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The influence of exposure to various concentrations of five antimicrobial agents on intracellular cytotoxin B production in *Clostridioides difficile*

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Abstract:

Background: *Clostridioides difficile* is an important cause of healthcare-associated diarrhea. Several anti-microbial agents are known to promote *C. difficile* infection (CDI). The impact of various concentrations of ampicillin (AMP), cefotaxime (CTX), clindamycin (CC), metronidazole (MTZ) and vancomycin (VAN) on intra-cellular cytotoxin B production was investigated in this study.
Methodology: Six clinical strains of *C. difficile* were grown at minimum inhibitory concentration (MIC) and sub-MIC concentrations of these antibiotics. Inoculum standardization was performed by Miles and Misra method. Intracellular toxin B production was detected using Vero cell cytotoxicity assay in sonicated cultures on days 1, 2, 3, 4, 5 and 7 days of incubation.
Results: There was a heterogeneous relationship between antibiotic exposure and the intra-cellular toxin production by the toxigenic strains. Clinical strains of *C. difficile* when exposed to MIC and sub-inhibitory concentrations of certain antibiotics produced high cytotoxin levels. All toxigenic isolates produced increased levels of cell-bound cytotoxin after exposure to antibiotics but there was no consistent pattern and the response to different doses varied considerably. Metronidazole was the most potent inducer of cell-bound cytotoxin followed by cefotaxime and clindamycin. Vancomycin induced the least amount of cytotoxin activity.
Conclusion: The effects of sub-inhibitory concentration of antibiotic that predispose to *C. difficile* infection may partially suppress the normal gut flora, allowing colonization and growth of *C. difficile*, and may affect the level of toxin produced.

Keywords: *Clostridioides difficile*, antibiotic exposure, intracellular cytotoxin B.

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L'influence de l'exposition à diverses concentrations de cinq agents antimicrobiens sur la production de cytotoxine B intracellulaire chez *Clostridioides difficile*

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Résumé :

Contexte: *Clostridioides difficile* est une cause importante de diarrhée nosocomiale. Plusieurs agents antimicrobiens sont connus pour favoriser l'infection à *C. difficile* (ICD). L'impact de diverses concentrations d'ampicilline (AMP), de céfotaxime (CTX), de clindamycine (CC), de métronidazole (MTZ) et de vancomycine (VAN) sur la production de cytotoxine B intracellulaire a été étudié dans cette étude.
Méthodologie: Six souches cliniques de *C. difficile* ont été cultivées à une concentration minimale inhibitrice (CMI) et à des concentrations sous-CMI de ces antibiotiques. La standardisation de l'inoculum a été réalisée par

la méthode de Miles et Misra. La production intracellulaire de toxine B a été détectée à l'aide d'un test de cytotoxicité sur cellules Vero dans des cultures soniquées aux jours 1, 2, 3, 4, 5 et 7 jours d'incubation. **Résultats:** Il existe une relation hétérogène entre l'exposition aux antibiotiques et la production intracellulaire de toxines par les souches toxigènes. Les souches cliniques de *C. difficile* lorsqu'elles étaient exposées à des concentrations de CMI et sous-inhibitrices de certains antibiotiques produisaient des niveaux élevés de cytotoxine. Tous les isolats toxicogènes ont produit des niveaux accrus de cytotoxine liée aux cellules après exposition aux antibiotiques, mais il n'y avait pas de tendance constante et la réponse aux différentes doses variait considérablement. Le métronidazole était l'inducteur le plus puissant de la cytotoxine liée aux cellules, suivi du céfotaxime et de la clindamycine. La vancomycine a induit le moins d'activité de cytotoxine.
Conclusion: Les effets de la concentration sous-inhibitrice d'antibiotique qui prédisposent à l'infection à *C. difficile* peuvent supprimer partiellement la flore intestinale normale, permettant la colonisation et la croissance de *C. difficile*, et peuvent affecter le niveau de toxine produite.

