International Journal of Antimicrobial Agents 54 (2019) 154–158

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Change in antimicrobial susceptibility and PCR ribotypes of *Clostridioides difficile* in a hospital over 5 years: Correlation analysis with antimicrobial consumption

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ARTICLE INFO

Article history:
Received 15 February 2019
Accepted 25 May 2019

Editor: Jean-Marc Rolain

Keywords:
Clostridioides difficile
PCR ribotype
Minimum inhibitory concentration
MIC
Piperacillin/tazobactam
Antibiotic consumption

ABSTRACT

Clostridioides difficile infection (CDI) is a major concern in hospital settings. Antimicrobial resistance is a key contributing factor in CDI outbreaks. This study analysed the antimicrobial susceptibility and PCR ribotypes (RTs) of 745 *C. difficile* isolates collected at a single institution over 5 years. Seventeen known RTs were identified in 643 isolates (86.3%), of which RTs 018, 017, 015, 001 and 002 were the most prevalent. Reduced susceptibility to metronidazole (MTZ) and vancomycin (VAN) was rare (2.0% and 0.7%, respectively). Resistance to rifaximin (RFX), moxifloxacin (MXF) and clindamycin (CLI) was high in multiple RTs (29.3%, 67.0% and 69.4% of total isolates, respectively). Antimicrobial susceptibility varied among RTs. Whilst non-susceptibility to VAN, RFX, MXF, CLI and piperacillin/tazobactam (TZP) mostly occurred in commonly identified RTs, MTZ resistance was observed in diverse RTs. Correlation analysis between the MICs of the six antimicrobials for annual isolates and antimicrobial consumption in the hospital by year showed variable degrees of correlation; significant positive correlation for TZP ($P = 0.037$), significant negative correlation for VAN ($P < 0.001$) and no significant correlation for the other antimicrobials. MIC creep of TZP occurred during the study period with the appearance of 19 isolates with TZP intermediate-resistance mostly in 2013 (89.5%; 17/19), and three RTs containing TZP-intermediate-resistant isolates, including RT015 ($n = 4$), RT002 ($n = 12$) and RT112 ($n = 1$), increased over time ($P = 0.010$). These findings suggest an association of antibiotic consumption and resistant *C. difficile* strains and question TZP use for limiting CDI in hospitals.

1. Introduction

Clostridioides difficile infection (CDI) is one of the most important nosocomial infections [1,2]. High levels of antibiotic consumption and resultant antimicrobial resistance are important factors in the selection of dominant *C. difficile* strains in hospitals, and most prevalent *C. difficile* strains are resistant to many antimicrobials that do not target *C. difficile* [3–5]. The risk of CDI has been increasing with the use of high-risk antibiotics such as cephalosporins, fluoroquinolones and clindamycin, whereas some agents, including piperacillin/tazobactam, have been found to be protective against CDI among antimicrobials not targeting *C. difficile* [6,7]. Of the antibiotics targeting *C. difficile*, minimum inhibitory concentrations (MICs) of metronidazole and vancomycin have remained relatively low over the years despite changes in antimicrobial resistance and PCR ribotype (RT) prevalence [3,8].

In this study, the antimicrobial susceptibility and PCR RTs of 745 *C. difficile* isolates obtained at a single institution over a 5-year period (2009–2013) were analysed and the findings were compared with annual levels of antimicrobial consumption.

2. Materials and methods

2.1. Study design

All *C. difficile* isolates obtained from patients with CDI over 5 years from January 2009 to December 2013 at Hanyang University Hospital, a 900-bed tertiary care facility in Seoul, South Korea, were included in this study. CDI was defined as previously described [5]. The study was approved by the Institutional Review Board of Hanyang University Hospital. Informed consent was waived by the board.

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https://doi.org/10.1016/j.ijantimicag.2019.05.022

2.2. Isolation of Clostridioides difficile and detection of toxin genes by multiplex PCR

Following alcohol shock treatment, stool specimens were cultivated on *C. difficile* moxalactam–norfloxacin–taurocholate agar (CDMN-TA agar; Oxoid Ltd., Cambridge, UK) supplemented with 7% horse blood. Colonies of *C. difficile* were identified using Rapid ID 32A (bioMérieux SA, Marcy-l'Étoile, France). Multiplex PCR was performed to identify toxin genes using template DNA as described previously [9]. Positive controls were *C. difficile* ATCC 43598 (PCR RT017), ATCC 9689 (PCR RT027), VPI 10643 (ATCC 43255, PCR RT087) and ATCC 700057, representing $A^-B^+CDT^-$, $A^+B^+CDT^+$, $A^+B^+CDT^-$ and $A^-B^-CDT^-$ strains, respectively.

