MOLECULAR STUDY: Nitroreductase enzyme and NIM genes effect on AMR to metronidazole in *Clostridium difficile*

ABSTRACT

Nitroreductase enzymes and *Nim* genes have been observed to be a factor predisposing to reduced susceptibility to metronidazole in anaerobic bacteria. Therefore, this study was determined the effect of nitroreductase enzymes and *nim* genes in metronidazole susceptibility in UK *C. difficile* isolates. Three UK *C. difficile*ribotypes 027, 001, 106 including metronidazole reduced susceptible (CDRM) and metronidazole susceptible (CDSM) *C. difficile* strains, and 2 control strains from ribotype 010 (CDRM) and 038 (CDSM) were used. Control strain *C. perfringens* ATCC 3626 previously identified to be nitroreductase positive, and positive control for *nimB*which was *Bacteroides fragilis* strain BF8 was also included in this research. These selected strains were observed for their nitroreductase activity and presence of *Nim* genes.*C. perfringens* ATCC 3626 demonstrated nitroreductase activity but was absent in *C. difficile* control. Three CDRM strains (001 and 106 ribotype) demonstrated nitoreductase activity. None of the CDSM strains analysed showed detectable nitroreductase activity. The *nim* gene PCR amplification was only observed in the *nim* positive control strain *BF8* (about 400 bp). All samples tested including ribotype 001 027 and 106 CDSM and CDRM strains showed no *nim* PCR product. In conclusion, despite the nitroreductase result obtained, inconsistencies observed during the assay showed methodology issues, thus using radio labelled metronidazole and monitoring the decline in the radiolabel signal proportional to the level of nitroreduction, maybe a preferred method in correlating nitroreductase activity to the CDRM phenotype. Also, this research observed the absence of *nim* genes in *C. difficile* UK ribotypes irrespective of metronidazole susceptibility patterns, depicting the inability of the universal primers for *nimA-NimE* to amplify a potential *nim* gene in *C. difficile* UK ribotypes. The universal primers were previously reported incapable of amplifying*nimJ* due to a different nucleotide sequence coding for same proteins as the universal primers.

1.0 INTRODUCTION

Clostridium difficile a spore forming, Gram positive anaerobic bacterium, has been observed to be the most prevalent infectious cause of antibiotic associated diarrhoea(AAD)

(Mutlu *et al*., 2007, Lynch *et al*., 2013). Systematic characterization of a world wide set of clinical trial *C. difficile* isolates from infected individuals reported the complexity of the genetic makeup *C. difficile* (Boekhoud*et al*., 2020, Zhao *et al*., 2021).Resistance to a variety of antibiotics has been observed to a variety of antibiotics (Wickramage*et al*, 2021)Reduced susceptibility in some *C. difficile* isolates has been observed to metronidazole, a frontline antibiotic for *C. difficile* infection, which has been observed in other anaerobic microbes such as *Bacteriodes fragilis.*Nitroreductase activity and the presence of *nim* genes have been previously identified as factors that could predispose to reduced susceptibility to metronidazole in earlier reports

1.1 Nitroreductase activity in predisposing to reduced susceptibility to metronidazole in anaerobic organisms

Nitroreductase are enzymes that have been observed to reduce nitrocompounds to nonmutagenic compounds and to activate prodrugs (Bryant *et al*, 1981). Nitroreductases could be involved in bioremediation e.g*pnrA*isa gene that encodes a nitroreductase observed to increase phytoremediation after it was introduced into a transgenic aspen used for phytoremediation of 2, 4, 6 trinitrotoluene an environmental pollutant (Van Dillewijn*et al*, 2008).

Nitrocompounds have been observed to be mutagenic to bacteria, as was confirmed in a research study involving 2,4 dinitrotoluene and 2,6 dinitrotoluene, which were shown to be mutagenic to *Salmonella typhimurium* (Padda *et al*, 2003). However, incidence of mutagenic stimulation may be lower than expected because of the conversion of nitrocompounds into their metabolites which are less harmful (Rafii *et al*, 1991). One bacterial genus observed to play a significant role in the conversions of nitro-compounds is *Clostridium* (Rafii *et al*, 1991). It was confirmed in an earlier research study which exposed faecal microbial flora of human origin to 1-nitropyrene, that organisms with clear zones around areas of microbial growth were isolated and identified. Though the experiment was limited in that the faecal material was from one human source, it served as platform for more research (Raffii*et al*, 1991). The organisms that metabolized 1-

nitropyrene as identified by the clear zones were *Clostridium clostridioforme,Clostridium paraputrificum, Clostridium leptum, Clostridium sp* and *Eubacterium sp.*

The presence of nitroreductase enzymes in microrganisms has been useful clinically in activation of prodrugs:, e.g. 5-(aziridin-1-yl)-2, 4-dinitrobenzamide (CB1954) is a therapeutic agent against tumour cells has been observed to be reduced by NfsA and NfsB, which are *E. coli*nitroreductase enzymes (Knox *et al*, 1988, Anlezark*et al*, 1992, Vass *et al*, 2009). Additionally, benznidazole is a prodrug that becomes active against *Trypanosomal cruzi* following its reduction initiated by a nitroreductase (Hall and Wilkinson, 2012).