Mots-clés: *Clostridioides difficile*, exposition aux antibiotiques, cytotoxine intracellulaire B

Introduction:

Clostridioides difficile is a major cause of healthcare-associated diarrhoea. Infections caused by this organism, called *C. difficile* infection (CDI), may manifest as asymptomatic carrier, mild self-limiting antibiotic-associated diarrhea (AAD), severe antibiotic-associated colitis (AAC) or pseudomembranous colitis (PMC). CDIs are sometimes complicated by ileus, toxic megacolon and gut perforation with peritonitis leading to potentially fatal consequences. The major virulence factors of most pathogenic *C. difficile* are the two large molecular weight toxins; toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin).

A wide range of antimicrobial agents has been implicated in the development of CDI. This is because they deplete the normal gut flora which normally provide colonization resistance and thus allow the overgrowth of *C. difficile* and production of potent toxins (1). Almost all antimicrobial agents can predispose to the development of CDI. However, cephalosporins, clindamycin and the new generation fluoroquinolones are known agents most often predisposing to CDI (2).

Toxin production varies among different toxigenic strains and it is influenced by different growth and environmental factors such as temperature, glucose, biotin limitation and amino acid concentrations. A previous study has shown that sub-inhibitory concentrations of clindamycin and cephaloridine, unlike tetracycline, stimulate enterotoxin production (3). In addition, the antibiotics are known to increase toxin A production. Drummond and colleagues (4) demonstrated that exposure of *C. difficile* to sublethal concentration of various antibiotics such as vancomycin, metronidazole, amoxicillin, clindamycin, cefoxitin and ceftriaxone has no consistent relationship between growth and toxin A production. Unlike clindamycin, sub-MIC levels of metronidazole, linezolid and vancomycin increased the transcription rate of toxin A and B genes (5). Emerson and colleagues (6) using microarray technology analyzed the transcriptional responses of *C. difficile* 630 strain to environmental shock and to the growth in the presence of sub-inhibitory concentrations of certain antibiotics such as amoxicillin, clindamycin and metronidazole. They found that amoxicillin and clindamycin increased the transcription of ribosomal protein genes and changed the transcription of genes encoding surface-associated proteins, while exposure of *C. difficile* to sub-inhibitory concentration of metronidazole resulted in minor changes in the transcription patterns.

The aim of this study was to investigate the effects of various concentrations of antibiotics known to predispose to CDI and, also, of those used in its therapy on the production of intracellular *C. difficile* toxin B in Vero cell line and to quantify the cytotoxic effects on cell bound cytotoxin B.

Materials and method:

Bacterial strains*:*

The six *C. difficile* strains used in this study were; local strains KM233D ribotype 078, and KM34A ribotype 097 (local toxigenic strains isolated from the stool samples of patients with PMC); strain KM175 ribotype 039 (a local non-toxigenic strain isolated from the stool of a patient with suspected AAD); strain KA11 ribotype 017 (toxin A-negative/toxin B-positive strain isolated from the stool of a patient with AAD); strain KM362C ribotype 046 (a local toxigenic strain isolated from a patient with AAC) and a UK strain, ribotype 001, isolated from stool of a patient involved in an outbreak of CDI in the UK (obtained from Professor BI Duerden and Dr JS Brazier of Anaerobe Reference Unit, Cardiff, UK).

All the strains were tested for their susceptibility to the five antibiotics by E-test (AB Biodisk, Solna, Sweden) on blood agar according to manufacturer's instructions. PCR ribotyping was previously performed (7). All the strains were stored at -70ºC until further testing.

Antimicrobial agents:

The following antibiotic powders were used; ampicillin (AMP), clindamycin (CC), ce-

fotaxime (CTX), metronidazole (MTZ) and vancomycin (VAN) (all from Sigma Pharmaceuticals). They were prepared in sterile distilled water as a 10x solution with reference to the highest concentration required.

Inoculum standardization:

The number of viable bacteria in the inoculum was determined by a modified Miles and Misra method (8). An inoculum containing 10^{11} CFU/ml which produced the highest cytotoxic activity was then used throughout the cytotoxin detection experiments.

Cytotoxin detection assay:

Strains were seeded onto blood agar and incubated anaerobically for 48 h at 37ºC. Then, a loopful of each strain was inoculated into 10ml of sterile liver broth (Oxoid Ltd, Basingstoke, Hampshire, UK), and incubated anaerobically overnight. This was diluted 1 in 10 corresponding to 10^{11} CFU/ml after which it was exposed to different concentrations of the test antibiotics i. e. MIC, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 MICs.

