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EMERGING METRONIDAZOLE RESISTANCE IN ANAEROBES AND MAPPING THEIR SUSCEPTIBILITY BEHAVIOUR

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ABSTRACT

In the present study, anaerobic clinical isolates of *Bacteroides fragilis*, *Escherichia coli*, *Staphylococcus aureus* and *Yersinia enterocolitica* were obtained from different clinical specimens and were subjected to molecular typing to detect the gene encoding metronidazole resistance in these isolates. Subsequently, antibacterial activity of drugs was tested against the selected clinical isolates. A total of 53 clinical isolates involving 18 obligate and 35 facultative anaerobic bacteria were recovered from clinical samples of 67 patients who were suspected to have anaerobic infection. A disk diffusion method was employed to screen for metronidazole-resistance among these isolates. PCR assay was used to detect the metronidazole resistant gene (*nim*). Susceptibility studies in metronidazole resistant clinical isolates as well as positive controls were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines. According to disc diffusion method, of 53 isolates, 21 isolates (39.6%) were found to be metronidazole resistant. Further screening of these isolates with PCR revealed only 13 isolates (24.5%) carry *nim* gene. Out of which 7 were of *B. fragilis*, 3 were of *Y. enterocolitica*, 2 were of *E. coli* and 1 was of *S. aureus.* The highest number of metronidazole resistant isolates were found in abscess (7) followed by intra-abdominal infection (5) and bone and joint infection (1). When metronidazole resistant isolates were subjected to screen for the presence of nim gene, all isolates were found to carry *nim* gene. According to minimum inhibitory concentration (MIC) data, among the tested antibacterial agents, Mebatic emerged as the most active antibacterial against metronidazole resistant isolates of *B. fragilis*, *E. coli*, *S. aureus* and *Y. enterocolitica* with MIC values 0.125 to 1.0 $\mu g\ mL^{-1}$. Similarly, Antimicrobial Susceptibility Test (AST) data also revealed that Mebatic was most efficacious in the metronidazole resistant organisms. From the above results, it is evident that Mebatic has enhanced *in vitro* antibacterial activity compared to other drugs in metronidazole resistant isolates thus can be a potent antibacterial agent for the treatment of infections caused by metronidazole resistant organisms.

Keywords: Anaerobes, Resistance, Clinical Isolates, Metronidazole, Mebatic, Nim Gene

1. INTRODUCTION

Obligate and facultative anaerobic bacteria are a common causes of infections, some of which can be serious and life-threatening. *Bacteroides* species are an important opportunist obligate anaerobic pathogens that are commonly isolated from human polymicrobial infections. Some common pathogenic facultative anaerobic bacteria include *Staphylococcus* spp., *Streptococcus* spp., *Shigella, Yertinia enterocolitica* and *Escherichia coli* (Gaetti-Jardim *et al.*, 2010; Guss *et al.*, 2011). Among the *Bacteroides* species, *Bacteroides fragilis* group is the most prevalent organism accounting for 41 to 78% of the isolates of this group. *B. fragilis* constitute major part of gastrointestinal flora (Brook, 2008) but comprising least common *Bacteroides* occur in

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fecal flora accounting only 0.5% of the bacteria present in stool. Obligate and facultative anaerobic bacteria have been reported to be involved in peritoneal infections (Levinson, 2010), bacteremia (Brook, 2010) intra-abdominal infections, diarrhea (Merino *et al*., 2011) and subcutaneous abscesses or burns near the anus (Brook, 2008). The pathogenicity of these groups of organisms is mainly due to capsular polysaccharide which makes it protective against phagocytosis (Levinson, 2010).

