance was calculated as 14.1%, which is non-significant (significance  $p \leq 5\%$ ) but still a low error-probability and explainable by the large standard deviations between the measurements of the respective strains (Tab. 8). Overall standard deviation is 13.59%, the standard error of arithmetic means 1.5%. The Levene Test (Tab. 10) shows that the greatest error probability is at 1  $\mu\text{g/ml}$ . In both cases, its values are non-significant with 25.4% (IC) and 38.6% (EC). Large deviations are also at 0  $\mu\text{g/ml}$  (EC) with 22.8% error probability.

## RESULTS

**Tab. 7:** Arithmetic means after treatment with pentamycin (94%)

Arithmetic mean of $\mu\text{g/ml}$ – all strains	1h	3h	6h
<b>IC<sub>50</sub></b>	1,04	0,82	0,65
<b>IC<sub>90</sub></b>	3,35	2,45	1,92
<b>EC<sub>50</sub></b>	2,19	1,46	1,09
<b>EC<sub>90</sub></b>	5,85	4,03	2,98

**Tab. 8:** Significance of consistency between the strains (marked bold).

Source	df (degree of freedom)	F	Significance
Constant Term	1	284,444	,000
Time	1	52,011	,000
Triplicate	1	3,531	,065
<b>Strain</b>	3	1,886	<b>,141</b>

**Tab. 9:** Arithmetic means of the sensitivity of strains with standard error.

Strain	Arithmetic mean (motile/vital cells in %)	Standard error
ATCC 30001	34,437	1,531
TV 2	32,284	1,531
ATCC 30236	29,810	1,577
ATCC 50138	34,188	1,531

## RESULTS

**Tab. 10:** Levene Test: consistency of error variance (bold: non-significant values).

	F	df1 (degree of freedom)	df2 (sample size)	Significance
0 µg/ml IC	8,544	3	67	,000
0,5 µg/ml IC	6,860	3	67	,000
<b>1 µg/ml IC</b>	1,388	3	67	<b>,254</b>
2 µg/ml IC	4,175	3	67	,009
3 µg/ml IC	4,121	3	67	,010
4 µg/ml IC	4,249	3	67	,008
5 µg/ml IC	7,029	3	67	,000
10 µg/ml IC	2,941	3	67	,039
15 µg/ml IC	.	3	67	.
Pent 20 µg/ml IC	8,071	3	67	,000
Metro 50 µg/ml IC	3,077	3	67	,033
<b>0 µg/ml EC</b>	1,479	3	67	<b>,228</b>
0,5 µg/ml EC	5,948	3	67	,001
<b>1 µg/ml EC</b>	1,027	3	67	<b>,386</b>
2 µg/ml EC	4,300	3	67	,008
3 µg/ml EC	5,022	3	67	,003
4 µg/ml EC	4,276	3	67	,008
5 µg/ml EC	6,187	3	67	,001
10 µg/ml EC	7,922	3	67	,000
15 µg/ml EC	.	3	67	.
Pent 20 µg/ml EC	3,636	3	67	,017
<b>Metro 50 µg/ml EC</b>	1,445	3	67	<b>,237</b>

## RESULTS

Summing up these results, a positive correlation of drug dose, incubation time and amount of impaired and lysed cells was measurable. 0.5 µg/ml pentamycin per 10<sup>6</sup> cells/ml is a sublethal, low dose that leads largely to discomfort and impairment and to a percentage of average 10% of dead cells. However, affected and though viable trichomonads are able to regenerate within 24 hours. 1 µg/ml is a critical threshold concentration as there were no viable cells observable after 24h of treatment, and it can be constituted as minimal lethal concentration (MLC). Up from 2 to 4 µg/ml, a serious effectiveness was observable as 30% and more cells could be killed within 1h. To kill 50% of trichomonads within 1h (EC<sub>50</sub>), a dose of 2.2 µg/ml would be necessary.

At 5 µg/ml, which can be assigned as a high dose, 100% of cells were lysed in the best case and 90% in the worst case. At 15 µg/ml, 100% of lysed cells within 1h can be stated. Treated cell cultures included impaired trichomonads after treatment with low, sublethal concentrations, showing uncoordinated, rotary movements and a few rounded, immotile forms. These forms appeared at low, sublethal doses. The higher the applied dose, the less of the round forms and more completely lysed cells were observable. At a dose of 10 µg/ml, nearly all cells were lysed. At a dose of 50 µg/ml, which is more than three times higher than the dose needed for an EC<sub>100</sub> within 1h, particles similar to granula were visible around the lysed cells.

At 1h post treatment, IC<sub>50</sub>s of the respective strains were between 0.86–1.35 µg/ml, IC<sub>90</sub>s between 2.45–3.78 µg/ml. EC<sub>50</sub>s were between 1.74–2.62 µg/ml, EC<sub>90</sub>s between 4.91–6.51 µg/ml. At 3h post treatment, IC<sub>50</sub>s of the different strains were between 0.68–0.94 µg/ml, IC<sub>90</sub>s between 2.08–2.90 µg/ml. EC<sub>50</sub>s were between 1.25–1.62 µg/ml, EC<sub>90</sub>s between 3.44–4.62 µg/ml. At 6h post treatment, IC<sub>50</sub>s of the

## RESULTS

different strains were between 0.53–0.77 µg/ml,  $IC_{90}$ s between 1.45–2.36 µg/ml.  $EC_{50}$ s were between 0.91–1.28 µg/ml,  $EC_{90}$ s between 2.36–3.62 µg/ml.

ATCC 30001 (highly metronidazole-sensitive) was the least susceptible strain, ATCC 50138 (metronidazole-resistant) the second-least susceptible, TV2 (metronidazole-sensitive) the second-most susceptible, and ATCC 30236 (metronidazole-sensitive) was the most susceptible strain. However, the arithmetic means are close to each other within a range of 5% (29–34%) and show no correlation to their sensitivity to metronidazole (Tab. 9). Regarding  $EC_{50}$ s and  $EC_{90}$ s, TV2 and ATCC 50138 were rather similar to each other, and closer to ATCC 30001 than to ATCC 30236. Interestingly, ATCC 30001 had the highest values of EC, whereas TV2 had the highest  $IC_{90}$ , and ATCC 50138 had the highest  $IC_{50}$ . ATCC 30236 was the most susceptible strain in all cases.

As the Levene-Test (Tab. 10) shows, the control “pentamycin 28% (20 µg/ml)” was highest significant with 0% at  $IC_s$  and significant with 1.7% at  $EC_s$ . The control “metronidazole (50 µg/ml)” was significant with 3.3% at  $IC_s$  but not significant with 23.7% at  $EC_s$  due to large deviations between the measurements.

### 3.4 Protein composition pre and post treatment

#### 3.4.1 Comparison of treated versus untreated cells

Fig. 28 shows the result of 1D SDS-PAGE with ATCC 30001 (highly metronidazole-sensitive), untreated and treated with 0.5 µg/ml pentamycin. The treated sample was lacking a protein band between 24 and 33 kDa (marked with dots).

Fig. 29 shows the repeat of the experiment with 2D SDS- PAGE, however, a protein spot (circled) was stronger expressed here in the treated sample, which is contrary to the previous result.

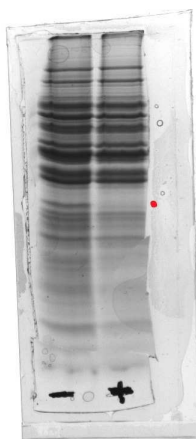
Fig. 30 shows the repeated experiment with both ATCC 30001 and TV2 (metronidazole-sensitive), treated with 0.5 µg/ml. The result displayed in the first experiment (Fig. 21) could be reproduced with ATCC 30001, TV2 showed no significant differences.

Fig. 31 and 32 show the reproduction of the previous experiment (Fig. 30), however, ATCC 30001 shows no significant differences here, and TV2 is differing in more and various spots, which is contrary to the first analysis too.

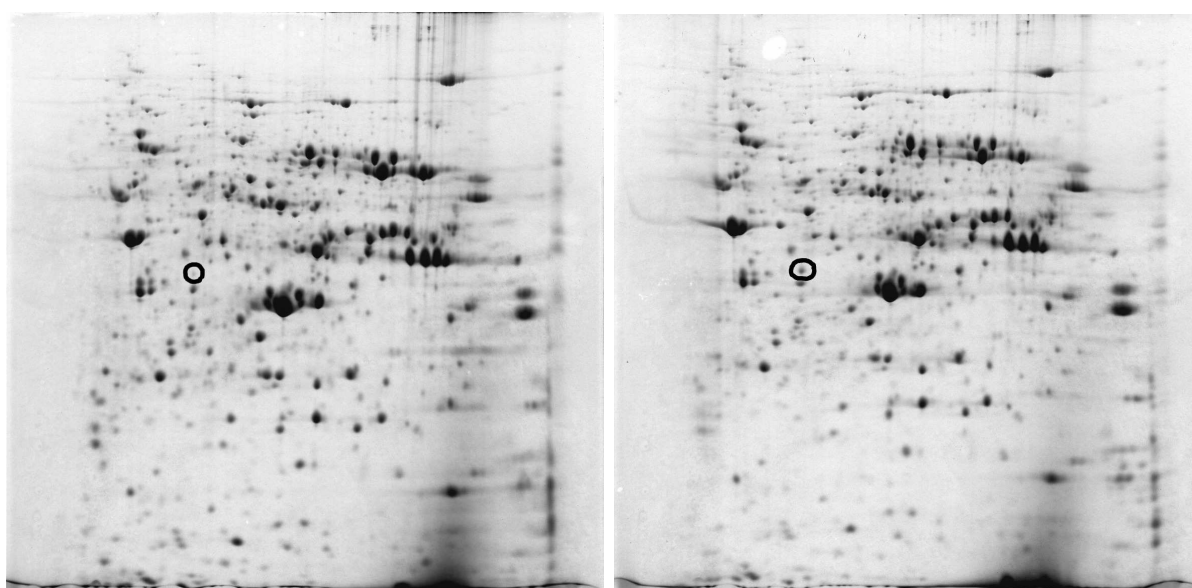
As displayed in Fig. 33, there were no visible differences in strains treated with 1 µg/ml at this time. However, it could be shown that higher doses like 2 µg/ml (Fig. 34) and 7 µg/ml (Fig. 35) led to weaker pronounced bandings in the treated samples.

After treatment with 10 µg/ml, most of the protein bands were diminished sharply (Fig. 36). Proteins with more than 33 kDa seemed to be more affected and, in general, the diminished proteins seemed to be affected randomly and not specifically.

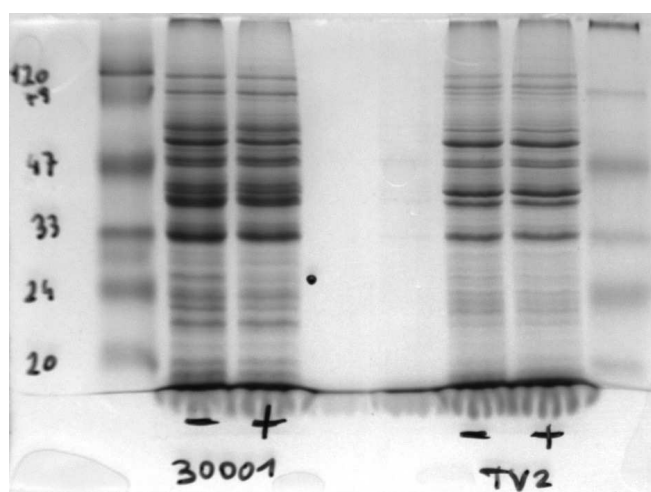
## RESULTS



**Fig. 28:** 1D SDS-PAGE gel of ATCC 30001 untreated (-) and treated with 0.5 µg/ml (+).



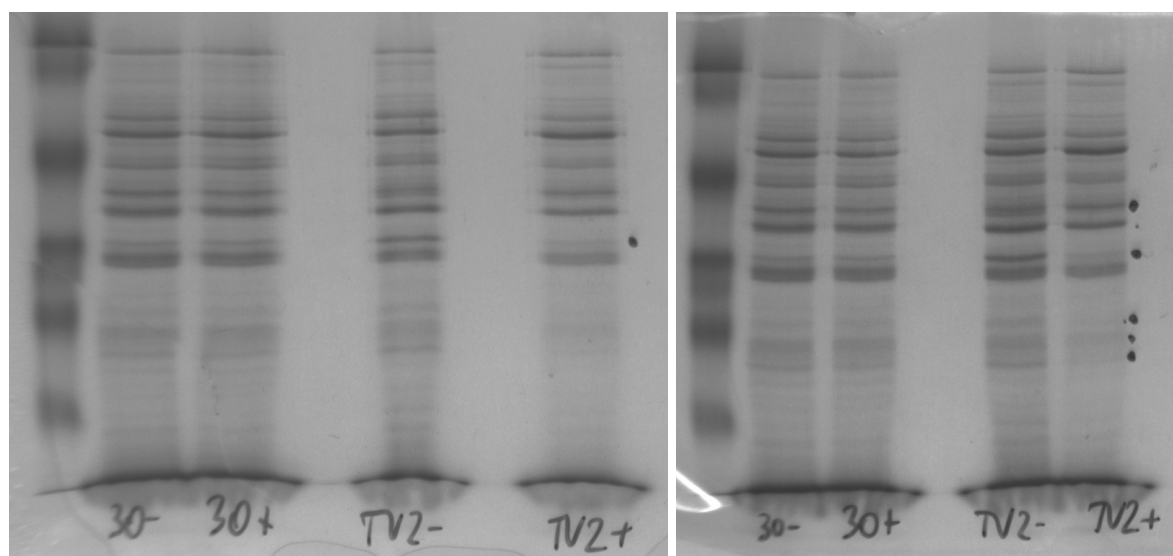
**Fig. 29:** 1D SDS-PAGE gel of ATCC 30001, untreated (-, left) and treated with 0.5 µg/ml (+, right).  
Performed by David Leitsch.



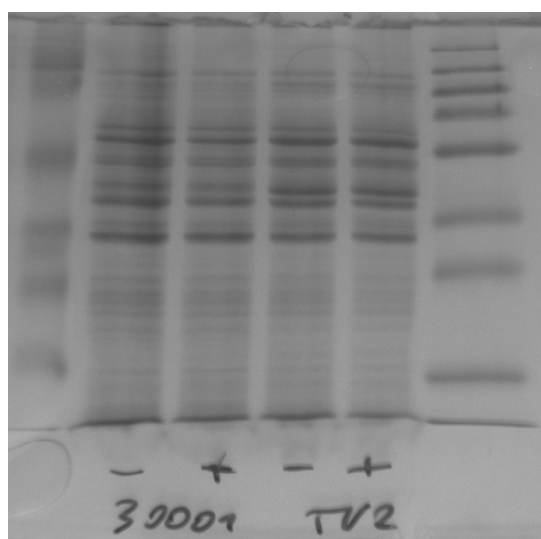
**Fig. 30:** ATCC 30001 and TV2, untreated (-) and treated with 0.5 µg/ml (+).



## RESULTS

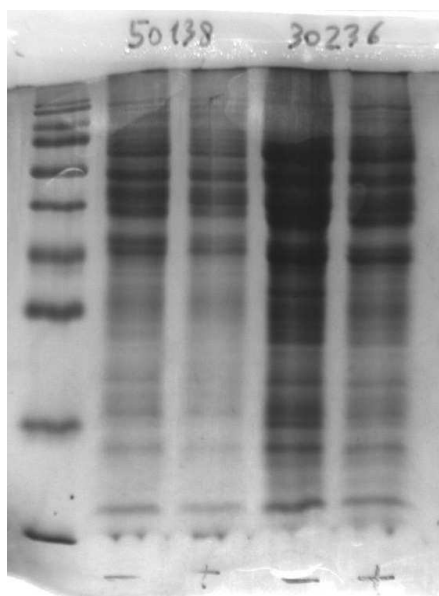


**Fig. 31 & 32:** ATCC 30001 (left) and TV2 (right), both treated with 0.5 µg/ml pentamycin.

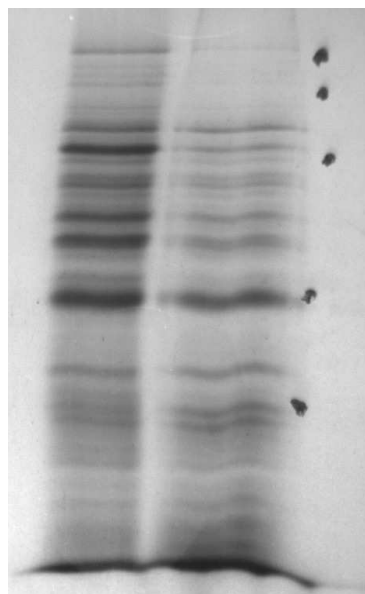


**Fig. 33:** ATCC 30001 and TV2 after treatment with 1 µg/ml pentamycin.

## RESULTS



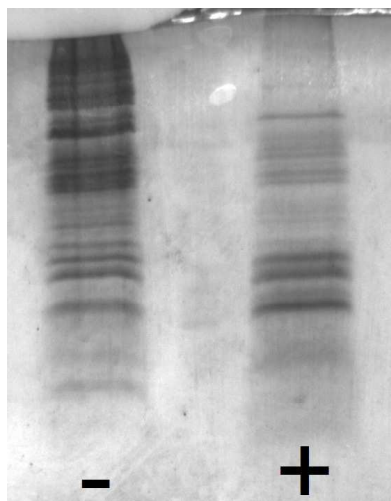
**Fig. 34**



**Fig. 35**

**Fig. 34:** ATCC 50138 and 30236 after treatment with 2 µg/ml pentamycin,

**Fig. 35:** ATCC 30001 after treatment with 7 µg/ml pentamycin.



**Fig. 36:** ATCC 30236 after treatment with 10 µg/ml pentamycin.

## RESULTS

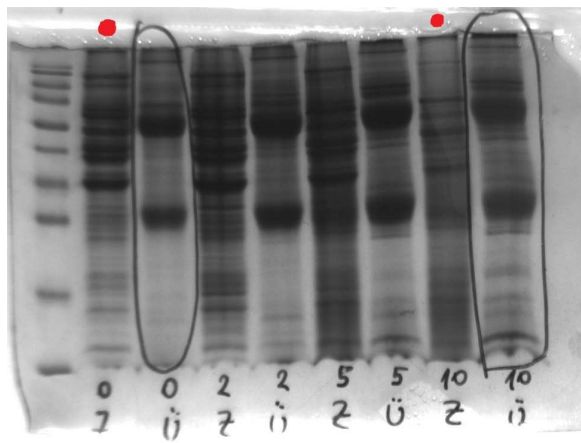
Our hypothesis is that these patterns are based on a loss of cytoplasmatic material. A degradation of proteins by proteases or similar mechanisms seemed not to be plausible, considering the chemical properties of pentamycin and the short time of incubation. To prove our hypothesis, we performed a protein diminished assay to compare the protein amount of centrifuged cell extract and supernatant.

### **3.4.2 Protein diminishment assay**

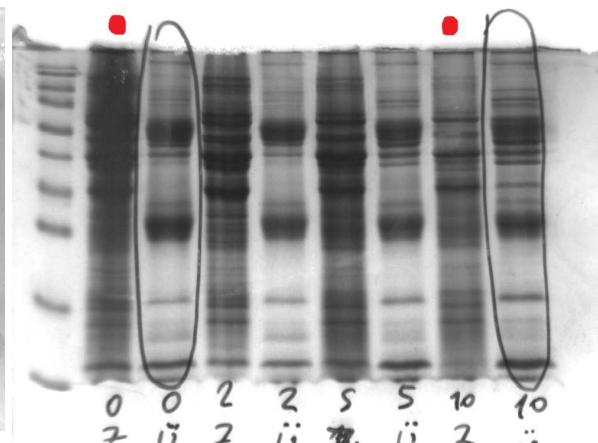
The comparison of untreated cells and cells treated with a highly toxic dose of 10 µg/ml showed that the amount of proteins was increased in the supernatant and diminished in the cell extract with rising concentrations of pentamycin (fig. 37). The differences between cell extracts (marked with a dot) and the supernatants (marked with a frame) are strongly visible after application of 0 and 10 µg/ml pentamycin. The protein bands of samples treated with 10 µg/ml are distinctly diminished compared to the negative control. Obviously nearly all proteins have disappeared or were degraded. Samples treated with 2 and 5 µg/ml showed diminished protein bands with intermediate intensity. The lanes of the supernatant show the converse effect: The supernatant of cells treated with 10 µg/ml displays more and stronger protein bands than the negative control. According to this, an accretion of proteins in the extracellular environment can be stated.

The presence of proteins in untreated cells is explainable as unavoidable entrainment of proteins when separating the supernatant from the cell pellets and the natural presence of lysed cells.

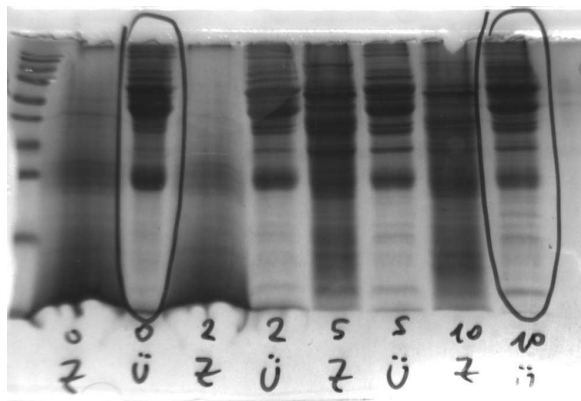
## RESULTS



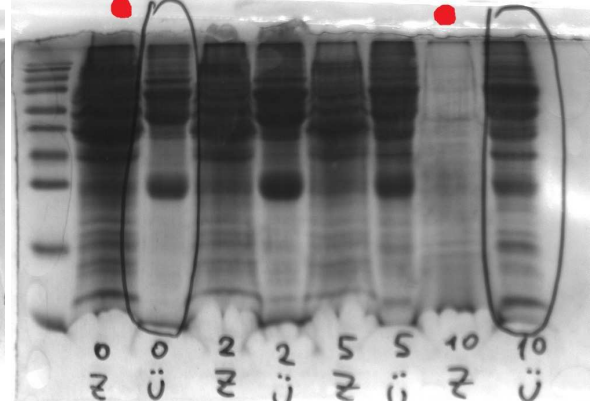
ATCC 30001



TV2



ATCC 30236



ATCC 50138

**Fig. 37:** From left to right: Protein marker “MW” (PeqLab), 0 µg/ml (cells), 0 µg/ml (supernatant), 2 µg/ml (cells), 2 µg/ml (supernatant), 5 µg/ml (cells), 5 µg/ml (supernatant), 10 µg/ml (cells), 10 µg/ml (supernatant). Z = cells, Ü = supernatant.

### **3.5 Long-term treatment and adaption**

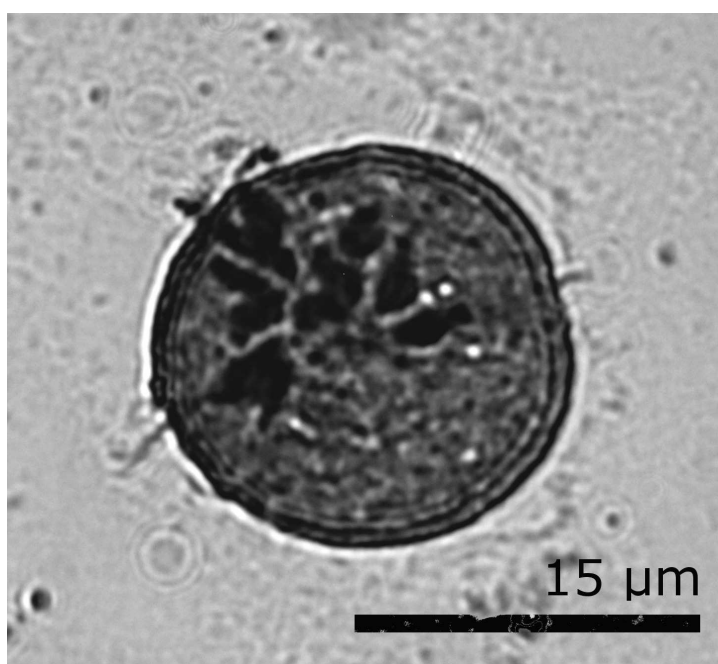
#### **3.5.1 Morphology of trichomonads in long-term treatment**

During continuous long-term treatment over six months, partial adaption could be observed. The drug admission with sublethal concentrations until 1 µg/ml had no effect on the appearance of the cells, however, up from 1 µg/ml cellular discomfort, impairment of motility and changes of morphology (round forms) were observable. However, the round forms did not lyse finally as it happened after treatment with higher doses, moreover, they were still motile and rotary, and after a certain time of regeneration they regained their vitality. Remarkably, they also appeared with multiple nuclei (Fig. 38).