1.11Nitroreductase in anaerobic organisms

Some nitroreductase genes have been isolated whose activation or inactivation has been associated with resistance to metronidazole. Metronidazole is a nitroimmidazole and a prodrug that requires the action of nitroreductases for its activation. Anaerobic bacteria such as *Helicobacter pylori* the cause of peptic ulcer, *Bacteroides fragilis* and*Clostridium* species which are normal colonic flora, that also could be pathogens, are sometimes being treated by antimicrobials requiring the activation by nitroreductase enzymes. As a result the activation and inactivation of the genes encoding these nitroreductase enzymes, could have an effect on efficacy of antimicrobial therapy.

Nitroreductase activity is naturally occurring within microorganisms, thus it does not require an external influence for its activity (Raffii*et al*, 1998) this is because it is involved in the normal cellular processes, e.g glucose metabolism. Nitroreductase involvement in normal metabolic processes was also reported in previous research by Britz and Wilkinson (1979), where the products of glucose metabolism in metronidazole resistant *B. fragilis* were compared with a metronidazole susceptible parent strain. It was observed that the increase in resistance to metronidazole was associated with a concurrent decrease in pyruvate dehydrogenase activity. The difference in production of other metabolic end products in the resistant *B .fragilis* strain relative to the wild type correlated to the decrease in pyruvate dehydrogenase activity (Britz and Wilkinson 1979).

The resistant mutants were of two levels of resistance at 25µg/ml MIC & highly resistant *B. fragilis* at 100µg/ml MIC (Britz and Wilkinson 1979). A quarter of the normal dehydrogenase level was produced in the lower level resistant strain; and pyruvate accumulation and increases in lactate concentrations were also observed. There was almost no dehydrogenase activity observed for the highly resistant *B. fragilis* strain and increased levels of CO_2 lactate and ethanol were produced. The results observed in the Britz and Wilkinson (1979) study suggested that resistant *Bacteroides fragilis* strains show lower energy yields obtained from glucose. When compared with normal *B. fragilis*, a decline in growth rates were observed in some resistant *B. fragilis*. The accumulation of pyruvate in the moderately resistant *B. fragilis* showed that it was unable to metabolize pyruvate via other pathways unlike the highly resistant *B. fragilis*.

However, conflicting data were reported by Tabeqchali*et al* (1983), where pyruvate dehydrogenase activity was observed to be active in metronidazole reduced susceptible *B. fragilis*, (MICs of 2-8mg/L) and also in highly resistant *B. fragilis* strain (MIC of 64mg/L) as well as metronidazole – susceptible *B. fragilis* strains with MIC 0.5mg/L). These observations question the indication that pyruvate dehydrogenase activity could be associated with reduction in susceptibility to metronidazole (Tabeqchali*et al*, 1983).

Nitroreductase activity is probably not the single cause of resistance to nitroimidazole, papers have also identified decreased uptake of antibiotics (Lacey *et al*, 1993). During therapy against *Bacteriodes fragilis* using metronidazole, *Enterococcus faecalis* has been observed to inhibit the susceptibility of *Bacteriodes fragilis* to metronidazole by inactivating metronidazole. Thus the resistance of *Bacteriodes fragilis* to metronidazole could also be influenced by other microbes in the environment. (Nagy and Foldes, 1991). Metronidazole resistance in *Bacteriodesfragilis* has also been observed to be conveyed by *nim* genes (Gal and Brazier, 2004).

As was stated earlier the bacteria frequently reported for metabolisingnitrocompounds that could be detrimental to the human health was isolated and identified to be of *Clostridium* genus. *Clostridia* were identified to metabolise -1- nitropyrene and associated compounds to amine derivatives (Rafii *et al*, 1991). This conversion was carried out by a nitroreductase which was confirmed using a nitroreductase assay. The

nitroreductaseenzyme was further characterised to be extracellular, requiring anaerobiosis for its activity as well as an optimal pH of 8 (Rafii *et al*, 1991). The isolates comprising of *Eubacterium sp,Clostridiumleptum, Clostridium clostridioforme, Clostridium paraputrificum and Clostridium sp*all showed nitroreductase activity without the addition of FAD except *Clostridium sp.* One distinct nitroreductase was identified per isolate (Rafii *et al*, 1991). The redox potential necessary for the reduction of metronidazole is -450mV (Edwards, 1980). The reduction of metronidazole not only activates the bactericidal effect of the drug but also increases its concentration intracellularly (Edwards, 1980)