Apart from Metronidazole which has been the drug of choice for the treatment of infections caused by anaerobic bacteria across the world for nearly 40 years (Cohen *et al*., 2010), anaerobic microbes show sensitivity to carbapenems, tigecycline, beta-lactam and beta-lactamase inhibitor combinations. However, in the past few years, metronidazole resistance among obligate anaerobes is a matter of concern as intermediate or high-level resistance to metronidazole have been reported (Alauzet *et al*., 2010). These species can also be resistant to a number of other antimicrobial agents such as beta-lactam agents (imipenem and cefoxitin), tetracycline and clindamycin (Boente *et al*., 2010; Nakano *et al*., 2011). Metronidazole resistance has been reported in Europe (Nagy *et al*., 2011; Hartmeyer *et al*., 2012), United states of America (USA) (Schapiro *et al*., 2004) and Africa (Buta *et al*., 2010).

It has been observed that resistance to beta-lactams in obligate and facultative anaerobic organisms is maily associated with Extended Spectrum Beta Lactamase (ESBL) and Metallo Beta Lactamse (MBL) production (Snydman *et al*., 2010). Whereas mechanism for metronidazole resistance in *B. fragilis* include the presence of *nim* genes, which encode 5-nitroimidazole reductases that convert metronidazole to a non-toxic amino derivative (Pumbwe *et al*., 2008), overexpression of the DNA repair protein, RecA (Steffens *et al*., 2010) and disruption of the electron transport chain (Lynch *et al*., 2013). 5-nitroimidazole resistance genes, *nimA* to *E*, have been identified to confer reduced susceptibility to 5-nitroimidazole antibiotics on species of the *B. fragilis* group (Hartmeyer *et al*., 2012). The *nim* genes encode a 5-nitroimidazole reductase (Nagy *et al*., 2011).

A very few data exist on the susceptibility of obligate and facultative organisms to antimicrobial agents commonly used for eradication of these pathogens. The reports of acquisition of metronidazole resistance by *B. fragilis* from India emphasizes the need for a study to assess more accurately the susceptibility profile of clinical isolates of obligate and facultative anaerobic organisms. Since antimicrobial resistance in anaerobes varies from one hospital to another and between different geographic locations, all hospitals must survey their sensitivity patterns and report any emerging resistance. Unfortunately, most of the Indian hospitals are ill equiped for anerobes culture, hence detection of anerobic resistance becomes even a more challanging issue.

This study involved screening of the presence of *nim* gene in clinical isolates collected from various hospitals of India and to study susceptibility of commonly used antibacterial agents against these *nim* positive isolates.

2. MATERIALS AND METHODS

2.1. Samples Collection

A total of 53 (18 obligate and 35 facultative) anaerobic bacterial isolates were obtained from various clinical samples of 67 patients who are suspected to have anaerobe infection and have been hospitalized in various hospitals of India (The name of hospitals can not be disclosed due to confidential agreement). The isolates were identified as: *B. fragilis* (n = 18), *Escherichia coli* (n = 17), *Staphylococcus aureus* (n = 11), *Yersinia enterocolitica* (n = 7). All the samples were collected with aseptic precautions from intra-abodominal infection, absess and bone and joint infections. The clinical specimens were taken into Hi-Media Anaerobe Transport Medium and have been delivered to the Venus Medicine Research Center, Baddi, Himachal Pradesh, India, at the shortest possible time. Subsequently, specimens were cultured in BBL CDC Anaerobe 5% Sheep Blood Agar plates (Becton, Dickinson, USA). Plates were incubated at 37°C for 48 h.

Clinical isolates were identified to species level using Vitek 2 system (BioMe´rieux, Marcy, France).

All these isolates were stored at 80°C and were cultured on an-aerobic agar medium (Hi-Media, Mumbai, India) supplemented with 6% horse blood in an anaerobic atmosphere [10 CO_2, 10 H2, 80% N2 (v/v)] at 37°C. *B. fragilis* ATCC25285 was included as a control organism. Organism collection, transport, confirmation of organism identification and development and management of a centralized database were coordinated by Venus Medicine Research Centre (VMRC), Baddi, India in assistance with Emerging Antimicrobial Resistance Society (EARS).

