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Haem is crucial for medium-dependent metronidazole resistance in clinical isolates of *Clostridioides difficile*

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Background: Until recently, metronidazole was the first-line treatment for *Clostridioides difficile* infection and it is still commonly used. Though resistance has been reported due to the plasmid pCD-METRO, this does not explain all cases.

Objectives: To identify factors that contribute to plasmid-independent metronidazole resistance of *C. difficile*.

Methods: Here, we investigate resistance to metronidazole in a collection of clinical isolates of *C. difficile* using a combination of antimicrobial susceptibility testing on different solid agar media and WGS of selected isolates.

Results: We find that nearly all isolates demonstrate a haem-dependent increase in the MIC of metronidazole, which in some cases leads to isolates qualifying as resistant (MIC >2 mg/L). Moreover, we find an SNP in the haem-responsive gene *hsmA*, which defines a metronidazole-resistant lineage of PCR ribotype 010/MLST ST15 isolates that also includes pCD-METRO-containing strains.

Conclusions: Our data demonstrate that haem is crucial for medium-dependent metronidazole resistance in *C. difficile*.

Introduction

Clostridioides difficile is a Gram-positive, anaerobic enteropathogen capable of causing a *C. difficile* infection (CDI) upon disruption of the normal intestinal microbiota by for instance antimicrobial therapy.[1,2] It is the primary cause of nosocomial diarrhoea, but is also found in cases of community-acquired disease.[2,3] Although the use of antibiotics is a risk factor for CDI, antimicrobials are also used to treat the infection. Until recently, metronidazole was considered the drug-of-choice for treatment of mild CDI. Though vancomycin and fidaxomicin are currently indicated as first-line therapeutics for the treatment of CDI,[4,5] metronidazole is still commonly used.[6,7]

Our understanding of the mechanisms of resistance to metronidazole in *C. difficile* is still limited. For Clostridia, studies are complicated by reports of unstable, inducible metronidazole resistance that is lost upon removal of antibiotic pressure or after the strain undergoes freeze-thawing cycles.[8,9] Recently, however, we have demonstrated that metronidazole resistance in diverse strains of *C. difficile* can be mediated by the plasmid pCD-METRO through a mechanism that is not yet understood.[10] Notably, the presence of pCD-METRO explains at least part of independently reported cases of metronidazole resistance.[11–13] However, strains that lack pCD-METRO can still be resistant to metronidazole. For instance, we have previously identified a pCD-METRO-negative strain that demonstrated medium-dependent metronidazole resistance.[10] This suggests that other, potentially chromosomal, determinants contribute to resistance.

Information on pathways that could contribute to resistance comes from laboratory strains with evolved resistance to

metronidazole. Using a laboratory-evolved PCR ribotype (RT) 027 strain exhibiting stable metronidazole resistance, mutations were identified in genes affecting electron transport and iron utilization, but the individual contribution of these mutations to the resistant phenotype was not further investigated.[14] More recently, a mutator strain defective in DNA mismatch repair was evolved in the presence of metronidazole.[15] The study discovered mutations in a gene encoding an iron transporter (*feoB1*) in metronidazole-resistant strains and showed that sequential mutations in *nifJ* [encoding the pyruvate-ferredoxin oxidoreductase ('PFOR')], *xdh* (encoding xanthine dehydrogenase) or *iscR* (encoding an iron–sulphur cluster regulator) could further increase metronidazole resistance.[15] Though studies with laboratory-evolved strains are informative, it is unclear how these findings translate to metronidazole-resistant strains isolated from subjects outside the laboratory.

Here, we leverage the potential of strains collected within a project to develop a detailed understanding of the epidemiology and clinical impact of CDI across Europe (COMBACTE-CDI) that were investigated for metronidazole resistance. Four strains demonstrated stable metronidazole resistance, but lacked pCD-METRO. We show that there is a haem-dependent increase in the MIC of metronidazole across PCR RTs and that a higher MIC, at least for a subset of strains, correlates with a specific mutational signature in the gene *hsmA*.

Materials and methods

Materials and methods for this study are described in the main text and details are available as Supplementary data at *JAC* Online.

Results

Metronidazole resistance is observed in the COMBACTE-CDI strain collection

Combatting Bacterial Resistance in Europe—*Clostridioides difficile* infections (COMBACTE-CDI) is a multicentre European-wide project with an aim to provide detailed understanding of the epidemiology and clinical impact of CDI across the whole healthcare economy in Europe. Sites testing both inpatient and community samples were recruited from 12 countries across Europe. All diarrhoeal faecal samples (regardless of tests requested by physician) were submitted to a central laboratory (Leeds, UK) on two selected days between July and November 2018. From these samples *C. difficile* was isolated and tested by PCR ribotyping. The metronidazole MICs for 213 clinical isolates (Belgium $n=3$, France $n=4$, Greece $n=4$, Ireland $n=1$, Italy $n=23$, Netherlands $n=8$, Poland $n=29$, Romania $n=37$, Slovakia $n=1$, Spain $n=43$, Sweden $n=12$, UK $n=48$) were determined by Wilkins-Chalgren agar dilution.[16] Of these, 16 isolates (7.5%) were found to be resistant to metronidazole using the EUCAST criteria as a cut-off (MIC > 2 mg/L) and were sent to the *C. difficile* reference laboratory of the Leiden University Medical Center for further study.[17] A further six isolates had an MIC of 2 mg/L. When possible, an RT-matched isolate with an MIC <2 mg/L from the COMBACTE-CDI collection was also provided. PCR RTs submitted were RT002 ($n=3$), RT010 ($n=7$), RT016 ($n=1$), RT018 ($n=3$), RT027 ($n=12$), RT176 ($n=1$), RT181 ($n=4$) and RT198 ($n=1$) (Table 1).