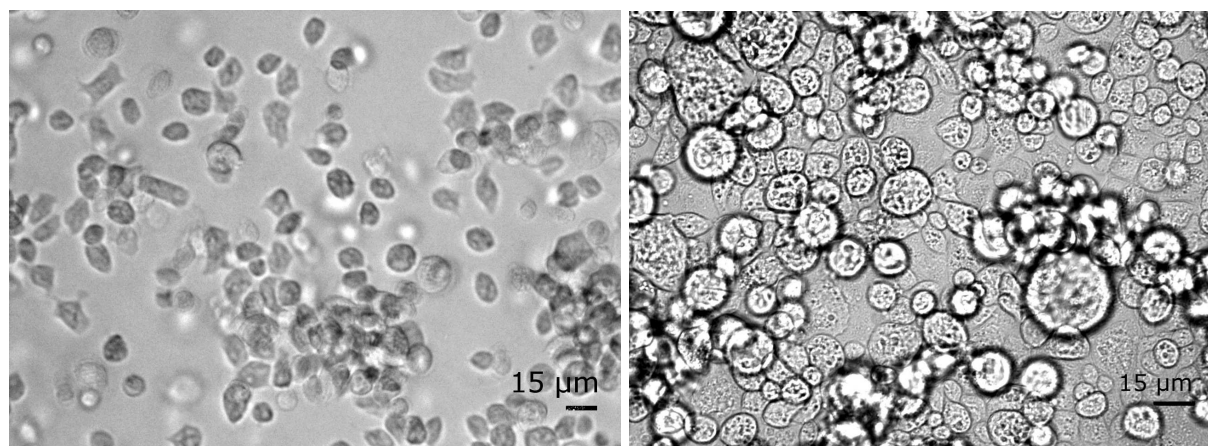
After the appliance of higher sublethal concentrations (between 1 and 3 µg/ml), the cells were impaired in their motility and vitality from several hours to days, but they were also able to regenerate from the stress after stopping the treatment. Partly, the cells appeared also larger (Fig. 39).

It was noticeable that pentamycin has a cumulative effect. The effect of adding 3 µg/ml for one time a day or 1 µg/ml for three times a day was all about the same. TV2 (metronidazole-sensitive) seemed to be more susceptible to the treatment than the other strains, however, this was only observed and not proved experimentally.

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**Fig. 38:** A rounded trichomonad cell with multiple nuclei and rotary motions after treatment with sublethal doses of pentamycin (Photo: M. Syrowatka).



**Fig. 39:** ATCC 30001; untreated and vital cells (left), cells after four months of resistance treatment (right).

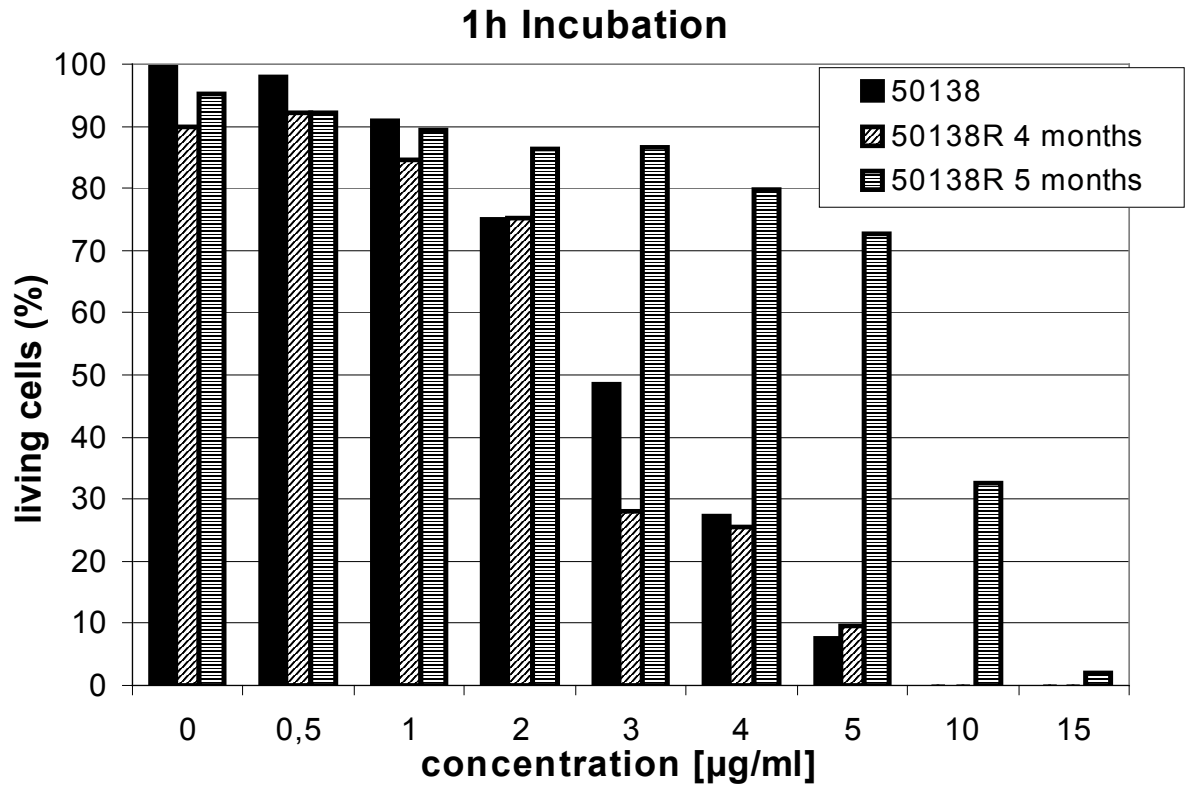
### 3.5.2 Recordable adaption to pentamycin

Fig. 40–42 and tab. 11–12 display the higher tolerance of strain ATCC 50138 (metronidazole-resistant) to pentamycin after 4 and 5 months of continuous treatment with sublethal doses (see 2.11). After 4 months, the cells did not show any distinctive differences: After 1h of incubation, their susceptibility was even 9% higher than the susceptibility of the untreated cells. 3h after incubation the susceptibility was about 12% lower, and after 6h it was about 33% lower. The values were far more distinctive after 5 months:

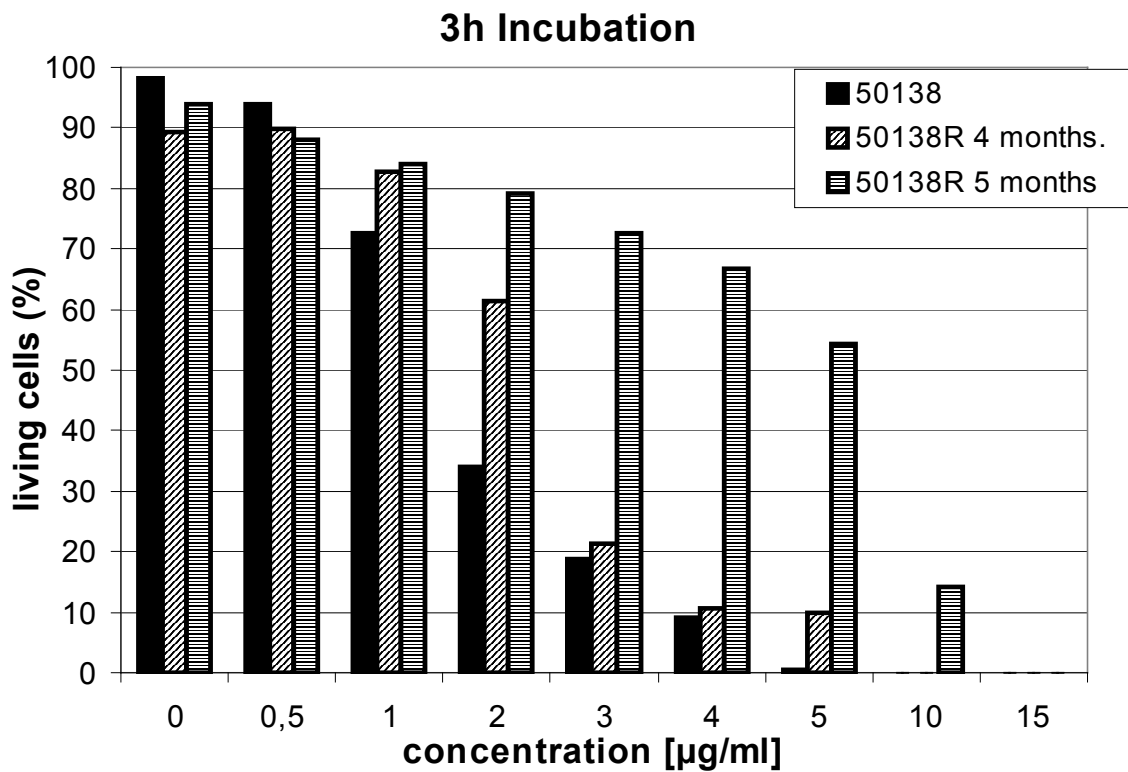
At 1h of post treatment the susceptibility was 44% lower, after 3h about 70% and after 6h about 85% lower. A percentage of cells was still vital at 10 µg/ml, a dose that leads to EC<sub>100</sub> in wildtypes. However, the susceptibility of the cells also increased with longer incubation time and finally led to 100% lysis at 15 µg/ml after 3 and 6 hours. All in all, after 4 months the susceptibility was reduced about 8.4% and after 5 months about 63%.

As shown in tab. 11 and 12, EC<sub>50</sub>s were higher – although not significantly – and have ascended after continuous treatment within 5 months. Thus, a lower susceptibility and adaption of trichomonads to pentamycin can be assumed.

## RESULTS



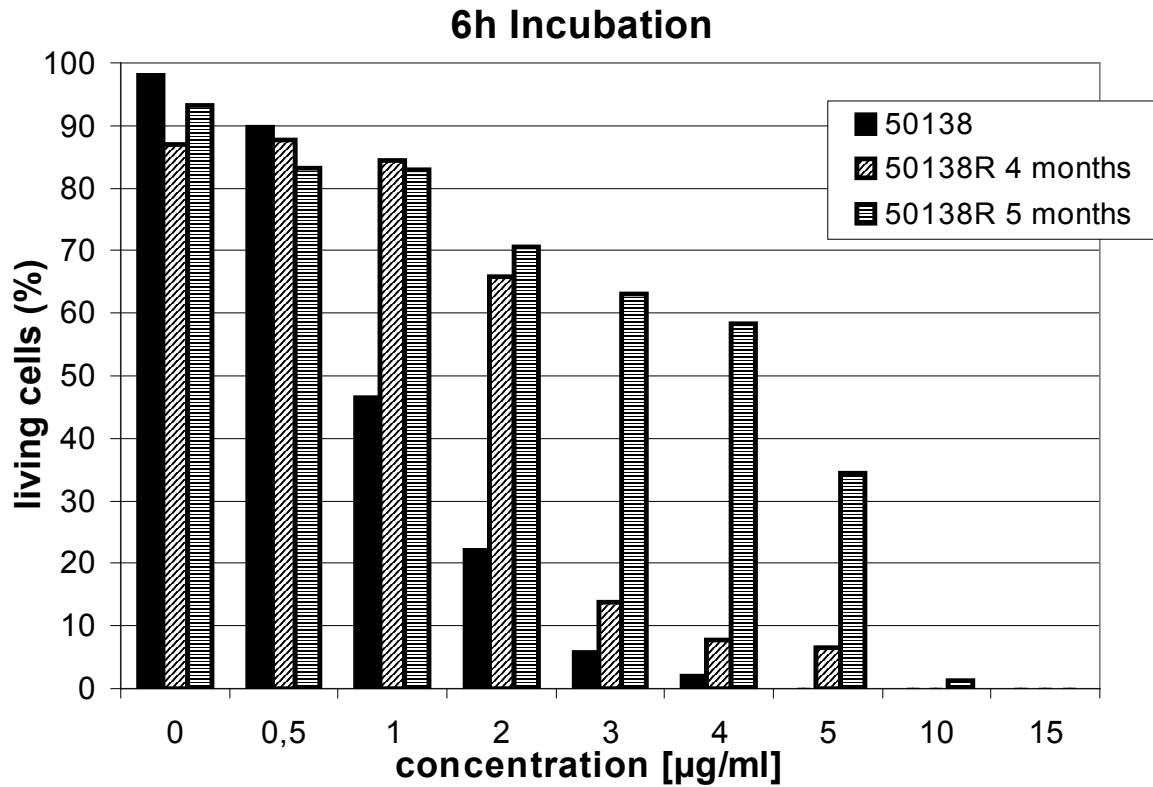
**Fig. 40:** Vitality of ATCC 50138 and 50138R, 1h after treatment with pentamycin.



**Fig. 41:** Vitality of ATCC 50138 and 50138R, 3h after treatment with pentamycin.



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**Fig. 42:** Vitality of ATCC 50138 and 50138R, 6h after treatment with pentamycin.

**Tab. 11:** EC<sub>50</sub> of ATCC 50138R after 4 and 5 months of treatment.

ATCC 50138 R	1h	3h	6h
EC <sub>50</sub> (4 months)	2,20	1,84	1,76
EC <sub>50</sub> (5 months)	6,40	4,17	4,42

**Tab. 12:** Comparative EC<sub>50</sub> of ATCC 50138 (wildtype, see also tab. 6).

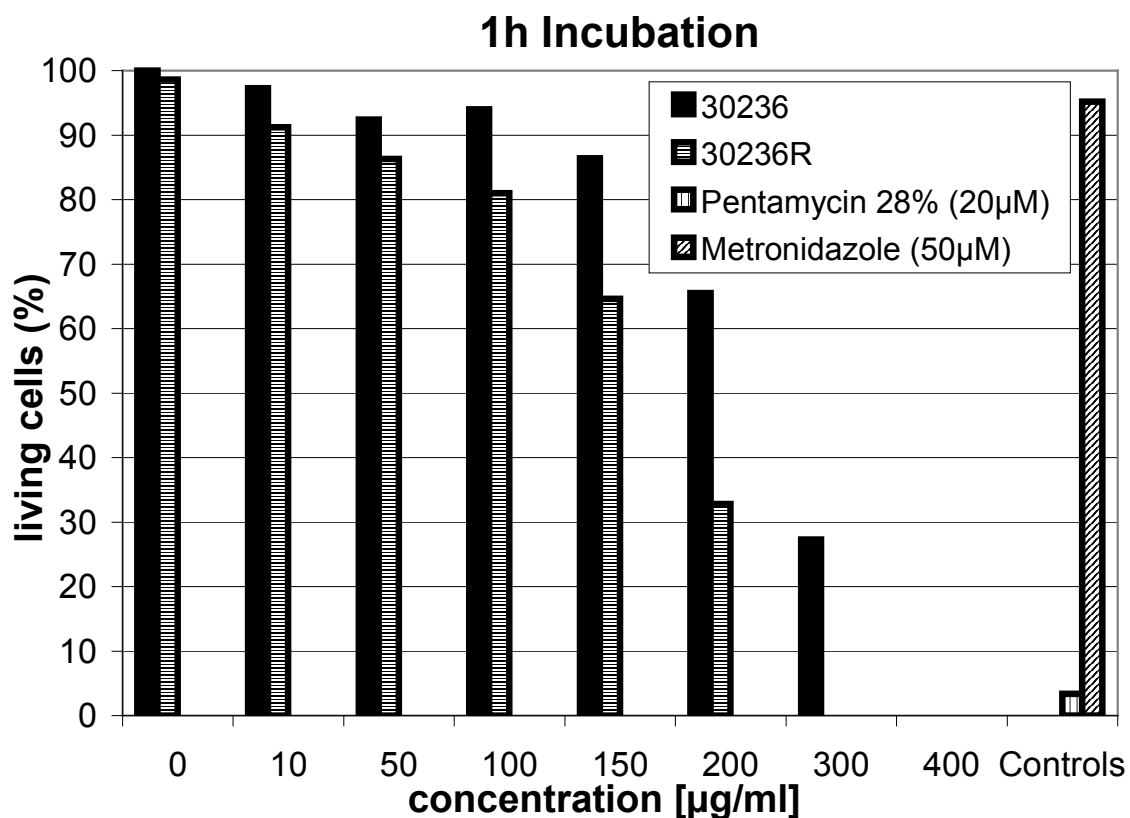
ATCC 50138	1h	3h	6h
EC <sub>50</sub>	2,62	1,51	1,06

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### 3.5.3 Cross resistance to amphotericin B

#### ATCC 30236

Fig. 43–45 and tab. 13–14 display the relative vitality of ATCC 30236 (metronidazole-sensitive), wildtype and R-strain, after 4 months of long-term treatment, both treated with various doses of amphotericin B. Remarkably, R-strains were about 23% more sensitive to amphotericin B in general, especially at higher doses – up from 200  $\mu\text{g/ml}$  after 1h, and up from 150  $\mu\text{g/ml}$  after 3 and 6h.



**Fig. 43:** Vitality of ATCC 30236 and 30236R, after 1h of treatment with pentamycin.

## RESULTS

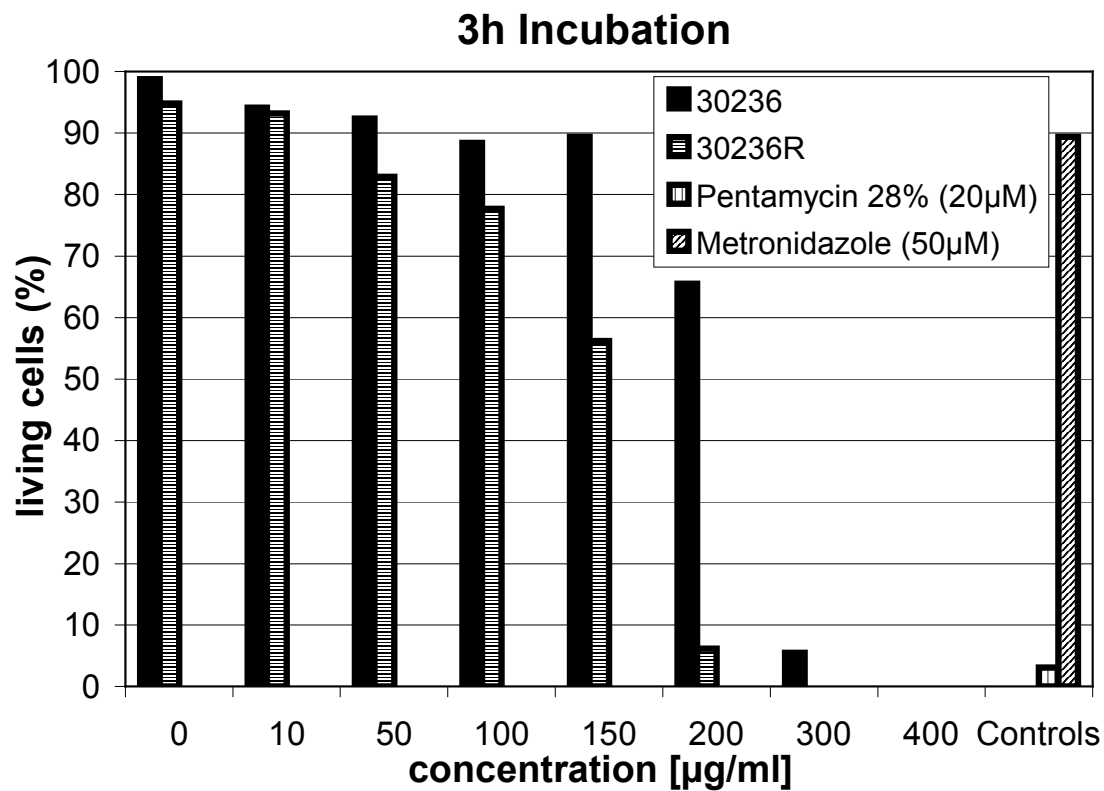


Fig. 44: Vitality of ATCC 30236 and 30236R, after 3h of treatment with pentamycin.

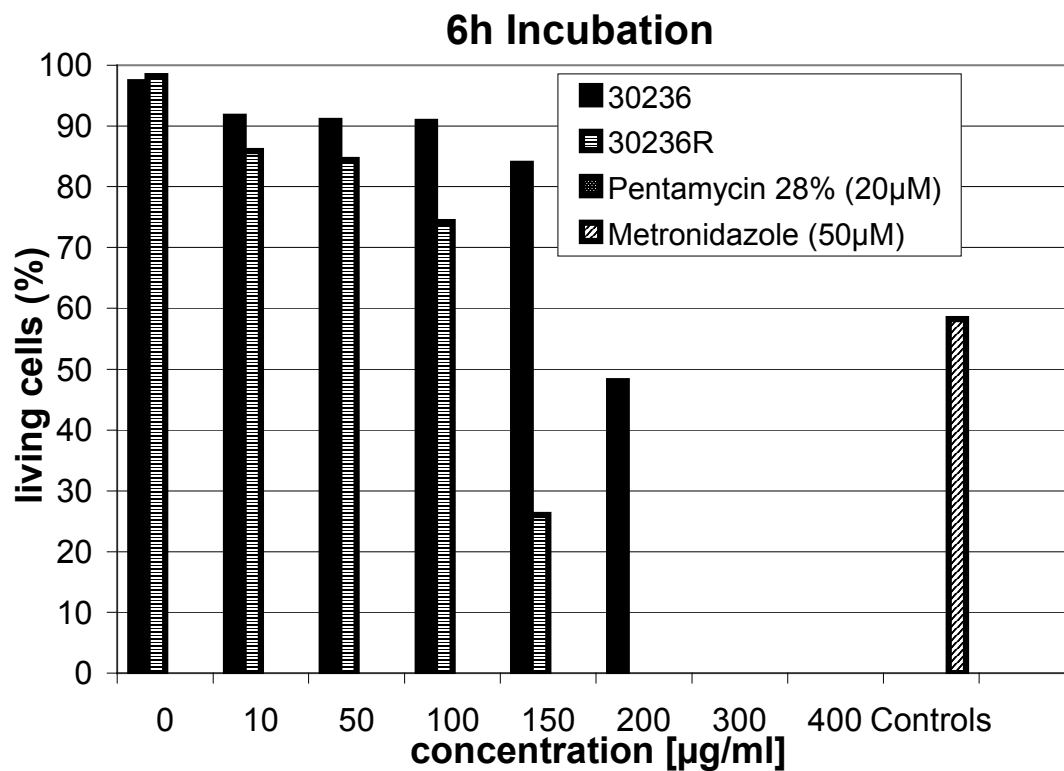


Fig. 45: Vitality of ATCC 30236 and 30236R, after 6h of treatment with pentamycin.

## RESULTS

**Tab. 13:** EC<sub>50</sub> of ATCC 30236R after treatment with amphotericin B.

30236R	1h	3h	6h
EC 50	121,33	103,96	76,41

**Tab. 14:** Comparative EC<sub>50</sub> of ATCC 30236 after treatment with amphotericin B (see also Tab. 3).

ATCC 30236	1h	3h	6h
EC 50	201,10	176,17	127,29

### TV2

Fig. 46–48 and tab. 15–16 display the relative vitality of TV2 (metronidazole-sensitive), wildtype and R-strain after 4 months of long-term treatment, both treated with various concentrations of amphotericin B. R-strains were more susceptible, especially at higher doses. Up from 300 µg/ml, R-strains have reached EC<sub>100</sub> within 1h, whereas wildtypes were still viable at 400 µg/ml. In general, the sensitivity of R-strains was about 25.8% higher than in wildtypes.