2.2. Antibacterial Agents

The following antibacterial agents were used in this study: Ofloxacin+ornidazole (Mebatic, a novel antibiotic adjuvant entity, Venus Remedies Limited, Panchkula, Haryana, India), ofloxacin (Oflonir, Aishwarya Healthcare, Mumbai, India), ornidazole (Nidazole, Sipra Remedies, Mumbai India), metronidazole

(Ivmetro, Torrent Pharmaceuticals Limited, Ahmedabad, Gujarat) and ciprofloxacin (Alcipro, Alkem Laboratories Limited, Mumbai, India).

2.3. Screening of Clinical Isolates for Metronidazole Resistance

Initial determination of the metronidazole susceptibility of each isolate was carried out by a standard disc diffusion test on CDC Anaerobe 5% Sheep Blood Agar plates with a 5mg metronidazole disc (Hi-Media, Mumbai, India).

2.4. Preparation of Nitroreductase Extract

Overnight grown cultures were centrifuged at 5000 rpm for 4 min. The pellet was weighed and equal amount of lysozyme was added and then pellet was suspended in phosphate buffer saline (PBS; 10 mM, pH 7.4) in order to disrupt bacterial cells. The solution was incubated at 37±2°C for 2 h and then centrifuged at 7000 rpm for 10 min. The supernatant (crude extracts) was use as a source of enzyme and stored at -70°C until use.

2.5. Nitroreductase Enzyme Assay

The nitroreductase enzyme assay was carried out according to the method described by Pal *et al.* (2009). The reaction mixture comprised of Tris-acetate (100 mM Tris-HCl, 50 mM acetate buffer, pH 7.0), 30 µM metronidazole as a substrate, half MIC of different drugs as inhibitors, 0.3 mM NADPH or NADH and enzyme in a 1 mL reaction volume. The assay was carried out at 25°C in a UV-visible spectrophotometer (Shimadzu, Japan) in quartz cuvettes with a 1-cm path length. The results of the assay were determined by measurement of the oxidation of NADPH or NADH at 340 nm (E 6.22 mM^{-1} cm^{-1}).

2.6. Deoxyribo Necleic Acid (DNA) Isolation

DNA from all metronidazole isolates was extracted according to the method described by Atashpaz *et al.*, (2010). Five ml of overnight anaerobic grown bacterial culture was used for the DNA isolation. DNA purity and concentrations were measured with spectrophotometer (260/280).

2.7. Polymerase Chain Reaction (PCR)

The *nim* gene was detected using previously reported primer (Verma *et al*., 2012). Primers were synthesized from Sigma Aldrich Chemicals Private Limited, Bangalore, India. Primers used for amplification were *nim*F-5'- ATGTTCAGAGAAATGCGGCGTAAGCG-3 and *nim*-R-5'-GCTTCCTTGCCTGTCATGTGCTC-3' that amplify a fragment of about 458 bp. PCR amplification was performed in a total volume of 20 µL containing 200 pg of DNA, 0.5 mM of dNTPs, 1.25 µM of each primer and 1.5 U of Taq polymerase (Banglore Genei). PCR amplification was carried out on a Eppendorf thermocycler (Germany) with cycling conditions: Initial denaturation at 96°C for 5 min followed by 32 cycles each of denaturation (94°C for 60 sec), annealing (52°C for 120 sec), extension (72°C for 120 sec) and final extension (72°C for 7 min), for the amplification of *nim* gene. After amplification, PCR products were separated on a 1% (w/v) agarose gel containing 25 µg of ethidium bromide in Tris-EDTA buffer and the gel was photographed under ultraviolet illumination using gel documentation system (Bio-Rad, USA). A 100-bp DNA ladder (GeNei, Banglore, India) was included in each run. PCR products from control strains *B. fragilis* 638R (kindly gifted by Dr. ND Chaurasiya, National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, MS, USA) was used as references for nim gene sizing analysis. The presence of an amplicon of 458 bp were considered as presumptive positive.

2.8. Determination of Minimum Inhibitory Concentrations (MICs)

MICs were determined by agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013) using the test organism at a concentration of 2×10^6 cfu mL^{-1}. *B. fragilis* ATCC25285 was used as reference strain.