Doubling dilutions were then made in 10ml of freshly prepared sterile Brain Heart Infusion (BHI) broth (Difco, Becton and Dickinson Company, Sparks, MD, USA) in 6 different bottles per concentration containing the specific concentrations of the test antibiotics. An inoculum of 100 µl containing approximately 10^{11} CFU/ml of the inoculated liver broth was added to each bottle. An antibiotic-free broth culture was used as control for each strain for baseline comparison. The inoculated broth cultures were incubated at 37ºC for 1, 2, 3, 4, 5, and 7 days anaerobically. At the end of each incubation period, cell-bound cytotoxin was assayed.

Cell-bound cytotoxin detection*:*

After incubation of each bottle of the broth cultures for each period (1 to 5, and 7 days), 5ml of the broth cultures was centrifuged at 3,500xg for 10 min and the sediment washed twice with 3ml of 50mM PBS (pH 7.0). The washed sediment was sonicated with an ultrasonic homogenizer (Labsonic U, B. Braun, Model 1254, Melsungen AG, W. Germany) for 5 min. The cellular debris was removed by centrifugation at 3,500 g for 10 min. The supernatant was filtered through a sterile membrane filter with 0.45µm pore size (Millex-AH, Millipore, Carrigtwchill, Co. Cork, Ireland) and assayed for cytotoxicity.

Assay for cytotoxicity:

Cytotoxicity was assayed as previously described (9) in a microliter plate using Vero cell line and dilution series from 1:40 to 1:5120. The highest dilution resulting in complete rounding of the cells was taken as the number of cytotoxic units (CU)/50µL sample (10) and the results were expressed as CU/ml. At the end of 7^{th} day incubation, the mean CUs/ml were calculated and tabulated. Analysis of the data was done by calculating the mean of the cytotoxic activities produced on all post-exposure days to various antibiotics.

Results:

The MICs of the five antibiotics tested against the 6 *C. difficile* strains investigated are shown in Table 1. The inoculum at which cell-bound cytotoxin production was maximal below the neat broth i. e. 10^{11}CFU/ml was used throughout the experiments (data not shown). The non-toxigenic strain, ribotype 039, did not induce cell-bound cytotoxin regardless of the presence or absence of any of the 5 antibiotics tested. There was no remarkable difference in the cytotoxic activity between 24 and 48 h incubation. Therefore, the data shown are those after 24 h incubation.

Table 1: Minimum inhibitory concentrations (µg/ml) of selected antibiotics against clinical strains of *Clostridioides difficile*

Antibiotics/breakpoints (µg/ml)	**MIC (µg/ml) of antibiotics against strains**					
	078	**039**	**097**	**017**	**046**	**001**
Clindamycin (4)	8.0	>256	>256	>256	4.0	8.0
Metronidazole (8)	0.25	0.06	0.06	0.25	0.25	0.25
Vancomycin (4)	0.5	1.0	1.0	1.0	0.5	2.0
Ampicillin (8)	0.5	0.5	1.0	1.0	1.0	2.0
Cefotaxime (32)	>256	>256	>256	>256	>256	>256