Sisson *et al*., 2002 determined the specificity of a range of nitroreductase enzymes e.g Pyruvate oxidoreductase (POR), RdxA, FrxA in activating of certain nitrocompounds: Metronidazole nitrofurans and nitazoxanide. It was elucidated that POR reduced nitrofurans but not to the same extent as FrxA, all three nitroreductases reduced nitazoxanide. The only nitroreductase that reduced metronidazole in *Helicobacter pylori*(*H. pylori)*was observed to be RdxA(Sisson *et al.*, 2002). This paper not only identified the nitroreductase specific for metronidazole but also showed that some nitroreductase enzymes can activate multiple substrates. The *rdxA* gene encodes an oxygen insensitive NADH nitroreductase activity and was also identified as a potent contributor to metronidazole resistance as a functional rdxA gene from *H. pylori* was transformed into normally metronidazole resistant *E. coli* and *H. pylori* and both strains became susceptible to metronidazole (Goodwin *et al*, 1998). Goodwin *et al*, (1998) paper also proposed that mutations in *rdxA* causing resistance could be as a result of continuous use of the drug and this supports Jenks *et al*, (1999) who worked on mice and found *rdxA*to bestable when not exposed to metronidazole. Resistance to metronidazole was linked to mutation in *rdxA* following exposure to metronidazole (Jenks *et al.*, 1999). However, the resistant phenotype was observed to be stable despite consecutive subcultures in the absence of metronidazole (Jenks *et al.*, 1999). Not all resistant *H. pylori* had a mutation in the *rdxA*gene, thus showing the involvement of other factors in conferring resistance in *H. pylori* (Jenks *et al*., 1999).

Another nitroreductase that has been implicated in metronidazole resistance is *frxA,* which encodes an oxygen insensitive NAD(P)H - flavin nitroreductase (Sisson *et al.*, 2002). Comparison of the gene sequence of 9 pairs of *rdxA* and *frxA* susceptible and resistant in

H. pylori, identified mutations linked to either of these genes in 8 of the pairs however one pair had no obvious mutation in *rdxA, frxA* and *recA* a gene for DNA repair in bacteria (Marais *et al*, 2003). RecAof*H. pylori* was identified to also contribute to metronidazole sensitivity by identifying the effects of mutant *recA* in *H. pylori* this as well affected DNA repair and transformations (Marais *et al*, 2003).

On frxA it was identified that *frxA* without mutation on *rdxA* gene can confer resistance to metronidazole in *H .pylori* as one of the 8 metronidazole resistant strains had mutation only in *frxA* gene not in *rdxA*. The role of *frxA* was further emphasized in a transformation experiment using *frxA* from a *H. pylori* susceptible to metronidazole, which was inserted into a metronidazole resistant *E. coli* which became susceptible. Thus it was suggested that *frxA* by itself could affect susceptibility metronidazole (Marais *et al*, 2003).

A similar experiment was performed by Kwon *et al*, (2000) transferring *frxA* from a metronidazole resistant *H. pylori* cloned gene into metronidazole sensitive *H. pylori* (minimum inhibitory concentrations (MIC)= 0.5-1 mg/L) yielded moderate level resistance to metronidazole (MIC =32mg/L).Metronidazole sensitive *H. pylori* strains had increased level of MIC (32mg/L) when their *rdxA* genes were inactivated (Kwon *et al*, 2000) also one strain was reported to have Metronidazole MIC increase to 128mg/L, thus showing the possibility of the inactivation of *rdxA*being involved in metronidazole resistance (Kwon *et al*, 2000).

Nitroreductase assay was carried out on *Clostridium* speciesresistant to nitrofurantoin. The cell free supernatant of the isolates mutants or wild type also metabolised nitrofurantoin which was detected by the disappearance of the nitrofurantoin peak using high performance liquid chromatography (HPLC). The use of cell free supernatant further indicates that nitroreductases are extracellular, and confirmation of nitroreductase presence was detected by the conversion 4- nitrobenzoic acid to 4 aminobenzoic acid (a known role of nitroreductases (Rafii *et al*., 1991). Using a Nitrofurantoin susceptible bacillus a bioassay was used to detect that nitrofurantoin was converted to a non-bactericidal substance by 2 of the mutants. This research confirms that both susceptible and resistant *Clostridium* species could produce nitroreductase but would convert substrate to different

metabolites (Rafii and Hansen, 1998). These same characteristics were observed in *Enterococcus casseliflavus*and *Enterococcus gallinarum*(Rafii *et al*, 2003).

Nitroreductase activity can be assayed for by measuring the conversion of 4- nitrobenzoic acid (PNBA) to 4-amino benzoic acid (PABA) which is an action carried out by the enzyme (Rafii *et al.*, 1991) the concentration of PABA is quantified by the production of a purple azo dye that occurs after a series of reaction that ends with addition of N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) to produce the azo dye that is measured using spectrophotometer.