Reported resistance rates for metronidazole vary from 0% to 18%, with a recent meta-analysis indicating a weighted pooled resistance of 1.9%.[18] Thus, the COMBACTE-CDI study resulted in the identification of a relatively high percentage of resistant isolates. With a ratio of resistant versus susceptible strains of <1/10, our findings underscore the importance for investigating large collections of clinical isolates to enrich for strains that are resistant, in order to investigate possible underlying causes of resistance.

Low-level resistance to metronidazole is not due to carriage of the pCD-METRO plasmid

We further investigated the clinical isolates from the COMBACTE-CDI strain collection ($n=32$; Table 1). To correct for inter-laboratory differences and to make the results directly comparable to our previous study,[10] we performed PCR ribotyping and antimicrobial susceptibility testing by agar dilution according to CLSI standards on Brucella Blood Agar (BBA) in a second laboratory.[10,19] A single strain showed discrepant results in PCR ribotyping and was therefore excluded from further analysis. We found 3/32 of the received COMBACTE-CDI strains to be resistant to metronidazole (10% of the preselected isolates; Table 1), all of which belonged to RT010 (MIC = 4 mg/L). One isolate had an MIC of 2 mg/L and belonged to RT016. All of these strains were identified as resistant in the initial susceptibility testing in Leeds. The inter-laboratory difference in antimicrobial susceptibility ($n=16$ in Leeds versus $n=3$ in Leiden) may be explained by differences in testing methodology or unstable/heterogeneous resistance,[8,20] but were not further investigated here.

To determine whether the observed resistance was due to the presence of the pCD-METRO plasmid, we performed a reference assembly of the sequence reads obtained from WGS of these isolates against the pCD-METRO reference sequence (obtained from the European Nucleotide Archive BioProject number PRJEB24167). No reliable mapping of reads to the reference sequence was found and—in line with this finding—the strains were negative in a PCR assay directed against pCD-METRO (data not shown).[10] We therefore conclude that these strains do not carry pCD-METRO and that a different mechanism confers metronidazole resistance in these isolates.

Resistance to metronidazole is medium-dependent

We have previously described a strain that demonstrated medium-dependent metronidazole resistance (MIC = 4 mg/L) independent of pCD-METRO.[10] In order to test if the metronidazole-resistant phenotype of the four strains from the COMBACTE-CDI study could similarly be medium-dependent, strain GSK241 (MIC = 4 mg/L) was plated on brain heart infusion (BHI) agar that is routinely used in our laboratory, BBA (containing 5% defibrinated sheep blood, 1 mg/L vitamin K and 5 mg/L haemin) and BHI blood agar (BHI agar supplemented with 5% defibrinated sheep blood, 1 mg/L vitamin K and 5 mg/L haemin) after which a metronidazole Etest was applied. We found that strain GSK241 was susceptible to metronidazole (MIC = 0.25 mg/L) on BHI medium, but resistant (MIC $\geq$ 2 mg/L) on both BBA agar and BHI blood agar (Figure 1). These results indicate that components present in blood agar are responsible for this medium-dependent resistant phenotype.

We wondered whether the medium-dependent change in MIC values was a general characteristic of *C. difficile*, irrespective of the

Table 1. Characteristics of the strains from this study

Isolate	Location of participant at time of sample	Country of origin	PCR RT	MIC of metronidazole[a] (mg/L)
GSK234	inpatient	Sweden	002	0.25
GSK6	community doctor	UK	002	0.25
GSK7	inpatient	UK	002	0.125
GSK22bis	inpatient	UK	010	0.125
GSK211	inpatient	Romania	010	0.25
GSK241	inpatient	France	010	4
GSK242new	inpatient	France	010	4
GSK246	inpatient	France	010	0.5
GSK313	inpatient	Spain	010	4
GSK184	inpatient	Poland	016	2
GSK39	community doctor	Italy	018	0.5
GSK302	inpatient	Italy	018	0.5
GSK303	inpatient	Italy	018	0.25
GSK54	inpatient	UK	027	0.25
GSK55	inpatient	Romania	027	0.25
GSK60	inpatient	Poland	027	0.5
GSK61	inpatient	Poland	027	0.5
GSK62	inpatient	Poland	027	0.5
GSK63	inpatient	Poland	027	0.5
GSK64	inpatient	Poland	027	0.5
GSK65	inpatient	Poland	027	0.5
GSK179	inpatient	Poland	027	0.5
GSK318	inpatient	Poland	027	0.5
GSK325	inpatient	Poland	027	0.5
GSK327	Inpatient	Poland	027	0.5
GSK258	Inpatient	Slovakia	176	0.5
GSK110	Inpatient	Romania	181	0.5
GSK113	Inpatient	Romania	181	0.25
GSK114	Inpatient	Poland	198	0.5
GSK180	community doctor	Romania	181	0.5
GSK190	Inpatient	Romania	181	0.25

[a]Determined by agar dilution at the Leiden University Medical Center (see the Supplementary data available at *JAC* Online).