MIC=Minimum inhibitory concentration

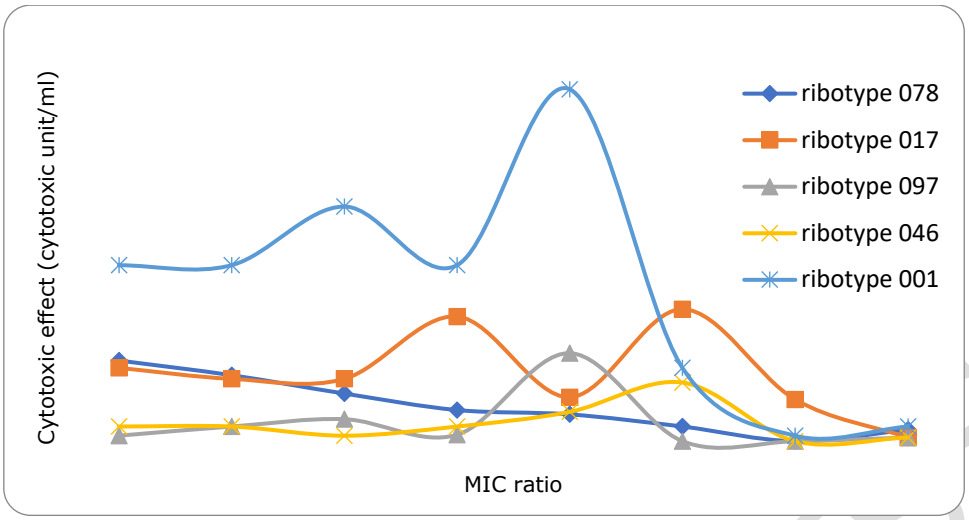

Fig 1: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to cefotaxime by all test strains

Cell-bound cytotoxic activity post exposure to cefotaxime (CTX):

For strain KM233D (ribotype 078), the effects of exposure to CTX on the cell-bound cytotoxic activities were not very remarkable as shown by the mean of cytotoxic unit/ml (CU/ml) in Fig 1. There was no increase in cytotoxin production at the MIC value. The increase observed at ½ MIC was marginal, 1.3x the control, but this rose gradually from 117.3 CU/ml (2.3x) at ¼ MIC to 352.0 CU/ml (7.0x) at 1/64 MIC. For cell-bound cytotoxin of strain KA11 (ribotype 017), the effect of exposure to CTX was considerably higher. It was 182.3 CU/ml and 320.0 CU/ml (11.3 and 20 times the control) at the MIC and 1/64 MIC, respectively. The greatest increase was noted at ½ and 1/8 MICs with production of cytotoxin measuring 576.1 (36.0x) and 544.0 CU/ml (34.0x), respectively.

Cefotaxime failed to induce cytotoxin production in strain KM34A (ribotype 097) at the MIC and ½ MIC but induced high level cytotoxin production of 384.2 CU/ml (24-fold) at 1/4 MIC and thereafter gradually waned off through 1/16 MIC (6x) to 1/64 MIC (1.5x). The antibiotic also did not induce cyto-toxin production in strain KM362C (ribotype 046) at the MIC but the maximum induction occurred at ½ MIC (256.0 CU/ml; 16-fold increase) then declined gradually to 64 CU/ml (4 x) each at 1/32 MIC and 1/64 MIC. With the UK ribotype 001, maximal induction occurred at ¼ MIC; 1532.1 CU/ml versus 64.0 CU/ml for the control which was a 24-fold increase. Another surge occurred at 1/16 MIC with the production of 1024.0 CU/ml (16-fold increase).

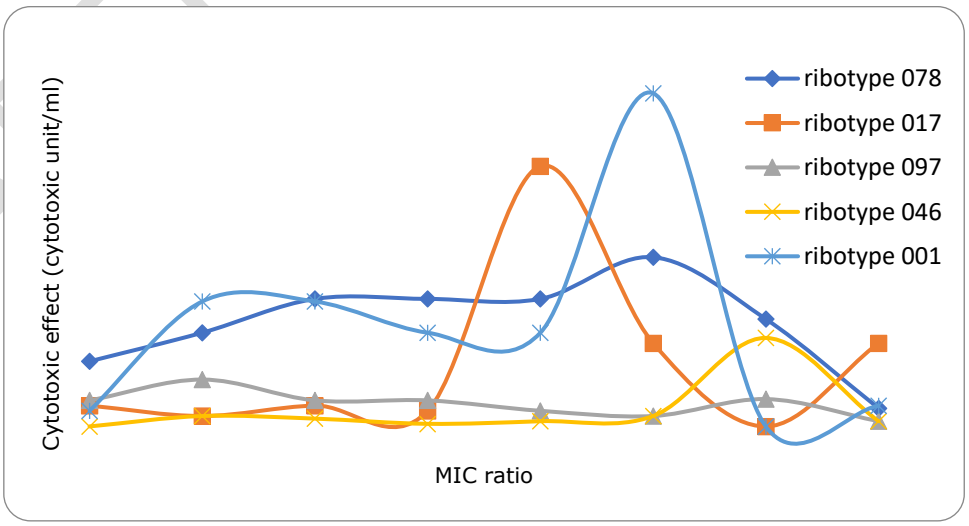

Fig 2: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to ampicillin by all test strains

