Investigating important aspects of antimicrobial resistance in *Bacteroides* strains and a direct qPCR method as a promising tool for *Mycoplasma* detection

Ph.D. Thesis

By

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1. INTRODUCTION

1.1. The current state of the antimicrobial resistance threat

Antimicrobial resistance (AMR) is an international public health situation that intimidates our ability to treat bacterial infections. AMR has been involved in 1.8 million deaths in 2020, which is anticipated to be one-third as many people as COVID-19 has killed. The inventor of penicillin, Sir Alexander Fleming himself, drew focus on the threat of underdosing resistance. Wherever antimicrobials are used, there will be reservoirs for resistance genes that will be used to render antibiotics ineffective.

1.2. Anaerobic bacteria and Bacteroides spp.

Anaerobes, the microorganisms that live and spread in settings lacking oxygen, play a considerable role in many processes in nature. They also play a vital function in human health and wellness regarding causing infections and making up essential microflora inside humans and animals. The human digestive tract microbiota is estimated to consist of 10^{18} CFU/g microorganisms, 10 times more than human cells. *Bacteroides fragilis* comprises around 0.1 to 0.5% of total gut bacteria but is the most frequently isolated anaerobe from peritoneal and abdominal abscesses and samples of bloodstream infections.

1.3. AMR in B. fragilis

AMR in anaerobes is complicated and facilitated by several mechanisms such as drug inactivation by enzymes, modification of target molecule through mutations and porin alterations. Metronidazole and carbapenem resistance is still low, but key antianaerobic agents are increasingly becoming less efficient. The chromosomally encoded genes *cfiA* and *ccrA* have been identified as coding carbapenem resistance. The antibiotic metronidazole is the most prescribed antibiotic for both prevention and treatment of Gram-negative anaerobic infections. These latter strains also show heterogeneous carbapenem resistance phenotypes usually identified through Etest antimicrobial susceptibility test (AST) experiments.

The best-known and most extensively examined resistance mechanism of *Bacteroides* against metronidazole is mediated by *nim* genes. The metronidazole MIC value of some *nimA*-positive strains does not correlate to the expression of genes. This is the case for other *Bacteroides* resistance genes, e.g., the carbapenem resistance *cfiA* gene. *Nim* genes are found to be preceded by insertion sequence elements that drive high expression of those same genes.

1.4. Bacterial tolerance, persistence, and heteroresistance

To stop resistant bacteria from growing, the antibiotic's minimum inhibitory concentration (MIC) must be much greater than for susceptible bacteria. In contrast to susceptible individuals, persistence and tolerance do not result in an increase in the MIC. Persistence is non-heritable, but the frequency of persister cells in a population is a heritable trait. An antibiotic-resistant cell has a resistance component by which it survives antibiotic treatment. Antibiotic persistence is always associated with a heterogeneous population, in which only a portion of the population is made up of tolerant cells.

1.5. Bacterial Heteroresistance

Heteroresistance (HR) is a phenomenon that describes variable responses of seemingly homogenous bacterial subpopulations to a specific antibacterial agent. HR has been proved to significantly impact the efficacy of antibiotic treatment in vitro and in vivo. Even though it has been known for a long time, HR is still a vague concept that lacks the precise definitions and guidelines to characterize or readily detect its presence.

The population analysis profile method (PAP) is considered the gold standard method for identifying HR. It involves quantifying the proportion of resistant cells existing within a culture at a variety of antibiotic concentrations. PAP is the most reproducible and reliable method for detecting HR, but it cannot be easily implemented into the clinical setting because it is tedious and time-consuming.

1.6. Toxin-Antitoxin (TA) pairs and their roles in persistence

When a plasmid encoding the TA system is removed from a cell, the toxin is unleashed from the existing TA complex. Type II systems are organized in operons, with the antitoxin protein generally placed upstream of the toxin. Toxins from these TA systems are thought to be involved in gene expression regulation, bacterial population control, and programmed cell death.

Toxin-antitoxin modules are gene loci that play a key role in the persister state, according to recent research. Bacterial persistence can be triggered through several factors, e.g., starvation. TA gene pairs, with their toxic and labile neutralizing antitoxin activities, can kill post-segregational plasmid-less daughter cells (if harbored by plasmids) or halt cell division.