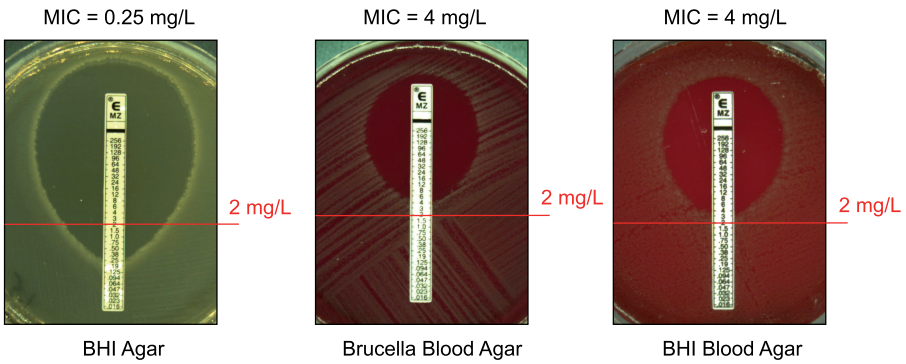

Figure 1. Medium-dependent resistance to metronidazole is independent of base medium. Strain GSK241 was grown and resuspended to a turbidity equivalent to that of a 2.0 McFarland standard and spread on BHI agar, Brucella Blood Agar (BBA) (containing 5% defibrinated sheep blood, 1 mg/L vitamin K and 5 mg/L haemin) and BHI blood agar (BHI agar supplemented with 5% defibrinated sheep blood, 1 mg/L vitamin K and 5 mg/L haemin). Etest strips were applied and plates were incubated for 48 h prior to imaging. Red lines indicate the epidemiological cut-off value for metronidazole as determined by EUCAST and used to indicate resistance in this study.[17] MIC values at the top of the image correspond to the reported MIC values on BHI agar, BBA and BHI blood agar.

Table 2. Metronidazole MICs on different agar media as determined by Etest

Strain name	PCR RT	Etest MIC BHI agar (mg/L)	Etest MIC BBA (mg/L)	Etest MIC BHI blood agar (mg/L)
GSK234	002	0.064	0.064	0.064
GSK241	010	0.25	4	6
GSK242new	010	0.25	6	6
GSK313	010	0.38	4	8
GSK246	010	0.125	1	1
GSK184	016	0.25	2	ND
GSK39	018	0.125	1.5	1
GSK318	027	0.125	1	1
GSK327	027	0.125	2	2
GSK325	027	0.125	1.5	ND
GSK60	027	0.125	4	ND
GSK61	027	0.125	2	1.5
GSK62	027	0.094	1.5	ND
GSK63	027	0.125	1.5	ND
GSK64	027	0.125	1.5	1.5
GSK65	027	0.094	1	ND
GSK179	027	0.125	1.5	ND
GSK258	176	0.16	1	1.5
GSK110	181	0.125	1	1.5
GSK180	181	0.094	1	1
GSK114	198	0.125	2	2

ND, not determined.

resistant phenotype, or specific to the resistant strains. For this reason, we tested selected COMBACTE-CDI strains by Etest on both BHI agar and BBA (Table 2). All strains were clearly susceptible on BHI agar (MIC <0.5 mg/L), but, with the exception of GSK234, showed a 4–32-fold increase in MIC when tested on BBA compared with BHI agar. This medium-dependent increase in MIC was not restricted to a specific RT, as the phenotype was seen for strains belonging to diverse types (RT010, RT016, RT018, RT027, RT176, RT181 and RT198).

Taken together, our data suggest that components present in BBA/BHI blood agar result in reduced susceptibility of strains to metronidazole through a general mechanism. In the case of strains GSK184, GSK241, GSK242new and GSK313 this leads to these strains qualifying as metronidazole resistant.

Haem is required for medium-dependent differences in metronidazole susceptibility

The fact that the medium-dependent increase in MIC was observed for both BBA and BHI blood agar (Figure 1) suggests that the phenotype is independent of the base medium and is likely to be mediated by the supplementation with vitamin K, haemin and/or blood.

For practical purposes, we evaluated the effect of haemin on the metronidazole-resistant phenotype of strains GSK64 (metronidazole susceptible) and GSK241 (metronidazole resistant). We found that supplementation of BHI with 5 mg/L haemin raised the metronidazole MIC to levels similar to those observed for the BBA and BHI blood agar plates (Table 2 and Figure 2a).

