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Short Communication

Lactate dehydrogenase activity in *Bacteroides fragilis* group strains with induced resistance to metronidazole

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ABSTRACT

The aims of this study were to induce in vitro metronidazole resistance in *nim*-negative *Bacteroides fragilis* group strains and to determine the lactate dehydrogenase (LDH) activity of the induced strains. A collection of *B. fragilis* group strains were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). Minimum inhibitory concentrations (MICs) for metronidazole were determined by the agar dilution technique. The presence of *nim* genes was screened by PCR. A sample of 52 *nim*-negative metronidazole-susceptible strains were selected at random and were exposed to metronidazole in the resistance induction experiment. LDH activity was measured by spectrophotometry. Of the 52 selected strains, 12 (23.1%) acquired resistance to metronidazole. MICs ranged from 8 mg/L to 96 mg/L. Eight of the twelve induced strains displayed decreased LDH activity, whilst only one expressed a significant increase in LDH activity with LDH values of 49.1 U/mg and 222.0 U/mg, respectively. In conclusion, in vitro induction of metronidazole resistance could be selected in *nim*-negative *B. fragilis* group strains. A statistically significant decrease in LDH activity was in contrast to previous findings in which, underlying higher metronidazole MICs, an increase in LDH activity compensated for the decreased activity of pyruvate-ferredoxin oxidoreductase (PFOR). These findings could be explained if the induction caused only physiological and not genetic changes. We believe that genetic mutations in the *B. fragilis* strain that demonstrated an emergent increase in LDH activity were responsible for the increased activity.

1. Introduction

Bacteroides spp. strains are the most numerous faecal microbiota. They colonise the upper part of the gastrointestinal and respiratory tracts as well as the lower part of the genital tract in females. As opportunistic pathogens, particularly *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*, they usually cause polymicrobial infections and septicaemia [1–3].

Metronidazole is one of the 5-nitroimidazoles and is the drug of choice for treating anaerobic infections [4]. It is assumed that the mechanism of action of metronidazole involves uptake of the prodrug via passive diffusion and then intracellular reduction of the nitro group leading to cytotoxic short-lived radicals that are able to damage DNA strands [5]. The pyruvate:ferredoxin oxidoreductase (PFOR) complex is considered to be crucial for determining susceptibility to metronidazole. The lower enzymatic activity of the PFOR complex and the compensatory increased activity of lactate dehydrogenase (LDH) are taken to be important factors in the occurrence of metronidazole resistance [6]. Other proposed resistance mechanisms could be inactivation of the drug by the *nim* gene products, enhanced activity of DNA repair enzymes, altered redox states with decreased glucose utilisation, and active efflux of the drug [7].

The objective of this study was to investigate the presence of increased LDH activity as a possible resistance mechanism in *nim*-negative *B. fragilis* group strains with induced resistance to metronidazole.

2. Materials and methods

2.1. Bacterial strains

A total of 155 non-duplicate *Bacteroides* spp. and *Parabacteroides* spp. strains were collected from various clinical samples from the University Clinical Hospital Centre Zagreb (Zagreb, Croatia)

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over a 2-year period. Columbia blood agar (BBL Columbia Agar Base; BD, Franklin Lakes, NJ) supplemented with 5% sheep blood, 5 mg/L haemin and 1 mg/L vitamin K_1 was used for the bacterial growth medium under anaerobic conditions (GasPak™ EZ Gas Generating Container System within anaerobic jar; BD) for 48 h at 35 °C. Routine phenotypic identification was carried out by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (MALDI Biotyper CA System; Bruker Corp., Billerica, MA). *Bacteroides fragilis* (n = 97) was the most commonly isolated *Bacteroides* spp., followed by *Bacteroides vulgatus* (n = 19), *B. thetaiotaomicron* (n = 16), *Bacteroides ovatus* (n = 9), *Bacteroides uniformis* (n = 4), *Bacteroides caccae* (n = 3), *Bacteroides stercoris* (n = 2) and *Bacteroides salyersiae* (n = 1). There were four *Parabacteroides distasonis* strains. Isolates were stored in Brucella broth (BBL Brucella Broth; BD) with 15% glycerine at −80 °C until use.

2.2. Susceptibility testing to metronidazole

Minimum inhibitory concentrations (MICs) for metronidazole were determined by the agar dilution technique as previously described [8] on freshly prepared supplemented Brucella agar plates (BBL Brucella Agar). The breakpoint panel of two antibiotic dilutions (4 mg/L and 8 mg/L) was used according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints interpretive criteria [9]. The full panel of antibiotic dilutions was additionally applied to strains with acquired metronidazole resistance. *Bacteroides fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used as control strains.