## RESULTS

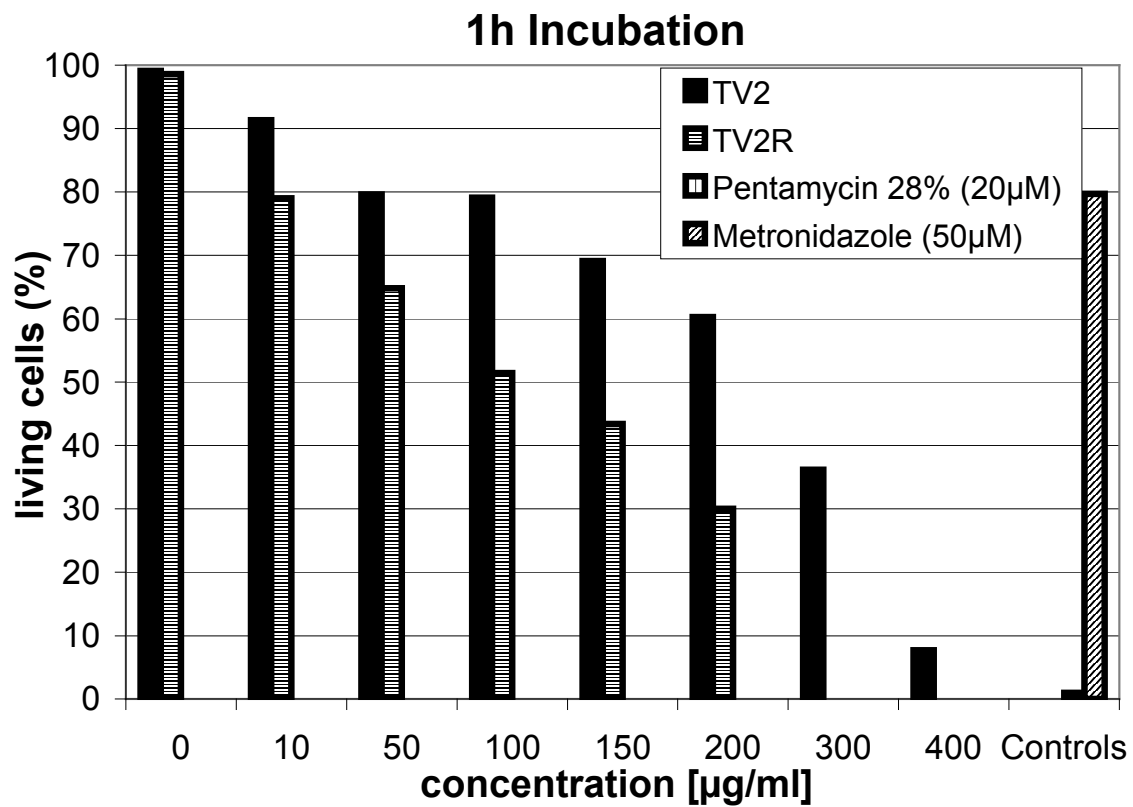


Fig. 46: Vitality of TV2 and TV2R, after 1h of treatment with pentamycin.

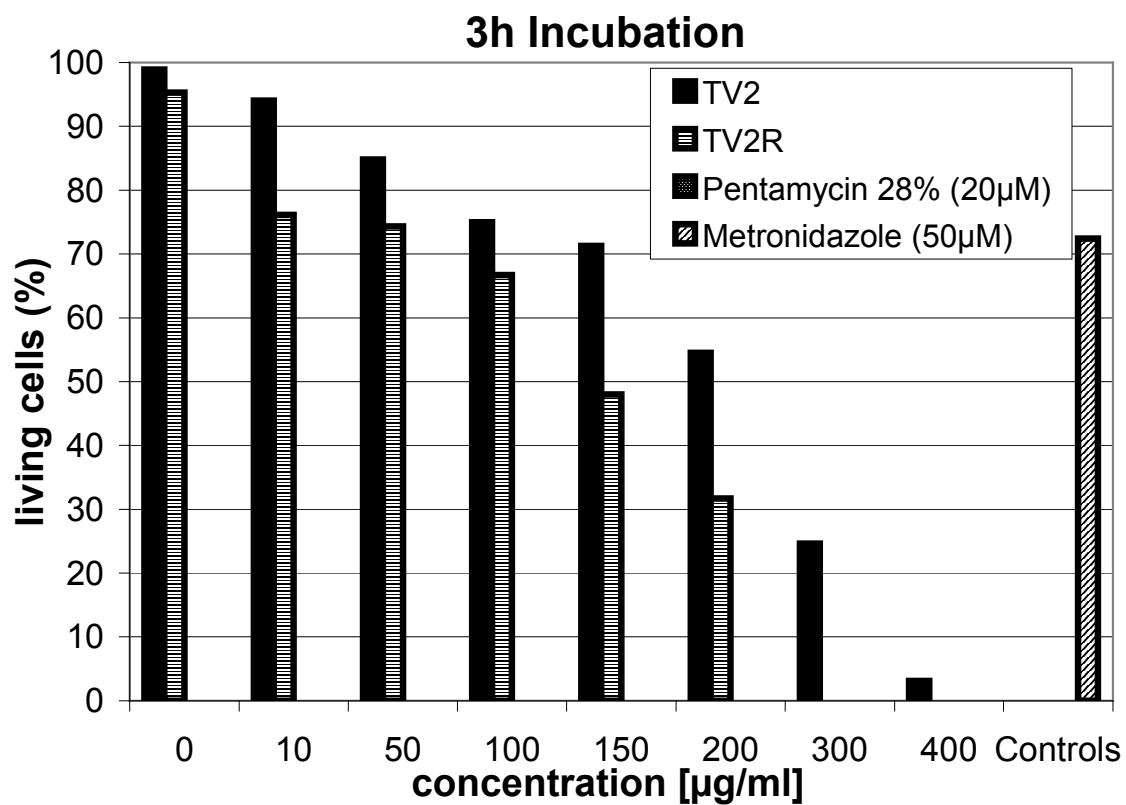
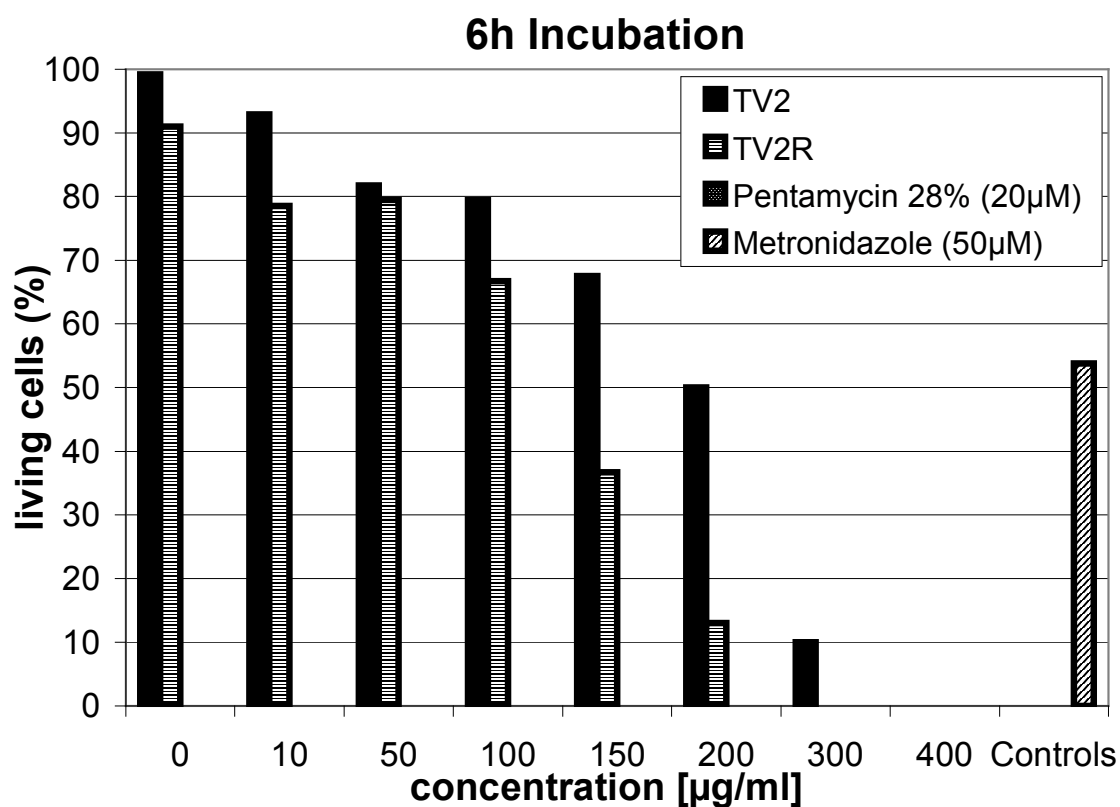


Fig. 47: Vitality of TV2 and TV2R, after 3h of treatment with pentamycin.

## RESULTS



**Fig. 48:** Vitality of TV2 and TV2R, after 6h of treatment with pentamycin.

**Tab. 15:** EC<sub>50</sub> of TV2R after treatment with amphotericin B.

TV2R	1h	3h	6h
EC 50	56,71	75,67	70,22

**Tab. 16:** Comparative EC<sub>50</sub> of ATCC 30236 after treatment with amphotericin B (see also Tab. 2).

TV2	1h	3h	6h
EC 50	162,46	153,85	108,18

## RESULTS

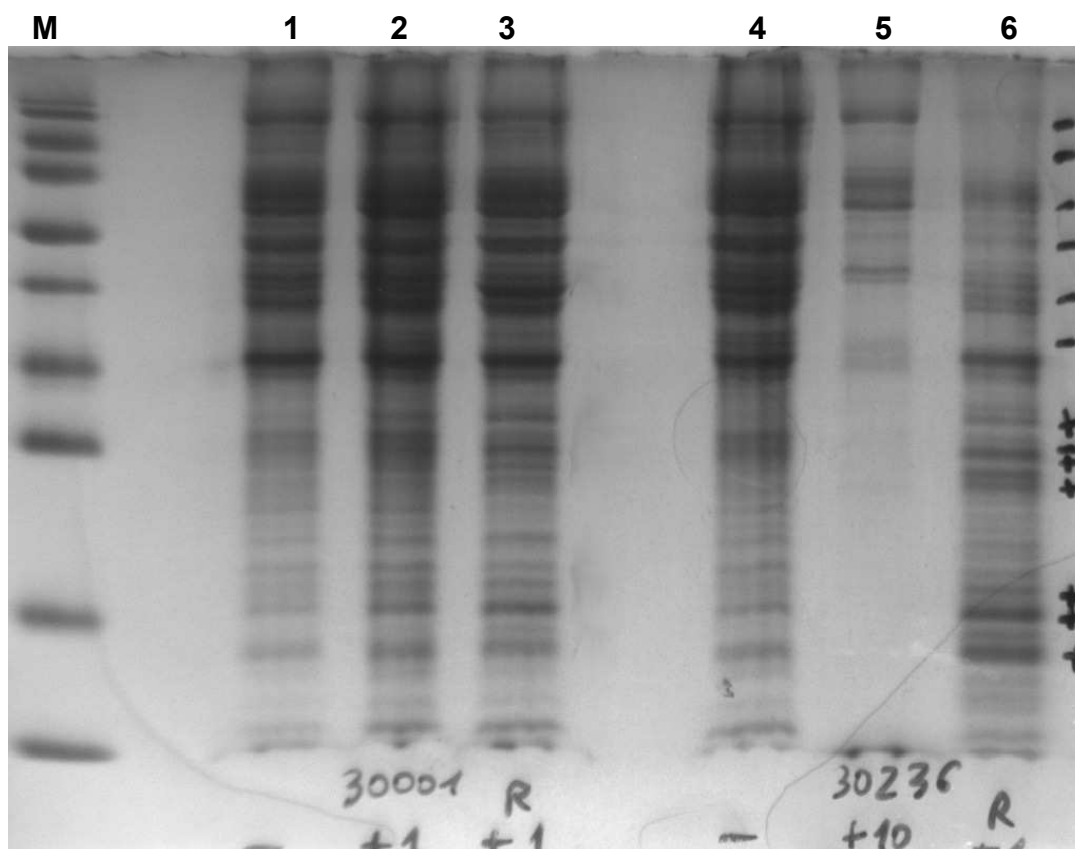
In both cases, EC<sub>50</sub>s of R-strains have decreased, comparing to wildtypes. ATCC 30236R was 2 times more susceptible to amphotericin B than wildtype, whereas TV2R was more than 3 times more susceptible after 1h. After 3h, the span between wildtype and R-strain was not so large anymore but still about 1.5 to 2 times in difference. ATCC 30236R showed higher EC values than TV2R in general. R-strains were more susceptible to a consecutive treatment with amphotericin B than untreated strains and a synergistic effect of both drugs can be stated.

### 3.5.4 Protein profile of wildtype and adapted strains

Fig. 49 shows a 1D SDS-PAGE gel, performed with both ATCC 30001 (highly metronidazole-sensitive) and ATCC 30236 (metronidazole-sensitive) to compare wildtype, single-time treated and long-term treated trichomonads. Whereas ATCC 30001 showed no significant alterations in its protein profile, ATCC 30236 showed remarkable differences. Lane 5, treated once with 10 µg/ml, displays the dramatic effect of high doses in comparison to its analogue, lane 2, which has been treated once with 1 µg/ml.

Long-term treated cells – in treatment for two months and at a dose level of 1 µg/ml at that time – showed a more variegated protein profile than single-time treated trichomonads, as displayed in lane 6: The upper, heavy-weighted proteins of a molecular mass between 250 kDa and 36 kDa seemed to be stronger diminished in treated cells whereas the light-weighted proteins between 36 kDa and 10 kDa were stronger expressed than those of untreated cells.

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**Fig. 49:** M: Marker; 1: ATCC 30001, untreated; 2: ATCC 30001, treated once with 1  $\mu\text{g/ml}$  pentamycin; 3: ATCC 30001 in resistance treatment after 2 months, last applied dose 1  $\mu\text{g/ml}$ ; 4: ATCC 30236, untreated; 5: ATCC 30236, treated once with 10  $\mu\text{g/ml}$  pentamycin; 6: ATCC 30236, in resistance treatment after two months, last applied dose 1  $\mu\text{g/ml}$ .

Fig. 50–54 show partly noticeable variations in the protein profile of wildtypes and R-strains. For example, fig. 53 shows a gel containing spot 4 which was not expressed on the other gels in that way. In the wildtype it was poorly expressed, whereas in the R-strain it was distinctly stronger expressed – a pattern that was not detectable in the other analyses where it was expressed weakly or was completely absent in wildtypes and R-strains. In fact, fig. 53 completely discerns from the other gels by its expression profile that is nearly completely diminished except spot 4.

The respective proteins were marked and numbered. Actin (1), lactate dehydrogenase (LDH) (2), enolase (3), unidentified protein spots (4), unidentified protein spot (5), pyruvate ferredoxin oxido-reductase (PFOR) (6), hydrogenosomal



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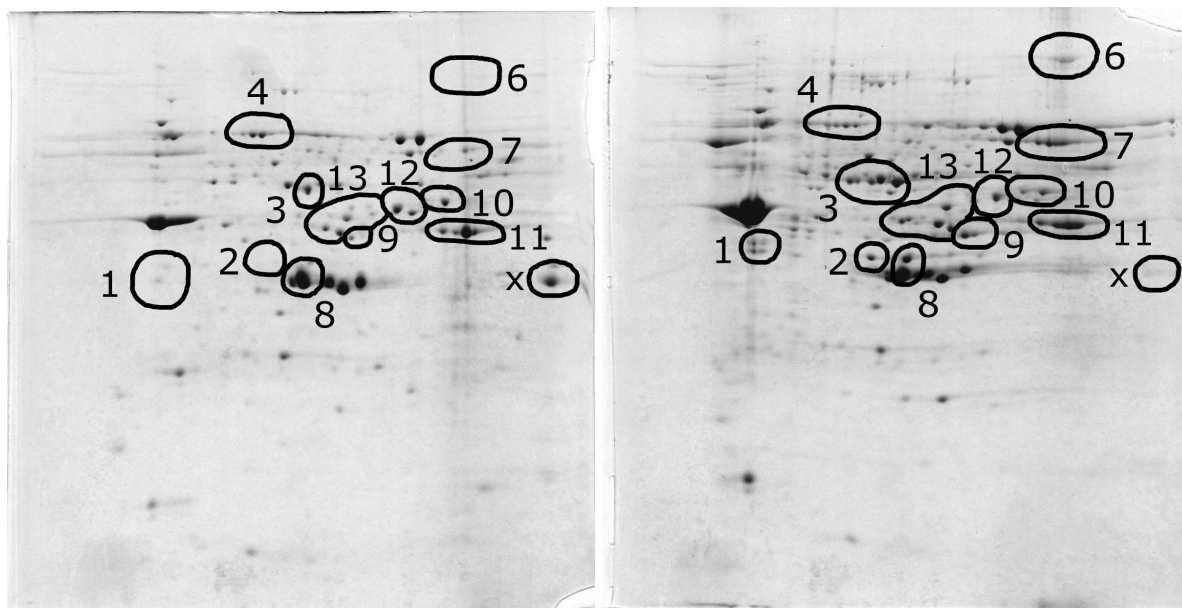
malate dehydrogenase (hMDH) (7), cytosolic malate dehydrogenase (cMDH) (8), alcohol dehydrogenase 1 (ADH1) (9), and further unidentified protein spots (10, 11, 12, 13).

Spots 1, 3, 7, 8, 9, 10, 11, 12, 13 were distinctly differing from their analogues in untreated cells, showing a visible fragmentation and degradation in R-strains, most probably due to long-term treatment. In 50% of cases, LDH was significantly stronger expressed in R-strains than in wildtypes (Fig. 50–52). PFOR was generally strongly diminished (Fig. 50–53). This key enzyme in the hydrogenosomal metabolism should normally be more intense. Indeed, in fig. 54 and 55 it was expressed more distinctive, due to the addition of iron (see 2.2).

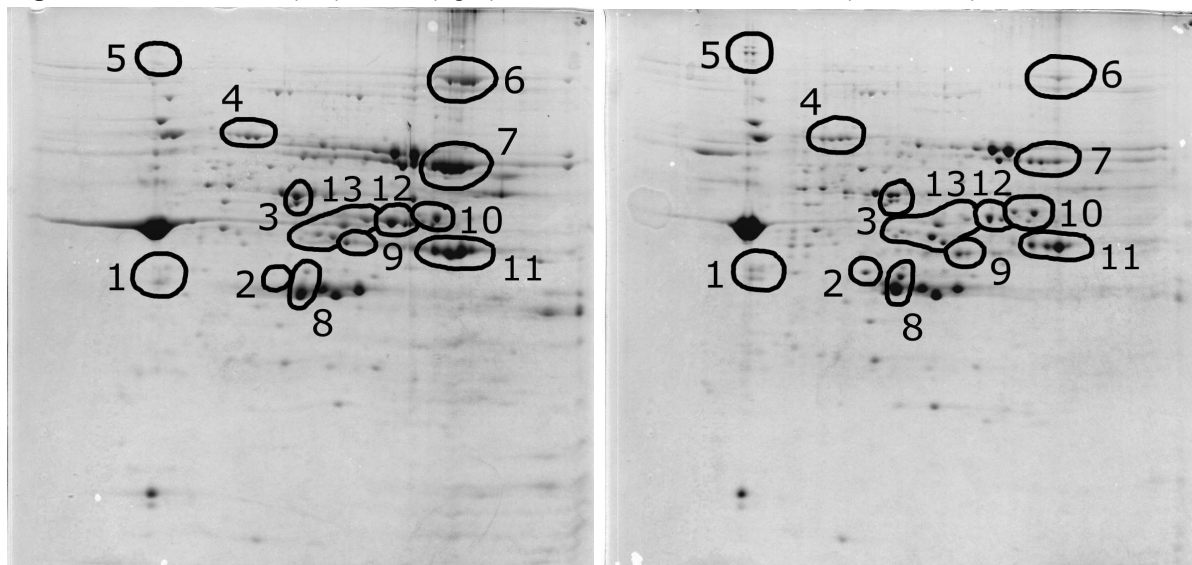
Spot 5, an unidentified protein, was only visible in fig. 51 and may probably be a fragmentation product of actin. Spot X was only detectable in fig. 50 (wildtype only) and also in fig. 55 in all strains. It failed to appear mostly, probably due to technical problems that occurred during performing the analysis.

cMDH also showed fragmentation, however, in several variations. In fig. 50 and 51, a shifted spot of a heavier protein fragment appeared additionally, whereat fig. 52 shows dissociation into proteins of equal molecular weight. In fig. 54, an additional spot of a dissociated, light-weighted protein fragment appeared under the original spot.