2.3. PCR ribotyping of Clostridioides difficile strains

PCR ribotyping was performed using genomic DNA as described previously [5]. Following electrophoresis of the amplified products, clustering of banding patterns was determined visually. Each pattern was used to identify the PCR RT by comparison with the patterns obtained from the reference strains RT027 (ATCC 9689) and RT017 (ATCC 43598) as well as standard strains from the European Centre for Disease Prevention and Control (ECDC) Brazier collection.

2.4. Antimicrobial susceptibility testing

MICs of six antimicrobials, namely metronidazole (MTZ), vancomycin (VAN), rifaximin (RFX), clindamycin (CLI), moxifloxacin (MXF) and piperacillin/tazobactam (TZP), were determined. *Brucella* agar containing hemin (5 μg/mL), vitamin K1 (10 μg/mL) and 5% horse blood was used for antimicrobial susceptibility testing as recommended by the Clinical and Laboratory Standards Institute (CLSI) [10]. MICs of CLI, MXF and VAN were determined by Etest (AB BIODISK, Solna, Sweden) and MICs of MTZ, RFX and TZP were determined by agar dilution (Sigma-Aldrich, St Louis, MO). *Clostridioides difficile* ATCC 700057 was used as a control strain for antimicrobial susceptibility testing. Epidemiological cut-off values of MTZ and VAN were adopted from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [11]; cut-off values of MXF, CLI and TZP were adopted from CLSI [10]; and the cut-off value of RFX was adopted from previous reports [12,13].

2.5. Measurement of antimicrobial consumption

Antimicrobials monitored in this study were those classified under J01 according to the Anatomical Therapeutic Chemical (ATC) classification system of the World Health Organization (WHO) [14]. These include systemic agents, administered orally or parenterally, and do not include topical agents. The amount of each antimicrobial type consumed was converted to a defined daily dose (DDD) using the ATC classification system and these amounts were then normalised to 1000 patient-days.

2.6. Statistical analysis

A linear regression model was used to assess the trend in antibiotic consumption, RT distribution and changes in geometric mean (GM) MICs of antimicrobials over time. The coefficient for time and the *P*-value for the trend in antibiotic consumption were extracted using measures on a monthly basis, whilst those for the trend in the proportion of each PCR RT were calculated on a yearly basis. The relationship between antimicrobial consumption and MIC of antimicrobials and the correlation among susceptibility to each of the six antimicrobials were evaluated by Spearman's rank correlation analysis (Spearman's ρ). Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics for Windows v.24.0 (IBM Corp., Armonk, NY).

3. Results

A total of 745 *C. difficile* were isolated during the 5-year period, comprising 116 in 2009, 176 in 2010, 154 in 2011, 164 in 2012 and 135 in 2013.

3.1. PCR ribotyping results

Of the 745 *C. difficile* isolates, 643 (86.3%) were classified into 17 known PCR RTs, whereas 102 (13.7%) were of unknown RTs. The distribution of known RTs was RT018 (27.9%), RT017 (26.8%), RT015 (6.3%), RT001 (5.1%), RT002 (4.8%), RT112 (4.3%), RT012 (3.5%), RT014 (3.5%), RT293 (1.5%), RT163 (0.7%), RT027 (0.4%), RT078 (0.4%), RT130 (0.4%), RT106 (0.3%), RT057 (0.1%), RT122 (0.1%) and RT267 (0.1%) (Fig. 1A). During the 5 years, RT018 and RT017 were consistently prevalent, whilst RT001, RT002, RT015 and RT112 were variable in abundance by year.