1.7. Mycoplasma and its role in cell-culture studies

Mycoplasma is a small cell-wall free prokaryotic bacterium with a remarkable diversity at the species level. Contamination of cell cultures is a frequent phenomenon, according to a survey by the DSMZ-German Collection of Microorganisms and Cell Cultures. It is hard to prevent/eradicate since the bacterium is less sensitive to antibiotics commonly applied in cell cultures. Its small size (0.3-1 μm) and non-rigid cell wall make it also hard to remove by filtration. According to the DSMZ-German Collection of Microorganisms and Cell Cultures survey, the prevalence of *Mycoplasma* contamination of cell lines was 28% including *Mycoplasma* species *M.orale, M. hyorhinis, M. arginini, M. fermentans, M. hominis* and *Acholeplasma laidlawii.*

1.8. Realtime PCR

Real-time polymerase chain reaction (PCR) is one of the most sensitive and reliable quantitative approaches for gene expression analysis. In traditional PCR, an end-point analysis is used to detect the amplified DNA product, or amplicon. In real-time PCR, the accumulation of amplification product is measured in real time as the reaction proceeds, with product measurement after each cycle.

1.9. Detection of Mycoplasma contamination

The high probability of introducing novel *Mycoplasma* infections into cell cultures means it is necessary to monitor cell culture ingredients and cell lines for contamination. A wide

variety of detection methods are available, including metabolism detection and genome detection by PCR and qPCR. Regular PCR has high sensitivity and specificity, but most cases require nucleic acid purification and gel electrophoresis. In this study, we eliminate the purification step and significantly shorten the protocol time required, but the inhibitory effect of the direct sample is always present.

2. Aims

I. **Directly detect *Mycoplasma* DNA in a U937 suspension cell culture without using DNA purification:**
- To make *Mycoplasma* contamination monitoring easier.
- To leave out the DNA purification step and develop a direct qPCR detection method that is suitable to detect *Mycoplasma* contamination within U937 cell cultures.

II. **Molecular characterization of metronidazole resistant *Bacteroides* strains from Kuwait:**
- To investigate the prevalence and function of *nim* genes that provide metronidazole resistance among clinical *Bacteroides* isolates and related upstream regulatory elements known as insertion sequences (*IS*).
- Using PCR and Southern blotting, plasmids were isolated, sequenced using NGS (Next-generation sequencing).
- Conduct genetic typing of the *nimE*-positive *B. fragilis* strains by means of ERIC PCR.

III. **Examine and characterize the carbapenem heteroresistance of *B. fragilis* by phenotypic and molecular methods and correlate them:**
- To characterize HR and investigate diagnostic issues in the set of *cfiA*-positive *B. fragilis* using phenotypic and molecular methods, AST's and the gold standard method PAP (population analysis profile).
- To screen the genes related to carbapenems resistance and their *IS* elements, and to measure the activity of the carbapenemase enzymes.
- To induce *B. fragilis* isolates using imipenem pressure, conduct PAP testing and compare the outcomes with the other strains.
- To trace the changes of RNA expression of the TA pair using qRT-PCR in clinical and induced strains.

3. MATERIALS AND METHODS

3.1. Direct qPCR optimization for Mycoplasma detection

3.1.1. Cell culture

Mycoplasma infected U937 human monocytic cells were grown in an RPMI 1640 medium containing 10% heat-inactivated FBS and 50 μg/ mL gentamicin at 37 °C in 5% CO2, all within a 25 cm^2 cell culture flask (Greiner Bio-One Hungary, Mosonmag- yaróvár, Hungary).

3.1.2. Mycoplasma elimination

Mycoplasma elimination was performed using *Mycoplasma* Elimination Reagent (Bio-Rad, Hercules, CA, USA). The reagent was added to the medium at a 0.5 μg/ml final concentration and the U937 cells were cultured for 7 days.

3.1.3. DNA extraction and qPCR

DNA was extracted from *Mycoplasma* infected U937 cell supernatants using the Qiagen QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) qPCRs with 20 µl final volume were performed using the Bio-Rad CFX Connect qPCR real-time system. A statistical comparison of qPCR cycle threshold (Ct) values was performed with Student's t-test.

3.2. Molecular characterization of metronidazole resistant Bacteroides

3.2.1. Bacterial strains, cultivation, identification and antimicrobial susceptibility tests

Twelve metronidazole-resistant strains of *B. fragilis* were selected from the 421 clinical *Bacteroides*/*Phocaeicola* isolates collected from 2006-2018 in an antibiotic resistance survey in Kuwait. The long-term storage and transfer of these strains from Kuwait to Hungary was done by lyophilization. The strains were revitalized in chopped-meat bouillon and made in a 20% glycerol-containing Brain-Heart Infusion (BHI) broth at -70 °C. Regular cultivation of the strains involved solid (Columbia agar supplemented with 10% defibrinated and 5% laked sheep blood, 0.3 g/l cysteine and 1 mg/l vitamin K1) or liquid media.

