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Molecular epidemiology and clinical risk factors for rifaximin-non-susceptible *Clostridioides difficile* infection in South Korea: a prospective, multicentre, observational study

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ABSTRACT

Objectives: This study was designed to investigate the molecular epidemiology of *Clostridioides difficile* isolates in South Korea and to evaluate risk factors for rifaximin-non-susceptible *C. difficile* infection (CDI).
Methods: A total of 413 patients with CDI from two sentinel hospitals in South Korea were enrolled in this study. Putative clinical risk factors for CDI were identified using digital medical records of the patients. Pathogen profiles, including antimicrobial susceptibility, toxin production and ribotype, were evaluated for each of the causative *C. difficile* isolates.
Results: Of the 413 *C. difficile* isolates, 81 (19.6%) were shown to be rifaximin-non-susceptible, with the most common ribotypes being 018 (56.8%; 46/81), 017 (16.0%; 13/81) and 027 (6.2%; 5/81). Rifaximin-non-susceptible *C. difficile* isolates exhibited higher non-susceptibility rates to most of the other drugs tested in this study compared with rifaximin-susceptible isolates. Previous history of pulmonary tuberculosis and prior rifaximin treatment were shown to be associated with the occurrence of rifaximin-non-susceptible CDI compared with susceptible CDI.
Conclusion: Non-susceptibility rates to rifaximin for the *C. difficile* isolates identified in this study were reasonably high with most of the resistant strains belonging to either ribotype 018 or 017. Widespread dissemination of these clones may be the result of antimicrobial selection pressure introduced by the widespread use of rifaximin. These results suggest that a sustainable surveillance programme for CDI and *C. difficile* resistance is needed in order to better control CDIs and to improve therapeutic efficacy.

1. Introduction

Clostridioides difficile is a spore-forming, Gram-positive anaerobe that is the most common causative pathogen of healthcare-associated diarrhoea. The incidence of *C. difficile* infection (CDI) has continuously increased over the last few decades, with both healthcare-associated and community-onset diarrhoea increasing exponentially throughout the world [1,2]. CDI is a toxin-mediated intestinal disease with a broad spectrum of clinical presentations, ranging from simple diarrhoea to pseudomembranous colitis, toxic megacolon and, in extreme cases, death [3]. CDI usually develops in patients after prolonged antimicrobial treatment that may disturb the balance of the normal intestinal microbiota. Antimicrobial resistance enables *C. difficile* to grow in the presence of antimicrobial drugs, and loss of the commensal microbiota barrier effect and the freeing of niches allow *C. difficile* to colonise and infect the intestine.

The antimicrobials of choice for the treatment of CDI are metronidazole and vancomycin as both of these compounds exhibit high clearance rates [3]. Resistance to these drugs is still rare and, to date, decreased susceptibility to metronidazole has not been linked to treatment failure [4]. However, high recurrence

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rates, ranging from 15–40%, have been reported for CDI. The reason might be that all of the breakpoints are based on serum drug concentrations but effective antimicrobial therapy of CDI requires intracolonic bactericidal concentrations [5], and the severity of diarrhoea may also affect intracolonic concentrations of metronidazole because it is mostly absorbed in the intestinal tract [6]. In addition, these recurrent illnesses could promote the development of resistance and result in the generation of more complex infections [7]. Fidaxomicin has been introduced as an active therapeutic alternative and has been shown to exhibit efficacy similar to those of the more established antimicrobial drugs, with sustained resolution of these infections [8].

Recently, rifaximin, a non-absorbable rifamycin antimicrobial, has been proposed as an adjunctive treatment to lower the recurrence of CDI [9]. However, the susceptibility of *C. difficile* to rifaximin has not been widely evaluated, with only a few studies describing the global non-susceptibility rates for this antibiotic in important *C. difficile* ribotypes (RTs) 018 and 027 [10,11]. In Poland, RT046 isolates exhibiting rifamycin non-susceptibility were recovered from patients who had been treated with antituberculosis drugs including rifampicin [12]. Here we conducted a multicentre, observational study designed to investigate the molecular epidemiology of rifaximin-non-susceptibility in *C. difficile* isolates from South Korea and to identify risk factors affecting the dissemination of rifaximin-non-susceptible clones.