3.2. Antimicrobial susceptibility testing

The susceptibilities of the isolates to six antimicrobials are presented in Tables 1 and 2. MTZ and VAN were active against 98.0% and 99.3% of isolates tested, respectively. Isolates with an MIC above the epidemiological cut-off of VAN were from hospital-prevalent RTs (four RT018 isolates and one RT017 isolate) and were found over the 5-year period, with two of the isolates obtained in 2009 and one isolate found in each of the years 2011, 2012 and 2013. The VAN MIC of three resistant RT018 isolates was 3 μg/mL, whilst the fourth isolate had an MIC of 4 μg/mL. The VAN-resistant RT017 isolate had an MIC of 6 μg/mL. Unlike VAN-resistant isolates, 15 isolates with MTZ resistance were from prevalent RTs (RT001 and RT014) as well as from RTs sporadically isolated [RT293, RT130 and unknown RT09 (UNK09)], and MTZ-resistant isolates were mostly from 2009 (12 isolates) and 2010 (3 isolates). In particular, RT130 and RT001 isolates frequently had MTZ resistance (66.7% and 21.1%, respectively), and the MTZ MIC of four RT001 strains reached 8 μg/mL, whilst the other isolates had MICs of 4 μg/mL.

RFX resistance was observed in multiple RTs; however, RT017 and RT015 presented the highest resistance rates (88.5% and 29.8%, respectively). Susceptibility to RFX for *C. difficile* has not been defined in CLSI or EUCAST standards, and the MICs of RFX are higher than the MICs of rifampicin (RIF) in most isolates [13,14]. When the cut-off value for intermediate resistance to RFX of 0.5–16 μg/mL was adopted, as suggested in a previous report [14], the percentage of isolates with intermediate-resistance to RFX was 4.7%. Most isolates with intermediate RFX resistance belonged to relatively common RTs in hospital settings such as RT001, RT002, RT012, RT014 and RT015. Resistance to MXF (67.0%) and CLI (69.4%) was common. The prevalence of RTs among isolates and the resistance rates to both antimicrobials correlated well, and GM MICs were highest in the most prevalent strains (RT018 and RT017).

Regarding TZP, 19 isolates (2.6%) exhibited intermediate-resistance to TZP and most of them were isolated in 2013 (17/19; 89.5%). TZP-intermediate-resistant isolates were observed among RT002 (12/36; 33.3%), RT015 (4/47; 8.5%) and RT112 (1/32; 3.1%).

In addition, the concordance of non-susceptibility among the six antimicrobials was analysed. Non-susceptibility to CLI, MXF, TZP and RFX was concordant with the non-susceptibility to each of them: CLI–MXF, $\rho = 0.691$, $P < 0.001$; CLI–TZP, $\rho = 0.12$, $P = 0.001$; CLI–RFX, $\rho = 0.188$, $P < 0.001$; MXF–TZP, $\rho = 0.16$, $P < 0.001$; MXF–RFX, $\rho = 0.209$, $P < 0.001$; and TZP–RFX, $\rho = 0.223$, $P < 0.001$.