1.2 *Nim* Genes: A Potential Mechanism of Reduced Susceptibility to Metronidazole in *C. difficile*

Nitroimmidazole resistance genes (*nim* genes) encode for nitroreductases that aid the reduction of metronidazole to non-antimicrobial compounds (Schapiro *et al,* 2004). The contribution of *nim* proteins to reduced susceptibility to nitroimmidazoles has been reported following a comparative study of *nim* positive and *nim* negative *Bacteroides fragilis* strains. Both strains were exposed to a dimetronidazole, which is a 5-nitroimidazole compound. The *nim* negative strain reduced dimetronidazole to a nitroso radical anion then to 5, 5' –azobis-(1,2-dimethylimidazole) but the *nim* positive strain reduced it to 5 amino-1, 2, dimethylimidazole which was nontoxic to *Bacteroides fragilis* (Carlier *et al,* 1997).Thus elucidating that the *nim* positive strains code for an enzyme that reduces metronidazole to a less harmful derivative compared to the *nim* negative strains (Carlier *et al,* 1997). Land and Johnson (1999) correlated the features associated with metronidazole decreased activity, in varying organisms including anaerobic bacteria, parasitic anaerobic protists as well as microaerophilic bacteria. It was concluded that metronidazole resistance was multifactorial, but a common link was observed to be due to metronidazole activation which is metronidazole inactivation or lack of activation (Land and Johnson, 1999). The above findings from Carlier*et al*, 1997 showing the roles of *nim* genes have supported these statements.

Mostly,*nim* genes have been observed in *Bacteroides fragilis* but there has been some controversy in their presence causing reduced susceptibility to metronidazole. Gal and Brazier (2004) analysed the susceptibilities of the *Bacteriodes*spp. sent to the Anaerobe Reference Laboratory (ARL, Cardiff, UK) and correlated susceptibility with the presence of specific *nim* genes. The total *Bacteriodes*species analysed were 206 isolates of human origin of which 142 were *Bacteriodes fragilis*. Fifty of these isolates were observed to be *nim* positive out of which *nimA* was more frequently observed (Gal and Brazier, 2004). The high occurrence of the *nimA*gene in *nim* positive strains analysed was also observed in a 2005 research, where of 30 *nim* positive *Bacteroides fragilis* strains the most common was *nimA*gene which was more than half of the population (Lofmark*et al*, 2005).

Another study by Schaumann *et al* (2005) examined metronidazole reduced susceptible, resistant and susceptible *Bacteroides fragilis* strains for the presence of *nim* genes. Some of these strains were reference strains of known *nim* activity. Those with reduced susceptibility were *nim* positive and those with susceptibility were *nim* negative. However, after serial passaging *B. fragilis* with the sub-inhibitory concentrations of metronidazole, all strains demonstrate increased MICs up to >256mg/L irrespective of the *nim* status (positive or negative).This was therefore suggestive of the need for further research in elucidating metronidazole reduced susceptibility mechanisms in *B. fragilis* without ruling out the association of *nim* genes. The decreased metronidazole susceptibility is likely to be a multifactorial process (Schaumann *et al*, 2005). Consequently, it could be that increase in metronidazole concentrations observed in patients following antimicrobial therapy has a way of switching on/off genes linked either to the activation, uptake, extrusion or inactivation of metronidazole (Schaumann *et al*, 2005).

The frequency of *Bacteroides fragilis* with decreased susceptibilityto metronidazole was relatively low when the published literature in this area were analysed (Lofmark*et al*, 2005).

Further to this, the frequency of *nim* positive strains has also been shown to be low, as observed in Lofmark*et al*, (2005) which showed only 30 strains out of 1502 *Bacteroides fragilis* strains analysed, were *nim* positive (2%) (Lofmark*et al*, 2005). It was also observed that *nim* positive strains had a higher propensity to show further decreases in

their susceptibility when exposed to metronidazole *in vitro* compared to strains without *nim* genes. The stability of the mechanism(s) responsible for these further elevations in metronidazole MICs was questionable, given that less than 20% of the strains that showed elevated MIC where irreversibly induced. The controversy on the role of nim genes in metronidazole reduced susceptibility/resistance was questioned in a study where 21 *nim* positive anaerobe strains were observed to be susceptible to metronidazole (Theron *et al,* 2004).

1.2.1 Presence of *Nim* Genes in Other Microorganisms

Nim genes have also been identified in anaerobic Gram positive cocci, mostly of *Peptostreptococcus*species, most of which were *nimB* positive. However, metronidazole susceptibility was also observed in these *nimB*positive strains (Theron *et al,* 2004) except for two strains of *F. magna* which were resistant to metronidazole with a MIC of >128mg/L. *nimK*was also observed in a metronidazole resistant clinical strain of *Prevotellabivia*(Ito et al, 2024)

A South African study identified the presence of *nimA* in a strain of *Clostridium bifermentans* following the analysis of 64 anaerobes, and reported the presence of *nim* genes in 35 anaerobes, including some strains of *Bacteriodes fragilis* and*Prevotellaspp*and *Actinomyces species* (Lubbe *et al,* 1999)