3.2.2. PCR experiments

DNA was extracted from 42 *Bacteroides* strains, including non-*fragilis* and *cfiA*-negative *B. fragilis* strains in addition to the strains from Kuwait. Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) typing, phylogenetic analysis (complete linkage, band-based clustering with Jaccard coefficients) and evaluation were carried out.

3.2.3. Plasmid screening, Southern blotting and plasmid sequencing

The plasmid profiles of the test *Bacteroides* and *P. dorei* strains were determined by the alkaline-SDS lysis procedure as described earlier. Their analysis was done in 0.7% agarose gels containing 0.5 mg/ml ethidium bromide in TBE buffer using a 12 V/cm voltage gradient. The plasmid DNA was transferred by capillary action to Hybond+ nylon membranes. The probe labeling, hybridization, and detection were carried out by North2South Labeling and Hybridization kits (Thermo Fisher Scientific). The sequences of *nim* gene-containing plasmids were deposited in GenBank under the accession numbers MW388914 (pPDQ1c), MW388913 (pBFQ6d), MW388915 (pBFQ8b), MW448185 (pBFQ10c) and MW448186 (pBFQ11c).

3.3. Studying B. fragilis heterogeneous resistance to carbapenems

3.3.1. Bacterial Strains and Cultivation

Cultivation of *B. fragilis* was performed on anaerobic Columbia blood agar plates (Columbia agar supplemented with 2.5% defibrinated sheep blood, 1.25% laked sheep blood and 300 mg/L L-cysteine and 1mg/L vitamin K1) at 37 °C under anaerobiosis (85% N2, 10% H2 and 5% CO2) in a cabinet (Concept400, Ruskinn, UK). Isolates were determined using the MALDI-TOF MS method (Microflex LP instrument and Biotyper 3.1 software package, Bruker Daltonics, Bremen, Germany).

3.4. MIC Determinations, Recording of Population Analysis Profiles and Time–Kill Curves

Cell suspensions were taken after 48 h cultivations on Columbia blood agar plates or overnight incubated BHIS broth culture. Optical density (at 600 nm) was measured by spectrophotometer (Thermo Scientific) for later normalization. Ten-fold dilutions of PBS and 100 µL inocula were spread on Wilkins-Chalgren Agar plates with an appropriate concentration of imipenem (from the 0.008–1024 µg/mL range) to yield 50–500 countable colonies per plate.

3.5. Imipenemase Activity Measurement and Induction of HR by Imipenem Treatment

Imipenemase activity was determined by centrifuging BHIS cultures of *B. fragilis* 3130 and CZE60 and exposing them to stepwise increments (0, 2, 8, 32 and 128 µg/mL) of imipenm concentrations. Protein concentrations were determined by the Qubit Protein Assay Kit (Thermo Fisher Scientific, Budapest, Hungary) in 1 mL UV-transparent plastic cuvettes in an Assay buffer (50 mM HEPES, 25 µM ZnSO4, pH 7) and an adjusted enzyme volume. The results were expressed by 1 pmole imipENem hydrolyzed per 1 min (U) and standardized by the protein content of the extracts (U/mg).

3.6. Conventional PCR, Nucleotide Sequencing and qRT-PCR Experiments

To examine the '*cfiA* element' constant gene ('GNAT', 'XAT' and *cfiA*) expression levels, total RNA was isolated (HighPure RNA Isolation Kit, Roche) from test strains. The 10 µL final volume PCR reactions contained 5 µL SYBR Green mastermix with ROX, 0.7 µM primers and 1 µL RNA sample.

3.7. Curve Plotting, Curve Parameter Calculation, Statistical Evaluation and Bioinformatics

Means and standard deviations were calculated after normalization of the OD_{600} values in MS Excel. For the saturation curves, the following equation (3-parameter sigmoid models) was used. The values of growth fraction for each imipenem concentration obtained this way were then plotted (Sigma plot 12) by direct axes (quasi hyperbolic curves), logarithmic x axis of imipenem concentrations and direct y axis of growth fraction ('saturation curves') and with both axes logarithmic (classical PAP curves). In addition, Spearman rank correlation calculations were performed to estimate congruences between recorded parameters (Sigma plot 12).

4. Results

4.1. Direct qPCR optimization for Mycoplasma detection

To achieve optimal sensitivity and the shortest possible reaction time of direct qPCR, we followed a step-wise optimization of the PhoenixDx *Mycoplasma* Mix (Procomcure Biotech, Thalgau, Austria) protocol. First, we tested to see whether reducing the annealing/extension time might influence qPCRs. The results indicated that reductions from 60 to 20 s had little or no effect on performance. Next, we examined the effect of sample volume on performance, and found that samples with 6, 8 and 10 microns of

supernatants had similar Ct levels. The QIAamp DNA purification kit was used to isolate Myco-plasma DNA from U937 cell cultures (medium + cells).