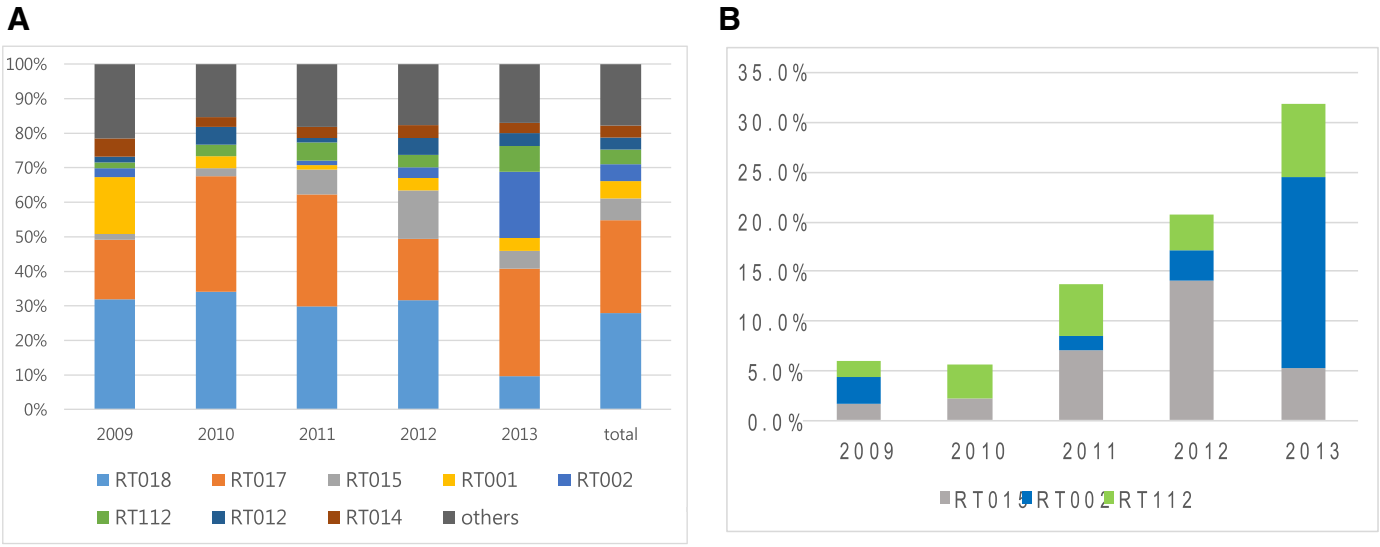

Fig. 1. Distribution of *Clostridioides difficile* PCR ribotypes (RTs) from 2009–2013. (A) Annual distribution PCR RTs. (B) Annual prevalence of RT002, RT015 and RT112 isolates that contained TZP-intermediate-resistant strains. The prevalence of three the RTs showed a significant increase over the 5-year period (linear regression, $\beta = 6.68$, $P = 0.010$). TZP, piperacillin/tazobactam.

Table 1
Susceptibility of *Clostridioides difficile* isolates ($n = 745$) to six antimicrobial agents.

	MTZ	VAN	RFX	MXF	CLI	TZP
MIC range (μg/mL)	<0.06–8	<0.016–6	<0.0019 to >64	<0.002 to >32	<0.016 to >256	<0.25/4–64/4
MIC_{50} (μg/mL)	0.25	0.25	0.0156	>32	>256	16/4
MIC_{90} (μg/mL)	0.5	0.75	>64	>32	>256	32/4
Percent susceptibility (breakpoint in μg/mL)						
%S	98.0 (≤2)	99.3 (≤2)	70.7 (<32)	32.9 (≤2)	26.7 (≤2)	97.4 (≤32/4)
%I	–	–	–	0.1 (4)	3.9 (4)	2.6 (64/4)
%R	2.0 (>2)	0.7 (>2)	29.3 (≥32)	67.0 (≥8)	69.4 (≥8)	–

MTZ, metronidazole; VAN, vancomycin; RFX, rifaximin; MXF, moxifloxacin; CLI, clindamycin; TZP piperacillin/tazobactam; MIC, minimum inhibitory concentration; $MIC_{50/90}$, MICs for 50% and 90% of the isolates, respectively; %S, percent susceptible; %I, percent intermediate; %R, percent resistant.
The cut-off value for MTZ and VAN were defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and those for MXF, CLI and TZP were defined by the Clinical and Laboratory Standards Institute (CLSI); the cut-off value of RFX was adopted from previous reports [12,13].

Table 2
Antimicrobial susceptibility and geometric mean minimum inhibitory concentrations (GM MICs in μg/mL) of *Clostridioides difficile* PCR ribotypes (RTs) from 2009–2013.

PCR RT	*n* (%)	MTZ		VAN		RFX		MXF			CLI			TZP		
		%R	GM MIC	%R	GM MIC	%R	GM MIC	%I	%R	GM MIC	%I	%R	GM MIC	%I	%R	GM MIC
018	208 (27.9)	0	0.3	1.9	0.1	8.2	0.01	0.0	100.0	50.3	0.0	98.6	420.3	0.0	0	9.8
017	200 (26.8)	0	0.2	0.5	0.2	88.5	40.2	0.5	99.0	56.7	0.0	100.0	472.8	0.0	0	10.9
015	47 (6.3)	0	0.2	0	0.2	29.8	0.2	0.0	53.2	4.8	0.0	70.2	38.0	8.5	0	14.0
001	38 (5.1)	21.1	0.4	0	0.4	0	0.01	0.0	47.4	3.6	7.9	65.8	31.0	0.0	0	6.1
002	36 (4.8)	0	0.3	0	0.1	0	0.01	0.0	77.8	16.4	5.6	41.7	9.4	33.3	0	21.0
112	32 (4.3)	0	0.2	0	0.1	3.1	0.02	0.0	3.1	0.5	3.1	6.3	0.2	3.1	0	12.9
012	26 (3.5)	0	0.3	0	0.2	0	0.01	0.0	0.0	0.3	19.2	42.3	7.4	0.0	0	8.4
014	26 (3.5)	3.8	0.3	0	0.2	3.8	0.03	0.0	19.2	1.3	11.5	11.5	0.6	0.0	0	9.6
293	11 (1.5)	18.2	0.3	0	0.1	0	0.01	0.0	0	0.1	9.1	0	0.1	0.0	0	5.5
163	5 (0.7)	0	0.2	0	0.2	0	0.01	0.0	0	0.6	20.0	0	0.4	0.0	0	18.4
027	3 (0.4)	0	0.5	0	0.5	0	0.02	0.0	0	1.6	100.0	0	4.8	0.0	0	16.0
078	3 (0.4)	0	0.4	0	0.2	33.3	0.2	0.0	66.7	11.6	33.3	0	1.6	0.0	0	12.7
130	3 (0.4)	66.7	1.6	0	0.5	0	0.00	0.0	0	1.0	33.3	0	2.3	0.0	0	6.3
Others	107 (14.4)	1.9	0.3	0	0.2	6.5	0.02	0.0	13.1	0.6	7.5	21.5	0.9	1.9	0	9.1