The *nim* gene primers were designed by Trinh and Resset in their 1996 research article. They designed two polymerase chain reaction (PCR) primers (*nim3* and *nim5*) which were observed to lead to the amplification of a product consisting of 458bp irrespective of gene location (plasmid or chromosome). These primers were also identified to be specific for *nim* genes due to the absence of cross reactivity with other regions of bacterial genomes. However, the sensitivity of the PCR assay was dependent on specificity and copy number of the *nim* target (Trinh and Reysset, 1996). The common *nim* genes identified in previous published research are *nimA - nimF*, however other *nim* genes have also been discovered including; *nimG, nimH, nimJ*, and *nimI.* NimI genes have been isolated in *Prevotella* species and not to be associated with previously identified *nim*-linked IS elements and were located on the chromosome (Alauzet*et al.,* 2010). NimG genes were observed in *Bacteriodesspp* (Gal and Brazier, 2004) and *nimJ* was identified in *Bacteriodes*

*fragilis*strains with a different sequence that could not be amplified from previously designed primers. Due to SNPs in the nucleotides encoding *nimJ*, the PCR was unsuccessful, however the amino acid sequences encoding nimJ were identical (Husain *et al.*, 2013). Nagy and foldes (1991) screened *Enterococcus faecalis* known for the inactivation of metronidazole for the presence of *nim* genes but these facultative anaerobes were found to be lacking *nim* (Trinh and Reysset, 1997). It may have been that enterococcal mediated metronidazole inactivation was not due to presence of *nim* genes, or the universal *nim* primers cannot amplify the sequences of the nim genes of *Enterococcus faecalis.*

As a result of the numerous associations of *nim* genes with resistance to metronidazole in *Bacteroides fragilis*, researchers have evaluated their presence inmetronidazole resistant *Clostridium difficile*. However, there is yet to be a research study that has positively identified *nim* genes in *Clostridium difficile* with a resistant or reduced susceptible phenotype to metronidazole though some efforts have been made at genomic level. The first *Clostridium difficile* strain identified in UK to show reduced susceptibility to metronidazole was reported to show a negative result to assay for *nim* gene (Brazier *et al.*, 2001). This *C.difficile* strain (*C.difficile* E4, ribotype010)was isolated in a care home in Leeds and the strain possessed a metronidazole MIC of 16mg/L when susceptibility to metronidazole was carried out with use of E-test (Brazier *et al.*, 2001). *NimB*is the only *nim* gene that has been associated with *C. difficile* in a proteomic assay (Chong *et al.*, 2014).

1.3 Aim

To analysemetronidazole reduced susceptible *C. difficile* strains (CDRM) from each ribotypes 001, 106 and 027 were selected along with metronidazole susceptible *C. difficile* strains (CDSM) strains and control strains for nitroreductase activity and *nim* genes.

To evaluate CDRM strains for the presence of nim genes. *NimA –E* was screened for in 3 ribotypes, ribotype 001, 027 and 106. Susceptible and reduced susceptible strains will be compared.

1.4. Objectives

1.4.1 Use of a spectrophotometry-based nitroreductase assay for the conversion of 4-nitrobenzoic acid (PNBA) to 4-aminobenzoic acid (PABA) in Metronidazole reduced susceptible *C. difficile* strains (CDRM) and Metronidazole susceptible *C. difficile* strains(CDSM)

1.4.2 Polymerase chain reaction experiments will be performed to analyse CDRM strains from ribotypes 001, 027 and 106 for the presence of *nimA – nimE* as the universal primers for these genes were used.

2.0 METHOD

The clinical strains were all isolated in the United Kingdom and were obtained from Professor Mark Wilcox (Leeds Teaching Hospitals NHS Trust and the University of Leeds).The strains were stored as spores in 50% ethanol which could germinate spontaneously in presence of germinants (e.g. bile salts) contained within the selective agar used.

Nitroreductase Assay

2.1 *Clostridium* strains

CDRM strains from ribotypes 001 (n=4), 106 (n=2), and 027 (n=5) were analysed in this experiment alongside CDSM strains 001 (n=2), 027 (n=1). Control strains 110 (E4) and 111(ATCC 70057) were incorporated into these experiments alongside *Clostridium prefringens* (Rafil*et al*, 1991) which was previously reported as a nitroreductase positive control (Rafil*et al*, 1991).

2.2 Nitoreductase assay

Research was carried out at the microbiology laboratory of University of Hertfordshire, Hatfield, UK. Overnight culture of *Clostridium difficile* strains in BHI broth (CM1135, Oxoid, Basingstoke, UK) were standardized to OD_{600} = 0.1 in sterile BHI broth. Then 500µg/ml of 4-nitrobenzoic acid (PNBA) (101553376 Sigma Aldrich, Poole UK) was added in a 1:10 dilution to new culture anaerobically and allowed to incubate for two hours. The culture mixture was then centrifuged for 30 mins at 4000rpm. Subsequently, the 4-aminobenzoic acid (PABA) (Sigma Aldrich, Poole UK) concentration in supernatant was detected by adding the following chemicals at 1 in 10 dilution as was described in Rafii *et al.,* 1991.