We extended this finding to a selection of COMBACTE-CDI strains and found that, with the exception of strain GSK234, all tested strains showed an 8–24-fold increase in metronidazole MIC on BHI supplemented with haemin compared with BHI alone (Table 2). These results mirror those obtained for the Etest on BBA, indicating that haemin is the main determinant of medium-dependent differences in metronidazole MIC for these strains. Under our experimental conditions, the haemin-dependent increase in MIC appears to be specific to metronidazole, as no increase in MIC was observed for vancomycin (Figure 2a) or several other tested antimicrobials (Table S1, available as Supplementary data at *JAC* Online).

We next assessed the MIC of both metronidazole and vancomycin for strains GSK64 and GSK241 with a range of 0–15 mg/L haemin. Both strains show a gradual increase in MIC of metronidazole that, in the case of GSK241, saturates at >5 mg/L haemin; the MIC for GSK64 appears to increase further at higher concentrations of haemin. In contrast, no increase in vancomycin MIC was seen, even in the presence of the highest concentrations of haemin tested (Figure 2b).

Altogether these results demonstrate that the presence of haem is crucial for a medium-dependent resistant phenotype in *C. difficile* and that this appears to be specific for metronidazole.

An hsmA genetic signature is associated with increased metronidazole MICs for PCR RT010 strains

Recent work has shown that four genes (*hsmA, hsmR, hatT* and *hatR*) are differentially regulated in response to haem and that the products of the *hsmAR* operon improve growth of a *C. difficile*

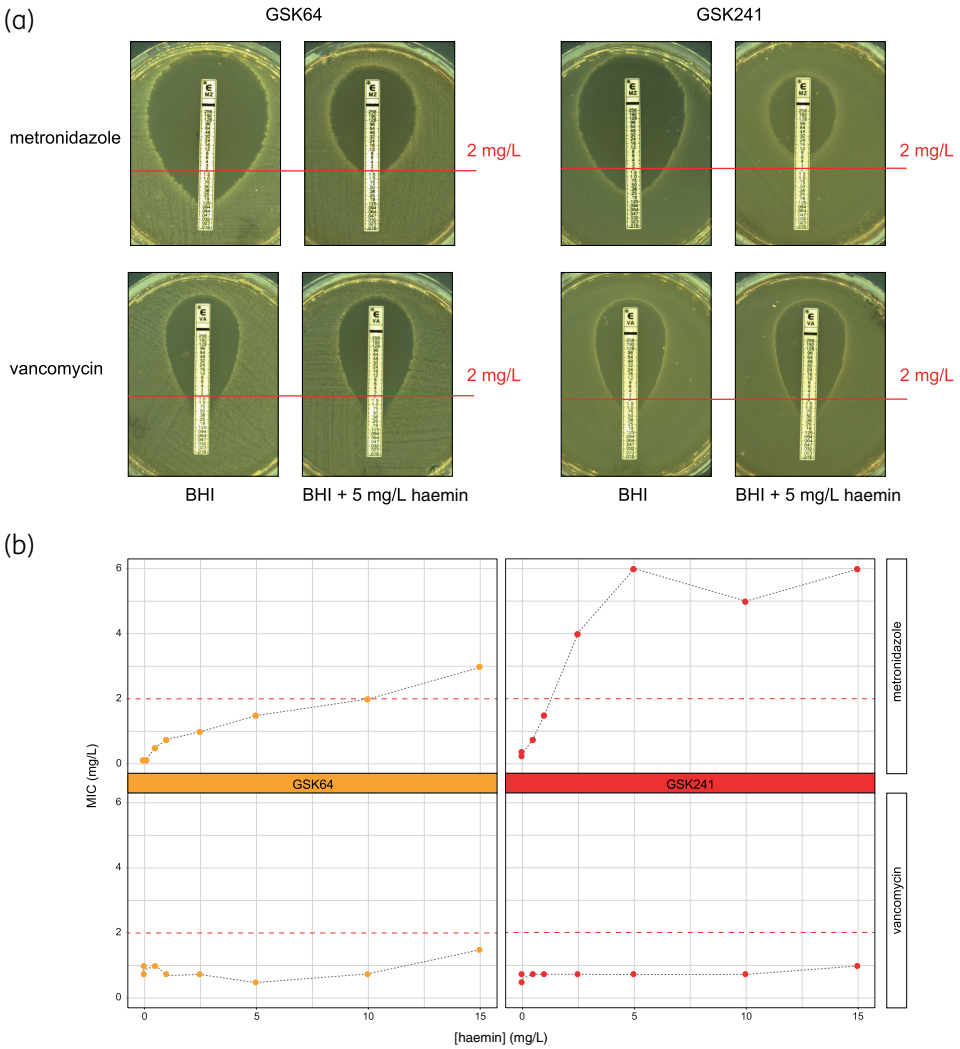

Figure 2. Haem supplementation increases the MICs of metronidazole, but not of vancomycin. (a) Strains GSK64 and GSK241 were resuspended in PBS to a turbidity equivalent to that of a 2.0 McFarland standard and plated on BHI agar and on BHI agar supplemented with 5 mg/L haemin. Etest strips for metronidazole and vancomycin were applied and plates were incubated for 48 h prior to imaging. Red lines indicate the epidemiological cut-off value for metronidazole as determined by EUCAST and used to indicate resistance in this study.[17] For a complete overview of the MICs of metronidazole for these strains on BHI agar and BHI agar supplemented with haemin, please see Table 2. (b) Typical metronidazole (top) and vancomycin (bottom) Etest results for strains GSK64 (orange) and GSK241 (red) and when grown on BHI supplemented with various concentration of haemin (0, 0.5, 1, 2.5, 5, 10 and 15 mg/L). The dashed line indicates the epidemiological cut-off value for metronidazole and vancomycin as determined by EUCAST (2 mg/L).