Cell-bound cytotoxic activity post exposure to ampicillin (AMP):

The quantified cytotoxin content of the sonicated cell effluents of the tested strains before and after exposure to ampicillin is shown in Fig 2. AMP did not induce cell-bound cytotoxin at MIC and ½ MIC in any of the strains. For strain KM233D (ribotype 078), the maximum cytotoxin production occurred at 1/16 with production of 520.0 CU/ml (9.3-fold increase over the control level). Thereafter production dropped to 392.0 CU/ml each at 1/32 and 1.64 MICs.

There was no apparent change in the cell-bound cytotoxic activity in KA11 (017), except at 1/4 MIC where the rise in production was 3.1-fold higher (800.0 CU/ml) than the control. Cell-bound cytotoxin production by strain KM34A (097) was almost in a linear fashion reaching a maximum of 80.0 CU/ml (a 5-fold increase) at 1/16 MIC.

With strain KM362C (046), cytotoxin production was maximal (272 CU/ml) at the ¼ MIC level (17-fold increase) and declined linearly to 24 CU/ml (1.5-fold increase) at 1/64 MIC. Cell-bound cytotoxin production was highest at ¼ MIC in the UK strain, ribotype 001; 1024.0 versus 64 CU/ml in control (16-fold increase).

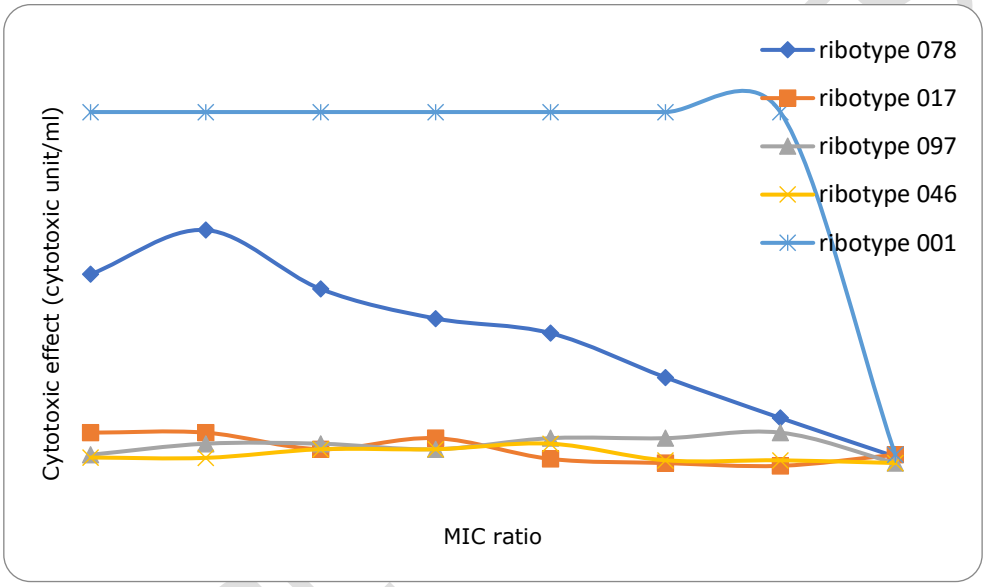

Fig 3: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to metronidazole by all test strains