2.9. Determination of Antimicrobial Susceptibility (AST) by Cup Plate Method

The cup-plate agar diffusion method, a modification described by chaudhary *et al.* (2012), was adopted to assess the antimicrobial susceptibility of the selected drugs. Inoculum containing 10^6 cfu mL^{-1} of test strain was spread with a sterile swab on a petri dish containing CDC blood agar plates and the cups were made in the agar plate using a sterile cork borer (6.5 mm). Then, 30 µL of the drug preparation was placed in the wells using a micro-pipette. The plates were incubated in an anaerobic atmosphere [10 CO_2, 10 H2, 80 N2 (v/v)] at 37°C for 48 h. After incubation the zone of inhibition around the wells was measured in mm (millimeter), averaged and the mean values were recorded.

3. RESULTS

3.1. Identification of Clinical Isolates

All of the clinical isolates obtained from various clinical specimens were identified to be *B. fragilis* (18), *E. coli* (17), *S. aureus* (11) and *Y. enterocolitica* (7) based on their morphological and biochemical characterization. Our data showed the highest number of *B. fragilis* isolates were recovered from intra-abdominal infection (9) followed by abscess (7) and bone and joint infection (2). For *E. coli*, the greatest number of isolates were recovered from intra-abdominal infection (9) followed by abscess (8). Of 11 *S. aureus* isolates, 6 were obtained from bone and joint infections and 5 were from abscess. *Y. enterocolitica* was only recovered from intraabdominal infection. Overall, the greatest number of isolates were recovered from intra-abdominal (25; 47.1%), followed by abscess (20; 37.7%) and bone and joint infections (8; 15.1%). When all of these 53 isolates were subjected for selection of metronidazole resistance by disc diffusion method, 21 isolates (39.6%) were found to be metronidazole resistant. When these metronidazole resistant isolates were subjected to PCR to screen for the presence of nim gene, only 13 isolates were confirmed to be positive with *nim* gene. Of which 7 were of *B. fragilis*, 3 were of *Y. enterocolitica*, 2 were of *E. coli* and 1 was of *S. aureus* (**Table 1 and Fig. 1**). The highest number of metronidazole resistant isolates were found in abscess (7) followed by intra-abdominal infection (5) and bone and joint infection (1). The *nim* gene positive isolates were used for further study.

3.2. Nitroreductase Enzyme Assay

The effect of all selected drugs on nitroreductase enzyme inhibition was determined and results showed that Mebatic brought about 86 to 89% inhibition in nitroreductase activity whereas other comparator drugs produced only 7 to 10% inhibition (**Table 2**).

3.3. MIC

As shown in the **Table 3**, Mebatic emerged as the most active antibacterial against metronidazole resistant facultative and obligate anaerobic isolates of *B. fragilis*, *E. coli*, *S. aureus* and *Y. enterocolitica* with MIC values 0.125 to 1.0 μg mL^{-1}. Ofloxacin was found to be second most active antibacterial agent with MIC values 1 to 8 μg mL^{-1} for *E. coli*, *S. aureus* and *Y. enterocolitica* whereas MIC for *B. fragilis* was 8 to 128 μg mL^{-1}. The MIC values for ciprofloxacin against *E. coli* and *S. aureus* was 2 to 16 μg mL^{-1} and 2 to 32 μg mL^{-1} and 8 to 128 μg mL^{-1} were observed for *Y. enterocolitica* and *B. fragilis*, respectively. Ornidazole MIC values for was 8 to 64 μg mL^{-1} for *B. fragilis* and *S. aureus* and 8 to 128 μg mL^{-1} for *E. coli* and *Y. enterocolitica*. The metronidazole MIC values for *B. fragilis* was 8 to 64 μg mL^{-1} whereas 16 to 128 μg mL^{-1} for *S. aureus* and 16 to 128 μg mL^{-1} for *E. coli* and *Y. enterocolitica*.