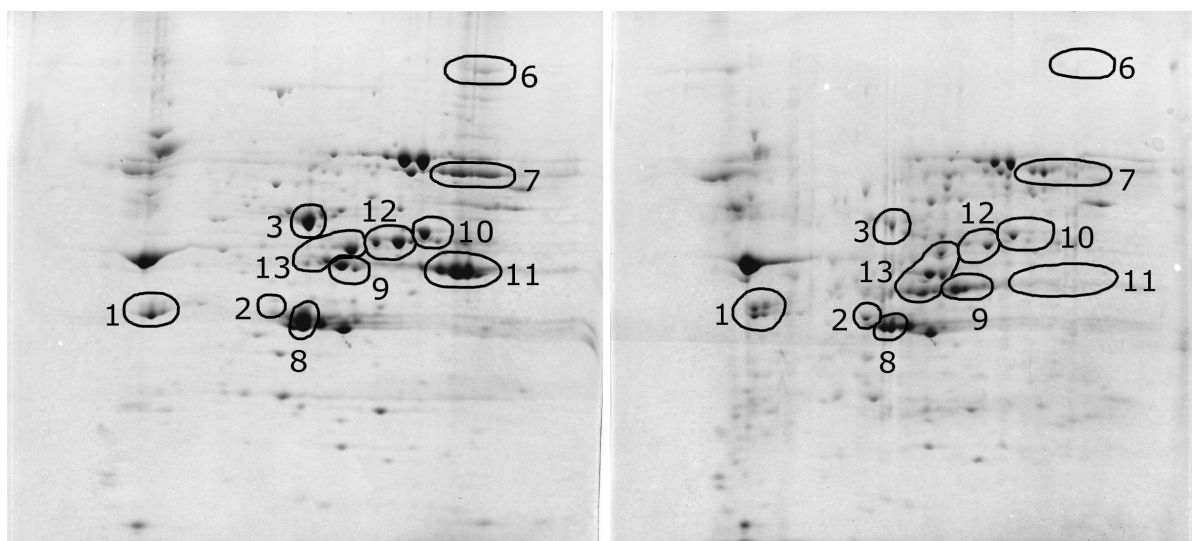
## RESULTS



**Fig. 50:** ATCC 30001 – (left) and R (right), after 10 weeks of treatment (21.1.2010).

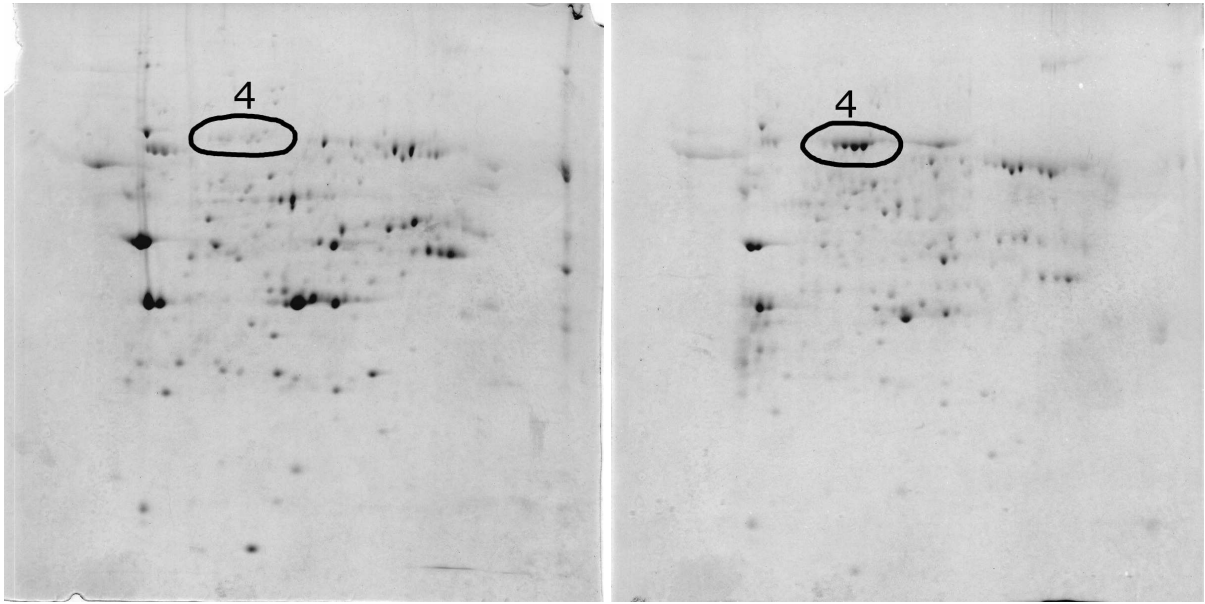


**Fig. 51:** ATCC 30001 – (left) and R (right), after 10 weeks of treatment (29.1.2010).

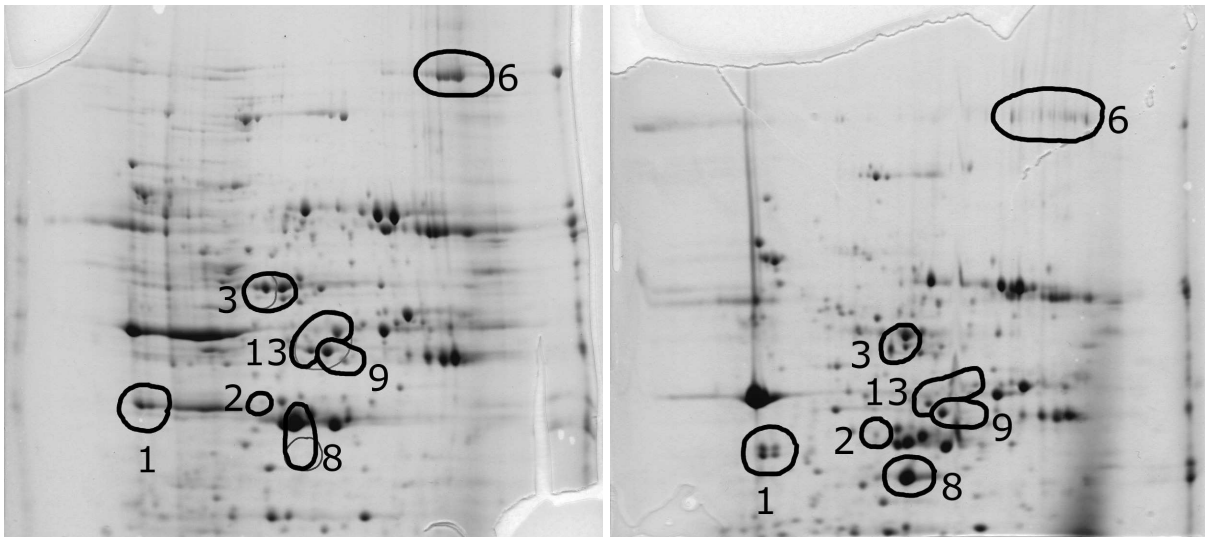


**Fig. 52:** ATCC 30236 – (left) and R (right), after 3 months of treatment (4.2.2010).

## RESULTS



**Fig. 53:** ATCC 30001 – (left) and R (right), after 14 weeks of treatment (18.2.2010).



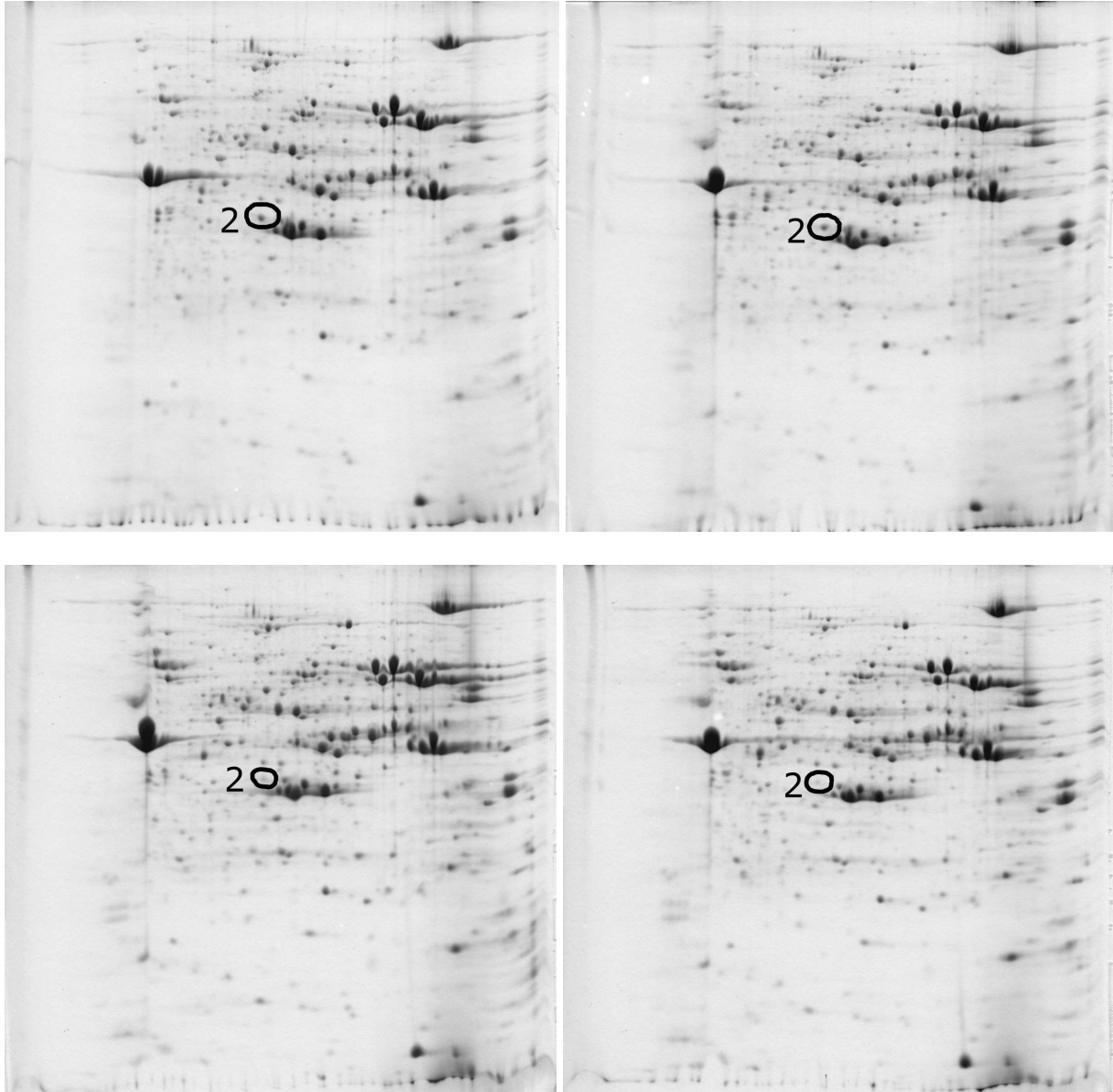
**Fig. 54:** ATCC 50138- (left) and R (right), after 6 months of treatment (26.3.2010).

The manual identification of these proteins refers to LEITSCH et al. (2009).

The final experiment (Fig. 55) was performed to exclude the possibility of protein fragmentation and proteolysis as an error source for variegated protein profiles (see 2.9). It shows that the fragmentation of proteins was not reproducible, even not by avoiding a previous treatment with pentamycin. Also the expected proof for an overexpression of LDH in treated trichomonads failed. Moreover, it is stronger expressed in the wildtype, both untreated and treated, and absent in the R-strains,

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both untreated and treated too. To prove its hereby role in indicating cell damage and proteolysis, a consecutive measurement of LDH activity was performed.



**Fig. 55:** ATCC 30001 wildtype, not treated (up left), treated with 2.5 µg/ml (up right), ATCC 30001 R-strain, not treated (bottom left), treated with 2.5 µg/ml (bottom right). Performed by David Leitsch.

### 3.5.5 Lactate dehydrogenase enzyme activity assay

Tab. 17 shows the results of the measurements of the LDH-induced NADH oxidation absorption rate of wildtypes and R-strains. In all cases, the values were very close to each other and even almost identical, so that a significant difference of the LDH expression grade in wildtypes and R-strains could be excluded.

**Tab. 17:** Measurements of the NADH oxidation absorption rate of ATCC 30001 wildtype and R-strain.

with substrate (pyruvate)		without substrate (blank)	
ATCC 30001	ATCC 30001 R	ATCC 30001	ATCC 30001 R
-0,0135	-0,0076	-0,008	-0,0025
-0,1165	-0,1257	-0,1004	-0,0509
-0,213	-0,247	-0,2006	-0,1254
-0,3226	-0,33	-0,311	-0,2057

## 3.6 Cell permeability assay

The following tables 18–24 display three different approaches in which the pH was measured, before and after addition of pentamycin and in certain time intervals. The values in bold show the lowest acidic pH which was at low, sublethal doses in all cases: mostly at 0.5 µg/ml, twice at 0.25 µg/ml and once at 1 µg/ml. The first experiment was a blank test and it was measured for one time after 30 minutes. Here, the most significant pH difference was at 0.5 µg/ml. In the next experiment, the result was also 0.5 µg/ml at 1, 3, and 6 h post treatment. In the final experiment, the most significant pH difference after 1 and 3 h was at 0.25 µg/ml and after 6h at both 0.25 and 1 µg/ml (bold).

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**Tab. 18:** pH changes 30 min after drug administration (strain TV2).

Pent. (µg/ml)	pH change
0	-0,002
<b>0,5</b>	<b>-0,06</b>
1	0
2	-0,005
3	0,02
4	0
5	-0,01
10	-0,001

**Tab. 19:** pH changes, 60 min after drug administration (strain TV2).

Pent. (µg/ml)	pH change
0	0
0,25	-0,002
<b>0,5</b>	<b>-0,04</b>
1	-0,015
2	-0,025
3	-0,025
5	-0,02
10	-0,025

**Tab. 20:** pH changes, 3 h after drug administration (strain TV2).

Pent. (µg/ml)	pH change
0	-0,005
0,25	0
<b>0,5</b>	<b>-0,04</b>
1	-0,02
2	-0,02
3	0
5	-0,01
10	0

## RESULTS

**Tab. 21:** pH changes, 6 h after drug administration (strain TV2).

Pent. (µg/ml)	pH change
0	-0,005
0,25	-0,01
<b>0,5</b>	<b>-0,04</b>
1	-0,03
2	-0,025
3	0,005
5	0,005
10	0,005

**Tab. 22:** pH changes, 60 min after drug administration (strain ATCC 30236).

Pent. (µg/ml)	pH change
0	-0,002
0,25	-0,001
<b>0,5</b>	<b>-0,002</b>
1	0
2	0,011
3	0,004
5	0,01
10	0,05

**Tab. 23:** pH changes, 3 h after drug administration (strain ATCC 30236):

Pent. (µg/ml)	pH change
0	0,001
<b>0,25</b>	<b>-0,002</b>
0,5	-0,001
1	0,011
2	0,019
3	0,055
5	0,05
10	0,095

## RESULTS

**Tab. 24:** pH changes, 6 h after drug administration (strain ATCC 30236):

Pent. (µg/ml)	pH change
0	0,001
<b>0,25</b>	<b>0</b>
0,5	0,005
<b>1</b>	<b>0</b>
2	0,022
3	0,035
5	0,038
10	0,09

### 3.7 Nile Red stain

The purpose of this experimental procedure was to observe altered membrane properties in pentamycin-treated trichomonads. The lipophilic character of Nile Red leads to a shift of emission from red to yellow according to the degree of hydrophobicity of lipids. Polar lipids like phospholipids appear red whereas neutral lipids like cholesterol appear yellow (DIAZ et al., 2008).

In this study, the analysis however did not produce useful results. Untreated and treated cells were stained to monitor a difference in the membrane composition. After the stain, cells appeared as orange with yellow dots but alterations of the cell shape and colour were not visible. However, due to the viscosity of glycerol, they appeared as blurry and were difficult to handle. Photographs were taken but the results were not successful as all photos were black after developing.



## 4 Discussion

### 4.1 Mode of action of pentamycin

#### 4.1.1 Phenotype of pentamycin-treated trichomonads

Motile, flagellated and pear-shaped trichomonads were observable in axenic cell culture under normal conditions (37°C). When attached to their host cells – or, in this case (*in vitro*), to the bottom of the culture flask – trichomonads alter their morphology to an amoeboid, amorphous shape (ARROYO et al., 1993). Additionally, they can also appear as round cells with or without flagella as well as with two and more nuclei (ABONYI, 1995).

We observed round, non-flagellated, enlarged, and comparably immotile trichomonads when they were exposed to effective concentrations of pentamycin or when they were stressed in general (cellular discomfort due to low temperature, high cell densities, low nutrient supply, old medium). However, high doses of 5 µg/ml (and above) pentamycin led to a fast disruption and lysis of the cell before the parasites could become rounded. ABONYI (1995) described these forms as “developmental stages preceding the appearance of mononuclear flagellate cells”, however, cell divisions were described as amitotic and without the characteristics of mitosis. Although the biology of these round forms is still unknown, it seems that stress may be the connection between our and ABONYI’S (1995) observation.

In 2008, MALIK et al. reported on the presence of homolog genes responsible for genetic recombination and meiosis in *T. vaginalis*. Amongst others, the authors discovered that 7 of 29 meiotic genes were expressed under stress (low-iron

conditions, cold), whereas three of them were expressed exclusively under these stress conditions. Moreover, one of these three genes (Msh5) is described to be meiosis-specific, directing Holliday junctions to a crossover (MALIK et al., 2008).

Comparing these findings with that of ABONYI (1995) and MALIK et al. (2008), stress seems to be the trigger for round forms and genetic recombination. It is thinkable that the developmental stages with multiple nuclei, which ABONYI (1995) described and which we could observe after drug administration, represent a stress-induced stage in which genetic recombination occurs, maybe as a response and reaction of the cells to stress. Further investigations of this phenomenon could be important in understanding the biology of *T. vaginalis* and other parasitic protozoa.

#### **4.1.2 Protein profiles of pentamycin-treated trichomonads**

Trichomonads treated with pentamycin showed an altered protein profile. Protein bands were diminished in their intensity or completely absent. The effect was more distinct, the higher the applied drug dose was. At low, sublethal doses there was either no visible effect, or only one or more single proteins were affected. After treatment with higher doses ( $> 5 \mu\text{g/ml}$ ), most of the proteins seemed to be degraded or absconded. Interestingly, the patterns of the affected proteins were not identical after repeating the experiments under the same conditions. Therefore, the drug does not seem to act specifically, but rather randomly. Hence, a direct interaction of pentamycin and the investigated proteins of the cell seems implausible. Also, epigenetic mechanisms like down-regulation of proteins or expression of proteinases are rather unlikely as a source because it would take more time of incubation until measurable results would be detectable.

## DISCUSSION

The protein diminishment assay at which drug administration resulted in an accretion of proteins in the supernatant and in a decrease of proteins in the cell extract – especially at high doses – provided evidence that proteins leak out of the cell rather than being degraded. Thus, it can be said that the source are cytotoxic effects that lead to cell lysis.

These observations and conclusions fit to previous studies on the mode of action of pentamycin, and polyenes in general. In studies in the 1960s, it was reported that polyenes such as nystatin and amphotericin B cause membrane distortion in yeast or *Candidia* cells and, consequently, a leak out of cytoplasmic material, which was considered as the primary reason for cell death (SUTTON et al., 1961; DEMEL & VAN DEENEN, 1965; ZYGMUNT, 1966). In the 1970s, these investigations on the mechanism of action of polyenes were continued. DE KRUIJFF et al. (1974) described the effect of several polyenes such as amphotericin B, nystatin and etruscomycin on *Acholeplasma laidlawii* cells as a formation of transmembrane pores with subsequent membrane disruption (DE KRUIJFF et al., 1974).

In 1978, it could be shown that an altered membrane permeability depends on the applied drug dose: At high doses, polyenes led to a complete disruption of yeast cells, whereas at low doses the cells were primarily not disrupted but lost potassium and other small ions as a consequence of membrane pore formation (PALACIOS & SERRANO, 1978). This also fits to the current study as we observed 100% of lysed cells at high doses (10 µg/ml and above) after a short time of incubation, whereas at lower doses (below 5 µg/ml) the cells were impaired, but not lysed. PALACIOS & SERRANO (1978) also measured a lower pH value in the cell environment after appliance of low and sublethal drug doses due to the exclusive outstream of small ions including H<sup>+</sup>. The cell permeability assay in our study also revealed the lowest

## DISCUSSION

pH values at 0.25 µg/ml and 0.5 µg/ml, respectively, after treatment with pentamycin in a range from 0.25 µg/ml to 10 µg/ml.

In general, the effect seems to depend on the molecular weight of the respective polyene and the applied dose. Polyene-induced membrane pores with loss of intracellular ions but no loss of viability have also been reported as well as formation of large planar aggregates between molecules of the membrane and the polyene leading to membrane instability (KERRIDGE, 1979; CASTANHO et al., 1992). For pentamycin in particular, it has been reported that it may enhance the effect of the cancer chemotherapeutic agent bleomycin by increasing the permeability of tumour cells for bleomycin which acts as an inhibitor of DNA, RNA, and protein synthesis (NAKASHIMA et al., 1974; TWENTYMAN, 1976).

These observations stand for a mode of action based on loss of membrane stability and resulting in leak out of cytoplasmic cell compartments, as demonstrated in this study.

#### **4.1.3 Mode of action of pentamycin at molecular level and compared to metronidazole**

It has been assumed that the mode of action of pentamycin is comparable to that of other polyenes and is based on an interaction with the eukaryotic cell membrane. However, the molecular mode of action and the induction of cell death have still not been entirely elucidated. Several studies from the 1960s to the 1980s have been published on this question that should be reviewed here shortly:

Polyenes, a subclass of the chemical class of the macrolides, have – in addition to the lactonized macrocyclic ring of carbon atoms – a series of two to seven conjugated double bonds. All organisms that are susceptible to polyenes contain sterols in their cell membrane and are eukaryotic; bacteria are unaffected by polyenes as they do not contain sterols and have a cell wall, in addition to their cell membrane (HAMILTON-MILLER, 1973). However, BOLARD (1986) has stated that sterols are not a primal requirement for polyene susceptibility. Apart from that, he could show that the addition of cholesterol to filipin causes a spectral change indicating a direct binding between these two molecules. Further, when sterols are in aqueous solution, the presence of a cholestane ring and a  $\Delta^{22}$  double bond is required for optimal interaction.

However, in the liposomal system – when the sterol is embedded in a natural or artificial membrane – an additional  $3\beta$ -OH bond on the sterol nucleus is required for optimal interaction (NORMAN et al., 1972). Evidence for a binding of polyenes to sterols has also been given by the addition of free sterols into the culture medium which prevented the drug from binding with the sterols of the membrane (GHANNOUM & RICE, 1999). Further criteria for a binding are the distribution of double bonds in the

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nucleus of the sterol that are related to differing polyene susceptibilities and the molecular weight of the polyene: polyenes with lower molecular weight are more toxic than those with higher molecular weight. This would mean, in particular, that pentamycin as a pentaene is more toxic than amphotericin B (a heptaene) but less toxic than dienes, trienes, and tetraenes (nystatin) (HSUCHEN & FEINGOLD, 1973; HOLZ, 1974; KERRIDGE, 1979).

Since it is known that the target of polyenes are sterols of the cell membrane, the mechanism of action has been proposed as following: in case of amphotericin B (heptaene) and nystatin (tetraene), hydrogen bonds are formed between their OH-group and that of the sterol. As this bond is linked together by van der Waals-forces, the hydroxyl side of the polyene faces inward which leads – together with a second polyene that is linked to the adjacent sterol – to formation of pores with a defined annulus. This configuration leads to damage of the cell membrane, an altered permeability with leakage of cytoplasmic material like proteins, and finally to cell death (KERRIDGE, 1979; GHANNOUM & RICE, 1999).

Apart from that, several authors (HAMMOND et al., 1974; HAMMOND & KLIGER, 1974; GALE, 1974; CHEN et al., 1978; JOHNSON et al., 1978) have stated that a dissociation of  $K^+$  permeability and leakage is a hallmark of the polyene interaction with the membrane, however, this action may not be primarily responsible for necrosis. Also, CHEN et al. (1978) have suggested that the induced aqueous pores may not be the primary reason for the death of the cell; the authors found vital cells with loss of cytoplasmic material but no loss of viability.

PALACIOS & SERRANO (1978) have demonstrated an alternative action mechanism basing on amphotericin B-induced inhibition of maltose fermentation of

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*Saccharomyces cerevisiae* and an increase of proton permeability of the cell membrane. This would also fit to the observation of  $K^+$  leakage which is linked to the proton gradient (KERRIDGE, 1979).

BOLARD (1986) has differentiated the action mechanism of polyenes into that of large polyenes which lead to alteration of membrane fluidity and permeability and that of short polyenes which lead to membrane disruption. Moreover, he proposed an additional influence of environmental factors: the ratio of antibiotics and sterols, the mode of addition of antibiotics, the incubation time and notably the gel state of the membrane, which is not identical in cells, making it sensitive for polyenes even if they do not contain sterols. Also the peroxidation of membrane lipids and inhibition of membrane-bound enzymes have been observed and assigned to the toxic effect of polyenes (BOLARD, 1986).

A recent study on germinating conidia of *Penicillium discolor* could show that natamycin (pentaene) works differently than nystatin (tetraene) and filipin (pentaene), which both act by disrupting the plasma membrane while natamycin binds to ergosterol but inhibits endocytosis and further fungal growth (VAN LEEUWEN et al., 2009).

In the context of polyenes against erythrocytes infected with *Plasmodium falciparum*, amphotericin B has been reported to be affine for oxidized cholesterol. This may derive from the oxidation of the erythrocyte membrane caused by the infection with the malaria parasite (WIEHART et al., 2006).

Summing up these studies, the described cytolytic mode of action is corroborated by the results of the current study. Although the members of the polyenes also have characteristic properties, the general mode of action is the same for all of them, thus,

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studies on amphotericin B, nystatin and filipin can provide a basis for investigations on pentamycin. Also the binding properties to ergosterol and cholesterol are comparable.

Thus, it can be stated that the antibiotic effect of pentamycin against fungal and protozoan cells relies on a membranolytic, osmotic mode of action as a consequence of an interaction with polyene and ergosterol (in fungi, *Trypanosoma* and *Leishmania*) or cholesterol in most other protozoa. Our observation of a more rapid cell lysis after treatment with higher doses fits also with BOLARD (1986) who described “larger pores, holes, or other sources of membrane damage” after “treatment with high doses” (BOLARD, 1986).

This mode of action of pentamycin, which also has a dramatic visible effect, is different to that of metronidazole, which is also an explanation for the non-selectivity of metronidazole-susceptible and -resistant strains of *T. vaginalis* to pentamycin. The mode of action of metronidazole can be summarized as following:

Metronidazole diffuses into the cell where it is reduced into its active form and leads to the release of free radicals which induce DNA damage and cell death within a few hours (FREEMAN et al., 1997). This mechanism takes action inside the cell and is also the reason for a different appearance of metronidazole-treated cells: They are observable as immotile and can be distinguished from vital cells by trypan blue stain, whereas pentamycin-treated trichomonads are visibly disrupted and lysed.

Indeed, this basic mechanism is generally accepted but there are diverse theories on the molecular mode of action of metronidazole. MÜLLER (1986) proposed an anaerobic reduction of the NO<sub>2</sub>-group of metronidazole by PFOR by concomitant oxidation of pyruvate to acetyl-CoA. Consequently, cytotoxic nitroradicals lead to



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DNA strand breaks, inhibition of mitosis, and finally to cell death. HRDY et al. (2005) postulated an alternative mechanism whereby the electron source is malate instead of pyruvate and the reducing enzyme is NAD:ferredoxin oxidoreductase instead of PFOR, however, the general pathway does not differ from that proposed by MÜLLER (1986).