2. Methods

2.1. Study design

This study was performed using samples from all CDI patients identified at two of the general hospitals participating in the Global Antimicrobial Resistance Surveillance System (GLASS) in South Korea (Kor-GLASS) over a 1-year period (January 2018 to December 2018) [13]. Clinical information, including demographic characteristics, underlying co-morbidities, previous antimicrobial usage, clinical symptoms, laboratory findings and CDI treatment, was investigated using the electronic medical records of each hospital. All clinical *C. difficile* isolates collected at each of the sentinel hospitals were transferred to the analysis centre and were stored at −70°C until evaluation. The requirement for informed consent from the participants was waived by the local ethical committees.

2.2. Definitions

Hospital-acquired infection was defined as culture-confirmed CDI occurring after ≥2 calendar days of hospitalisation. Antimicrobial exposure refers to a patient receiving a specific antimicrobial treatment within 3 months of sample collection. Severe CDI was defined as elevated serum albumin levels (>3.0 g/dL) accompanied by leukocytosis (>15 000/mm^3) or abdominal tenderness [14].

2.3. Microbiological analysis

Frozen isolates were thawed and were subcultured anaerobically on Brucella blood agar (Becton Dickinson, Cockeysville, MD, USA) for 48 h at 35°C. Bacterial identification was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) using a Bruker Biotyper (Bruker Daltonics, Leipzig, Germany). Minimum inhibitory concentrations (MICs) for ampicillin, cefotetan, clindamycin, imipenem, chloramphenicol, tetracycline, moxifloxacin, rifaximin, metronidazole and vancomycin were determined by the agar dilution method on Brucella agar supplemented with 5 μg/mL hemin, 1 μg/mL vitamin K1 and 5% laked sheep blood according to Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741 and *C. difficile* ATCC 700057 were used as quality control strains. CLSI interpretive criteria were used to determine non-susceptibility to most antimicrobial agents tested [16]. Because CLSI interpretive criteria for anaerobes are based on serum drug concentrations, the epidemiological cut-off values proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for the antimicrobials for the treatment of CDI such as vancomycin and metronidazole [17]. Finally, interpretive criteria for rifaximin were determined using the value of ≤0.015 μg/mL as susceptible.

Clostridioides difficile toxin genes including *tcdA, tcdB, cdtA* and *cdtB* were detected using PCR as previously described and the following primer pairs: *tcdA*-F and *tcdA*-R for *tcdA*; NK104 and NK105 for *tcdB;* *cdtA*-pos and *cdtA*-rev for *cdtA*; and *cdtB*-pos and *cdtB*-rev for *cdtB* [18]. Nucleotide sequences of the RNA polymerase β subunit gene *rpoB* were determined using PCR and sequencing using a previously described primer [11] and were compared with the sequences from the *C. difficile* reference strain CD630. For all *C. difficile* isolates, PCR ribotyping was performed using the CD1-CD1445 primers [19]. Comparison of PCR ribotyping patterns was performed visually with known standards (VPI 10463, RT078, ATCC9689, ATCC43598 and ATCC70057), and the RT patterns with at least one band difference were assigned to different RTs.

2.4. Statistical analysis

Statistical analyses were performed using R software v.3.4.3 (R Development Core Team 2017; http://www.R-project.org/). Differences between groups were analysed using the Mann–Whitney *U*-test and Fisher's exact test for continuous variables and categorical variables, respectively. Significance was assigned at a *P*-value of <0.05.