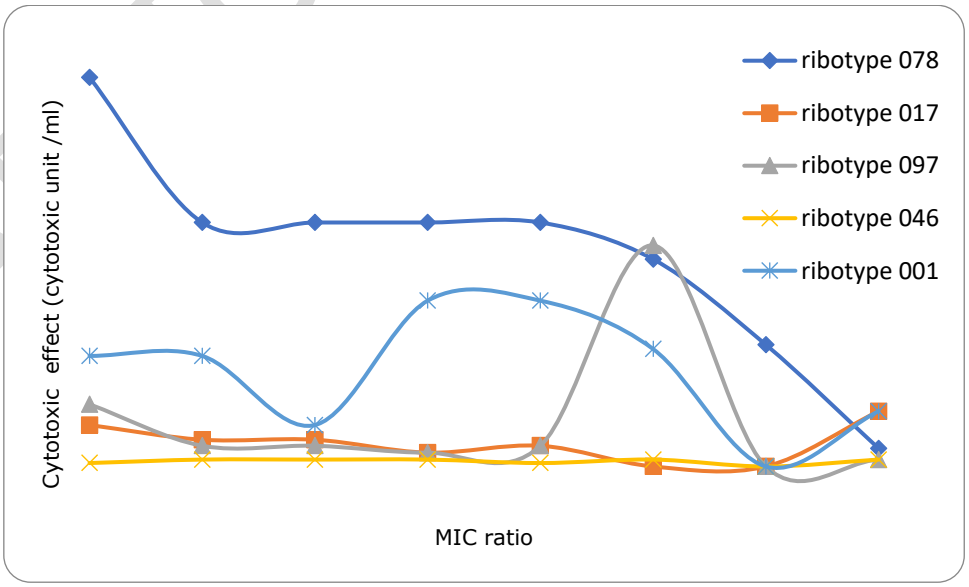

Fig 4: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to vancomycin by all test strains

Cell-bound cytotoxic activity post exposure to metronidazole (MTZ):

Fig 3 shows the amount of cell-bound cytotoxic unit (CU/ml) produced by the strains post-exposure to MTZ. There was a linear increase in the cell-bound cytotoxin production by strain KM233D (ribotype 078), in the presence of MTZ from 277.3 CU/ml at ½ MIC (4.8-fold increase), through 853.0 CU/ml at 1/16 MIC (14.7-fold) to 1365.3 CU/ml at 1/64 MIC (23.5-fold). For strain KA11 (017), cell-bound cytotoxin production was not as dramatic as there was only 2.5-fold (160.0 CU/ml) increase at 1/16 MIC reaching maximum level of 192.0 CU/ml at 1/64 MIC (3-fold increase).

Strain KM34A (097) yielded maximum production of 192.0 CU/ml at ¼ MIC declining to 128.0 CU/ml at 1/64 MIC. For strain KM362C (046), the maximum production was 128 CU/ml (8-fold increase) at 1/8 MIC. With the UK strain (ribotype 001), post-exposure production was massive with 2048.0 CU/ml (32-fold increase) at all concentrations.

Cell-bound cytotoxic activity post exposure to vancomycin (VAC):

As shown in Fig 4, the maximum cell-bound cytotoxin production by strain KM233D (ribotype 078) was 565.3 CU/ml (13.5-fold increase) which occurred post-exposure to VAC at 1/16, 1/32 and 1/64 MICs. There was no cytotoxin production at the MIC and ½ MIC. At ¼ MIC it was 282.6 CU/ml (6.7-fold rise). Strain KA11 (017) produced a relatively low level constitutive cytotoxin at all MIC and sub-MICs. VAC induced maximum production of cell-bound cytotoxin (512.0 CU/ml; 32-fold increase) at ¼ MIC and lowest (32.0 CU/ml; 2-fold) at 1/16 MIC in strain KM34A (097).

Post-exposure of strain KM362C (046) did not result in any appreciable cytotoxin production at all concentrations. Exposure of the UK strain (001) did not yield appreciate increase in cytotoxin production. At ½, ¼, and 1/64 MICs, the production was 272.0 (2.1-fold increase), 384 (3-fold) and 256.0 (2-fold) CU/ml, respectively.

Cell-bound cytotoxic activity *post exposure to* clindamycin (CLIN):

Fig 5 shows the cell-bound cytotoxic activities after exposure to clindamycin for the different strains. Cell-bound cytotoxic activity of strain KM233D (ribotype 078) after exposure to clindamycin at the MIC, was about 17.9-fold higher than the control. Then it increased gradually reaching a maximum of 32-fold at 1/16 MIC (1088.0 CU/ml). For ribotype KM11 (017), post-exposure to CLIN induced high cell-bound cytotoxin production at the MIC (272.0 CU/ml; 17-fold higher than the control), 264.0 CU/ml (16.5-fold increase) at 1/8 MIC and 256.0 CU/ml (16-fold) at 1/32 MIC. With strain KM34A (097), the cytotoxic activities were each 272 CU/ml (17-fold) at the MIC, ½, 1/8, and 1/32 MICs, and 288.0 (18-fold) at 1/16 MIC. Strain KM362C (046) induced 64 CU/ml (4-fold increase) at 1/8 MIC, 48 CU/ml (3-fold increase) at MIC, ¼ and 1/32 MICs.