MTZ, metronidazole; VAN, vancomycin; RFX, rifaximin; MXF, moxifloxacin; CLI, clindamycin; TZP, piperacillin/tazobactam; %R, percent resistant; %I, percent intermediate.

However, non-susceptibility to MTZ was negatively correlated with non-susceptibility to MXF and RFX, was positively correlated with non-susceptibility to TZP, and had no significant correlation with the other antibiotics (MTZ–MXF, $\rho = -0.157$, $P < 0.001$; MTZ–RFX, $\rho = -0.154$, $P < 0.001$; MTZ–TZP, $\rho = 0.09$, $P = 0.014$; MTZ–CLI, $\rho = -0.054$, $P = 0.142$; and MTZ–VAN, $\rho = 0.064$, $P = 0.079$). VAN non-susceptibility had no significant correlation with the other antibiotics (VAN–CLI, $\rho = 0.067$, $P = 0.069$; VAN–MXF, $\rho = 0.037$, $P = 0.319$; VAN–TZP, $\rho = -0.027$, $P = 0.466$; and VAN–RFX, $\rho = 0.051$, $P = 0.165$).

3.3. Five-year trend of antimicrobial consumption

Overall annual consumption of antimicrobial agents for systemic use from 2009–2013 showed a significant decreasing trend for extended-spectrum cephalosporins (range of annual