The elution volume was 100 µl, but we opted for the 6 µl sample volume. In a comparison of these samples, we found that the 6µl purified sample produced lower Ct values (~ 2 cycles) than the 0.36 µl direct sample. This suggests a low level of qPCR inhibition of the supernatant.

Direct qPCR method proved to be a quick and effective method for monitoring the decrease in *Mycoplasma* DNA during the elimination process. Our results showed that the supernatants containing removal agent or free from removal agent both resulted in nearly the same Ct levels (27.04 ± 0.24 and 26.94± 0.45, respectively). This indicated that the presence of removal agent did not influence qPCRs performance.

4.2. Metronidazole resistant strains from Kuwait

A high prevalence of *cfiA* (n=6, 60%) was encountered among the *B. fragilis* strains determined by MALDI-TOF MS, metronidazole and imipenem resistance values were determined by Etest. Among the 11 *nimE*-positive strains, 10 carried IS*Bf6* that could be mapped to the upstream region of the *nimE* genes. The distance between these elements was a constant 26 bp. From the plasmid sequences obtained, we could also deduce that only one of these strains had a common origin.

Although only one *nimE* plasmid type has been described to date, in our Southern blots, we detected 5 different *nimE* plasmids. A high prevalence of *cfiA* (n=6, 60%), by PCR and MALDI-TOF MS typing, was encountered among the *B. fragilis* strains. One representative of each of the different sized plasmids was also sequenced by high-throughput sequencing.

4.3. Characterization of heteroresistance to imipenem in Bacteroides fragilis

4.3.1. Phenotypic Characterization and PAP Experiments

The expression of imipenem HR phenotypes, as determined by gradient tests, varied from low to high. We analyzed the HR behavior of colonies in the partial inhibition zone. For strains showing a highly expressed HR phenotype, such as *B. fragilis* CZE60, some induction was observed as HR increased.

4.3.2. *PAP Curves, Assessment and Correlation of the Phenotypic Heteroresistance Parameters*

In addition to PAP plots, plots without logarithmic axes (x and y axis direct—hyperbolic curves) were also examined, allowing more insight into the nature of HR. The saturation curves displayed some meaningful properties: it was interesting that, sometimes, at the lowest imipenem concentrations, we obtained fewer colony-forming units (CFU) than on the next higher-concentration plate. We explained this by presuming that some dormant cells were present in the inoculating cell preparations (cultures suspended in PBS or BHIS) and that the higher, but non-selective, concentration induced the cells to exit dormancy.

The starting imipenem concentrations caused a smoothly decreasing curve, validating our sigmoid hypothesis, and the decrease in CFUs caused by increasing imipenem concentrations also tended to be somewhat continuous. The saturation curves widened as the HR increased and also produced the x_0 value, which was the inflection point or the

maximum value of their derivative, the density function. We also recorded imipenem MICs on WC agar plates since the latter was also used for PAP measurements. The PAP AUC ratios were also calculated.

Almost all test parameters showed potential for HR and were cross-correlated to assess the connections between them. We obtained a quite good rate of relatedness between the PBS and BHIS values and almost all the phenotypic parameters. An increased imipenemase production could be a cause of the HR phenotype.

4.3.3. *HR Induction by Imipenem and Correlation of the Molecular Characteristics of Heteroresistance*

A chromosomal segment ('*cfiA* element') containing the *cfiA* gene and a proposed TA gene pair with some insertional elements (MITE1, IS elements) was identified as being characteristic of *B. fragilis* strains. The 'GNAT' toxin gene showed a high homology to the elongation protein 3 ($e = 1.03 \times 10{-}40$) or to the AtaT-TacT-ItaT TA toxins in protein BLAST conserved domain searches. Imipenem stirred the HR strain cells from dormancy, we attempted to induce or increase HR by imipenem treatment. It can be seen that as imipenem concentration increased, HR, measured by E-tests and *cfiA* expression, 'GNAT' and 'XAT' genes and the 'GNAT-XAT' expression ratio, also showed increases.
CfiA gene expression showed a strong correlation with the composition of Lrp, as detected by the sequencing of its fragments by PCR amplification. 'GNAT' correlated with almost all the phenotypic traits, and imipenemase production correlated with the expressions of *cfiA* and 'XAT'. The cross-correlation of molecular traits also provided some insights into the possible action mechanism.