Exposure of UK strain (001) to clindamycin induced cell-bound cytotoxin production at all concentration but maximally at 1/4 and 1/16 MICs of 192 CU/ml (3-fold).

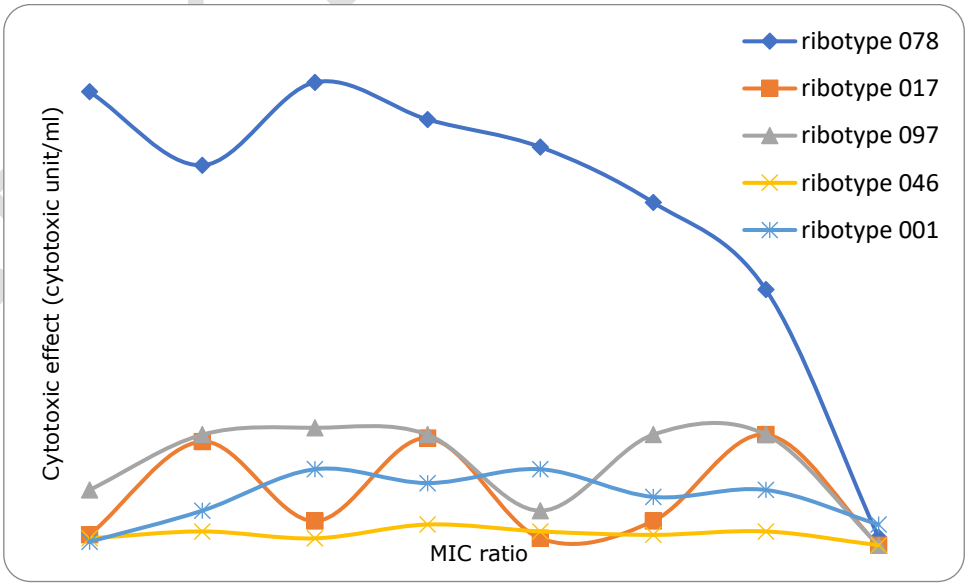

Fig 5: Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to clindamycin by all test strains

Discussion*:*

This study focused on the effects of MIC and sub-inhibitory concentrations of 5 different antibiotics, including those used for treatment of CDI and those known to predispose to CDI occurrence, on the production of cytotoxin B by 6 different strains of *C. difficile*. The study showed clearly that there is a heterogeneous relationship between antibiotic exposure and toxin B production intracellularly by the toxigenic strains of *C. difficile*. The non-toxigenic strain from a symptomatic patient with diarrhea, ribotype 039, did not produce toxin B inside the cell in the absence or presence of any antibiotic. This may be related to the absence of the genes that encode the toxin or the absence of other virulence factors.

The results showed that certain strains, when exposed to the MIC and sub-inhibitory concentrations of certain antibiotics, are capable of producing high level of cytotoxin compared to the antibiotic-free control. This may be due to the stress that an organism experiences in the presence of antibiotics. Early reports suggested that the stress may induce extracellular toxin production. For example, Onderdonk et al., (11) reported that raised temperature leads to higher cytotoxin production and toxin production increased in the presence of sub-inhibitory concentration of vancomycin and penicillin. A study by Karlsson and colleagues (12) showed that temperature may act as a controlling factor for the expression of toxin A and TcdD. Therefore, toxin production may be enhanced by environmental stress. Emerson et al., (6) showed that exposure of *C. difficile* to environmental stress such as heat shock and acid shock, lead to upregulation of certain genes that allow the vegetative form of *C. difficile* to tolerate this type of environmental stress. In addition, *C. difficile* respond to oxidative stress by upregulation of electron transporters (6). The same investigators found that exposure to amoxicillin and clindamycin increased the transcription of ribosomal protein genes and altered transcription of genes encoding surface-associated proteins, while minor changes in the transcription occurred after exposure to metronidazole (6).