PCR RT027 strain in the presence of metronidazole.[21,22] For this reason we performed an SNP analysis on the genes *hatR, hatT, hsmR* and *hsmA* using the sequences from the RT027 strain R20291 (GenBank accession number FN545816) as a reference.

Using variant positions, we identified signatures for these genes for each strain from the COMBACTE-CDI collection. In the case of *hatR, hatT* and *hsmR* these signatures were conserved within a PCR RT and even across closely related PCR RTs (e.g. RT027, RT198, RT181 and RT176 share the same signatures) (Table S2). However, for *hsmA*, we observed two distinct, but related, signatures within a single PCR RT. SNPs were identified in the 399 bp *hsmA* gene at positions 129, 249, 366, 372 and 392, resulting in a 5 bp signature sequence. This signature is GGCAT in the RT027 and RT027-like isolates, TGTAC in the RT002 and RT018 isolates and TATAC in some RT010 isolates (Figure 3a). The first three variable nucleotide positions (129, 249 and 366) result in the same amino acid sequence compared with the RT027 reference, whereas the fifth variant position results in a change of a valine residue to an alanine without a marked effect on protein characteristics (Figure S1). Interestingly, the signature sequence TAT-C was found in all three metronidazole-resistant RT010 isolates. The deletion at position 372 results in a frameshift and alters the primary amino acid

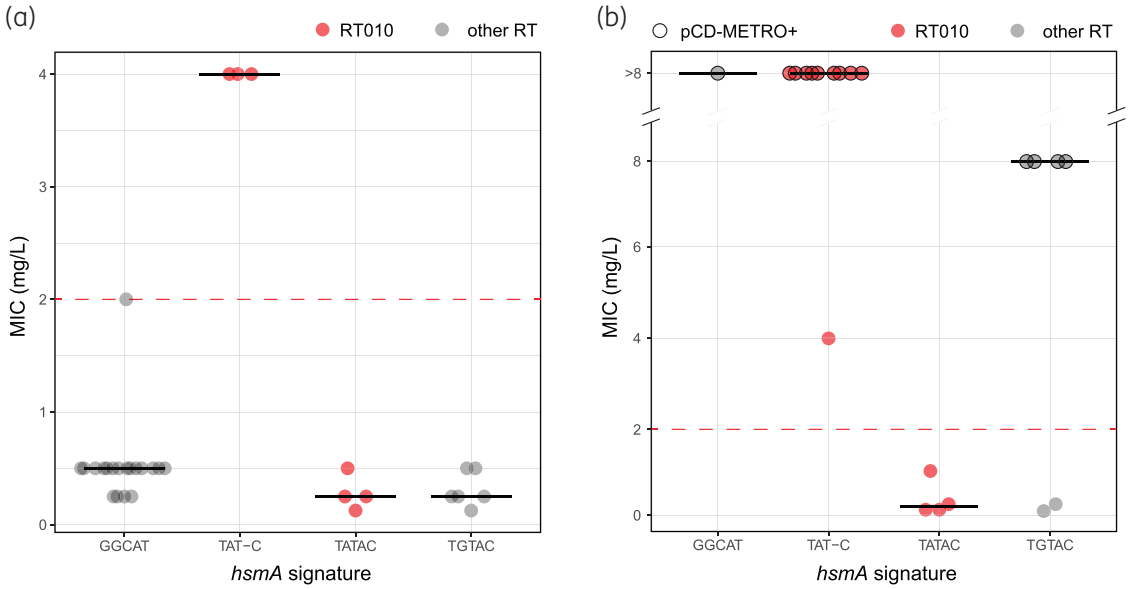

Figure 3. TAT-C signature in *hsmA* correlates to metronidazole resistance in RT010 isolates. The horizontal black bars represent the median MIC. (a) *hsmA* signature based on SNP analysis in the COMBACTE-CDI clinical isolates. Strains containing the GGCAT signature belong to RT016, RT027, RT176, RT181 and RT198. The TAT-C and TATAC signatures correspond to RT010 in this study, whereas RT002 and RT018 contain sequence TGTAC. (b) *hsmA* signature based on SNP analysis in the clinical isolates sequenced in the pCD-METRO study.[10] The signature sequence GGCAT is found in RT027, the signature sequences TAT-C and TATAC are found in RT010 and the signature sequence TGTAC is found in RT012 and RT020.

sequence as well as the hydrophobicity profile of the C-terminus of the HsmA protein (Figure S1).