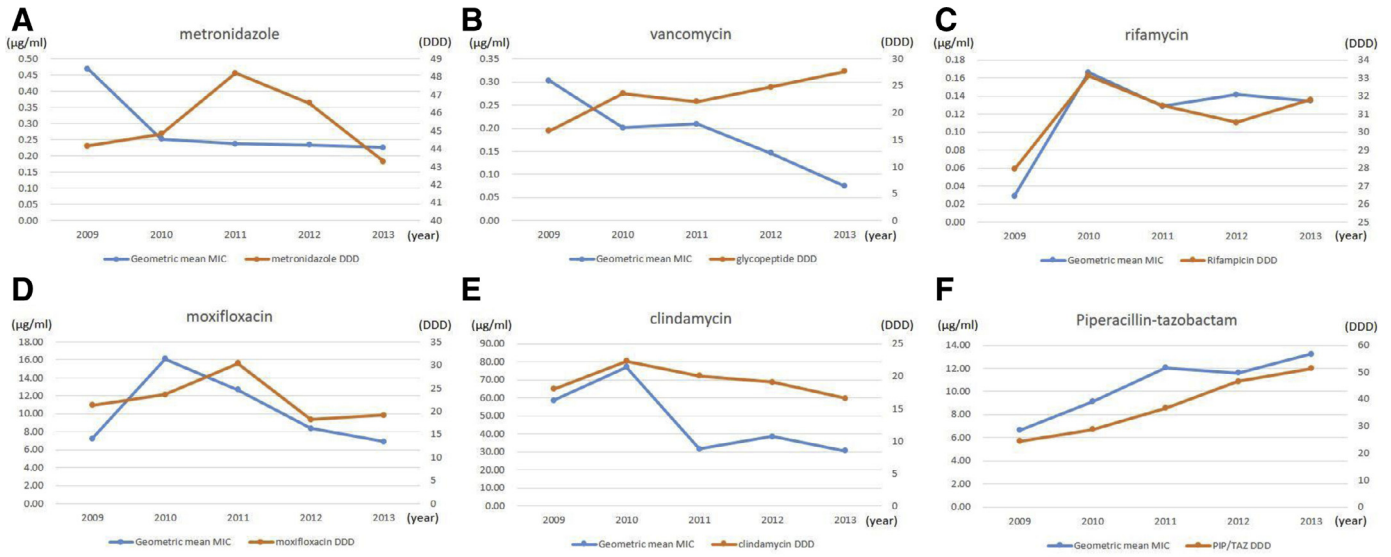

Fig. 2. Association of antibiotic consumption and minimum inhibitory concentration (MIC) of each antibiotic for yearly isolates of *Clostridioides difficile* during the 5-year period (2009–2013). (A) Metronidazole (MTZ). (B) Vancomycin (VAN): antibiotic consumption is measured by vancomycin plus teicoplanin, and the geometric mean (GM) MIC is for vancomycin. (C) Rifamycin: antibiotic consumption is measured by rifampicin, and the GM MIC is for rifaximin (RFX). (D) Moxifloxacin (MXF). (E) Clindamycin (CLI). (F) piperacillin/tazobactam (TZP). The *y*-axis on the left side presents the GM MIC for the yearly isolates of *C. difficile* (blue line) and the *y*-axis on the right presents the annual consumption of antibiotics presented as defined daily doses (DDD)/1000 patients-day (orange line). Antibiotic consumption appears to influence the GM MIC of each antibiotic for isolates collected over time, but the correlation was not significant for MTZ, RFX, MXF or CLI (Spearman's correlation analysis, $\rho = -0.051$, $P = 0.935$; $\rho = 0.667$, $P = 0.219$; $\rho = 0.600$, $P = 0.285$; and $\rho = 0.600$, $P = 0.285$, respectively). However, TZP showed a positive correlation between antibiotic consumption and GM MIC for yearly isolates ($\rho = 0.900$, $P = 0.037$), and VAN showed a negative correlation ($\rho = -1.000$, $P < 0.001$).

consumption, 185.1–190.0 DDD/1000 patient-days; coefficient for time –0.469, $P < 0.001$), MXF (range of annual consumption, 18.2–30.3 DDD/1000 patient-days; coefficient for time –0.144, $P = 0.005$), CLI (range of annual consumption, 16.6–22.3 DDD/1000 patient-days; coefficient for time –0.107, $P = 0.005$) and MTZ (range of annual consumption, 43.3–48.2 DDD/1000 patient-days; coefficient for time –0.136, $P = 0.004$). In contrast, consumption of TZP (range of annual consumption, 24.4–51.4 DDD/1000 patient-days; coefficient for time 0.428, $P < 0.001$) and glycopeptides (range of annual consumption, 16.7–24.7 DDD/1000 patient-days; coefficient for time 0.133, $P < 0.001$) showed a significant increasing trend. Consumption of levofloxacin (range of annual consumption, 27.9–33.1 DDD/1000 patient-days; coefficient for time 0.081, $P = 0.201$) and RIF (range of annual consumption, 27.9–33.1 DDD/1000 patient-days; coefficient for time –0.034, $P = 0.501$) remained stable throughout the study period (Supplementary Fig. S1).

3.4. Correlation of antibiotic use with antimicrobial susceptibility and PCR ribotypes of Clostridioides difficile in the hospital

Fig. 2 shows the correlation of antimicrobial consumption in the hospital with the antimicrobial susceptibility of *C. difficile* isolates. Decreased consumption of MTZ, MXF and CLI did not show a significant correlation with the GM MICs of the three antimicrobials for yearly *C. difficile* isolates. The GM MIC of TZP significantly increased with increased TZP use over 5 years (Spearman's $\rho = 0.900$, $P = 0.037$). In contrast, the GM MIC of VAN decreased despite the mildly increasing consumption of VAN ($\rho = -1.000$, $P < 0.001$).

To determine whether changes in PCR RT distribution influenced the increase in the GM MIC of TZP, changes in the PCR RT distribution during the 5-year period were analysed. No significant change in the prevalence of RTs could be found, except for a marginal increase of RT112. However, the annual prevalence of the three RTs that contained TZP-intermediate-resistant isolates (RT015, RT002 and RT112) had a significantly increasing trend from 2009 to 2013 (linear regression, $\beta = 6.68$, $P = 0.010$) (Fig. 1B).