In 2009, LEITSCH et al. suggested a novel and completely different mode of action of metronidazole. The authors discovered that the antioxidative enzyme thioredoxin reductase reduces metronidazole which subsequently forms covalent adducts with thioredoxin reductase and further proteins that are involved in the thioredoxin-mediated redox regulation. Hence, the activity of these enzymes is sharply decreased. Since thioredoxin is essential for decomposition of damaging peroxides, the toxic effect of metronidazole seems to be based rather on the obviation of efficient cell detoxication (LEITSCH et al., 2009).

Although these points of metronidazole action remain still unclear, it is generally accepted that it harms the cell via release of radicals and oxidative stress by its metabolism. In this case, a benefit for pentamycin as an anti-trichomonal pharmaceutical drug may be its faster and more visible effect, whereas its disadvantage may be its non-selectivity to target cells which would require a local, topical application form.

## 4.2 Dose-response relationship

### 4.2.1 Microscopic observations

As mentioned before, a positive correlation of dosage regimen, incubation time, and impaired and lysed cells can be stated. At lower doses from 0.5–4 µg/ml, more inhibited cells – that were partially rounded and resembled to blebs – than lysed cells were observable. With increasing dose, the amount of lysed cells was increasing too whereas the amount of rounded, inhibited cells was diminishing. It is obvious that a higher dose led to faster cell death; therefore, the incubation time might be too short for rounding and blebbing. As nearly all cells treated with 10 µg/ml and above were lysed, it seems plausible that the amount of pentamycin molecules that are able to bind to membrane cholesterol correlates with the severity of cell damage as more locations with pores may lead to faster necrosis.

We also observed granula-like particles around the cells after a dose of 50 µg/ml – which is more than a ternary lethal overdose. These particles only appeared at this dose. Their origin is unclear, however, redundant pentamycin molecules – which might be thinkable after such an high dose as there would be more molecules of pentamycin than free sterols – may not bind to membrane cholesterol, but form aggregations with lipids from the leaked cytoplasm or from ingredients of the medium.

The morphology of pentamycin-treated trichomonads resembles to that of miltefosine-treated trichomonads which also resulted in “rounding up, blebbing, and total lysis of the organism” (BLAHA et al., 2006). Miltefosine, an antileishmanial antibiotic, belongs to the alkylphosphocholines but has a similar mode of action to

pentamycin as it also interacts with the cell membrane. Also, low doses require longer incubation time whereas high doses induce necrosis within in a short time and “without prior rounding up”, like in this current study (BLAHA et al., 2006).

The blebbing and rounding of the cells might also have another possible background. According to ABONYI (1995), BLAHA et al. (2006) and MALIK et al. (2008), these stages are physiological reactions to stress such as drug administration and nutrient depletion. A further explanation may be that the round forms are connected with apoptosis. It was postulated that drug treatment leads to programmed cell death with its characteristic features (such as nuclear condensation and reduced cytoplasm) in protozoans, like metronidazole in *Blastocystis hominis* (NASIRUDEEN et al., 2001, 2004). Recently, apoptosis – which was formerly believed to be a feature of multicellular organisms – was also proved in *T. vaginalis* cells exposed to stress conditions as well as formation of round pseudocysts with internalized flagella (BENCHIMOL, 2008). The protozoan parasites seem to react to toxic substances in the environment and induce apoptosis as a defense strategy for the welfare of the whole cell population. A smaller cell density increases the probability to evade the hosts' immune system (NASIRUDEEN et al., 2004). Especially when protozoans are assembled in dense batches, this might be a plausible action and resembles also to the purpose of apoptosis in multicellular organisms: to protect the population of cells with programmed cell suicide. It seems that the parasites response to the presence of cytokines and antibiotic drugs with the same physiological reaction; but in contrary to the immune system, antibiotic drugs are not deceivable in that way.

#### 4.2.2 Efficacy of pentamycin

The impact of pure pentamycin is strong and fast, requiring only a small dose of 15 µg/ml to eradicate 100% of trichomonads within 1h. It is a formidable effectivity compared to that of pentamycin with less purity (28%) and metronidazole. There are other antibiotic drugs that have a similar efficacy but also more disadvantages.

N-chlorotaurine, an oxidant produced by human immune cells to combat infections and inflammations which has also been in test use against *T. vaginalis*, also showed lower effectory concentrations than metronidazole, however, it does not reach the efficacy of pentamycin (EC<sub>50</sub> after 1h: 1.636 mg/ml for the highly metronidazole-sensitive strain ATCC 30001, and 5.08 mg/ml for the metronidazole-resistant strain ATCC 50138, respectively). Moreover, N-chlorotaurine is not applicable as an antitrichomonal drug due to its low stability (FÜRNKRANZ et al., 2010).

Also Hamycin, a polyene similar to amphotericin B, showed a similar trichomonicidal activity of 1 µg/ml but also killed mammalian tissue cell culture lines HeLa and BHK-21 at this concentration, which makes it inapplicable against trichomoniasis too (LUSHBAUGH et al., 1995). The efficacy of pentamycin against *T. vaginalis* is most likely comparable to miltefosine which showed similar values (EC<sub>50</sub> of 3.26 µg/ml after 30 min) (BLAHA et al., 2006) but also shows partially severe side effects.

The remarkable low inhibitory and effectory concentrations of pentamycin (94%) compared to pentamycin (28%) and metronidazole may be explainable by the higher purification grade of pentamycin (94%) on the one hand, and by its direct and expeditious mode of action on the other hand. The low dose that is required for an effective eradication of trichomonads may be advantageous for a topical application.

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Because of its affinity for membrane sterols, it would also affect human cells, thus, it seems important to avoid side effects by a narrow sphere of action.

A study on the safety, tolerability and pharmacokinetics of intravaginal pentamycin reported of “mild or moderate vaginal discharge and mild symptoms of vaginal irritation (mainly pruritus or vaginal sensation)” (FREY TIRRI et al., 2010) after the application of the drug. The patients received doses from 3 to 100 mg for a duration of 6 days (FREY TIRRI et al., 2010). In this case, a drug dose that can be adjusted to the number of trichomonads could help to avoid side effects. Also a re-formulation could alleviate adverse events. Amphotericin B, for instance, is far less toxic for the patient when applicated as a liposomal formulation (GHANNOUM & RICE, 1999).

There was no significant difference between the four investigated strains in their sensitivites to pentamycin. All differently metronidazole-susceptible strains had nearly the same susceptibility to pentamycin as investigated by the dose-effect relationship tests. Thus, the sensitivity of trichomonads to metronidazole does not correlate with that to pentamycin. This is due to the different modes of action these drugs have and is a high advantage of pentamycin, as it can be used in patients infected with metronidazole-resistant strains. Hence, resistant trichomonads would be eliminated in the same way as metronidazole-sensitive strains.

These findings are comparable to the *in vitro* activity of miltefosine against *T. vaginalis* where also four differently metronidazole-susceptible strains did not differ in their sensitivity to miltefosine (BLAHA et al., 2006).

### 4.3 Resistance

#### 4.3.1 Phenotype of adapted trichomonads

Trichomonads, treated continuously with sublethal concentrations of pentamycin over a period of six months, appeared partly larger, impaired, and slower in their motility and their growth rate. These findings correlate with the observations in the reviews of HAMILTON-MILLER (1973) and GHANNOUM & RICE (1999), who reported of polyene-resistant *Candida* strains that grow slower than the parental strains, have a greater cell size, and are less virulent. There is also remarked that polyploidy correlates directly with resistance to nystatin and cell size (HAMILTON-MILLER, 1973).

A slower growth was also observed in metronidazole-resistant trichomonads (LEITSCH et al., 2010). These phenotypic appearances also correlate with the observations of ABONYI (1995) who described the round forms and assumed a developmental stage. It is likely that stress, in this case the treatment with pentamycin, is the source for rounding and polyploidy as it occurs in this stage, and also for genetic recombination that has been reported from *T. vaginalis* previously by MALIK et al. (2008). The authors discovered that the parasite expresses a set of 18 meiotic genes of which 7 were expressed exclusively under stress – 5 under low iron conditions and 2 in cold-induced pseudocysts. This set comprises genes involved in chromosome assembly and segregation, direction of Holliday junction to crossover, sister chromatid cohesion, DNA repair and more (MALIK et al., 2008). The pseudocysts induced by low temperature seem to be identical with the hereby observed round forms, not least because at lower temperatures (26°C, 20°C) we also found more rounded trichomonads.

It is thinkable that the round trichomonads are the link between induced stress and observed genetic recombination, which is in fact the same principle as in sexual reproduction: exchange of genetic material to enhance the fitness of the next generation. Although trichomonads are haploid and asexual organisms, a one-step meiosis that is confined to homologous recombination may be possible, as it was observed in *Giardia intestinalis* too (POXLEITNER et al., 2008).

#### **4.3.2 Establishment of an adaption to pentamycin and cross resistance to amphotericin B**

It is known that trichomonads can develop true resistance against metronidazole *in vitro* and *in vivo* whereas the effective mechanism of resistance is still controversial (KULDA, 1999; LEITSCH et al., 2010). In the current study, we wanted to monitor the process of long-term treatment with pentamycin and if trichomonads have the potential to develop resistance to pentamycin within six months. It could be shown that true resistance does not seem to occur. Cells continuously treated with higher doses – increasing by 0.5 µg/ml every other day – rather died than developed resistance, at the latest at 6 µg/ml. However, after stopping the treatment a partial recovery was observable after 24 hours. This may have its reason in cells that were not affected by the drug as they were inside in a cell cluster and could proliferate; a phenomenon called pseudo-resistance or passive resistance.

The procedure of applying sublethal concentrations 2–3 times a day – starting at 0.25 µg/ml and increasing cautiously the dose by about 0.1 µg/ml, depending on the health of trichomonads – led to an adaption to pentamycin. After 4 months, the sensitivity to pentamycin was found to be reduced about 8.5% and after 5 months

even about 63%. This proves an adaption of trichomonads to the drug, at least until doses of 15 µg/ml and more that still led to 100% lysis. These follow-up assays were performed two times with the metronidazole-resistant strain (ATCC 50138).

True resistance – that also means a hereditary transmission – did not develop within six months which resembles previous investigations in polyene resistance. In the 1970s, it was reported that even after 15 years of polyene antibiotic use against *Candida*, resistance never was a serious problem and only a few strains showed the ability to express resistance (HAMILTON-MILLER, 1973). In 1999, almost 30 years later, resistance – which was mainly investigated against amphotericin B and nystatin – was still rare and confined to less common species of *Candida* (GHANNOUM & RICE, 1999). Recently, it could be shown that only one isolate of several *Candida* species expressed resistance against amphotericin B (AMRAN et al., 2011). Due to similarities between pentamycin and amphotericin B in chemical structure and mode of action, these properties could be allocated to pentamycin too.

Although it is noted that “only a slight increase in resistance to polyenes would render them virtually useless as chemotherapeutic agents” (HAMILTON-MILLER, 1973), it is unclear whether the hereby observed increase of adaption would give a cause for concern. In the current study, doses of 15 µg/ml and above, always led to a complete eradication of trichomonads *in vitro*, even in adapted strains. Up from 5 µg/ml, no adaption was observable. Moreover, adaption was immediately lost when subcultured in fresh medium without the drug.

Also, no cross resistance between the two polyenes pentamycin and amphotericin B was found in this study. On the contrary, after four months of continual, daily treatment with sublethal doses of pentamycin, strains expressed a higher sensitivity



to amphotericin B, namely about a fourth. Actually, the sensitivity of both strains resembled each other as ATCC 50138 (metronidazole-resistant) had an increased susceptibility about 23.2% and TV2 (metronidazole-sensitive) about 25.8%.

Altogether, cross resistance can not only be excluded but a combination of both drugs would act in concordance which is simply explainable by the action mechanism of both polyenes and the increased accumulation of cholesterol-interacting molecules. Also because there are no reports of amphotericin B-resistant trichomonads until now, these findings look advantageous for a clinical use of pentamycin.

#### **4.3.3 Protein profile of long-term treated trichomonads**

Long-term treatment with pentamycin altered the protein profile of trichomonads but differently than single-dose treatment. Whereas single-dose treatment resulted in a diminishment and leak out of proteins, the consequence of long-term treatment seemed to be proteolysis as most of the altered protein spots disappeared or were shifted. We could exclude a change in the protein profile that might be an evidence for a physiological reaction of the cell and therefore resistance. Moreover, the patterns of the protein profiles were differing from each other, indicating an unspecific mode of action.

It can be assumed that pentamycin and stress caused by long-term treatment, respectively, affect and impair proteins and lead to fragmentation. Thus, the spots were either missing (complete proteolysis) or partitioned and shifted in their position (fragmentation of a protein into smaller and light-weighted parts). For instance, actin,

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a protein of the cytoskeleton, partly seemed to dissociate, as in treated cells more spots of actin and also a novel, unidentified actin spot (spot 5), were visible.

Furthermore, some spots in treated trichomonads were more intense, which might be interpretable via stronger protein expression or adduct formation of protein fragments. As shown previously, covalent adducts are identifiable as novel protein spots and shifts of the respective proteins (LEITSCH et al., 2009; LEITSCH et al., 2010). However, the patterns of the spots in these experiments can be interpreted generally as a result of cellular discomfort caused by stress induced by drug administration.

Indeed, LDH was most frequent altered protein and was continuously present in 50% of the gels. It was stronger expressed in treated cells but it was not possible to assign this to an increased protein expression or adduct formation. In clinical diagnosis, LDH is a parameter for damage of tissue and organs due to lysed cells and migration of cytoplasmic content into blood, plasma and serum. However, LDH can also be a reaction of the cell to insufficient energy production: LDH steps in when glycolysis expires as it produces lactic acid and  $\text{NAD}^+$  of pyruvate and NADH.  $\text{NAD}^+$  can be used as electron acceptor from glucose to form NADH and to continue energy production (GOODSELL, 2008). As mentioned before, “mellow” effects of polyenes also include the inhibition of glycolysis (PALACIOS & SERRANO, 1978), thus, the increased expression of LDH could possibly refer to that.

Also remarkable was the poor expression of PFOR in general. This iron-sulfur protein is an enzyme in the hydrogenosomal metabolic pathway and oxidizes pyruvate to acetyl-CoA via reducing ferredoxin and releasing  $\text{CO}_2$ . Ferredoxin transfers electrons to hydrogenase which converts  $\text{H}^+$  to  $\text{H}_2$  (MÜLLER, 1993). PFOR is assumed to be involved in the activation of metronidazole as reduced ferredoxin subsequently

reduces metronidazole to its nitroradical anion (MÜLLER, 1986). Furthermore, also a role of PFOR in resistance against metronidazole was proposed, due to its decreased or absent activity in resistant strains (KULDA, 1999).

When iron is lacking, PFOR – which has a high uptake rate of iron – also shows diminished activity which might be a reason for its poor presence here (LEITSCH et al., 2009). Stress caused by drug treatment may lead to a higher demand for iron for essential metabolic functions of the cell, thus lowering the activity of PFOR. This is also in agreement with the biological attribute of *T. vaginalis* responding to varying iron availability by mechanisms of differential gene expression (repression or overexpression of iron-depending enzymes to adapt itself to low nutrient supply) (GOMEZ et al., 2010).

After cultivating trichomonads with additional iron supplement for four weeks, a stronger expression of PFOR was observable. The metronidazole-resistant strain (ATCC 30001) showed a remarkably increased susceptibility to metronidazole when incubated with additional iron. The novel, alternative mechanism of resistance assigns high-level metronidazole resistance to an absent thioredoxin reductase activity (LEITSCH et al., 2010). This finding supports the hypothesis of anaerobic metronidazole resistance which relies on a minimal PFOR activity (KULDA, 1999). It is also thinkable that true metronidazole resistance may be based not only on one single mechanism. This question still remains to be resolved, equally to the question of resistance to pentamycin.

In the current study, the respective protein profiles of pentamycin-treated cells were rather the consequence of stress and harming effects caused by treatment and, to a certain extent, also due to manufactural differences between the 2D SDS-PAGE gels.

## DISCUSSION

A final experiment was performed to investigate the role of LDH, and also to distinguish between wildtypes and R-strains that were treated 2h before and 48h before, respectively. Differences between these two approaches were expected to appear, in fact, all four gels were almost identical and no differences between any spot could be observed. Even LDH, that was stronger expressed in 50% of the previous analyses, appeared to be equally distinctive in all cases. Therefore, the hypothesis of a stronger expression of LDH in treated cells as well as an influence of drug administration shortly before harvesting could not be verified.

Finally, long-term treatment during six months had no impact on the protein profile at all – although it cannot be excluded –, rather it may have been the consequence of inaccuracies in the 2D SDS-PAGE experimental procedure. The subsequent LDH activity assay supports this conclusion as no differences in the enzyme activity between wildtypes and R-strains could be detected.

In summary, the majority of the identified proteins that were affected by long-term treatment are essential enzymes in metabolic pathways:

Enolase catabolizes 2-phosphoglycerate to phosphoenolpyruvate which is an essential step in glycolysis. As low dose of pentamycin may inhibit glycolysis (PALACIOS & SERRANO, 1978), a fragmentation and impairment of enolase could refer to that.

Actin, a structure protein, is essential in maintenance of the cytoskeleton. A degradation of actin may also affect the stability of the cell. Both cytosolic (cMDH) and hydrogenosomal malate dehydrogenase (hMDH) are responsible for catabolising malate to pyruvate, and especially hMDH is associated tightly with PFOR.

ADH1 belongs to the enzyme class of oxidoreductases, detoxifying alcohols to the respective aldehydes and ketones. An impairment of these proteins (and also the above mentioned LDH and PFOR) would obviously lead to malfunctions and damages of the cell.

#### **4.3.4 Mechanisms of adaption**

Although there were no significant differences in the protein profiles of untreated trichomonads and those after long-term treatment, differences in morphology and susceptibility to pentamycin were detectable. Adaption to pentamycin after six months of long-term treatment can be stated. In general, resistance to polyene antibiotics is rare and a build-up of a full established resistance can be a process of years (GHANNOUM & RICE, 1999).

Knowledge in polyene resistance comes mainly from studies on fungi and ergosterol-containing protozoa with amphotericin B, nystatin and also filipin. Exclusive resistance studies on pentamycin and cholesterol-containing protozoa have not been published yet, accordingly in this current study we were considering the hitherto reported equalities and similarities between the respective polyenes and of cholesterol- and ergosterol-containing organisms.

In the 1970s, numerous studies have been published on the resistance to polyenes whereas a few should be cited here: To this date, resistance to polyenes did not present a serious problem, even after 15 years of polyene use and, as mentioned before, resistance did not occur except in few strains of *Candida* (HAMILTON-MILLER, 1973).

From observations on laboratory produced *S. cerevisiae*-mutants resistant to nystatin it was proposed that zymosterol – a cholesterol intermediate – may have replaced ergosterol due to a defective production of ergosterol and accumulation of zymosterol in the cell membrane, which finally should prevent the drug from binding (THOMPSON et al., 1971). Furthermore, studies on polyene-resistance in *Microsporum* revealed that a lower susceptibility correlates with a decreased sterol content (CAPEK & SIMEK, 1972). However, mutants lacking entirely ergosterol are very unlikely to develop in nature.

Regarding the mechanism of drug resistance in general, there are two controversial theories: FRYBERG (1974) has suggested that “development of resistance occurs by selection of naturally occurring resistant cells, present in small numbers in the population” (GHANNOUM & RICE, 1999). Concerning polyenes, this would mean that sterols in these naturally resistant cells are modified so that the affinity for polyenes is reduced. In such populations these cells would replace the majority of non-resistant ones. On the contrary, ATHAR & WINNER (1971) have stated that resistance results rather from mutations – such as induced by drug treatment and consecutive differentiated gene expression as feedback mechanism – than from selection.

Although many open questions remain, an explanation for the process of gradual adaption could also be a small naturally resistant population that spreads via lateral gene transfer (transformation, transduction) as well as partially altered sterol molecules that still allow a – hindered – polyene binding.

Whether the basic mechanism may be selection or mutation, it can be stated for polyenes that the level of resistance is proportional to changes in the sterol content of the cell membrane, either qualitative or quantitative. According to HAMILTON-MILLER

(1973), “resistant cells with altered sterol content should bind smaller amounts of polyene than do susceptible cells” (HAMILTON-MILLER, 1973).

In case of *Candida albicans*, this phenomenon could be attributed to the total ergosterol content of the cell, replacement of some or all polyene-binding sterols by substitution of less-well binding sterols, e.g. by a 3-hydroxy or 3-oxo derivative, or reorientation or masking of ergosterol that changes sterically or thermodynamically dependent binding (GHANNOUM & RICE, 1999). A decrease in the ergosterol content to 74–85% in *C. albicans* has been demonstrated (DICK et al., 1980) as well as a higher affinity of polyenes to  $\Delta^{5,7}$ - and  $\Delta^7$ -sterols than to  $\Delta^8$ -sterols (FRYBERG, 1974).

Moreover, an amphotericin B-resistant strain of *Cryptococcus neoformans* showed a defect in  $\Delta^{8,7}$ -sterol isomerase, leading to accumulation of ergosta-5,8,22-dienol, ergosta-8,22-dienol, fecosterol and ergosta-8-enol, with a final depletion of ergosterol (KELLY et al., 1994). These findings fit to a previous study of 1974 in which *Candida* strains resistant to amphotericin B and nystatin were found to possess altered sterols with a lower affinity to polyenes. Especially a lack of ergosterol and  $\Delta^{8,7}$ -sterols was described (WOODS et al., 1974).

MBONGO et al. (1998) have demonstrated that amphotericin B-resistant *Leishmania donovani* promastigotes show increased membrane fluidity and possess cholesta-5,7,24-trien-3 $\beta$ -ol (an ergosterol precursor) as major sterol in their membrane instead of ergosterol. Additionally, RAKOTOMANGA et al. (2005) have discovered that miltefosine-resistant *Leishmania donovani* promastigotes show alterations of their fatty acid and sterol metabolism, resulting in a lower membrane fluidity due to a lower amount of unsaturated phospholipid alkyl chains compared to those of wildtypes.

## DISCUSSION

A similar mechanism would be plausible for the build-up of pentamycin resistance in *T. vaginalis* due to the reported alterations in the sterol metabolism and altered membrane cholesterol.

The synthesis of cholesterol, from acetyl-CoA to precursor molecules like isoprenoids and squalenes, provides many possible ways for the cell to intervene in the action metabolism and forming sterol derivatives that lead to resistance. If truly resistant strains could be established, knock-out mutants of the candidate gene(s) could possibly show more knowledge of the mechanism of resistance.

It was shown, that there is no cross resistance between amphotericin B and pentamycin, in contrast, these drugs seem to act synergistically. Cross resistance of metronidazole-resistant *T. vaginalis* to nitazoxanide, furazolidone, toyocamycin and 2-fluoro-2'-deoxyadenosine has been described (WRIGHT et al., 2010) and cross resistance of amphotericin B-resistant *Leishmania* to azoles (although these drugs act differently to polyenes by inhibition of ergosterol biosynthesis) as well (OUELLETTE & WARD, 2003). Cross resistance of metronidazole to pentamycin seems unlikely due to the complete different mode of action, however, as amphotericin B is very similar to pentamycin, further research on the interaction of both drugs could be promising. In *Leishmania* and *Trypanosoma* inhibition of fatty acid and sterol biosynthesis and further depletion of membrane sterols can be achieved by azole and triazole compounds (ROBERTS et al., 2003).



#### **4.4 Relevance of temperature**

Lower temperatures did not only impair the motility, vitality and reproducibility of trichomonads, but also seemed to make them less susceptible to pentamycin. The reversibility of this effect could be an evidence for a better affinity of pentamycin to the membrane of the parasite when it finds optimal living conditions.

The function of sterols in the cell membrane is to keep up its integrity – which has been attributed to specialised microdomains called “lipid rafts” and caveolae that contain increased amounts of cholesterol (SIMONS & TOOMRE, 2000) – and also to increase its flexibility and fluidity over the range of physiological temperatures. At higher temperatures, cholesterol makes the membrane more rigid, impairing the motility of the phospholipids, while at lower temperatures it keeps up its fluidity.

An altered membrane fluidity caused by non-lethal temperature changes could eventually impair the correct binding of pentamycin and cholesterol which could be recovered again at physiological temperature settings.