Trichloroacetic acid (0.21% w/v) (11964921 Fisher Scientific, Loughborough, UK) thereafter, sodium nitrite (0.007%w/v) (563218, Sigma Aldrich, Poole, UK) was added and incubated at room temperature for 20 minutes. Next ammonium sulfamate (0.04%w/v) (51512, Sigma Aldrich, Poole, UK) was added to neutralize the sodium nitrite following a

3min incubation period at room temperature. After NEDD N-(1- Naphthyl) ethylenediamine dichloride(NEDD 0.35%w/v) (1001844308, Sigma Aldrich Poole, UK) was added to a mixture to yield a purple dye. The PABA concentration was then determined using a Spectrophotometer (Cecil instruments, Cambridge, England) set to an absorbance of 540nm (Rafil*et al,* 1991)., one unit of enzyme is one microgram of PABA.

Detection of *nim* genes

2.3 *Clostridium difficile* strains

Thirteen, eight and seven strains were selected of ribotypes 001, 027 and 106. These strains were inclusive of two *C. difficile* strains known to have being susceptible to metronidazole (CDSM strains) for each ribotype. Two control strains where also involved E4 and ATCC 700057 of ribotype 010 and 038 respectively, as well as the positive control for *nimB*which was *Bacteroides fragilis* strain BF8 (Soki *et al.,* 2006).

2.4 *Nim* gene detection

Research was carried out at the microbiology laboratory of University of Hertfordshire, Hatfield, UK. A suspension of *C. difficile* colonies for each strain was made in sterile water (100µl) (Eitel *et al.,* 2013). The suspension was incubated for 15mins in 95^{o}C then it was centrifuged at 14000 rpm for 2mins to obtain the DNA template. This was performed for all sample strains as well as the positive control for *nimB*which was BF8 (Soki *et al.,* 2006). PCR mixture contained

1µl of DNA template, 1µl of primers (Primers NIM3 (5′-ATG TTC AGA GAA ATG CGG CGT AAG CG-3′) NIM5 (5′-GCT TCC TTG CCT GTC ATG TGC TC-3′)), 12.5µl of AmplitaqGold^{TM}PCR mastermix (10289234, Applied Biosystems^{TM}, Thermo Fisher Scientific, Loughborough UK) and 9.5-10µl of sterile distilled water. The primers used were the universal primers NIM-3 and NIM-5 known to be used for the amplification of *nimAnimE.*

The *nim* genes PCR cycling conditions used were 35 cycles of: 94^{o}C for 15seconds, 62^{o}C for 30secs, and 72^{o}C for 30 seconds. The cycle was done (Eitel *et al,* 2013) in a thermal

cycler (Biocompare, sanFrancisco, California, US). Then each PCR product was mixed with 2.5µl of loading dye and loaded in a tris boris EDTA gel, for gel electrophoresis alongside a 100bp ladder (1002389655, Sigma Aldrich, Poole, UK). Gel red (Biotium, Hayward, California, US) was incorporated in the agarose gel to enable detection of PCR products following exposure to UV light.

3.0 RESULTS

Nitroreductase Assay

Table 3.1 Nitroreductase (Nit) activity demonstrated by *Clostridium difficile* strains from ribotypes 001, 027, 106 using a spectrophotometry based PNBA reduction assay at 540nm.

***C. difficile* ribotype**	**Number of CDRM strains nitroreductase positive (%)**	**Number of CDSM strains nitroreductase positive (%)**
001	1(25)	0(0)
027	0(0)	0(0)
106	2(100)	No strains analysed

The strains with positive enzyme activity were all approximately one enzyme unit which was very low. Variability was also observed in this assay.

C. perfringens ATCC 3626 demonstrated nitroreductase activity in the assay but the two internal *C. difficile* control strains did not. Only a low proportion of CDRM strains demonstrated nitoreductase activity. None of the CDSM strains analysed showed detectable nitroreductase activity.

3.2 Nim Genes

The *nim* gene PCR amplification was only observed in the *nim* positive control strain *BF8* (about 400 bp). All samples tested including ribotype 001, 027 and 106 CDSM and CDRM strains showed no *nim* PCR product (Figure 3.1)