4.3.4. Time–Kill Curves

'GNAT-XAT' formed a TA pair that may also cause persister phenotypes. The curves were straight, reminiscent of antibiotic tolerance even for the *B. fragilis* 638R control strain. If a strain is tolerant to one antibiotic, it can display tolerance to others through slow growth. This was not the case for our strains. This was reinforced, as imipenem induction did not cause alterations in the curves of imipenem-induced and non-induced strains.

5. DISCUSSION

5.1. Direct qPCR is a sensitive approach to detect Mycoplasma contamination in U937 cell cultures

While various methods exist for the detection of *Mycoplasma* contamination, probably the most frequently used ones are biochemical detection of *Mycoplasma* metabolism and PCR-based detection of *Mycoplasma* DNA. Though the biochemical detection of *Mycoplasma* ATP generation (Mycoalert, Lonza, Basel, Switzerland) is a quick protocol, it has certain disadvantages that should be mentioned, including requiring that reagents be reconstituted and brought to 22 °C before each measurement and requiring a luminometer for ATP detection. Aspecificity due to ATP generated by other cells may lead to a high background and eventually false negative measurements. The *Ureaplasma* species which are also a common contaminant in a cell culture cannot be detected by Mycoalert as their own ATP production relies on the hydrolysis of urea. Finally, the sensitivity of biochemical detection has been shown to be lower than that for PCR or qPCR methods.

There are a variety of kits on offer based on regular PCR, followed by gel electrophoresis. The major advantage of these kits is the wide availability of regular PCR and electrophoresis equipment. However, decreased specificity compared to probe-based qPCR, the additional electrophoresis step, and the inability to quantitatively monitor the decrease in *Mycoplasma* genome concentration during treatment are clear drawbacks. Intercalation-based (e.g., SYBR Green) qPCR kits such as MycoSEQ *Mycoplasma* Detection Assay (Thermo Fisher, Waltham, MA, USA) eliminate the electrophoresis step and provide quantitative information about *Mycoplasma* genome concentration. The disadvantages of intercalation-based qPCR kits compared to probe-based kits are a lower specificity, lack of internal control and the potential effect of cell culture composition, ionic composition and ionic strength to change the melting temperature of the qPCR product. Since this melting temperature is the basis for evaluating specificity in intercalation-based qPCRs, changing it can be problematic. Probe-based qPCRs such as PhoenixDx (Procomcure Biotech, Thalgau, Austria), Microsart RESEARCH *Mycoplasma* (Sartorius, Göttingen, Germany) and qPCR Detection Kit (XpressBio, Frederick, MD, USA) avoid these problems and due the additional requirement of the binding of the probe sequence, these kits provide a higher specificity than regular PCRs and intercalation-based qPCRs.

Noting the advantages of probe-based qPCRs, we optimized the Procomcure PhoenixDx kit to perform a direct qPCR with a *Mycoplasma* infected U937 cell culture. Our results indicates that the optimal temperature was the same as that in the original protocol, so the primer + probe binding was not affected by the presence of the direct template. The fact that the optimal template volume was 6 µl (30% of the total qPCR volume) meant that the direct sample did not have a significant inhibitory effect on the qPCR. A major optimization step that we performed was decreasing the annealing/extension time from 60 s to 20 s, thus saving 40 s in each cycle. Interestingly, this decrease led to only a minor decrease in the sensitivity (~ 0.6 Ct level increase). In addition, decreasing the number of cycles from 50 to 40, reduced the total qPCR time required to 65 min. When we used the optimized qPCR protocol with direct and purified cell culture templates, we found that Ct levels of a 6 µl direct template was almost identical to that of purified DNA from a 60 µl cell culture. The reason for this is mainly due to a dilution of the original DNA content during the elution step at the end of DNA purification. Overall, in our case, direct qPCR sensitivity was higher than qPCR with a purified template, with a saving in the cost/time of DNA purification. We monitored the elimination of *Mycoplasma* contamination from the U937 cell culture using the optimized direct qPCR protocol. One of the concerns using pathogen DNA detection is that the non-viable pathogen's DNA can also be detected and lead to a false positive signal. In our case however, the *Mycoplasma* DNA content dropped to ~ 20% of the original concentration after 1 day of treatment, and though days 1 and 2 contained a similar level of DNA, this decrease continued on day 3. In summary, with direct qPCR we were able to monitor the elimination of *Mycoplasma* over the treatment period.

5.2. Molecular characterization of metronidazole resistant Bacteroides strains from Kuwait

Kuwait has a high prevalence of *Bacteroides* and *P. dorei* strains that are resistant to metronidazole. This figure is high compared to the resistance prevalent in northern countries but is in the described range for countries south of Europe. Essentially, non-prudent use can be held responsible for this phenomenon.