#### **4.5 Advantages of pentamycin as an antitrichomonal drug**

The results of this study show that pentamycin has the potential as a pharmaceutical drug against *T. vaginalis*. As mentioned before, the mode of action of pentamycin and the established drug metronidazole is entirely different. A previous, less pure preparation of pentamycin with a dose regimen of 3 mg daily up for 10 days or 6 mg daily up for 5 days (BALMER, 2009) shows a greater effectiveness and lower necessary application rate *in vivo* than metronidazole with a dosage regimen of 2 g

once or 250 mg three times daily for 7 days (CUDMORE et al., 2004). The MLC (minimal lethal concentration) after 24h for pentamycin was shown to be 1 µg/ml for all four included strains in this study. Compared to metronidazole, the metronidazole-resistant strain (ATCC 50138) showed an MLC *in vitro* of 4 µg/ml (anaerobic) to 150 µg/ml (aerobic) and the highly metronidazole-sensitive strain (ATCC 30001) showed an MLC *in vitro* of 0.5 µg/ml (anaerobic) to 2 µg/ml (aerobic) (MÜLLER & GORRELL, 1983). Regarding this, pentamycin is four times more effective than metronidazole.

Moreover, the increasing resistance of trichomonads against metronidazole – two different mechanisms have been described (KULDA, 1999; LEITSCH et al., 2010) – still remains a problem. In case of pentamycin, the situation looks different: As described for polyenes in general, resistance does hardly occur (HAMILTON-MILLER, 1973; GHANNOUM & RICE, 1999), further, in this current study it could also be shown that true resistance does not develop, not even after six months of permanent resistancy stress. However, we could show an adaption of *T. vaginalis* to low sublethal doses that was not hereditary and lost after stopping the treatment.

Finally, the side effects of metronidazole, which is usually applied orally, can be severe to some extent (CUDMORE et al., 2004). Pentamycin was shown to be generally well-accepted with a few single cases of mild vaginal irritations (BALMER, 2009; FREY TIRRI et al., 2010). Also, its chemical structure and high molecule weight makes it optimally applicable for a topical, intravaginal use (BALMER, 2009).

Concluding, the results of this study suggest high suitability of pentamycin for pharmaceutical use in the treatment of trichomonosis. Clinical studies on its tolerability and application are underway.

## 5 Summary

In this study, the effect of pentamycin – a polyene antibiotic currently mainly used in the treatment of fungal infections – against the sexually transmitted human parasite *Trichomonas vaginalis* was examined. The three major aims were to investigate the molecular mode of action of the drug with focus on the protein composition of the cell; further to determine the relationship between applied drug dose and incubation time on four differently metronidazole-sensitive *T. vaginalis* strains; and finally to monitor the effects of long-term treatment and establishment of resistance. The investigated preparation of pentamycin has a purity grade of 94%.

The primary methods that were used in this study include microscopy and staining techniques (Giemsa) to observe the effects of drug treatment in cell culture visually, and 1D and 2D SDS-PAGE to analyse the protein composition before and after the treatment. Further, it was worked with microtiter assays to determine the dose-effect relationship between pentamycin and trichomonads. Finally, statistic analyses were performed with Excel and SPSS.

It could be shown that the mode of action of pentamycin is based on a binding to sterols of the cell membrane, leading to membrane instability and formation of pores. The effects vary from general cellular discomfort and rounded, spinning, and immotile cells at lower doses to severe structural damage, total lysis and cell death at higher doses. The proteome compared of treated and untreated cells has shown dramatically altered protein profiles which is attributed to the effluence of proteins out of the cell, which correlates positively with higher doses of pentamycin.

## SUMMARY

The general effect can be described as very fast and highly effective. One hour after treatment, an EC<sub>100</sub> of 15 µg/ml and an EC<sub>50</sub> of 2.2 µg/ml was significant, which is an remarkable low dose compared to metronidazole, the standard drug in treatment of trichomonosis. Moreover, all four investigated strains showed almost the same susceptibility to pentamycin, which makes pentamycin applicable against metronidazole-resistant trichomonads.

Six months of long-term treatment resulted in a reversible adaption of trichomonads to pentamycin, at which sensitivity turned out to be lower, especially at sublethal concentrations until 5 µg/ml. However, a dose of 15 µg/ml was still lethal. True resistance as well as cross resistance of pentamycin to the polyene amphotericin B was not observable, moreover, a synergistic effect of both drugs could be shown.

Protein profiles of adapted trichomonads was shown to be different from those of wildtypes and may have derived most probably from proteolysis and harming effects of long-term treatment with sublethal doses of pentamycin. An altered metabolism as a reason which might be an indication for resistance can be excluded.

Altogether, this study confirms previous knowledge of antibiotics of the polyene group and reveals new insights in the mode of action of pentamycin against *T. vaginalis*. The results are promising for a pharmaceutical use of pentamycin in the medical treatment of trichomonosis. It showed a much lower incubation time, together with a higher efficacy and different action mechanism than metronidazole.

## 6 Appendix

### 6.1 Glossary

**18S rRNA:** Part of the small eukaryotic ribosomal 40 S rRNA subunit (18S: 18 Svedberg ( $10^{-13}$  seconds for sedimentation)), widely used in molecular analysis, especially for evolutionary history of invertebrates, due to its slow mutation rate.

**1D SDS-PAGE:** A method to separate previously denatured proteins by their molecular mass as the negatively charged proteins migrate in an electric field to the positively charged anode. The proteins with a higher molecule mass migrate slower, whereas the proteins with lower molecule mass migrate faster. After they were stained, a specific pattern is visible and comparable with a standard marker.

**2D SDS-PAGE:** This method separates proteins by their isoelectric point first ("isoelectric focusing") whereas the proteins migrate until the pH where they are charged neutrally ("isoelectric point") This is called the "first dimension" or "horizontal separation". The "second dimension" or "vertical separation" is the same principle as the 1D SDS-PAGE. By this method, it is possible to identify proteins by their IEP and mass which is an advantage to 1D SDS-PAGE which does not allow to identify proteins with the same mass.

**Adenylate kinase:** Catalyzes the reaction  $2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$  and plays therefore an important role in cellular energy homeostasis.

**Amphotericin B:** Antifungal polyene drug, used also against leishmaniosis, primary amoebic meningoencephalitis and trichomonosis.

**Anaerobic:** Organisms that do not require oxygen for their growth and may even die if oxygen is present (depending on obligate, facultative or aerotolerant anaerobic).

**Apoptosis:** Programmed cell death with characteristic changes of the cell morphology, in contrary to necrosis.

**Bromophenol blue:** 3',3'',5',5''-tetrabromophenolsulfonphthalein, used as a dye and a pH indicator in electrophoresis. It makes the protein samples visible at their migration and indicates the pH which is also important for a successful analysis.

**Commensalism:** Relationship between two organisms wherea one benefits but the other is not affected.

**Complementary DNA (cDNA):** An synthesized DNA strand from an mRNA template catalized by the enzymes reverse transcriptase and DNA polymerase. It is often used to create a cDNA bank and to express eukaryotic genes in prokaryotes. Retroviruses also produce cDNA.

**Concentration:** The relationship of a scientific unit to another, mostly larger unit; in this study the most frequently used concentration was µg/ml.

**Coomassie Brilliant Blue:** Two similar triphenylmethane dyes (G-250 and R-250) that are used for staining proteins. Also referred as "Coomassie" only (named after the town Kumasi in Ghana).

**Cross resistance:** Resistance to a usually toxic substance as a consequence of exposure to another, similar acting substance.

**Cysteine proteinase:** Polypeptide degrading enzymes with a common catalyzing step that involves nucleophilic cysteine thiol. They play an ubiquitary role in physiology and development, such as growth, immune responses, and apoptosis.

**Cytochrome:** A group of membrane-bound hemoproteins, responsible for electron transport.

**Cytostome:** A part of the cell that features structures for ingestion (a sort of cell mouth).

**Dictyosome:** Single stack of the Golgi apparatus.

**Dimethylsulfoxide (DMSO):**  $(\text{CH}_3)_2\text{SO}$ , a polar atropic solvent, used here for dissolving and storage of pentamycin, amphotericin B and metronidazole. It also alleviates the penetration through the membrane in the target organism.

**Dose:** The concentration related to the size of the organism, in this study it was the cell number; the most frequently used dose was  $\mu\text{g/ml}$  related to  $10^6$  cells.

**Dyspareunia:** Painful sexual intercourse.

**Elongation factor (EF):** Set of proteins that facilitate the connection of peptide bonds in the step of protein translation elongation.

**Effective concentration (EC):** Concentration which leads to a distinct effect on an organism, in this case necrosis. The most important value is the  $\text{EC}_{50}$  – the concentration at which 50% of cells are vital – as it shows the comparably lowest

standard deviation. The other calculated value in this study was  $EC_{90}$ , at which 90% of the cells are dead.

**Electrophoresis:** The motion of dispersed charged particles in a fluid through a constant electric field, caused by the difference in charge of particle and surrounding fluid. Here, it is part of the SDS-PAGE technique.

**Endosymbiosis:** Relationship between two organisms where one is living within the body or cell of the other and both benefit from each other. Examples are nitrogen-fixing bacteria, and mitochondria and chloroplasts that are believed to be of prokaryotic origin.

**Enolase:** Catalizes the conversion step of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in glycolysis. Also known as phosphopyruvate dehydratase.

**Epigenetics:** A field in genetics that focuses on changes in the phenotype and gene expressed caused by other mechanisms than changes in the underlying DNA sequence (Greek: epi: above, other).

**Equibrilation:** The process in which a system has reached balance. In this study, equilibration means a step in 2D SDS-PAGE in which reduction of cysteine groups with dithiothreitol (DTT) and alkylation of reduced cysteines with iodacetamide (IAA) is required to obtain defined protein spots.

**Eukaryote:** Cells with high organisation level, they contain nucleus with genetic material and other membrane-bound organelle like Golgi-apparatus, mitochondria,....(Prokaryotes contain no nucleus and organelle, are more primitive



and usually smaller in size).

**Excavata:** One of the six supergroups of the eukaryotes including *Trichomonas*. It contains multi-flagellated organisms with a characteristic cell mouth (cytostome).

**Glycocalyx:** The outer layer on the cell membrane of eukaryotes and the cell wall of prokaryotes, consisting of polysaccharides bound covalently with glycoproteins and glycolipids. In prokaryotes it forms a cyst, providing protection from the environment whereas in eukaryotes it builds up a slime surface and also determines the blood group and rhesus factor.

**Glycolysis:** The main step in the catabolism of monosaccharides that involves the conversion from glucose to pyruvate. In further steps, pyruvate becomes catabolized to  $\text{H}_2\text{O}$  and  $\text{CO}_2$  whereas the function is to obtain energy in form of ATP (“cellular respiration”).

**Golgi apparatus:** A membrane-bound organelle complex in eukaryotes that processes and packages macromolecules after their synthesis and before secretion.

**Heat shock proteins:** An ubiquitary class of functionally related proteins whose expression is increased when cells are exposed to high temperatures and stress, protecting cellular proteins from denaturation.

**Heterotrophy:** The property of organisms to need organic compounds as a carbon source (The opposite is autotrophy: The ability to fix carbon and organic compounds with energy from sunlight or inorganic compounds).

**Hydrogenase:** Enzyme that catalyzes the oxidation of hydrogen:  $\text{H}_2 \leftrightarrow 2\text{H}^+ + 2\text{e}^-$

**Hydrogenosomes:** Cell organelle in anaerobians that are analogues to mitochondria and function in cell respiration. It is believed that hydrogenosomes have secondarily evolved from mitochondria as their host cells have adapted to anaerobic environments.

**Incidence:** A term used in medical statistics. It means the number of new cases of an infection or a disease in a population group related to a certain time (usually 1 year).

**Inhibitory concentration (IC):** Concentration which leads to an inhibitory effect (motility) on an organism. The most important value is the  $IC_{50}$  – the concentration at which 50% of cells are inhibited – as it shows the comparably lowest standard deviation. The other calculated value in this study was  $IC_{90}$ , at which 90% of the cells are inhibited.

**Internal transcribed spacer (ITS):** Sequence of non-functional rRNA between structural rRNA sequences on a precursor script; shows a high degree of variation and is used in studies that involve closely related species.

**Isoelectric focusing (IEF):** A technique that allows to separate proteins by their different electric charges whereas the charge changes with the pH of the surroundings.

**Isoelectric point (IEP):** The individual pH value of a molecule where it is neutrally charged and stops to migrate in the electric field of the pH gradient of the IEF.

**Jaundice:** Yellow pigmentation of the skin caused by increased levels of bilirubin in the blood, also known as Icterus.

**Lactate dehydrogenase (LDH):** A ubiquitary enzyme that catalyzes the conversion from pyruvate to lactate in the cell respiration with simultaneous oxidation of NAD<sup>+</sup> from NADH. It also plays an important role as marker for tissue or cell breakdown, f.e. necrosis leads to higher LDH levels.

**Leukocyte:** White blood cell

**Lipophosphoglycan (LPG):** The major glycoconjugate on the surface of *T. vaginalis* and important in pathogenesis. It is also found in *Leishmania* promastigotes where it promotes the protection of the parasite from the host's immune responses.

**Macrolide:** Group of drugs (mostly antibiotics) containing a macrocyclic lactone ring.

**Malate dehydrogenase (MDH):** An enzyme in the citric acid cycle, converting malate into oxaloacetate. In *T. vaginalis*, there are isozymes in the cytosol and in the hydrogenosomes. MDH also supported the theory that hydrogenosomes may have a mitochondrial origin.

**Mastigont** (also kinetid): A unit in flagellates, comprised of the flagella and their basal bodies, attached flagellar roots and associated centrosomal structures.

**Meiosis:** Specialised cell division, necessary for sexual reproduction, localised in the primary sexual organ. A cell with a diploid set of chromosomes will be divided into four cells with a haploid set of chromosomes (gametes in animals, spores in fungi).

**Messenger RNA (mRNA):** The RNA transcript from a DNA template, providing the needed information for transcription into amino acids and finally proteins. The mRNA may underlie many modification and converting steps.

**Metronidazole:** A 5-nitroimidazole drug, applied against bacteria and anerobic protozoa.

**Microaerophilic:** Organisms that are actually anaerobians but tolerate or even grow best at little amounts of oxygen.

**Mitochondria:** Cell organelle in aerobic eukaryotes that are responsible for cell respiration. They are the analogue to the hydrogenosomes in anaerobians.

**Mitosis:** The process of the division of the nucleus in eukaryotic cells. With the subsequently following cytokinesis or cell division that produces two daughter cells from one cell, it compasses the M-phase within the cell cycle.

**Monophyly:** A term for a group (clade) of organisms that descend all from one common ancestor.

**Monoxenous:** The property of a pathogenic organism to infect one host only.

**NAD: ferredoxin oxidoreductase:** An enzyme that catalyzes the reaction of reduced ferredoxin +  $\text{NAD}^+$  to oxidized ferredoxin +  $\text{NADH} + \text{H}^+$ .

**Necrosis:** The premature traumatic and death of cells, in contrary to apoptosis.

**Neutropenia:** Abnormally low number of neutrophils (white blood cells)

**Nile Red** (9-diethylamino-5H-benzo [α] phenoxazine-5-one): Selective fluorescent lipophilic dye, produced by boiling of Nile Blue with sulfuric acid and used to visualize intracellular lipid droplets. Stained neutral lipids fluoresce yellow (590 nm), polar lipids fluoresce orange (600 nm).

**Nitroimidazole:** Aromatic heterocyclic molecule ( $C_3H_4N_2$ ) with an additional nitrogroup.

**Nucleoside:** Glycosylamines consisting of a nitrogenous bound to a ribose or desoxyribose, f.e. the nucleobases of DNA and RNA.

**Nucleotide:** A nucleotide bound to one to three phosphate groups; the structural unit of DNA/RNA.

**Ortholog:** Genes in different species that are related and originate from a common ancestor.

**Osmosis:** The movement of water through a semi-permeable (biological) membrane from the area with low solute concentration to the area with high solute concentration with the purpose to reach equal solute concentration.

**Parabasal:** A class within the Excavata, described by Honigberg, 1973. Characteristics are their parabasal apparatus, anterior flagella cluster, anaerobic habitus and lack of mitochondria.

**Parasitism:** A relationship between two organisms whereas one benefits from the other without killing it. The parasite can be temporary or permanently, living in or on the mostly larger host.

**Parasthesia:** Loss of sensation (temporary or permanent)

**Polymerase Chain Reaction (PCR):** A standard technique in molecular biology that allows to amplify DNA fragments millions of times, making them visible and identifiable.

**Pentamycin:** A polyene macrolide antibiotic, obtained from *Streptomyces pentaticus*. It is used against fungal infections and trichomonosis.

**Peptidase (Protease):** An enzyme that cleaves other proteins by hydrolysis of their peptide bonds.

**Phagocytosis:** The process of ingestion of solid particles by the cell membrane and subsequent forming of a vesicle with the intaked particles in the cell.

**Pinocytosis:** The process of ingestion of fluid particles by the cell membrane and subsequent forming of a vesicle with the intaked particles in the cell.

**Polyene:** Chemical group of poly-unsaturated organic molecules with one or more alternating double and single carbon-carbon bonds.

**Polyploidy:** The property of organisms to contain a set of more than two pairs of homologous chromosomes.

**Prevalence:** A term used in medical statistics. It means the number of new cases of an infection or a disease divided by the number of individuals in the population.

**Promastigotes:** A morphological form of protozoan parasites f.e. *Leishmania*, *Trypanosoma*. It is the most common morphology in the insect host (mostly primary host).

**Protein:** Organic macromolecule, assembled of linear arranged amino acids that are connected together by peptide bonds between the amino and carboxyl groups and are folded into a globular form. The organic tissue and cells of all organisms consist of proteins. A protein may also termed as gene product as it contains the information of one or more genes after mRNA processing and translation.

**Proteolysis:** Degradation of proteins by peptidases or other factors.

**Proteome:** The entire set of proteins in respective biological system (cell, tissue, or organism).

**Proteomics:** The science of the function and structure of proteins.

**Purine:** Aromatic heterocyclic molecule consisting of a pyrimidine connected with an imidazole ring f.e. guanine (G) and adenine (A) of the DNA, caffeine, uric acid.

**Pyrimidine:** Aromatic heterocyclic molecule with two N-atoms at position 1 and 3 of the six-carbon ring, f.e. thymine (T) and cytosine (C) of the DNA.

**Pyruvate ferredoxin oxidoreductase (PFOR):** Enzyme that catalyzes the reaction  

$$\text{pyruvate} + \text{CoA} + 2 \text{ oxidized ferredoxin} \leftrightarrow \text{acetyl-CoA} + \text{CO}_2 + 2 \text{ reduced ferredoxin} + 2 \text{ H}^+$$

**Retinol binding protein 1 (RBP1):** A carrier protein involved in the transport of retinol from the liver storage site to peripheral tissue. Encoded by the RBP1 gene.

**Resistance:** Resistance against a drug is to ability of the organism to survive or to be more tolerant to highly toxic doses as a consequence of protective mechanisms developed by the organism.

**Ribosomal RNA (rRNA):** A specific sort of RNA in the ribosomes and responsible for the protein manufacturing by providing peptidyl transferase activity.

**RNA interference (RNAi):** A system in cells regulating the activity genes by small RNA molecules that bind to other RNAs.

**R-strain:** A term used exclusively in this study for cell cultures of *T. vaginalis* that are exposed to sublethal drug treatment permanently with the aim to generate resistant trichomonads.

**Sensitivity:** Percentage of true positive events (recall rate)

**Specificity:** Percentage of true negative events

**Struma:** Swelling in the thyroid gland; in humans it has medical importance whereas in birds it is part of their natural anatomy.

**Symbiosis:** Relationship between two organisms where both benefit from each other.

**Thioredoxin reductase:** Ubiquitary enzyme class that reduces thioredoxin by oxidation of NADPH; essential for cell growth and survival.



**Transfer RNA (tRNA):** The intermediate RNA molecule in transcription as it links each base triplet of the mRNA with a corresponding amino acid.

**Triosephosphate isomerase:** An enzyme involved in glycolysis as it catalyzes the reversible interconversion of the triosephosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate.

**Valyl tRNA synthetase:** An enzyme (ligase) that catalyzes the reaction of  $\text{ATP} + \text{L-valine} + \text{tRNA}^{\text{Val}} \leftrightarrow \text{AMP} + \text{Diphosphate} + \text{L-valyl-tRNA}^{\text{Val}}$ . It plays a major role in biosynthesis of the amino acids valine, leucine and isoleucine.

## 6.2 Abbreviations

**1D SDS-PAGE:** One dimensional sodium dodecylsulfate polyacrylamide gelelectrophoresis

**2D SDS-PAGE :** Two dimensional sodium dodecylsulfate polyacrylamide gelelectrophoresis

**Acetyl-CoA:** Acetyl coenzyme A

**ADH1 :** Alcohol dehydrogenase 1

**ADP:** Adenosine diphosphate

**AP:** Adhesion protein

**APS:** Ammoniumpersulfate

**ATCC:** The Global Bioresource Center ([www.atcc.org](http://www.atcc.org))

**ATP:** Adenosine triphosphate

**CDF:** Cell detaching factor

**cDNA:** complementary DNA

**CHAPS:** 3-[(3-Cholamidopropyl) dimethylammonio]-propane- sulfonate

**cMDH:** cytosolic malate dehydrogenase

**DMSO:** Dimethylsulfoxide

**DNA:** Desoxyribonucleic acid

**DTT:** Dithiothreitol

**EC:** Effective concentration

**EDTA:** Ethylenediaminetetraacetic acid

**EF:** elongation factor

**H<sup>+</sup>:** hydrogen ion

**HIV:** Human Immunodeficiency Virus

**IAA:** Iodacetamide

**IC:** Inhibitory concentration

**IEF:** Isoelectric focusing

**IEP:** Isoelectric point

**Ig:** Immunoglobuline

**IPG:** Immobilized pH gradient

**ITS:** Internal transcribed spacer

**K<sup>+</sup>:** potassium ion

**kbp:** Kilo basepairs

**LDH:** Lactate dehydrogenase

**LPG:** Lipophosphoglycan

**MALDI-TOF:** Matrix-assisted laser desorption/ionisation – time of flight

**Mb:** megabases

**MIC:** minimal inhibitory concentration

**MLC:** minimal lethal concentration

**MOPS:** 3-N-morpholino-propanesulfonic acid

**mRNA:** messenger RNA

**tRNA:** transcription RNA

**NAD<sup>+</sup>:** Nicotinamid adenine dinucleotide, oxidized form

**NADH:** Nicotinamid adenine dinucleotide, reduced form

**NO<sub>2</sub>:** nitrogen dioxide

**PBS:** Phosphate buffered saline

**PCR:** Polymerase Chain Reaction

**PFOR:** Pyruvate ferredoxin oxidoreductase

**pH:** pondus hydrogenii / potentia hydrogenii

**RBP1:** Retinol binding protein 1

**rDNA:** ribosomal DNA

**RNA:** Ribonucleic acid

**RNAi:** RNA interference

**rRNA:** ribosomal RNA

**SM buffer:** buffer containing sucrose and MOPS

**STD:** Sexually Transmitted Disease

**TCA:** Trichloroacetic acid

**TEMED:** N, N, N', N' – tetramethylethylenediamine

**TYM:** Trypticase Yeast Maltose

### 6.3 Chemical reagents

The chemical reagents used in the experiments were obtained by the following companies:

3-N-morpholino-propanesulfonic acid (Sigma), acetic acid (Merck), acetone (Merck), acrylamide (Sigma), ammoniumpersulfate (Sigma), amphotericin B, solubilized (Sigma), BioLyte 3-10 Buffer 100x (BioRad), BBL trypticase peptone: Pancreatic digest of casein (BD), Cell Freezing Medium-DMSO (Sigma), CHAPS minimum 98% TLC (SigmaUltra), Coomassie Brilliant Blue R-250 (Serva), Diamond Vitamin Tween 80 Solution (40x) (Sigma), dimethylsulfoxide (Sigma), dipotassium-hydrogenphosphate (Merck), DL-dithiothreitol (Sigma), ethanol (Merck), EDTA (Merck), glycine (Serva), glycerol (Merck), HCl (Merck), Horse serum heat-inactivated (Sigma), iodacetamide (Sigma), isopropanol (Merck), Giemsa's azur-eosin-methylenblue solution (Merck), L-ascorbic acid (Sigma), L-cysteine hydrochloride monohydrate (Merck), maltose monohydrate (Merck), methanol (Merck), NADH (Sigma), NaOH (Merck), N,N,N',N'-tetramethylethylenediamine (Merck), penicillin G-sodium 10 Mega IE (Sandoz), pentamycin (Carbogen Amcis), phosphate buffer solution after "Weise" (Sigma), potassiumdihydrogenphosphate (Merck), Prestained Protein Marker MW (PeqLab), pyruvate (Sigma), silver nitrate (Merck), sodium carbonate (Merck), Sodium dodecyl sulphate / SDS (Merck-Schuchardt), sodium thiosulfate (Merck), sucrose (Sigma), thiourea (GE Healthcare), trichloroacetic acid (Sigma-Aldrich), Trizma base (Sigma), Tris-HCl (Sigma), Trypan blue solution, 0.4% in 0.81% sodium chloride and 0.06% potassium phosphate (Sigma), urea (Merck), water deionized, yeast extract (Merck)

## 6.4 Equipment

Biorad 1D and 2D-gelelectrophoresis units, Eppendorf tubes 1.5 ml, Falcon 12.5 cm<sup>2</sup> tissue culture flasks, Fuchs-Rosenthal hemacytometer, GFR shaker, Heraeus incubator, Heraeus thermo centrifuge, Ikamag magnetic stirrer, Inolab 730 pH-electrode, Jouan centrifuge, liquid nitrogen deep freezer, Nikon phase contrast microscope, Nunc 12- well and 24-well microtiter plates, Nunc cryopreservation tubes, Safe 2020 sterile bench, Sarstedt 50 ml tubes, standard chemicals, pipettes and tips, vacuum pump, - 85°C deep freezer

## 6.5 Literature

ABONYI A (1995): Examination of nonflagellate and flagellate round forms of *Trichomonas vaginalis* by transmission electron microscopy. – *Applied Parasitology* 36 (4): 303–310.