2.3. PCR screening for nim genes

Rapid DNA extraction and PCR amplification for the presence of *nim* genes were performed according to previously described procedures [10]. The positive control strains containing *nim* genes were *B. fragilis* 638R(pIP417) (*nimA*), *B. fragilis* BF-8 (*nimB*), *B. fragilis* 638R(pIP419) (*nimC*), *B. fragilis* 638R(pIP421) (*nimD*) and *B. fragilis* 388 (*nimE*). *Bacteroides fragilis* ATCC 25285 was used as a negative control for the *nim* gene. PCR products were resolved by electrophoresis on 2.0% agarose gels (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) using a molecular DNA weight standard (100 bp ladder; Roche Diagnostics GmbH, Mannheim, Germany) in a working solution of 1× TAE buffer (40 mM Tris–acetate, 1 mM ethylene diamine tetra-acetic acid, pH 8.0). The running time was 60 min at 100 V. The DNA gels were stained with ethidium bromide (0.5 μg/mL) and were visualised under an ultraviolet (UV) light transilluminator in a UVIdoc gel documentation system (UVItec, Cambridge, UK).

2.4. Inducible metronidazole resistance

The resistance induction experiment was performed by a previously described procedure [11] using a sample of 52 randomly selected *nim*-negative metronidazole-susceptible strains. Briefly, a powder of known activity (Sigma–Aldrich) was used to prepare a stock solution containing 10 mg/mL metronidazole that was needed for preparing the required concentrations that were then incorporated into supplemented Brucella agar. Bacterial growth proceeded on agar through a series of exposures to gradually doubling metronidazole concentrations. First at twice, then at four, eight, or more times the original MIC, and in the case of poor bacterial growth, an exposure of one-half of the neighbouring values was used. Resistance was induced after multiple passages under anaerobic conditions for 48–72 h at 35 °C.

2.5. Measurement of lactate dehydrogenase activity

The induced and uninduced strains were grown in 8 mL of brain–heart infusion broth supplemented with 0.25% yeast extract, 10 mg/L haemin and 1 mg/L vitamin K_1 until the stationary phase (uninduced strains overnight and induced strains for 72 h) with and without added metronidazole (16 mg/L for induced and 0.125 mg/L for uninduced strains). The cultures were chilled on ice and were centrifuged at 4 °C for 15 min at 3000 rpm, were washed in 3 mL of 40 mM $NaPO_4$ buffer (pH 7.0) and were recentrifuged for 5 min using the same conditions. The cell pellets were re-suspended in the same buffer as described above and were sonicated. After removal of cell debris by centrifugation at 4 °C for 10 min at 3000 rpm, the protein content of the cellular preparation was measured using a Qubit Fluorometer and Quant-iT Protein Assay Kit (Life Technologies, Carlsbad, CA). LDH enzymatic assays were carried out in 1 mL of UV-transparent plastic cuvettes (Merck, Darmstadt, Germany) applying two technical parallels containing 20 mM Na-pyruvate, 0.24 mM reduced nicotinamide adenine dinucleotide (NADH) and 20–40 μL of enzyme preparation, which led to linear absorbance change at 340 nm during 10 min incubations in 50 mM MOPS buffer, pH 6.8 [12]. Enzymatic activity was expressed as nmol NADH oxidised per min and the different measurements were standardised by the protein content of the cell preparations.

2.6. Statistical analysis

Statistical analyses (t-test and Spearman's correlation) were carried out using SigmaPlot 12 software (Systat Software GmbH, Erkrath, Germany). The threshold of significance was set at $P = 0.05$.

3. Results

All 155 collection strains were sensitive to metronidazole (MIC ≤ 4 mg/L) and were *nim*-negative.

Metronidazole resistance was induced in 12 (23.1%) of the 52 selected strains after 9–17 passages on an agar plate. Metronidazole MICs for the induced strains ranged from 8 mg/L to 96 mg/L, which was 4–96× the original MICs (Table 1).

The specific LDH activity values are shown in Table 2. The specific LDH activity of the *B. fragilis* NCTC 9343 strain (34.5 U/mg) was practically the same as that measured by Narikawa et al. [13], so we can rule out that the LDH activity changes would be in vitro artefacts. Metronidazole exposure was applied because of the need to maintain the induced state of the strains. In the uninduced strains, the LDH activities with sub-MIC metronidazole exposure showed a decreasing tendency, whilst in the cases of induced strains, the sub-MIC metronidazole exposure gave rise to LDH activities that were comparable with the values of the non-exposed cultures.

4. Discussion

In the present study, none of the collection strains harboured *nim* genes or showed phenotypic resistance to metronidazole. The same findings were observed by Eitel et al. in a collection of 161 *B. fragilis* group strains [1]. Sóki et al. reported changes in the frequency of *nim* genes in Europe, describing only three *nim*-positive strains out of 640 *Bacteroides* strains reported in a 2008–2009 European antibiotic susceptibility survey [7]. In contrast, a Brazilian study by Nakano et al. [14] revealed the presence of *nim* genes in 7.8% of 114 *Bacteroides* spp. and *P.* distasonis strains from children's intestinal microbiota, indicating differences in the geographical distribution of *nim* genes.