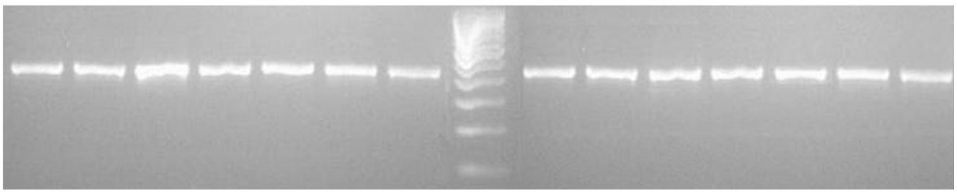

Fig. 1. Agarose gel showing PCR amplified products of nim gene in clinical isolates. Lane H 100 bp DNA size marker; Lane A to G and I to N nim (458 bp); lane O nim Positive control B. fragilis 638R (458 bp)

Table 1. Source of clinical isolates

Name of isolates	Sources: Intra abdominal	Sources: Abscess	Sources: Bone and joint infections	Total (53)	Metronidazole resistant isolates by phenotypic method (%)	Metronidazole resistant isolates by PCR (%)
Bacteroides fragilis	9	7	2	18	10 (55.5)	7 (38.9)
Escherichia coli	9	8	0	17	4 (23.5)	2 (11.7)
Staphylococcus aureus	0	5	6	11	2 (18.2)	1 (9.1)
Yersinia enterocolitica	7	0	0	7	5 (71.4)	3 (42.8)
Total	25	20	8	53	21(39.6)	13 (24.5)

Table 2. Effect of drugs on nitro-reductase activity of nim positive isolates

Name of drugs	% inhibition of nitro-reductase activity *Bacteroides fragilis*	*Escherichia coli*	*Staphylococcus aureus*	*Yersinia enterocolitica*	*B. fragilis* 638R
Mebatic (Ofloxacin+ Ornidazole+VRP006)	86.3±6.5	87.5±7.3	86.8±8.1	89.3±8.5	88.3±7.8
Ornidazole	10.1±1.2	8.4±0.9	9.3±1.1	9.4±0.9	8.1±0.9
Ofloxacin	9.8±1.1	8.6±1.0	8.5±0.9	8.7±1.0	8.4±0.8
Metronidazole	8.8±1.3	8.6±0.9	9.2±1.1	8.6±0.9	8.7±0.7
Ciprofloxacin	7.4±0.8	7.5±0.8	8.5±1.0	10.3±0.9	7.9±8.3

Table 3. Minimum inhibitory concentration test of drugs against anaerobic strains

Name of drugs	MIC (µg/mL) *Bacteroides fragilis*	*Escherichia coli*	*Staphylococcus Aureus*	*Yersinia enterocolitica*	*B. fragilis ATCC25285*	*B. fragilis 638R*
Mebatic (Ofloxacin +Ornidazole+VRP006)	0.25-1.0	0.125-1.0	0.25-1.0	0.125-1.0	0.13	0.25
Ornidazole	8.0-64	8.0-128	8.0-64	8.0-128	2.00	16.0
Ofloxacin	8.0-128	1.0-8.0	1.0-8.0	1.0-8.0	1.00	16.0
Metronidazole	8.0-64	16-28	16-64	16-128	0.25	16.0
Ciprofloxacin	8.0-128.0	2.0-6	2.0-16	2.0-32.0	2.00	32.0

Table 4. Antimicrobial susceptibility of drugs against anaerobic strains

Name of drugs	Zone of inhibition (mm) *Bacteroides Fragilis*	*Escherichia coli*	*Staphylococcus aureus*	*Yersinia enterocolitica*	*B. fragilis ATCC25285*	*B. fragilis 638R*
Mebatic Ofloxacin +Ornidazole +VRP006) (5:12.5 µg)	22.7±2.1	26.5±2.4	24.7±2.3	25.9±2.2	29.2±2.4	26.3±2.3
Ornidazole (30 µg)	15.5±1.1	No zone	No zone	No zone	20.4±1.9	10.5±1.6
Ofloxacin (5 µg)	No zone	19.4±1.8	18.2±1.2	17.4±1.5	22.4±2.2	11.2±1.3
Metronidazole (30 µg)	11.2±1.1	8.4±1.0	7.2±0.8	7.4±1.0	22.2±2.1	8.2±1.0
Ciprofloxacin (5 µg)	8.6±1.2	14.6±1.1	13.5±1.2	12.1±1.0	21.6±1.7	12.5±1.1