In this study, strains KM233D, KA11 and UK strain, ribotypes 078, 017 and 001, respectively were the most responsive to antibiotic induction of cytotoxin production. KA11 (ribotype 017) was an isolate from an AAD patient while KM233D (078) was an isolate from a patient with PMC. UK strain, (ribotype 001) was a strain associated with CDI outbreak in the UK. Surprisingly, metronidazole was the most potent inducers of cytotoxin in our hands. It provoked an increase in the cell-bound toxin in all toxigenic strains. This was followed by cefotaxime and clindamycin which produced almost the same level of cytotoxin. Ampicillin and vancomycin induced the least amount of cytotoxic activity on all the strains. Metronidazole and ampicillin induced cell-bound cytotoxin for all toxigenic isolates at various degrees especially ribotypes 078 and 001. Although ribotypes 078 and 097 strain were isolated from patients with PMC, metronidazole and ampicillin induce more cell-bound cytotoxin in ribotype 078 rather than 097. Vancomycin induced *C. difficile* ribotype 078 and 001 to produce a large amount of intracellular cytotoxin. This may indicate that vancomycin increases cytotoxin release from within the cell rather than enhances synthesis for *C. difficile* ribotypes 078 and 001.

CDI is not only enhanced by usage of antibiotics but also by physiological changes that affect the pathogenicity of *C. difficile* (13). Previously, Onderdonk et al., (14); Honda et al., (3) and Drummond et al., (4) reported that certain antibiotics enhance synthesis of toxin A and/or cytotoxin B, which are the main virulence factors. Onderdonk et al., (14) in 1981, compared the effect of clindamycin and its metabolites in a hamster model of *C. difficile*-associated colitis and did not find any correlation between clindamycin potency and $AACD_{50}$ (toxin lethal to 50% of the animals). Other investigators described a human gut model of *C. difficile* infections and they demonstrated that *C. difficile* germinate and produce cytotoxin in response to clindamycin (15), cefotaxime with and without its metabolites, desacetylcefotaxime (16), metro -nidazole (17) and fluoroquinolones such as ciprofloxacin and levofloxacin (18). Recently, Baines et al., (19) demonstrated that vancomycin reduced the vegetative forms and cytotoxin titers of epidemic strains of *C. difficile* but did not have any anti-spore activity. In addition, the same researchers showed that vancomycin was more effective than metronidazole in reducing *C. difficile* PCR ribotype 027 numbers and cytotoxin titer (19). In contrast, germination and cytotoxin production was not observed in the human gut model after exposure to piperacillin-tazobactam or tigecycline (20,21)

There appears to be no correlation between the severity of symptoms and level of cytotoxin produced in Vero cell line. For example, cefotaxime induced more cytotoxic effect in Vero cells from ribotype 017 which caused only AAD in contrast to ribotype 097 that was associated with PMC. Moreover, ampicillin induced more cytotoxic effects on Vero cells by ribotype 017 isolated from a patient with diarrhea (AAD) than ribotype 046 isolated from a patient with colitis (AAC)

and ribotype 097 isolated from a PMC case. This suggests that the quantity of the cytotoxin produced is not enough to explain the gut pathology caused by the different strains of *C. difficile.*

Our results showed that there is a general fluid relationship between antibiotics and toxin production by *C. difficile*. All isolates showed increase in cytotoxin production after exposure to antibiotics but there was no consistent pattern and the response to different doses varied considerably. Antibiotics that are used for treatment and those that precipitate the disease have different effects on different *C. difficile* isolates, therefore the relationship may be complex. The effects of sub-inhibitory concentration of antibiotic that predispose to CDI development may suppress the normal gut flora partially and allow colonization and growth of *C. difficile* and may affect the level of toxin produced.

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Contributions of authors:

WJ conceptualized the study, carried out the laboratory work, and wrote the initial manuscript; BD and VOR conceptualized the study research, supervised and corrected the final manuscript draft. All authors approved the final manuscript submitted.

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Conflict of interest:

Authors declare no conflict of interest

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