In order to validate the significance of the signature, we performed the same *hsmA* SNP analysis on whole-genome sequences obtained from another collection of isolates enriched for metronidazole resistance.[10] This collection contains RT010, RT020 and RT027 isolates that have been characterized with respect to metronidazole MIC and pCD-METRO carriage. We expected to find a similar clustering of the *hsmA* signature sequence and PCR RT and predicted that the previously described RT010 isolate showing medium-dependent resistance (MIC = 4 mg/L) would carry the 1 bp deletion in *hsmA*. Indeed, we found this was the case (Figure 3b). Strikingly, all highly resistant RT010 strains that carried pCD-METRO also contained the TAT-C *hsmA* signature sequence (Figure 3b).

We wondered how frequently this deletion could be found in RT010 isolates, as metronidazole resistance is most commonly observed in this RT. For this reason, we performed whole-genome SNP ('wgSNP') analysis through the Enterobase platform on sequences available in the Sequence Read Archive (SRA) of MLST ST15 (which includes RT010) ($n = 57$) and ST15-like strains ($n = 4$) (Table S3) as well as the sequences of the RT010 isolates described earlier in this study ($n = 21$).[10,23–25] We found that the TAT-C signature in *hsmA* was detected in a specific lineage of ST15 and ST15-like strains originating from different countries (Figure 4). One out of 61 (1.6%) of the ST15 and ST15-like isolates was found to contain the 1 bp *hsmA* deletion (accession number ERR125985), but no metadata were available for this strain in the SRA to confirm metronidazole resistance. Interestingly, pCD-METRO carriage is distributed throughout the lineage with the TAT-C *hsmA* signature, suggesting that pCD-METRO may be preferentially acquired in strains with pre-existing low-level metronidazole resistance.

Altogether our results demonstrate that a specific signature of *hsmA*, resulting in an altered C-terminal protein sequence, is associated with haem-dependent metronidazole resistance as well as pCD-METRO carriage in RT010 strains of *C. difficile*.

Discussion

In this study we describe a collection of clinical *C. difficile* isolates that demonstrate a haem-dependent increase in the MIC of metronidazole and make the observation that four strains determined to be resistant to metronidazole required haem supplementation for this phenotype. Additionally, we show that a C-terminal deletion at position 372 in *hsmA* correlates to metronidazole resistance in RT010 isolates.

The observation that the MICs of certain antibiotics vary depending on the type of medium used has been well documented in other organisms, but little to no data are available for *C. difficile*.[10,26] One of the best known examples is the effect of divalent calcium on the daptomycin susceptibility of a variety of organisms, but other examples have been documented as well.[27] For instance, *Escherichia coli* was susceptible to bleomycin in LB broth, but was resistant to this antibiotic in glucose minimal medium, though the mechanism behind this difference remains unclear.[28] Similar results were obtained for *Moellerella wisconsensis* and *Proteus* spp. for fosfomycin resistance when comparing MICs in Iso-Sensitest broth and (cation-adjusted) Mueller–Hinton broth.[29,30] Additionally, medium-dependent activity of gentamicin sulphate against enterococci has also been encountered, showing that the medium-dependent activity of antibiotics against bacteria is not a phenomenon restricted to Gram-negative organisms.[31]

In this study we observed that almost all strains showed an increase in metronidazole MIC when grown on blood agar compared