ABRAHAM MC, DESJARDINS M, FILION MJ, GARBER JE (1996): Inducible immunity to *Trichomonas vaginalis* in a mouse model of vaginal infection. – *Infection and Immunity* 64: 3571–3575.

ACKERS JP (2001): Trichomonads. – In: GILLESPIE SH, PEARSON RD (eds): *Principles and Practice of Clinical Parasitology*. John Wiley & Sons, Ltd.: 243–268.

ADL SM, SIMPSON AG, FARMER MA, ANDERSEN RA, ANDERSON OR, BARTA JR, BOWSER SS, BRUGEROLLE G, FENSOME RA, FREDERICQ S, JAMES TY, KARPOV S, KUGRENS P, KRUG J, LANE CE, LEWIS LA, LODGE J, LYNN DH, MANN DG, MCCOURT RM, MENDOZA L, MOESTRUP O, MOZLEY-STANDRIDGE SE, NERAD TA, SHEARER CA, SMIRNOV AV, SPIEGEL FW, TAYLOR MF (2005): The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. – *The Journal of Eukaryotic Microbiology* 52 (5): 399–451.

ALDERETE JF, GARZA GE (1985): Specific nature of *Trichomonas vaginalis* parasitism of host cell surfaces. – *Infections and Immunity* 50: 701–708.

ALDERETE JF, PROVENZANO D, LEHKER MW (1995): Iron mediates *Trichomonas vaginalis* resistance to complement lysis. – *Microbial pathogenesis* 19: 93–103.

ALLSWORTH JE, RATNER JA, PEIPERT JF (2009): Trichomoniasis and Other Sexually Transmitted Infections: Results From the 2001–2004 National Health and Nutrition Examination Surveys. – *Sexually Transmitted Diseases* 36 (12): 738–744.

ALVAREZ-SÁNCHEZ ME, SOLANO-GONZÁLEZ E, YAÑEZ-GÓMEZ C, ARROYO R (2007): Negative iron regulation of the CP65 cysteine proteinase cytotoxicity in *Trichomonas vaginalis*. – *Microbes and Infections* 9: 1597–1605.

AMRAN F, AZIZ MN, M IBRAHIM H, NORHALIM A, SABARATNAM B, M RAMDZAN N, ISMAIL MI (2011): *In vitro* antifungal susceptibilities of *Candida* isolated from patients with invasive candidiasis in Kuala Lumpur Hospital, Malaysia. – Journal of Medical Microbiology 1: [Epub ahead of print].

ANDREOLI TE, MONAHAN M (1968): The Interaction of Polyene Antibiotics with Thin Lipid Membranes. – The Journal of General Physiology 52: 300-325.

ARROYO R, GONZALEZ-ROBLES A, MARTINEZ-PALOMO A, ALDERETE JF (1993): Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesion synthesis follows cytoadherence. – Molecular Microbiology 7: 299–309.

ASPÖCK H (1994): Protozoen als Erreger von Krankheiten des Menschen: Übersicht und aktuelle Probleme in Mitteleuropa. – In: Katalog OÖ Landesmuseum N.F. 71: 219–266.

ASPÖCK H, WALOCHNIK J (2002): Was sind Parasiten? – In: ASPÖCK H (Hrsg.): Amöben, Bandwürmer, Zecken...Parasiten und parasitäre Erkrankungen des Menschen in Mitteleuropa. – Denisia 6, Biologiezentrum der OÖ Landesmuseen (Hrsg.): 2–12.

ASTELBAUER F (2010): Anti-protozoal activity of plant-derived flavaglines, acridones and sulfur-containing amides. PhD thesis, Medical University of Vienna, Austria.

ATHAR MA, WINNER HI (1971): Development of resistance by *Candida* species to polyenes antibiotics in vitro. – Journal of Medical Microbiology 4: 505–517.

BALMER JA (2009): Treatment of Vaginal Infections with Intravaginal Pentamycin in Clinical Practice. – The Internet Journal of Gynecology and Obstetrics 11 (1) ([http://www.ispub.com/journal/the\\_internet\\_journal\\_of\\_gynecology\\_and\\_obstetrics/volume\\_11\\_number\\_1\\_6/article/treatment\\_of\\_vaginal\\_infections\\_with\\_intravaginal\\_pentamycin\\_in\\_clinical\\_practice.html](http://www.ispub.com/journal/the_internet_journal_of_gynecology_and_obstetrics/volume_11_number_1_6/article/treatment_of_vaginal_infections_with_intravaginal_pentamycin_in_clinical_practice.html), 7.6.2011).

BASTIDA-CORCUERA FD, OKOMURA CY, COLOCOUSSI A, JOHNSON PJ (2005): *Trichomonas vaginalis* Lipophosphoglycan Mutants Have Reduced Adherence and Cytotoxicity to Human Ectocervical Cells. – *Eucaryotic Cell* 4 (11): 1951–1958.

BENCHIMOL M (2008): Cell death in trichomonads. – In: PEREZ MARTIN JM: *Programmed Cell Death in Protozoa*. *Molecular Biology Intelligence Unit* 23: 97–115. DOI: 10.1007/978-0-387-76717-8\_9.

BJELLQVIST B, EK K, RHIGETTI PG (1982): Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. – *Journal of Biochemical and Biophysical Methods* 6 (4): 317–339.

BLAHA C, DUCHENE M, ASPÖCK H, WALOCHNIK J (2006): *In vitro* activity of hexadecylphosphocholine (miltefosine) against metronidazole-resistant and – susceptible strains of *Trichomonas vaginalis*. – *Journal of Antimicrobial Chemotherapy* 57 (2): 273–278.

BLUM H, BEIER H, GROSS HJ (1987): Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. – *Electrophoresis* 8 (2): 93–99.

BOLARD J (1986): How do the polyene macrolide antibiotics affect the cellular membrane properties. – *Biochimica et Biophysica Acta* 864 (3-4): 257–304.

BRENIER-PINCHART MP, PELLOUX H, DEROUICH-GUERGOUR D, AMBROISE-THOMAS P (2001): Chemokines in host-protozoan-parasite interactions. – *Trends in Parasitology* 17: 292–296.

BÜTSCHLI O (1880–1889): Erster Band. Protozoa.– In: Dr. H.G. BRONNS *Klassen und Ordnungen des Thier-Reichs*. Winter'sche Verlagshandlung, Leipzig.

CAPEK A, SIMEK A (1972): Antimicrobial agents. XIV. Content of ergosterol and the problem of transient resistance and microbial degradation of antimycotics. – *Folia Microbiologica* 17: 239–240.



CARLTON JM, HIRT RP, SILVA JA, DELCHER AL, SCHATZ M, ZHAO Q, WORTMAN JR, BIDWELL SL, ALSMARK UCM, BESTEIRO S, SICHERITZ-PONTEN T, NOEL CJ, DACKS JB, FOSTER PG, SIMILLION C, VAN DE PEER Y, MIRANDA-SAAVEDRA D, BARTON GJ, WESTROP GD, MÜLLER S, DESSI D, FIORI PL, REN Q, PAULSEN I, ZHANG H, BASTIDA-CORCUERA FD, SIMOES-BARBOSA A, BROWN MT, HAYES RD, MUKHERJEE M, OKUMURA CY, SCHNEIDER R, SMITH AJ, VANACOVA S, VILLALVAZO M, HAAS BJ, PERTEA M, FELDBLYUM TV, UTTERBACK TR, SHU C, OSOEGAWA K, DE JONG PJ, HRDY I, HORVATHOVA L, ZUBÁČOVÁ Z, DOLEZAL P, MALIK S, LOGSDON JR. JM, HENZE K, GUPTA A, WANG CC, DUNNE RL, UPCROFT JA, UPCROFT P, WHITE O, SALZBERG SL, TANG P, CHIU C, LEE Y, EMBLEY TM, COOMBS GH, MOTTRAM J, TACHEZY J, FRASER-LIGGETT CM, JOHNSON PJ (2007): Draft Genome Sequence of the Sexually Transmitted Pathogen *Trichomonas vaginalis*. – Science 315: 207–212.

CARTER RF (1969): Sensitivity to amphotericin B of a *Naegleria* sp. related from a case of primary amoebic encephalitis. – Journal of Clinical Pathology 22: 470–474.

CARTER JE, WHITHAUS KC (2008): Neonatal respiratory tract involvement of *Trichomonas vaginalis*: A case report and review of the literature. – The American Journal of Tropical Medicine and Hygiene 78 (1): 17–19.

CASTANHO MARB, COUTINHO A, PRIETO MJE (1992): Absorption and Fluorescence Spectra of Polyene Antibiotics in the Presence of Cholesterol. – The Journal of Biological Chemistry 267 (1): 204–209.

CAVALIER-SMITH T (1987): Eucaryotes with no mitochondria. – Nature 326: 332–333.

CAVALIER-SMITH T (1998): A revised six-kingdom of life. – Biological Reviews of the Cambridge Philosophical Society 73: 203–266.

CAVALIER-SMITH T (2002): The phagotrophic origin of eucaryotes and phylogenetic classification of Protozoa. – International Journal of Systematic Evolutionary Microbiology 52: 297–357.

CEPICKA I, HAMPL V, KULDA J (2010): Critical Taxonomic Revision of Parabasalids with Description of one New Genus and three New Species. – *Protist* 16: 400–433.

CHEN CC, CHOU DL, FEINGOLD DS (1978): Dissociation between ion permeability and the lethal action of polyene antibiotics on *Candida albicans*. – *Antimicrobial Agents and Chemotherapy* 13: 914.

CLARK CG, DIAMOND L (2002): Methods for Cultivation of Luminal Parasitic Protists of Clinical Importance. – *Clinical Microbiology Reviews* 15 (3): 329–341.

CONRAD M, ZUBÁČOVÁ Z, DUNN LA, UPCROFT J, SULLIVAN SA, TACHEZY J, CARLTON JM (2010): Microsatellite polymorphism in the sexually transmitted pathogen *Trichomonas vaginalis* indicates a genetically diverse parasite. – *Molecular & Biochemical Parasitology* 175: 30–38.

COSAR C, JULOU L (1959): Activity of 1-(2'-hydroxyethyl)-2-methyl-5-nitroimidazole (8823 RP) against experimental *Trichomonas vaginalis* infection. – *Annales de l'Institut Pasteur* 96: 238–241.

COTCH MF, PASTOREK JG 2<sup>ND</sup>, NUGENT RP, HILLIER SL, GIBBS RS, MARTIN DH, ESCHENBACH DA, EDELMAN R, CAREY JC, REGAN JA, KROHN MA, KLEBANOFF MA, RAO AV, RHOADS GG (1997): *Trichomonas vaginalis* associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. – *Sexually Transmitted Diseases* 24: 353–360.

CUDMORE SL, DELGATY KL, HAYWARD-MCCLELLAND SF, PETRIN DP, GARBER GE (2004): Treatment of Infections Caused by Metronidazole-Resistant *Trichomonas vaginalis*. – *Clinical Microbiology Reviews* 17 (4): 783–793. DOI: 10.1128/CMR.17.4.783–793.2004.

CUI J, DAS S, SMITH TF, SAMUELSON J (2010): *Trichomonas* Transmembrane Cyclases Result from Massive Gene Duplication and Concomitant Development of Pseudogenes. – *Public Library of Science* 4 (8): e782, 1–11.

DE JESUS JB, CUERVO P, JUNQUEIRA M, BRITTO C, SILVA-FILHO FC, SOARES MJ, CUPOLILLO E, FERNANDES O, DOMONT GB (2007): A further proteomic study on the effect of iron in the human pathogen *Trichomonas vaginalis*. – *Proteomics* 7 (12): 1961–1972.

DE JESUS JB, CUERVO P, BRITTO C, SABOIA-VAHIA L, E SILVA-FILHO FC, BORGES-VELOSO A, PETROPOLIS DB, CUPOLILLO E, DOMONT GB (2008): Cysteine Peptidase Expression in *Trichomonas vaginalis* Isolates Displaying High- and Low-Virulence Phenotypes. – *Journal of Proteome Research* 8: 1555–1564.

DE KRUIJFF B, GERRITSEN WJ, OERLEMANS A, DEMEL RA, VAN DEENEN LLM (1974): Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by polyene antibiotics. – *Biochimica et Biophysica Acta* 26, 339 (1): 30–43.

DELGADO-VISCOGLIOSI P, VISCOGLIOSI E, GERBOD D, KULDA J, SOGIN ML, EDGCOMB VJ (2000): Molecular phylogeny of parabasalids based on small subunit rRNA sequences, with emphasis on the Trichomonadinae subfamily. – *Journal of Eukaryotic Microbiology* 47: 70–75.

DEMEL RA, VAN DEENEN LLM (1965): Penetration of Lipid Monolayers by Polyene Antibiotics. – *The Journal of Biological Chemistry* 240 (6): 2749–2753.

DEPLAZES P, ECKERT J (2005): Parasitologie – In: KAYSER FH, BÖTTGER EC, ZINKERNAGEL RM, HALLER O, ECKERT J, DEPLAZES P: Taschenlehrbuch Medizinische Mikrobiologie, 11. Überarbeitete und erweiterte Auflage. Georg Thieme Verlag (Stuttgart, New York): 502–504.

DIAMOND LS (1986): In vitro cultivation of the Trichomonadidae: a state of the art review. – *Acta Univ. Carol. Biol.* 30: 221–228.

DIAZ G, MELIS M, BATETTA B, ANGIUS F, FALCHI AM (2008): Hydrophobic characterization of intracellular lipids in situ by Nile Red red/yellow emission ratio. – *Micron* 39: 819–824.

DIAZ N, DESSI D, DESSOLE S, FIORI PL, RAPPELLI P (2010): Rapid detection of coinfections by *Trichomonas vaginalis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* by a new multiplex polymerase chain reaction. – *Diagnostic Microbiology and Infectious Disease* 67: 30–36.

DICK JD, MERZ WG, SARAL R (1980): Incidence of polyene-resistant yeasts recovered from clinical specimens. – *Antimicrobial Agents and Chemotherapy* 18: 158–163.

DUREL P, ROIRON V, SIBULET A, BOREL LJ (1960): Systemic treatment of human trichomoniasis with a derivate of Nitroimidazole. – *British Journal Of Venereal Diseases*: 21.

EL-SHAZLY AM, EL-NAGGAR HM, SOLIMAN M, EL-NEGERI M, EL-NEMR HE, HANDOUSA AE, MORSY TA (2001): A study on *Trichomonas vaginalis* and female infertility. – *Journal of the Egyptian Society of Parasitology* 31: 545–553.

EMBLEY TM, HIRT HP (1998): Early branching eukaryotes? *Current Opinion in Genetetics and Development* 8: 624–629.

EMBLEY TM, VAN DER GIEZEN M, HORNER DS, DYLAL PL, BELL S, FOSTER PG (2003): Hydrogenosomes, mitochondria and early eukaryotic evolution. – *IUBMB Life* 55 (7): 387–395.

FIORI PL, RAPPELLI P, ADDIS MF (1999): The flagellated parasite *Trichomonas vaginalis*: new insights into cytopathogenicity mechanisms. – *Microbes and Infection* 2: 146–156.

FOUTS AC, KRAUS SJ (1980): *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. – *Journal of Infectious Diseases* 141: 137–143.

FREEMAN CD, KLUTMAN NE, LAMP KC (1997): Metronidazole. A therapeutic review and update. – *Drugs* 54: 679–708.

FREY TIRRI B, BITZER J, GEUDELIN B, DREWE J (2010): Safety, tolerability and pharmacokinetics of intravaginal pentamycin. – *Chemotherapy* 56(3): 190–196.

FRYBERG M (1974): Sterol biosynthesis in antibiotic-resistant yeast: nystatin. – *Archives of Biochemistry and Biophysics* 160: 83–89.

FÜRNKRANZ U, NAGL M, GOTTARDI W, DUCHENE M, ASPÖCK H, WALOCHNIK J (2010): In vitro activity of N-chlorotaurine (NCT) in combination with NH<sub>4</sub>Cl against *Trichomonas vaginalis*. – *International Journal of Antimicrobial Agents* 37: 171–173.

GALE EF (1974): The release of potassium ions from *Candida albicans* in the presence of polyene antibiotics. – *Journal of General Microbiology* 80: 451.

GARBER GE, LEMCHUK-FAVEL LT, BOWIE WR (1989): Isolation of a cell detaching factor from *Trichomonas vaginalis*. – *Journal of Clinical Microbiology* 29: 1847–1849.

GHANNOUM MA, RICE LB (1999): Antifungal Agents: Mode of action, Mechanisms of Resistance, and Correlation of These Mechanisms with Bacterial Resistance. *Clinical Microbiology Reviews* 12 (4): 501–517.

GÖRG A, KLAUS A, LÜCK C, WEILAND F, WEISS W (2007): Two-Dimensional Electrophoresis with Immobilized pH Gradients for Proteome Analysis – A Laboratory Manual (<http://www.wzw.tum.de/proteomik>).

GOMEZ C, RAMIREZ ME, CALIXTO-GALVEZ M, MEDEL O, RODRIGUEZ MA (2010): Regulation of Gene Expression in Protozoa Parasites. – *Journal of Biomedicine and Biotechnology*, Article ID 726045, 24 pp. DOI: 10.1155/2010/726045

GOODSELL D (2008): Molecule of the Month June 2008: Lactate Dehydrogenase – RSCB Protein Data Bank (<http://www.rcsb.org/pdb/101/motm.do?momID=102>). DOI: 10.2210/rcsb\_pdb/mom\_2008\_6. (14 July 2011).

GUNDERSON J, HINKLE G, MORRISON HG, STICKEL SK, ODELSON DA., BREZNAK JA, NERAD TA, MULLER M, SOGIN ML (1995): Phylogeny of trichomonads inferred from small-subunit rRNA sequences. – *Journal of Eukaryotic Microbiology* 42: 411–415.

HACKSTEIN JHP, AKHMANOVA A, VONCKEN F, VAN HOEK A, VAN ALLEN T, BOXMA B, MOON-VAN DER STAAY SY, VAN DER STAAY G, LEUNISSEN J, HUYNEN M, ROSENBERG J, VEENHUIS M (2001): Hydrogenosomes: convergent adaptations of mitochondria to anaerobic environments. – *Zoology* 104: 290-302.

HAIDO RMT, BARRETO-BERGTER E (1989): Amphotericin B-induced damage of *Trypanosoma cruzi* epimastigotes. – *Chemico-Biological Interactions* 71 (1): 91–103.

HAMILTON-MILLER JMT (1973): Chemistry and Biology of the Polyene Macrolide Antibiotics. – *Bacteriological Reviews* 37 (2): 166–196.

HAMMOND SM, KLIGER BM (1974): Studies on the role of the cell wall of *Candida albicans* in the mode of action of polyene antibiotics. – *Bacteriological Reviews* 37: 166.

HAMMOND SM, LAMBERT PA, KLIGER BN (1974): The mode of action of polyene antibiotics; induced potassium leakage in *Candida albicans*. – *Journal of General Microbiology* 81: 325.

HAMPL V, VANACOVA S, KULDA J, FLEGR J (2001): Concordance between genetic relatedness and phenotypic similarities of *Trichomonas vaginalis* strains. – *BMC Evolutionary Biology* 1: 11.

HEMERKA G (2008): „Die Trichomonade“ – In: Die Trichomonade – Ein Geißeltierchen als Geißel der Menschheit: 26.

HEMPHILL A, GOTTSTEIN B (2006): Parasitische Protozoen. – In: HIEPE T, LUCIUS R, GOTTSTEIN B: Allgemeine Parasitologie mit den Grundzügen der Immunologie, Diagnostik und Bekämpfung. Parey (Stuttgart): 80–109.

HERNÁNDEZ-GUTIÉRREZ R, AVILA-GONZÁLEZ L, ORTEGA-LÓPEZ J, CRUZ-TALONIA F, GÓMEZ-GUTIERREZ, ARROYO R (2004): *Trichomonas vaginalis*: characterization of a 39-kDa cysteine proteinase found in patient vaginal secretions. – *Experimental Parasitology* 107: 125–135.

HERNÁNDEZ-ROMANO P, HERNÁNDEZ R, ARROYO R, ALDERETE FJ, LÓPEZ-VILLASEÑOR I (2010): Identification and characterization of a surface-associated, subtilisin-like serine protease in *Trichomonas vaginalis*. – *Parasitology* 137 (11): 1621–1635.

HIEPE T (2006): Parasitismus als Lebensform – eine Einführung. – In: HIEPE T, LUCIUS R, GOTTSTEIN B: *Allgemeine Parasitologie mit den Grundzügen der Immunologie, Diagnostik und Bekämpfung*. Parey (Stuttgart): 1–23.

HIRT RP, NOEL CJ, SICHERITZ-PONTEN T, TACHEZY J, FIORI PL (2007): *Trichomonas vaginalis* surface proteins: a view from the genome. – *Trends in Parasitology* 23 (11): 540–547.

HÖHNE O (1916): *Trichomonas vaginalis* als häufiger Erreger einer typischen colpitis purulenta. – *Zentralblatt für Gynäkologie* 40: 4–14.

HOLZ RW (1974): The effects of the polyene antibiotics nystatin and amphotericin B on thin lipid membranes. – *Annals of the New York Academy of Sciences* 235: 469–479.

HONIGBERG BM (1978): Trichomonads of importance in human medicine. – In: KREIER JP (ed.): *Parasitic Protozoa*. Academic Press: New York; 275–454.

HRDY I, CAMMACK R, STOPKA P, KULDA J, TACHEZY J (2005): Alternative Pathway of Metronidazole Activation in *Trichomonas vaginalis* Hydrogenosomes. – *Antimicrobial Agents and Chemotherapy* 49 (12): 5033–5036.

HSUCHEN C-C, FEINGOLD DS (1973): Selective Membrane Toxicity of the Polyene Antibiotics: Studies on Natural Membranes. – *Antimicrobial Agents and Chemotherapy* 4 (3): 316–319.

HUANG KY, CHIEN KY, LIN, YC, HSU WM, FONG IK, HUANG PJ, YUEH YM, GAN RRC, TANG P (2009): A proteome reference map of *Trichomonas vaginalis*. – Parasitology Research 104: 927–933.

JACOB SW (1985): „Pharmacology of DMSO” – In: JACOB SW, HERSCHLER RW, SCHELLENKAMP H: The Use of DMSO in Medicine (Springer) ([www.dmsso.org](http://www.dmsso.org), 18 June 2011).

JOHNSON B, WHITE RJ, WILLIAMSON GM (1978): Factors affecting the susceptibility of *Candida albicans* to the polyenic antibiotics nystatin and amphotericin B. – Journal of General Microbiology 104: 325.