This is not unexpected as it has been experienced before, that in other strains MICs can change through metronidazole induction or withdrawal. Differences in the regulation of *cfiA* and nim genes can be suspected. Almost all strains that had resistance to metronidazole MIC in Kuwait were nim gene-positive, so we still expect a functional role for nims in resistance. We also found a highly imipenem resistant strain (*B. fragilis* Q10) for which we have a good explanation.

The nim gene typing by sequencing also showed that the 11 nim positive strains harbored the *nimE* type, which is also characteristic of strains in southern countries. This gene was found predominantly in metronidazole resistant *Bacteroides* strains in India, Afghanistan and in an earlier report for Kuwait.

Of the 11 *nimE* genes, 9 harbored IS*Bf6* or IS*612B* in their upstream region. When we could PCR-map the elements to each other, their distances were constant (26 bp). This implied that there was no independent insertion of IS*BF6* in the different plasmids, but rather a preformed *nimE*-IS*Bf6* configuration which then mutated and spread. This implied to us that there was no independent insertion of IS*Bf6* in the different plasmids, but rather a preformed *nimE*-IS*Bf6* configuration inserted into a plasmid, and that then mutated and spread later as suggested in Sóki et al. The *nimE* positive *B. fragilis* strains did not have a common origin to indicate the mobility and horizontal transfer of these plasmids. This origin hypothesis can be discussed together with earlier findings that chromosomal *nim* gene carrying strains tend to also harbor the *cfiA* gene. The most notable are pPDQ1c and pBFQ11c where only a sub-segment of IS*Bf6* and IS*612B* respectively were in the upstream regions of the *nimE* genes. The *nimE* and *cfiA*-positive strains were different from the '*B. fragilis* BF8 multidrug-resistant cluster' as shown by ERIC typing, indicating that they emerged from a different background. Additionally, the association between *nim* and *cfiA* genes invites further investigation, crucially in terms of multidrug-resistance phenotypes. One of our *nimE* and *cfiA*-positive strains was highly imipenem resistant, and all our strains can be regarded as multidrug resistant to at least three groups of antibiotics (most β-lactams, clindamycin and metronidazole).

5.3. Characterization of heteroresistance to imipenem in Bacteroides fragilis

This study revealed that carbapenem heteroresistance is a characteristic phenotype of some *cfiA*-positive *B. fragilis* strains. The phenotypic parameters studied as the saturation curve x_0 and b, the PAP curve dilution and AUC, the imipenem MIC and HRI values and the specific imipenemase activities of the strains were interconnected and predicted HR well. The most important and central parameter was the imipenemase production, which could mediate the resistance and affect the other phenotypic parameters as it was proportional to the other parameters observed: agar dilution MICs, PAP AUC ratios and the saturation curve (simpler PAP curve) extension in PAPs of agar plate-grown cells. In our opinion, all the studied parameters could predict HR, but PAP AUC was the best. However, since most

of these parameters were continuous in form from low to high imipenem MICs and HR parameters, continuing to use PAP curve extensions could also be regarded as a very good method. The PAP AUC method was also suggested as a good prediction parameter for the reduced glycopeptide susceptibility of *staphylococci*.

We related the HR phenotype to stochastic processes. According to our hypothesis, this was due to the action of a proposed toxin ('GNAT') that may stop growth, but also allows viability under antibiotic-exposed circumstances. The primary finding supporting this was the widening of the PAP saturation curve parameter, b, which could be regarded as a standard deviation parameter. To improve the discussion of HR and persistence, Brauner and Balaban suggested the term heterotolerance for HR. In more thoroughly investigated tolerance and persistence mechanisms, it has also been suggested that wider distributions, and those above a certain persistence factor threshold, yield more persisters. We believe this is true for the carbapenem heteroresistance of *B. fragilis*, as we also detected a widening of our saturation curves. Imipenemase activity values, as an effector mechanism, also correlated with most of the phenotypic parameters of HR.

We propose an HR mechanism of *cfiA*-positive *B. fragilis* strains as follows: (i) *cfiA* is expressed proportionally to HR, and (ii) the parallel expression of 'GNAT-XAT' allows reduced cellular activities. We conclude the same from experiments in which we induced imipenem HR by imipenem; however, to obtain a more detailed picture about this, experiments are under way in our laboratory to determine the promoters of *cfiA* and 'GNAT-XAT', how they act individually and in conjunction with other promoters, what is the biochemical nature of 'GNAT' and 'XAT', do they form a TA pair and what is the role of the lysine-rich peptide in the '*cfiA* element'. It is conceivable through the above that 'GNAT' acts through the acetylation of a lysine in a ribosomal protein or in tRNA-Lys molecules and Lrp may modify these actions.