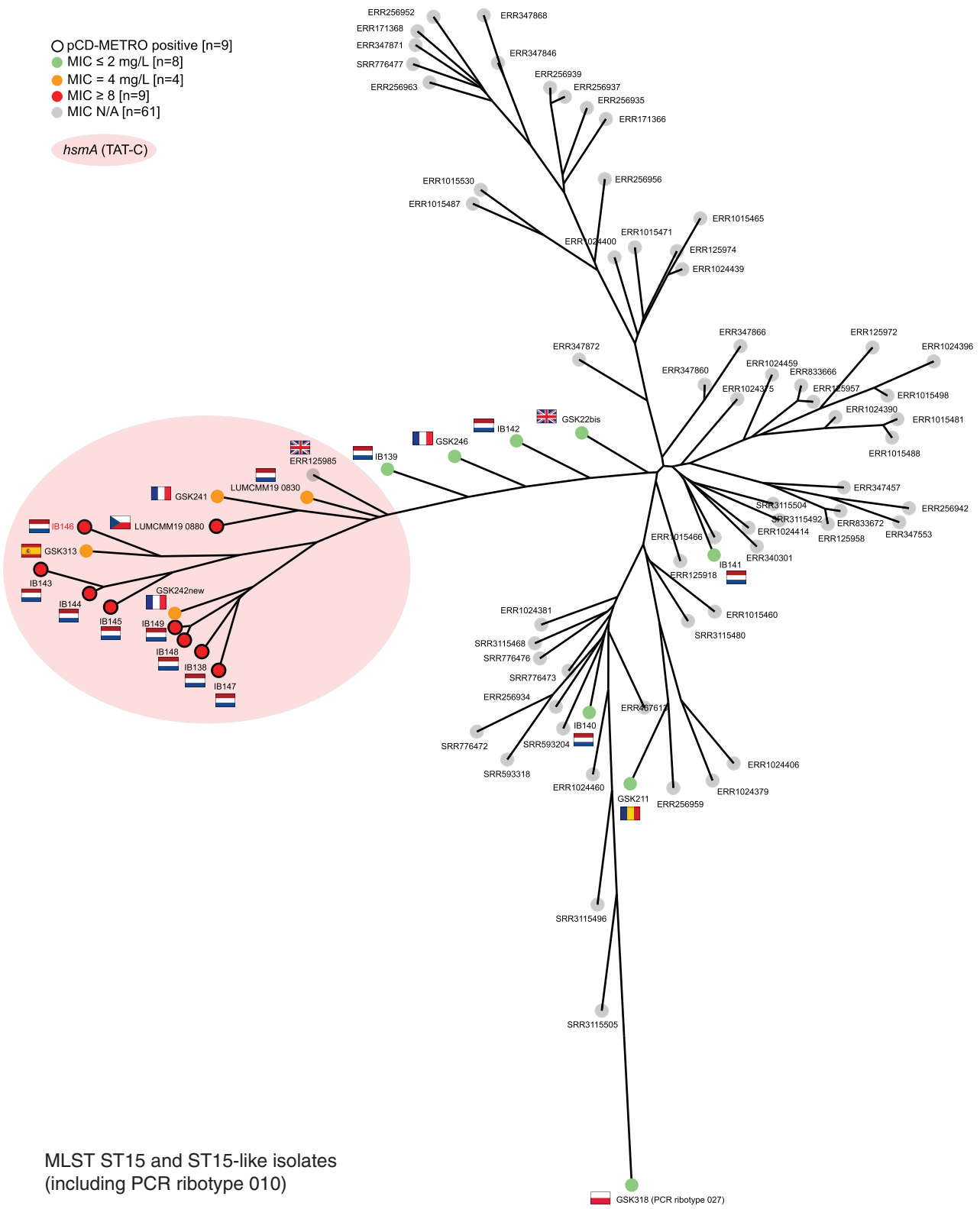

Figure 4. The C-terminal deletion in *hsmA* is associated with a lineage of ST15 and ST15-like isolates. When available, metronidazole susceptibility data and pCD-METRO carriage are indicated. Country flags prior to strain names indicate strain origin, when known. Strain names[10] or SRA accession numbers are included. Distances in tree are shown in logarithmic scale. N/A, not available (i.e. no MIC metadata are deposited with the sequences and the isolates were not available for testing).

with BHI (Figure 1 and Table 2). We note the limitation that the COMBACTE-CDI collection we analysed here does not encompass an unbiased collection of PCR RTs and, in particular, clade 2 strains (RT016, RT027, RT176, RT181 and RT198) appear to be overrepresented. This may also have contributed to the relatively high percentage of metronidazole-resistant strains initially identified as metronidazole resistant within COMBACTE-CDI, though we note that if we assume the three confirmed resistant isolates are the only stably resistant isolates within this collection, the number is in line with the literature (3/213 = 1.4%).[18] We found that haem supplementation of the blood agar plates was the causative determinant for this phenotype (Figure 2 and Table 2), presumably through the ability of haem to detoxify the nitro-radicals generated by metronidazole activation.[32] As lethal concentrations of antimicrobials are thought to generate toxic radicals by altering cellular metabolism, we expected to find haem-dependent alterations in antibiotic susceptibility for antibiotics other than metronidazole.[33,34] However, no haem-dependent reduction in susceptibility towards vancomycin and several other antimicrobials was found in our study, suggesting that the effect of haem shows specificity for metronidazole under the conditions tested.

Elegant work by Knippel *et al.*[21] has demonstrated that reduced metronidazole susceptibility upon haem supplementation for R20291 was largely mediated by the *hsmRA* operon, leading to the question of whether the presence/absence or sequence variants of these genes underlies haem-dependent resistance in other RTs. Based on the present dataset, however, we were unable to identify specific sequence variants of this operon (or in the *hatRT* operon also involved in haem detoxification) that could explain why the vast majority of strains are less susceptible to metronidazole upon haem supplementation. The genes appear to be (near-)universally conserved amongst different *C. difficile* types (data not shown) and the same signature is found in strains that do or do not respond to haem supplementation and those that do or do not qualify as resistant (Table 1, Table 2 and Table S2). These results imply that factors other than the sequence of the *hatRT* and *hsmRA* operons can contribute to the haem-dependent reduction in metronidazole susceptibility.

Nevertheless, we did identify a C-terminal deletion in *hsmA* that correlated to haem-dependent metronidazole resistance in RT010 (ST15) isolates (Figure 3, Figure 4 and Table S2). Isolates without this deletion did become less susceptible to metronidazole in the presence of haem, but did not exceed the EUCAST criterion for resistance to this antibiotic.[17] We validated our findings in the collection of clinical isolates used in the pCD-METRO study.[10] At present, there is no structural information on the HsmA protein, though homology between the protein and haem-containing cytochromes has been noted.[21] We also found a structural homology with helices 2–5 of human duodenal cytochrome b (PDB: 5ZLG; Figure S1). The putative location of the C-terminus of HsmA outside of the membrane suggests that the C-terminal extension as result of the frameshift is also located extracellularly (Figure S1). HsmA has been postulated to act through sequestration of haem, but, as it is unclear how HsmA binds haem and whether residues involved in haem binding in cytochrome b are conserved in HsmA, the effect of the altered C-terminal sequence on the affinity for haem remains to be elucidated.