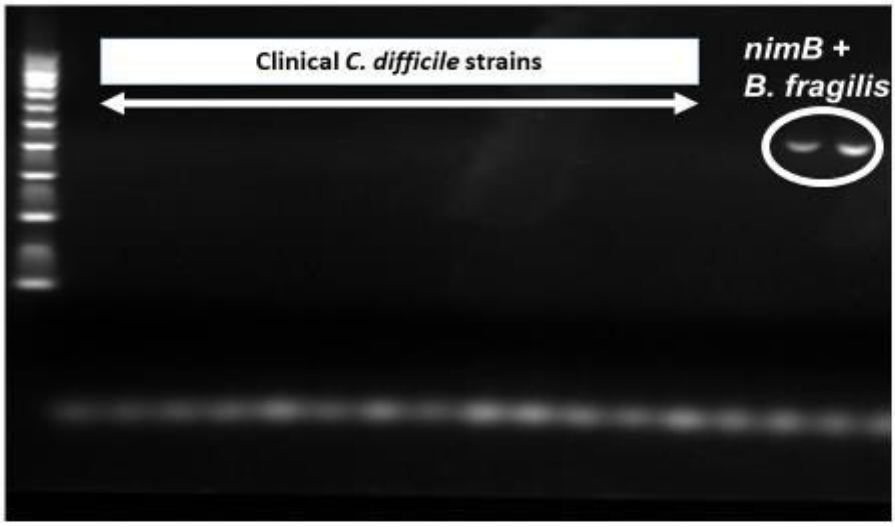

Figure 3.1: PCR amplification of *C. difficile* strains and *nimB* positive control *BF8*.The first well contained 100 base pair ladder, other wells contained *C. difficile* strains except the last 2 wells that contained the positive control *BF8*

4.0 DISCUSSION

4.1 Nitroreductase Assay

This research has shown that majority of *C. difficile* strains were nitroreductase negative with the exception of three strains using the method of detection described by Rafii *et al.,* 1991 where *Clostridium* species actually showed positive nitroreductase activity. Although the nitroreductase assay used in this experiment yielded positive nitroreductase data for the *C. perfringens* strain used as a positive control, there was inconsistency in the data generated from *C. difficile* strains, both clinical CDRM isolates and CDSM isolates and in the internal control strains used.

Rafii *et al.,* 1991 stated that the nitroreductase activity was extracellular and constitutive however metronidazole activation has been reported to occur intracellularly(Edwards and Mathison, 1970). It has also been stated previously that nitroreductase enzymes are substrate specific (Sisson *et al,* 2002) thus there is a possibility that the nitroreductase enzyme responsible for conversion of PNBA to PABA is extracellular and that of metronidazole is intracellular. The method used in the present study was following the method reported for the detection of nitroreductase activity in wild type bacteria and nitrofurantoin resistant mutants (Raffii*et al,* 1998). Nitrofurantoin is a nitrofuran antimicrobial agent and this group of nitro compounds has been identified to have redox potentials ranging from -250mV to -270mV, nitazoxanide has a redox potential of -360mV while that of metronidazole is -485mV (Sisson *et al.,* 2002). The substrate specificity was observed to be largely linked to the redox potential requirement of each substrate. Therefore, in *Helicobacter pylori,* POR was more active to reduce nitazoxanide, RdxA reduced metronidazole and FrxA reduced nitrofurans, while both RdxA and FrxA were also observed to reduce nitazoxanide(Sisson *et al.,* 2002). Furthermore, Raffii*et al.,* (2003), has observed nitroreductase activity in metronidazole resistant *Enterococcus* species, which metabolised metronidazole to a non-bactericidal metabolite and the nitroreductase activity was detected by measuring PABA production. It was also elucidated that PNBA conversion was not affected by the presence of metronidazole thus there was no substrate competition which should not be the case when PNBA was

converted and metronidazole metabolised (Raffii*et al.*, 2003), this further emphasizes the likelihood of different reductase enzymes involved in the nitroreduction in bacteria. Thus indicating that perhaps the conversion of PNBA to PABA might be performed by specific nitroreductases, distinct from those responsible for the activation of metronidazole, or perhaps this conversion could occur by a wide range of nitroreductases. This present research and previously published literature have shown that due to the substrate specificity of different nitroreductase enzymes, any experiment with the aim of detecting nitroreductase activity has to be modified with respect to the substrate and potentially the microorganism under study.

4.2*Nim* genes

Nim genes, as earlier mentioned, have been implicated in previous reports as being involved in contributing to decreased susceptibility to metronidazole in anaerobic organism such as *Bacteroides fragilis*. *nimA* was proposed to prime *B. fragilis* towards an alternative metronidazole resistant pathway (Paunkov et al, 2022). In the present study *C. difficile* PCR ribotypes 001, 027 and 106 were screened for the presence of *nimA-E* genes. Most of the strains screened were strains observed to be CDRM and the study also included CDSM for each of the ribotypesanalysed. This research confirmed the absence of *nim* genes in *C. difficile* UK ribotypes irrespective of metronidazole susceptibility patterns. As shown in Figure 7.1, no *nim* genes were amplified in any of the ribotypes irrespective of susceptibility of the strains analysed. PCR amplification of putative *nim* was only observed in the *nim* positive control strain of *Bacteriodes fragilis* (*BF8*). *Bacteriodes fragilis* (*BF8*) as was earlier mentioned is *nimB* positive, positive control strains for the remaining *nim* genes could not be sourced as part of this study however, given that these cycling conditions have successfully amplified other nim genes previously (Eitel *et al*, 2013), had these *nim* genes been present then the PCR process would have successfully amplified them, as the primers used were universal primers for *nimA-E*. It is interesting that *nimB* was absent in the *C. difficile* strains evaluated, since research by Chong and colleagues (2014) suggested that *nimB* was present in both metronidazole susceptible and resistant ribotype 027 variants of *C. difficile.*