At present, no particular common mechanism was found to explain HR in other bacteria. However, in some cases, regulatory proteins were involved as well, which we believe may also produce stochastic regulation. Additionally, monoclonal heteroresistance could also emerge by the tandem duplication of DNA segments of the effector genes of *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* or *Streptococcus pneumoniae*. However, this latter mechanism can result in a variable number of repeats in the cells of a given population, which can be both stochastic and difficult to detect. Recently, for the aminoglycoside HR of *A. baumannii* in a *recA*-negative background, a modest copy number variation of the *aadB* gene-containing integron was linked to HR. The step causing the copy number increase was hypothesized as a stochastic process. In earlier experiments, we did not observe that *cfiA* or the '*cfiA* element' had copy number variations or that the *cfiA* promoter was invertible (data not shown), as CPS promoters usually are in *B. fragilis*. The role of global regulatory systems ((p)ppGpp, relA, spoT) in bacterial persistence was proven, something which we would like to examine regarding the HR of *B. fragilis*.

For TA systems, the prominent role of governing persistence was attributed, but some parallels between HR and persister phenotypes could also be drawn, HR can be regarded as concentration-dependent, while persistence can be regarded as a time-dependent survival phenomenon. In our opinion, the stochastic hypothesis of carbapenem HR of *B. fragilis* may facilitate research into this being the case in other HR systems as well.

7. NEW FINDINGS:

I. Direct qPCR for *Mycoplasma* detections:

- We demonstrated firstly the successful direct use of the *Mycoplasma* qRT-PCR kit.
- A novel user-friendly probe-based qPCR was developed to detect *Mycoplasma* contamination method.
- The developed direct qPCR method eliminates purification, maintains sensitivity, and is 65 minutes long.
- The developed direct qPCR method could be implemented to detect the presence of other microorganisms.

II. Metronidazole resistance in Kuwait:

- In Kuwait, majority of metronidazole resistance in *B. fragilis* is due to plasmid-carried *nimE* genes preceded by IS-provided promoters.
- The prevalence of metronidazole resistant *Bacteroides* strains is around 4% in Kuwait.
- *nimE*-positive strains also carry the *cfiA* gene, causing severe issues linked to multidrug-resistance, particularly in sub-Saharan Africa.

III. Heteroresistance to carbapenems in *B. fragilis:*

- This is the first work to investigate HR in anaerobes and successfully determined some of the main factors involved in this phenomenon in *B. fragilis.*
- The study shows that the saturation curve, PAP AUC, agar dilution and imipenemase activities are good predictors of HR.
- The expression of the '*cfiA* element' genes ('GNAT', 'XAT', and *cfiA*) can act as a toxin causing dormancy.
- Imipenem HR could be induced by exposure to imipenem.
- Carbapenem heteroresistance of the *B. fragilis* strains is stochastically regulated and is mediated by the altered imipenemase production.
- The proposed factors causing HR in *B. fragilis* could provide a strong basis to conduct more research and establish a better understanding of this ambiguous phenomenon.

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This Ph.D. thesis is dedicated to my lifelong friends Uncle Ali and Senan, who passed away during the period of this Ph.D.

9. PUBLICATIONS

1. PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS

This thesis is based on results published in the following papers:

I. **Baaity, Z.**, Breunig, S., Önder, K. and Somogyvári, F. (2019) Direct qPCR is a sensitive approach to detect *Mycoplasma* contamination in U937 cell cultures. BMC Research Notes 12 (1), 720:
https://doi.org/10.1186/s13104-019-4763-5
(IF_{2019}: 1.66), Rank: Q2 *Citations: 1*

II. **Baaity, Z.**, Jamal, W., Rotimi, V.O., Burián, K., Leitsch, D., Somogyvári, F., Nagy, E. and Sóki, J. (2021) Molecular characterization of metronidazole resistant *Bacteroides* strains from Kuwait. Anaerobe 102357: https://doi.org/10.1016/j.anaerobe.2021.102357
(IF_{2021}: 3.331), Rank: Q2 *Citations: 1*

III. **Baaity, Z.**, Loewenich, F., Nagy, E., Orosz, L., Burián, K., Somogyvári, F. and Sóki, J. (2022) Phenotypic and molecular characterization of carbapenem heteroresistant *Bacteroides fragilis* strains. Antibiotics (MDPI). https://doi.org/10.3390/antibiotics11050590
(IF_{2022}: 4.639), Rank: Q1 *Citations: -*
Cumulative Impact factor = 9.63