JORDAN JA, LOWERY D, TRUCCO M (2001): TaqMan-based Detection of *Trichomonas vaginalis* DNA from female genital specimens. – Journal of Clinical Microbiology 39: 3819–3822.

KAUR S, KHURANA S, BAGGA R, WANCHU A, MALLA N (2008): Antitrichomonas IgG, IgM, IgA, and IgG subclass responses in human intravaginal trichomoniasis. – Parasitology Research 103: 305–312.

KELLY SL, LAMB DC, TAYLOR M, CORRAN AJ, BALDWIN BC, POWDERLY WG (1994): Resistance to amphotericin B associated with defective  $\Delta^{8,7}$ -sterol isomerase in a *Cryptococcus neoformans* strain from an AIDS patient. – FEMS Microbiology Letters 122: 39–42.

KERRIDGE D (1979): The polyene macrolide antibiotics. – Postgraduate Medical Journal 55: 653–656.

KIERNAN JA (2008): Histological and histochemical methods. Theory and praxis: 334–335. 4<sup>th</sup> ed., Scion Publishing Ltd.

KIM YS, SONG HO, CHOI IH, PARK SJ, RYU JS (2006): Hydrogenosomal activity of *Trichomonas vaginalis* under different iron conditions. – Korean Journal of Parasitology 44 (4): 373–378.



KNIPPERS R (2001): „Genomik: Forschungen über die Struktur und Expression komplexer Genome“ – In: KNIPPERS R: Molekulare Genetik, 8. neu bearbeitete Auflage: 510–511 (Thieme).

KOTLER-BRAJTBURG J, MEDOFF G, KOBAYASHI GS, BOGGS S, SCHLESSINGER D, PANDEY RC, RINEHART JR. KL (1979): Classification of Polyene According to Chemical Structure and Biological Effects. – Antimicrobial Agents and Chemotherapy 15 (5): 716–722.

KRASHIN JW, KOUMANS EH, BRADSHAW-SYDNOR AC, BRAXTON JR, EVAN SECOR W, SAWYER MK, MARKOWITZ LE (2010): Trichomonas vaginalis prevalence, incidence, risk factors and antibiotic-resistance in an adolescent population. – Sexually Transmitted Diseases 37 (7): 440–444.

KULDA J (1999): Trichomonads, hydrogenosomes and drug resistance. – International Journal of Parasitology 29: 199–212.

LAB TESTS ONLINE (2007): Trichomonas – Der Test (author unknown). (<http://www.labtestsonline.de/tests/Trichomonas.html>)

LAEMMLI UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. – Nature 227: 680–685.

LAGA M, MANOKA A, KIVUVU M, MALELE B, TULIZA M, NZILA N, GOEMAN J, BEHETS F, BATER V, ALARY M (1993): Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. – AIDS 7: 95–102.

LEITSCH D, RADAUER C, PASCHINGER K, WILSON IBH, BREITENEDER H, SCHEINER O, DUCHENE M (2005): *Entamoeba histolytica*: analysis of the trophozoite proteome by two-dimensional polyacrylamide gel electrophoresis. Experimental Parasitology 110: 191–195.

LEITSCH D, KOLARICH D, BINDER M, STADLMANN J, ALTMANN F, DUCHENE M (2009): *Trichomonas vaginalis*: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. – *Molecular Microbiology* 72 (2): 518–536.

LEITSCH D, KOLARICH D, DUCHENE M (2010): The flavin inhibitor diphenyleneiodonium renders *Trichomonas vaginalis* resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways. – *Molecular and Biochemical Parasitology* 171 (1): 17–24.

LEKHER MW, ALDERETE JF (1999): Resolution of six chromosomes of *Trichomonas vaginalis* and conservation of size and number among isolates. – *Journal of Parasitology* 85: 976–979.

LEÓN-SICAÍROS CR, LÉON-FÉLIX J, ARROYO R (2004) : *tvcp12*: a novel *Trichomonas vaginalis* cathepsin L-like cystein proteinase-encoding gene. – *Microbiology* 150: 1131–1138.

LEWIN LC (2007): Sexually transmitted infections in preadolescent children. – *Journal of pediatric health care* 21 (3): 153–161.

LOPEZ JV, YUHKI N, MODI W, MASUDA R, O'BRIEN SJ (1994): Numt, a recent transfer and tandem amplification of mitochondrial DNA in the nuclear genome of the domestic cat. – *Journal of Molecular Evolution* 39: 171–190.

LUCIUS R, LOOS-FRANK B (2008): *Biologie von Parasiten*, 2. Auflage (Springer): I-X + (1)–552.

LUSHBAUGH WB, CLEARY JD, FINLEY RW (1995): Cytotoxicity of hamycin for *Trichomonas vaginalis*, HeLa and BHK-21. – *Journal of Antimicrobial Chemotherapy* 36 (5): 795–802.

MALIK SB, PIGHTLING AW, STEFANIAK LM, SCHURKO AM, LOGSDON JR. JM (2008): An Expanded Inventory of Conserved Meiotic Genes Provides Evidence for Sex in *Trichomonas vaginalis*. – Public Library of Science 3 (8): e2879, 1–13.

MBONGO N, LOISEAU PM, BILLION MA, MALKA R-G (1998): Mechanism of Amphotericin B Resistance in *Leishmania donovani* Promastigotes. – Antimicrobial Agents and Chemotherapy 42 (2): 352–357.

MENÉNDEZ C, CASTELLSAGUÉ X, RENOM M, SACARLAL J, QUINTÓ L, LLOVERAS B, KLAUSTERMEIER J, KORNEGAY JR, SIGAUQUE B, BOSCH FX, ALONSO PL (2010): Prevalence and Risk Factors of Sexually Transmitted Infections and Cervical Neoplasia in Women from a Rural Area of Southern Mozambique. – Infectious Diseases in Obstetrics and Gynecology: ID 609315, 9 pp.

MORENO-BRITO V, YAÑEZ-GÓMEZ C, MEZA-CERVANTES P, AVILA-GONZÁLEZ L, RODRIGUEZ MA, ORTEGA-LOPEZ J, GONZÁLEZ-ROBLES A, ARROYO R (2005): A *Trichomonas vaginalis* 120 kDa protein with identity to hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by iron. – Cellular Microbiology 7 (2): 245–258.

MÜLLER M (1986): Reductive activation of nitroimidazoles in anaerobic microorganisms. – Biochemical Pharmacology 35: 37–41.

MÜLLER M (1990): “Biochemistry of *Trichomonas vaginalis*” – In: HONIGBERG BM: Trichomonads parasitic in humans: 53–83 (Springer).

MÜLLER M (1993): The hydrogenosome. – Journal of General Microbiology 139: 2879–2889.

MÜLLER M, GORRELL TE (1983): Metabolism and Metronidazole Uptake in *Trichomonas vaginalis* Isolates with Different Metronidazole Susceptibilities. – Antimicrobial Agents and Chemotherapy 24 (5): 667–673.

MURKEN J, GRIMM T, HOLINSKI-FEDER E (2006): Grundlagen der Genetik; Von der DNA zum Protein; Lebensdauer von Proteinen – In: MURKEN J, GRIMM T, HOLINSKI-FEDER E: Taschenlehrbuch Humangenetik, 7. Auflage: 40 (Thieme).

NAKASHIMA T, KUWANO M, MATSUI K, KOMIYAMA S, HIROTO I, ENDO H (1974): Potentiation of Bleomycin by an Antifungal Polyene, Pentamycin, in Transformed Animal Cells. – Cancer Research 34: 3258–3261.

NASIRUDEEN AMA, TAN KSW, SINGH M, YAP EH (2001): Programmed cell death in a human intestinal parasite, *Blastocystis hominis* – Parasitology 123 (3): 235–246.

NASIRUDEEN AMA, HIAN YE, SINGH M, TAN KSW (2004): Metronidazole induces programmed cell death in the protozoan parasite *Blastocystis hominis*. – Microbiology 150 (1): 33–43. DOI: 10.1099/mic.0.26496-0.

NIAID (2003): Prevalence and Incidence of Trichomoniasis – <http://www.cureresearch.com/t/trichomoniasis/intro.htm>. (18 June 2011).

NIX DE, TYRRELL R, MÜLLER M (1995): Pharmacodynamics of Metronidazole Determined by a Kill-Time Assay for *Trichomonas vaginalis*. – Antimicrobial Agents and Chemotherapy 39 (8): 1838–1852.

NORMAN AW, DEMEL RA, DE KRUYFF B, VAN DEENEN LLM (1972): Studies on the Biological Properties of Polyene Antibiotics. – The Journal of Biological Chemistry 247(6): 1918–1929.

NYIRJESI P (1999): Managing resistant *Trichomonas* Vaginitis. – Current Infectious Disease Reports 1(4): 389–392.

OLLER LZ (1969): Prevention of post-metronidazole candidosis with amphotericin B pessaries. – British Journal of venereal Diseases 45: 163–166.

OUELLETTE M, WARD SA (2003): « Drug resistance in parasites » – In: MARR JJ, NILSEN TW, KOMUNIECKI RW: Molecular medical parasitology: 397ff (Elsevier Science Ltd).

PAGE FC (1991): Nackte Rhizopoda – In: MATTHES D: Protozoenfauna, Band 2 (G. Fischer, Stuttgart).

PAILA YD, SAHA B, CHATTOPADHYAY A (2010): Amphotericin B inhibits entry of *Leishmania donovani* into primary macrophages. – Biochemical and Biophysical Research Communications 399: 429–433.

PALACIOS J, SERRANO R (1978): Proton permeability induced by polyene antibiotics. A plausible mechanism for their inhibition of maltose fermentation in yeast. – FEBS letters 9 (2): 198–201.

PERAZZI BE, MENGHI CI, COPPOLILLO EF, GATTA C, ELISETH MC, DE TORRES RA, VAY CA, FAMIGLIETTI AMR (2010): Prevalence and Comparison of Diagnostic Methods for *Trichomonas vaginalis* Infection in Pregnant Women in Argentina. – The Korean Journal of Parasitology 48 (1): 61–65.

PETRIN D, DELGATY K, BHATT R, GARBER G (1998): Clinical and microbiological aspects of *Trichomonas vaginalis*. – Clinical Microbiology Reviews 11: 300–317.

PIPERAKI ET, THEODORA M, MENDRIS M, BARBITSA L, PITIRIGA V, ANTSAKLIS A, TSAKRIS A (2010): Prevalence of *Trichomonas vaginalis* infection in women attending a major gynaecological hospital in Greece: a cross-sectional study. – Journal of Clinical Pathology 63 (3): 249–253.

POXLEITNER MK, CARPENTER ML, MANCUSO JJ, WANG CJ, DAWSON SC, CANDE WZ (2008): Evidence for karyogamy and exchange of genetic material in the binucleate intestinal parasite *Giardia intestinalis*. – Science 319: 1530–1533.

PRITHAM EJ, PUTLIWALA T, FESCHOTTE C (2006): *Mavericks*, a novel class of giant transposable elements widespread in eukaryotes and related to DNA viruses. – Gene 390 (2007): 3–17.

RAJEBALI A (2008): Trichomoniasis – An Underrated STI. – Indian Journal for the Practising Doctor 5 (1): 2008-03–2008-04 (<http://www.indmedica.com/journals.php?journalid=3&issueid=124&articleid=1649&action=article>, 7.6.2011).

RAKOTOMANGA M, SAINT-PIERRE-CHAZALET M, LOISEAU PM (2005) : Alteration of Fatty Acid and Sterol Metabolism in Miltefosine-Resistant *Leishmania donovani* Promastigotes and Consequences for Drug-Membrane Interactions. – Antimicrobiol Agents and Chemotherapy 49 (7): 2677–2686.

RASOLOSON D, VAŇÁČOVÁ Š, TOMKOVÁ E, RÁZGA J, HRDÝ I, TACHEZY J, KULDA J (2002): Mechanisms of in vitro development of resistance to metronidazole in *Trichomonas vaginalis*. – Microbiology 148: 2467–2477.

REDIGUIERI CF, PORTA V, NUNES DSG, NUNES TM, JUNGINGER HE, KOPP S, MIDHA KK, SHAH VP, STAVCHANSKY S, DRESSMAN JB, BARENDT DM (2011): Biowaiver monographs for immediate release solid oral dosage forms: Metronidazole. – Journal of Pharmaceutical Sciences 100 (5): 1618–1627.

RIVERA WL, ONG VA, MASALUNGA MC (2009): Molecular characterization of *Trichomonas vaginalis* isolates from the Philippines. – Parasitology Research 106: 105–110.

ROBERTS CW, MCLEOD R, RICE DW, GINGER M, CHANCE ML, GOAD LJ (2003): Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. – Molecular & Biochemical Parasitology 126: 129–142.

ROBINSON RA, LAWSON B, TOMS MP, PECK KM, KIRKWOOD JK, CHANTREY J, CLATWORTHY IR, EVANS AD, HUGHES LA, HUTCHINSON OC, JOHN SK, PENNYCOTT TW, PERKINS MW, ROWLEY PS, SIMPSON VR, TYLER KM, CUNNINGHAM AA (2010): Emerging Infectious Disease Leads to Rapid Population Declines of Common British Birds. – Public Library of Science One 5(8): e12215 (published online).

RYU JS, MIN DY (2006): *Trichomonas vaginalis* and trichomoniasis in the Republic of Korea. – Korean Journal of Parasitology 44 (2): 101–116.

SAIKI RK, GELFAND DH, STOFFEL S, SCHARF SJ, HIGUCHI R, HORN GT, MULLIS KB, ERLICH HA (1988): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. – Science 239 (4839): 487–491.

SAMBROOK J, FRITSCH EF, MANIATIS T (2001): Molecular Cloning: A Laboratory Manual – Second Edition, Cold Spring Harbor, NY Cold Spring Harbor Laboratory Press.

SCHWEBKE JR, AIRA T, JORDAN N, JOLLY PE, VERMUND SH (1998): Sexually transmitted diseases in Ulaanbaatar, Mongolia. – International Journal of STD & AIDS 9 (6): 354–358.

SEÑA AC, MILLER WC, HOBBS MM, SCHWEBKE JR, LEONE PA, SWYGARD H, ATASHILI J, COHEN MS (2007): *Trichomonas vaginalis* Infection in Male Sexual Partners: Implications for Diagnosis, Treatment, and Prevention. – Clinical Infectious Diseases 44: 13–22.

SIMHAN HN, ANDERSON BL, KROHN MA, HEINE RP, DE TEJADA BM, LANDERS DV, HILLIER SL (2007): Host immune consequences of asymptomatic *Trichomonas vaginalis* infection in pregnancy. – American Journal of Obstetrics & Gynecology 196: 59.e1–59.e5.

SIMONS K, TOOMRE D (2000): Lipid rafts and signal transduction. – Nature Reviews Molecular Cell Biology 1: 31–39.

SORVILLO F, SMITH L, KERNDT P, ASH L (2001): *Trichomonas vaginalis*, HIV, and African-Americans. – Emerging Infectious Diseases 7: 927–932.

STARY A, KOPP W, SÖLTZ-SZÖTS J (1991): Medical health care for Viennese prostitutes. – Sexually Transmitted Diseases 18 (3): 159–165.

STORCH V, WELSCH U (1997): Protozoa, 2. Ordnung Trichomonadida – In: STORCH V, WELSCH U: Systematische Zoologie. 5. Auflage, Gustav Fischer Verlag (Stuttgart, Jena, Lübeck, Ulm): 14.

STRATAKIS DF, LANG SM, EICHENLAUB S, LOSCHER T, STEIN R & HUBER RM (1999): Pulmonary trichomoniasis: diagnosis based on identification of irritation in Bronchoalveolar Lavage. – *Pneumologie* 53: 617–619.

SUTCLIFFE S, ALDERTE JF, TILL C, GOODMAN PJ, HSING AW, ZENILMAN JM, DE MARZO AM, PLATZ EA (2009): Trichomonosis and subsequent risk of prostate cancer in the Prostate Cancer Prevention Trial. – *International Journal of Cancer* 124 (9): 2082–2087.

SUTCLIFFE S, NEWMAN SB, HARDICK A, GAYDOS CA (2010): Prevalence and correlates of *Trichomonas vaginalis* infection among female US federal prison inmates. – *Sexually Transmitted Diseases* 37 (9): 585–590.

SUTTON DD, ARNOW PM, LAMPEN JO (1961): Effects of high concentrations of nystatin upon glycolysis and cellular permeability in yeast. – *Proceedings of the Society of Experimental Biology and Medicine* 108: 170–175.

TAYLOR-ROBINSON D (1998): *Mycoplasma hominis* parasitism of *Trichomonas vaginalis*. – *The Lancet* 352 (9145): 2022–2023.

THOMASON JL, GELBART SM (1989): *Trichomonas vaginalis*. – *Obstetrics & Gynecology* 74 (3): 536–541.

THOMPSON ED, STARR PR, PARKS LW (1971): Sterol accumulation in a mutant of *Saccharomyces cerevisiae* defective in ergosterol production. – *Biochemical and Biophysical Research Communications* 43: 1304–1309.

THURMAN AR, DONCEL GF (2011): Innate immunity and Inflammatory Response to *Trichomonas vaginalis* and Bacterial Vaginosis: Relationship to HIV Acquisition. – *American Journal of Reproductive Immunology* 65: 89–98.



TOVAR J, FISCHER A, CLARK CG (1999): The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. – *Molecular Microbiology* 32 (5): 1013–1021.

TWENTYMAN PR (1976): The effect of bleomycin and pentamycin in combination on the survival of EMT6 mouse tumour cells in vitro and in vivo. – *British Journal of Cancer* 33: 459.

UMEZAWA S, TANAKA Y, OOKA M, SHIOTSU S (1958): A new antifungal antibiotic, pentamycin. – *The Journal of Antibiotics* 11: 26–29.

VALLELY A, PAGE A, DIAS S, SIBA P, LUPIWA T, LAW G, MILLAN J, WILSON DP, MURRAY JM, TOOLE M, KALDOR JM (2010): The Prevalence of Sexually Transmitted Infections in Papua New Guinea: A Systematic Review and Meta-Analysis. – *Public Library of Science* 5 (12): e15586.

VAN BELKUM A, VAN DER SCHEE C, VAN DER MEIJDEN WI, VERBRUGH JA, SLUITERS HJ (2001): A clinical study on the association of *Trichomonas vaginalis* and *Mycoplasma hominis* infections in women attending a sexually transmitted disease (STD) outpatient clinic. – *FEMS Immunology and Medical Microbiology* 32: 27–32.

VAN LEEUWEN MR, GOLOVINA EA, DIJKSTERHUIS J (2009): The polyene antimycotics nystatin and filipin disrupt the plasma membrane, whereas natamycin inhibits endocytosis in germinating conidia of *Penicillium discolor*. – *Journal of Applied Microbiology* 106: 1908–1918.

VATANSHENASSAN M, REZAEI S, MOHEBALI M, NIROMAND N, KAZEMI B, BABAEI Z, REZAEIAN M (2010): *Trichomonas vaginalis*: Investigation of a novel diagnostic method in urine samples using cysteine proteinase 4 gene and PCR technique. – *Experimental Parasitology* 126: 187–190.

WALOCHNIK J, ASPÖCK H (2002): *Trichomonas vaginalis* – ein parasitärer Erreger einer Geschlechtskrankheit. – In: ASPÖCK H (Hrsg.): Amöben, Bandwürmer, Zecken...Parasiten und parasitäre Erkrankungen des Menschen in Mitteleuropa. Denisia 6, Biologiezentrum der OÖ Landesmuseen (Hrsg.): 145–161.

WALOCHNIK J, ASPÖCK H (2004): Pränatale, perinatale und neonatale Protozoen-Infektionen des Menschen: Überblick und aktuelle Probleme. – Nova Acta Leopoldina NF 89, Nr. 334: 187–207.

WENDEL KA, WORKOWSKI KA (2007): Trichomoniasis: Challenges to Appropriate Management. – Clinical Infectious Diseases 44 (3): 123–129.

WIEHART UIM, RAUTENBACH M, HOPPE HC (2006): Selective lysis of erythrocytes infected with the trophozoite stage of *Plasmodium falciparum* by polyene macrolide antibiotics. – Biochemical Pharmacology 71: 779–790.

WOLFF EDS, SALISBURY SW, HORNER JR, VARRICCHIO DJ (2009): Common Avian Infection Plagued The Tyrant Dinosaurs. – Public Library of Science ONE 4 (9), e7228: 1–7.

WOODS RA, BARD M, JACKSON IE, DRUTZ DJ (1974): Resistance to Polyene Antibiotics and Correlated Sterol Changes in Two Isolates of *Candida tropicalis* from a Patient with an Amphotericin B-resistant Funguria. – The Journal of Infectious Diseases 129(1): 53–58.

WHO – WORLD HEALTH ORGANIZATION (2001): Global prevalence and incidence of selected curable sexually transmitted diseases. – [www.who.int/docstore/hiv/GRSTI/006.htm](http://www.who.int/docstore/hiv/GRSTI/006.htm). (18 June 2011)

WRIGHT JM, DUNN LA, KAZIMIERCZUK Z, BURGESS A, KRAUER KG, UPCROFT P, UPCROFT JA (2010): Susceptibility in vitro of clinically metronidazole-resistant *Trichomonas vaginalis* to nitazoxanide, toyocamycin and 2-fluoro-2'-deoxyadenosine. – Parasitology Research 107: 847–853. DOI 10.1007/s00436-010-1938-3.

ZHANG K, BANGS JD, BEVERLEY SM (2010): Sphingolipids in parasitic protozoa. – *Advances in Experimental Medicine and Biology* 688: 238–248.

ZUBÁČOVÁ Z, CIMBŮREK Z, TACHEZY J (2008): Comparative analysis of trichomonad genome sizes and karyotypes. – *Molecular & Biochemical Parasitology* 161: 49–54.

ZYGMUNT WA (1966): Intracellular Loss of Potassium in *Candida albicans* after Exposure to Polyene Antibiotics. – *Applied Microbiology* 14 (6): 953–956.

## **6.6 Curriculum vitae**

Name: Markus Kranzler  
Born: 7.11. 1982 in Vienna

### **Education**

1989–1993: Primary school in Vienna

1993–2001: Realgymnasium in Vienna

18. 6. 2001: Matura

2001–2003: Kolleg for Chemistry (Biochemistry & Genetic Engineering),

HTL Rosensteingasse Vienna

2.9. 2003: Kolleg Diploma

2003–2011: Study of Biology / Anthropology, Human Genetics & Microbiology

2006: Degree in General Biology

2008: Erasmus Semester at the Georg-August University Göttingen, Germany

### **Scientific work experience**

10/2003–12/2003: Laboratory work experience at the Department of Plant Genetics  
of the Institute of Applied Genetics and Cell Biology / University of Agricultural  
Sciences Vienna

06/2005–07/2005: Internship in the research project Türktas et al. (2005): “Toward  
the determination of the genetic basis of heavy metal accumulation in six wild  
metallicolous and non-metallicolous *Salix caprea* populations” at the Department of  
Plant Genetics of the Institute of Applied Genetics and Cell Biology / University of  
Agricultural Sciences Vienna

2004–2007: Maintenance service of the collecting points for problematic waste  
substances in Vienna

2009–2011: Diploma Thesis at the Department of Parasitology of the Institute for  
Specific Prophylaxis and Tropical Medicine / Medical University of Vienna

## APPENDIX

01/2011: Tutor of Univ.-Prof. Dr. Andreas Hassl in the lecture “Krankheit, Manifestation und Wahrnehmung, allgemeine Arzneimitteltherapie” / Medical University Vienna

05/2011: Assistance of Dr. Robert Hofrichter at the marinebiological field station of “mare mundi” in Krk, Croatia

2010–2011: Scientific assistance of Univ.-Prof. Dr. Horst Aspöck

### Conferences

2007: Summer School Alpbach on *Astrobiology*. Interdisciplinary group project: Biochemical and Geophysical Field Study on Mars

2009: “Giftiger Samstag”

2009: ÖGTP- Advanced training courses 02/2009, 04/2009, 05/2009

2009: Parasitologische Fachgespräche

2009: 43th Annual Conference of Austrian Society for Tropical Medicine and Parasitology. Presentation: “Mode of action of pentamycin on *T. vaginalis*”

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