As earlier stated there has been some controversy correlating *nim* to decrease in metronidazole activity. This controversy is further confirmed in an earlier research where 2% of *Bacteriodes* spp. from Hungary where reported to have reduced susceptibility to metronidazole. However just 5% of these decreased susceptibility strains were *nim* positive with the *nimA* and *nimB* genes (Urban *et al*, 2002). The present study is also in line with a previous research that involved observing twenty four isolates of *Clostridium difficile* for the presence of *nim* genes. Fourteen out of these isolates were identified to be metronidazole resistant, however *nim* genes were not found in the isolates (Pelaez *et al*, 2008). Whole genome sequencing was used also to analyse three *C. difficile* strains of ribotype 010 originally from Spain, one of the strains was reduced susceptible to metronidazole, the other metronidazole resistant, as well as susceptible strain. *Nim* genes were not observed in the genomic analysis of these *C. difficile* isolates (Moura *et al*, 2014).

Conversely, a proteomic assay was carried out on three 027 ribotype strains which were metronidazole susceptible, reduced susceptible and resistant to metronidazole. *NimB* was detected and *NimB* expression was observed to be 3-fold higher in the reduced susceptible and metronidazole resistant strain compared to the susceptible strain (Chong *et al*., 2014). However, these same strains had been earlier sequenced using next generation sequencing and *nim* genes were absent (Lynch *et al*, 2013). The possibility of *nimB* gene is further emphasized in the reclassification of *C. difficile* into the family of *Peptostreptococcacaee* that led to its renaming as *Peptoclostridium difficile* (Yutin& Galperin, 2013). The *Peptostreptococci* have previously been reported to possess *nimB* genes (Theron *et al*, 2004).

The present study has confirmed earlier findings showing the absence of *nim* in *C. difficile*, thus indicating that metronidazole reduced susceptibility may be due to factors other than the conversion of metronidazole into a less harmful derivative as facilitated by *nim* genes. It also, along with observations in published literature depicts the potential inability of the *nim* universal primers to identify *nim* sequences in all bacterial species. This issue was previously encountered in *Bacteriodes fragilis,* where the universal primers could not amplify *nimJ* because it had a different nucleotide sequence even though the proteins where same with the universal primers. Therefore, this would have given a false *nim* negative result if the research had been limited to PCR amplification using the

universal primers (Hussain *et al*, 2013). Whole genome sequencing on *C. difficile* has identified *nimB* and shown 45% similarity to *B. Fragilis NimB*(Olaitan et al, 2023)

however the reason for its inability to be amplified with universal primers was not stated, but this further buttresses the elucidation of different primer sequences from universal primers. Though the target of study was on fluoroquinolone-resistant *Clostridioides difficile* (Olaitan et al, 2023)

5.0 Conclusion and future work

In conclusion the method employed was not efficient to detect the nitroreductase enzyme reduction of metronidazole,since the method detects extracellular nitroreductase. Devising a method using radio labelled metronidazole (Tally *et al.*, 1978) and monitoring the decline in the radiolabel signal, which is proportional to the level of nitroreduction, maybe a more useful method in correlating nitroreductase activity to the CDRM phenotype.This approach may also provide an insight into the level and rate of activity of reductases. Thus would likely be more sensitive in the detection of nitroreductase activity.

There is yet to be a nitroreductase enzyme associated with *Clostridium difficile* with respect to metronidazole activation but as a frontline therapy for CDI, this warrants further investigations. Future work on the identification of nitroreductases, both intracellularly and extracellularly expressed are warranted in *Clostridium difficile* in order to more fully understand the process of nitroreduction in *Clostridium difficile*. Whole genome sequencing in *C. difficile* would identify putative nitroreductases, potential mutations (SNPs or deletions), and mutagenesis experiments and gene knockouts (Jeong *et al*, 2000, Jeong *et al*, 2001) targeted at the nitroreductases would yield valuable data on the role of these enzymes in *C. difficile* physiology and in the activation of metronidazole.

This research has also shown inability to amplify *nim* gene using the universal primers. Though, the present study did not assess at the proteomic level for the presence of Nim as in the study of Chong *et al* (2014), therefore the presence of Nim in the *C. difficile* proteome of strains in the present study cannot be ruled out. The proteomic assay which demonstrated *nimB* expression that was not identified at genomic level (Lynch et al., 2013 and Chong *et al* 2014) indicates a different gene sequence that could have similar proteins

as *nimB*. Thus this could be an aspect for future research as genomic assays is yet to identify *nim* presence in *Clostridium difficile*.

The submission of the *C. difficile* Nim protein sequence to Phyre Protein Fold recognition server could also aid in the comparison with other *nim* protein structures using Dali server (Hussain *et al*, 2013). As well as converting the *C. difficile* Nim protein sequence to its gene sequence, which would aid identification of genetic differences with other *nimB* sequences in published literature.

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