PUBLICATIONS NOT RELATED TO THE TOPIC OF THE THESIS

I. **Baaity, Z.**, Almahmoud, I. and Khamis, A. (2017) Prevalence of extended spectrum β lactamases (ESBL) in *E. coli* at Al-Assad Teaching Hospital. Research Journal of Pharmacy and Technology 10 (7), 2433-2436:
http://dx.doi.org/10.5958/0974-360X.2017.00430.9
(IF_{2017}: 0.53), Rank: Q3 *Citations: 12*

II. Ismail, R., **Baaity, Z.** and Csóka, I. (2021) Regulatory status quo and prospects for biosurfactants in pharmaceutical applications. Drug Discovery Today https://doi.org/10.1016/j.drudis.2021.03.029
(IF_{2021}:7.851), Rank: D1 *Citations: 2*

III. Kincses, A.; Rácz, B.; **Baaity, Z.**; Vásárhelyi, O.; Kristóf, E.; Somogyvári, F.; Spengler, G. (2021) The Relationship between Antibiotic Susceptibility and pH in the Case of Uropathogenic Bacteria. Antibiotics (MDPI), 10, 1431. https://doi.org/10.3390/antibiotics10121431
(IF_{2021}:4.639), Rank: Q1 *Citations: -*

IV. Rutai, A., Zsikai, B., Tallósy, S., Érces, D., Bizánc, L., Juhász, L., Poles, M., Sóki, J., **Baaity, Z.**, Fejes, R., Varga, G., Földesi, I., Burián, K., Szabó, A., Boros, M., and Kaszaki, J. (2022) A porcine sepsis model with numerical scoring for early prediction of severity. Frontiers in Medicine.
https://doi.org/10.3389/fmed.2022.867796
(IF_{2022}:5.091), Rank: Q1 *Citations: -*

Cumulative Impact factor = 18.111

PRESENTATIONS AND ABSTRACTS RELATED TO THE TOPIC OF THE THESIS

- ***Verbal presentations***

1. Csajbók, D., Nagy, V., **Baaity, Z.**, Ferenc Somogyvári. (2019) ECCMID 29th: Rapid screening for the bacterial pathogens from serum in the case of sepsis., Amsterdam, Netherlands.
2. **Baaity, Z.**, Somogyvári, F., and Sóki, J. (2020) EUGLOH 1st: (online) Challenging antibiotic resistance mechanisms in the 21st century – the case of *Bacteroides* spp. Szeged, Hungary.
3. **Baaity, Z.**, Jamal, W., Rotimi, V.O., Burián, K., Leitsch, D., Somogyvári, F., Nagy, E. and Sóki, J. (2020) MMT 2020 (Hungarian Society for Microbiology) Metronidazole-resistant *Bacteroides* strains from Kuwait: a molecular study. Kecskemét, Hungary.

- ***Poster and abstract presentations***

I. **Baaity, Z.**, Jamal, W., Rotimi, V.O., Burián, K., Leitsch, D., Somogyvári, F., Nagy, E. and Sóki, J. (2020) ECCMID 30th: (Online) Molecular analysis of metronidazole resistant *Bacteroides* strains from Kuwait. Paris, France.

II. **Baaity, Z.**, Burián, K., Somogyvári, F., Nagy, E. and Sóki, J. (2020) 15th Biennial Congress of the Anaerobe Society of the Americas (ASA): (Online) Investigations into the phenotypic characteristics and molecular mechanisms of the Heterogeneous carbapenem resistance of *Bacteroides fragilis* strains. Seattle, Washington USA.

III. **Baaity, Z.**, Jamal, W., Rotimi, V.O., Burián, K., Leitsch, D., Somogyvári, F., Nagy, E. and Sóki, J. (2020) 15th Biennial Congress of the Anaerobe Society of the Americas (ASA): (Online) Molecular characterization of metronidazole resistant *Bacteroides* strains from Kuwait. Seattle, Washington USA.

IV. **Baaity, Z.**, Nagy, E., Sóki, J. (2020) MMT 2020 (Hungarian Society for Microbiology): Phenotypic and molecular characterization of carbapenem heteroresistant *Bacteroides fragilis* strains. Kecskemét, Hungary.

V. **Baaity, Z.**, Burián, K., Hamasalih, B., Nagy, E., and Sóki. (2021) ECCMID 31st (Online) Characterization of heteroresistance to imipenem in *Bacteroides fragilis* strains. Vienna, Austria.