The COMBACTE-CDI collection analysed here includes a limited number of strains per RT (Table 1). It will be interesting to see whether targeted analyses of larger collections of specific RTs will reveal additional sequence variants of *hsmAR* associated with reduced susceptibility or resistance to metronidazole.

Our data hint at a possible cumulative effect of chromosomal and extrachromosomal determinants in metronidazole resistance as strains carrying the pCD-METRO plasmid are dispersed over the resistant ST15/ST15-like (RT010) lineage characterized by the TATC *hsmA* signature (Figure 4). Strains that possess both the C-terminal adenine deletion in *hsmA* and the pCD-METRO plasmid have a higher metronidazole MIC (MIC $\geq$8 mg/L versus 4 mg/L for the pCD-METRO-negative RT010 isolates). As no pCD-METRO-positive RT010 isolate containing the TATAC signature sequence was present in this collection, we do not know if pCD-METRO carriage without the deletion can still result in an MIC of $\geq$8 mg/L, though this appears to be the case in RT020 and RT027.[10] Irrespective of the effect on metronidazole MICs, pCD-METRO carriage is associated with the 1 bp deletion in *hsmA* in RT010 isolates. Though this might result from a selection bias (by preferentially characterizing isolates with higher MICs), it is conceivable that the deletion facilitates pCD-METRO carriage in some way.

Our findings suggest that the haem-dependent reduction in metronidazole susceptibility is common in *C. difficile* (Table 2). Though haem levels can be elevated at the host–pathogen interface during CDI,[22] pathogenicity does not seem to be a requirement for the haem-dependent reduction in MIC as it is also found in non-toxigenic strains such as those belonging to RT010. For the same reason, it is unlikely that extensive and continued use of metronidazole provided the selective pressure for the acquisition and/or persistence of this phenomenon during evolution.[6,7,35] In the majority of the strains the MIC will likely not be raised over the EUCAST cut-off (2 mg/L) for resistance,[17] but they clearly do become less susceptible to metronidazole. Due to absorption in the small intestine and sequestration or inactivation of the microbiota, levels of metronidazole at the end of the colon are potentially low as determined by concentrations found in faecal material.[36–39] It is therefore quite possible that a moderate increase in metronidazole MIC could in fact facilitate growth of *C. difficile* in patients treated with metronidazole. Whether or not haem-dependent reduced susceptibility plays a role in treatment failure (that does not appear to correlate with metronidazole resistance) remains to be established.[10,40]

An important implication of our findings is that a well-described testing method in diagnostic antimicrobial susceptibility testing is of the utmost importance. The type of media and supplementation used can influence the MIC of certain antibiotics as we have demonstrated for haem supplementation and metronidazole susceptibility. It is our experience that small inter-laboratory differences in standard operating procedures exist despite conforming to CLSI standards, which may explain the differences in MICs sometimes found between institutions. As medium-dependent differences in antimicrobial susceptibility may be both antibiotic- and organism-specific, this argues for a standardized method per genus rather than a standard testing method for all anaerobic organisms.

In conclusion, we have demonstrated that haem is the causative agent of medium-dependent reduction in metronidazole susceptibility in clinical *C. difficile* isolates of different RTs, but does not influence vancomycin susceptibility. Additionally, we have found a

deletion in the C-terminal part of *hsmA* that correlates to metronidazole resistance in RT010 isolates.

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Members of the COMBACTE-CDI Consortium

The COMBACTE-CDI Consortium consists of academic partners and European Federation of Pharmaceutical Industries and Associations (EFPIA) partners. Academic partners: Marc Bonten (University Medical Center Utrecht, The Netherlands), Kerrie A. Davies (Scientific Lead and European Coordinator Laboratory, University of Leeds, UK), Ed J. Kuijper (Leiden University Medical Center, The Netherlands), Maja Rupnik (National Laboratory of Health, Environment and Food, Maribor, Slovenia), Sebastian Wingen-Heiman (University Hospital of Cologne, Germany), Evelina Tacconelli (Karls Eberhard University Tübingen, Germany), Tuba Wilken (University of Antwerp, Belgium) and Nicolla Petrosillo (Lazzaro Spallanzani National Institute for Infectious Diseases, Rome, Italy). EFPIA partners: Pfizer, GSK, bioMérieux, Sanofi Pasteur and Da Volterra. The management board of COMBACTE-CDI consists of Marc Bonten (University Medical Center Utrecht), Philippe Cleuziat (bioMérieux), Chris Webber (Pfizer) and Mark Wilcox (University of Leeds).

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Transparency declarations

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Author contributions

Conceived the study: I.M.B., E.J.K., J.F. and W.K.S. Performed experiments: I.M.B., C.H., I.M.J.G.B.-S., W.S., E.C. and J.F. Analysed data: I.M.B., S.N., I.S., V.V., K.D., J.F. and W.K.S. Drafted manuscript: I.M.B. and W.K.S. All authors edited and approved the final version of the manuscript.

Supplementary data

Materials and methods, Tables S1 to S3 and Figure S1 are available as Supplementary data at *JAC* Online.

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