S =≥21, I= 16-20, R= ≤15

3.4. AST

The zones of inhibition were calculated in millimetre for all strains and presented in the **Table 4.** Mebatic was found to be most effective against all clinical isolates as evident by zone of inhibition values between 22 to 26 mm. Other tested drugs including ofloxacin, ornidazole, metronidazole and ciprofloxacin displayed intermediate to resistant profile against all of the clinical isolates.

4. DISCUSSION

The implications of anaerobic microbes in the human infections is well known. The infections caused by anaerobic bacteria are some of the most important causes of morbidly and mortality in developing countries (Akhi *et al*., 2013). The development of resistance to metronidazole leading to treatment failure during the course of metronidazole therapy in a patient has been reported (Buta *et al*., 2010; Soki *et al*., 2013). Numerous studies have demonstrated the incidence of *metronidazole resistance* in recent years (Alauzet *et al.*, 2010; Lynch *et al.*, 2013; Abdollahi *et al.*, 2011). In a study conducted by Datta *et al*. (2005) reported that 85% strains of *Helicobacter pylori* were resistant to metronidazole. In other previous studies, metronidazole resistance in *Bacteroides* spp. has been noted to be varied from 5 to >15% (Gal and Brazier, 2004).

Our PCR data revealed that 13 (24.5%) isolates were metronidazole resistant suggesting nim genes are quite common in metronidazole resistant clinical isolates. Intrestingly, our results showed that in addition to *B. fragilis*, nim gene was detected across a number of facultative anaerobes specieses, *E. coli*, *S. aureus* and *Y. enterocolitica* which indicates transferability of *nimA* gene from one species to another in anaerobic environments. It has also been reported earlier that metronidazole resistance could be a consequence of the activation of nim gene as a result of point mutation (Gal and Brazier, 2004). We observed that the metronidazole resistant isolates were mainly found in abscess 53.8% (7/13) followed by intra-abdominal 38.5% (5/13) and bone and joint infection 7.7% (1/13). This study is hospital based and does not reflect the prevalence or incidence of metronidazole resistance in the community. Leiros *et al.* (2010) studied the hydrophobicity profile of the nim gene products and reported that it decreases penetration of metronidazole into the cells. It has also been noted that the nim genes encode a 5-nitroimidazole reductase that converts 5-nitroimidazole to its non-toxic amino derivative, thus preventing the accumulation of toxic nitroradicals (Lofmark *et al*., 2010).

In our study, Mebatic was found to be most efficacious antibacterial agent against metronidazole resistant organisms. The enhanced antimicrobial efficacy of Mebatic against these isolates may be due to the synergistic effect of ofloxacin and ornidazole along with a non antibiotic adjuvant VRP006 (disclosed by Venus Remedies on request) which enhanced the penetration of the drug inside the bacterial cells and inhibits the nitro reductase activity thus prevents the conversion of non toxic form of the drug.

It has been reported that metronidazole-resistant and susceptible strains of *B. fragilis* became more virulent following exposure to low doses of metronidazole. Probably low concentration of Metronidazole may paved the way for the development of either non-stable phenotypes or permanently resistant mutants (Diniz *et al*., 2003), therefore, rapid identification of Metronidazole resistance is essential for early initiation of appropriate antimicrobial therapy and to limit the inappropriate use of antibacterial agents.

5. CONCLUSION

In conclusion, from the above results, it is evident that Mebatic has synergistic*in vitro* antibacterial activity as compared to other drugs in Metronidazole resistant isolates thus can be a potent antibacterial agent for the treatment of infections caused by Metronidazole resistant organisms.

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