4. Discussion

In this study, hospital-prevalent RT018, RT017, RT015, RT001 and RT112 isolates were highly resistant to commonly used antimicrobials such as MXF and CLI, and non-susceptibility to CLI, MXF, TZP and RFX was present among each isolate because of concordant exposure to the common antimicrobials. VAN-resistant isolates also appeared in hospital-prevalent RTs (RT018 and RT017), although concordance with resistance to other antimicrobials was not demonstrated because of the small number of VAN-resistant isolates. However, MTZ resistance seemed to appear among diverse RTs discordantly with resistance to other antimicrobials in RTs commonly identified in the hospital (RT001 and RT014) as well as in non-epidemic RTs (RT293, RT130 and UNK09). Interestingly, a recent report described the decrypting plasmids that mediate stable MTZ resistance in *C. difficile* strains, and carriage of the plasmids is common in non-epidemic RTs [15]. Studying the carriage of plasmids in isolates in the current study will be of interest.

During the 5-year period, TZP use gradually increased, and MIC creep of TZP in *C. difficile* isolates appeared with this increase in TZP consumption. Hospital-wide TZP use is recommended instead of antimicrobials traditionally known to place patients at greater risk for CDI because the activity of TZP against *C. difficile* is mostly maintained, even in the intestine, which decreases CDI in hospitals [6,7,16,17]. Many reports have described the successful replacement of CDI-prone antimicrobials such as third-generation cephalosporins with TZP, resulting in a decrease of CDI in hospitals [6,7]. Although there were no TZP-resistant strains among the isolates in the current study and the clinical significance of TZP-intermediate-resistance is not clear yet, there is a possibility of the appearance of isolates with TZP resistance with MIC creep. The results presented here show for the first time an increase in isolates

with TZP-intermediate-resistance in a hospital with increasing consumption of TZP, suggesting that TZP use may not work as a replacement for CDI-prone antimicrobials in hospitals for very long.

Regarding VAN, consumption increased during the 5-year study period and the level of consumption in the hospital was similar to CLI and MXF. However, the GM MIC of VAN for the yearly isolates decreased significantly over time. VAN is known to be excreted partially into the intestine through bile, and it was previously reported that the concentration of VAN in bile or stool ranged from 3.3–94.8 μg/mL after 5 days of treatment with 1 g of VAN every 12 h [18]. Therefore, the explanation for this paradox is not clear and it would be necessary to observe this phenomenon over a longer period.

In this report, changes in the MICs of several antibiotics and PCR RTs during 5 years in our institution were observed, and an increase in TZP MIC occurred with the increase of three PCR RTs containing TZP-intermediate-resistant strains. Interestingly, we previously reported an increase or decrease in MICs for antimicrobials in multiple strains derived from a single clone of RT018 [19]. With these findings, we understand that *C. difficile* survives under antibiotic selection pressure by shifting to advantageous PCR RTs as well as by development of resistance in individual cells over time.

This study has an advantage of the cohort to observe changes in antimicrobial susceptibility and PCR RTs of *C. difficile* in a single institution; all CDI patients and their isolates from an institution during the study period were enrolled. However, there are also limitations to this study. First, although the study showed a correlation between antibiotic consumption and antimicrobial susceptibility for *C. difficile* isolates, the presence of a causal effect could not be evaluated. Second, as this is a single-centre study, we cannot generalise the findings to other hospitals. Third, the 5-year period is relatively short to observe change in antimicrobial susceptibility and PCR RTs.

5. Conclusion

To summarise, antimicrobial susceptibility of *C. difficile* varied among PCR RTs and correlated with antimicrobial consumption. MIC creep of TZP occurred along with increased TZP consumption, and an abundance of TZP-intermediate-resistant strains appeared in our hospital. MIC creep of TZP occurred with shifting of PCR RTs that contained TZP-intermediate-resistant strains.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education [2016R1A6A3A11934276].

Competing interests

None declared.

Ethical approval

This study was approved by the Institutional Review Board (IRB) of Hanyang University Hospital (Seoul, South Korea) [IRB no. 2016-01-031]. Informed consent was waived by the board.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.05.022.

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