3. Results

3.1. Patient characteristics (Table 1)

The median age of the 413 patients with CDI was 76 years (interquartile range, 64–81 years). The most common underlying comorbidity was malignancy (16.0%; $n = 66$), followed by diabetes mellitus (15.3%; $n = 63$), end-stage renal disease (15.3%; $n = 63$) and cerebrovascular disease (10.2%; $n = 42$). Only five patients (1.2%) had a history of pulmonary tuberculosis. Two-thirds of the patients (66.8%; $n = 276$) had a hospital-acquired infection. Most of the patients (82.3%; $n = 340$) had a history of antimicrobial usage, and the most common antimicrobial agent was a β-lactam/β-lactamase inhibitor combination (34.6%; $n = 143$), followed by extended-spectrum cephalosporins (24.5%; $n = 101$) and fluoroquinolones (16.7%; $n = 69$). Antimicrobial therapy was discontinued in 26.6% of patients ($n = 110$), and metronidazole and vancomycin were administered to 61.3% ($n = 253$) and 36.1% ($n = 149$) of patients, respectively. Only 42 cases of recurrent CDI in 29 patients (7.0%) were observed within the 3-month follow-up period, and all-cause in-hospital mortality of patients with CDI was 14.8% ($n = 61$).

3.2. Characteristics of Clostridioides difficile isolates (Table 1)

The most common RTs of the *C. difficile* isolates were RT018 (21.8%; $n = 90$), RT014/020 (15.5%; $n = 64$), RT002 (7.7%; $n = 32$), RT012 (6.5%; $n = 27$) and RT017 (5.6%; $n = 23$). Only five RT027 isolates (1.2%) were identified, and the remaining 104 *C. difficile* isolates were included in 42 different RTs. Most of the *C. difficile* isolates were non-susceptible to clindamycin (91.5%; $n = 378$) and imipenem (92.0%; $n = 380$), and almost one-half of them were non-susceptible to cefotetan (47.5%; $n = 196$) and moxifloxacin

Table 1
Clinical characteristics of patients with culture-confirmed *Clostridioides difficile* infection (CDI) and pathogen profiles, stratified according to their non-susceptibility to rifaximin

Variable	Total (n = 413; 100%)	Rifaximin-NS (n = 81; 19.6%)	Rifaximin-S (n = 332; 80.4%)	P-value[a]
Patient characteristics				
Age (years) [median (IQR)]	76 (64.0–81.0)	77.0 (70.0–83.0)	76.0 (63.0–81.0])	0.192
Male sex	177 (42.9)	36 (44.4)	141 (42.5)	0.844
Underlying co-morbidities				
Malignancy	66 (16.0)	11 (13.6)	55 (16.6)	0.625
Diabetes mellitus	63 (15.3)	12 (14.8)	51 (15.4)	0.999
Cardiovascular disease	16 (3.9)	4 (4.9)	12 (3.6)	0.816
Cerebrovascular disease	42 (10.2)	9 (11.1)	33 (9.9)	0.914
End-stage renal disease	63 (15.3)	12 (14.8)	51 (15.4)	0.999
Liver cirrhosis	11 (2.7)	2 (2.5)	9 (2.7)	0.999
Chronic pulmonary disease	29 (7.0)	3 (3.7)	26 (7.8)	0.289
Pulmonary tuberculosis	**5 (1.2)**	**5 (6.2)**	**0 (0)**	**–**
Hospital-acquired infection	276 (66.8)	60 (74.1)	216 (65.1)	0.158
Fever	61 (14.8)	13 (16.0)	48 (14.5)	0.909
Accompanying PMC	75 (18.2)	14 (17.3)	61 (18.4)	0.946
Severe CDI	81 (19.6)	14 (17.3)	67 (20.2)	0.539
Laboratory findings				
WBC count >15 000 cells/μL	84 (20.3)	16 (19.8)	68 (20.5)	0.999
Albumin <2.5 g/dL	107 (25.9)	24 (29.6)	83 (25.0)	0.477
Proton pump inhibitor	46 (11.1)	12 (14.8)	34 (10.2)	0.358
Previous antibiotic usage	340 (82.3)	71 (87.7)	269 (81.0)	0.194
β-Lactam/β-lactamase inhibitors	143 (34.6)	28 (34.6)	115 (34.6)	0.999
1/2G cephalosporins	24 (5.8)	4 (4.9)	20 (6.0)	0.913
3/4G cephalosporins	101 (24.5)	23 (28.4)	78 (23.5)	0.438
Carbapenems	59 (14.3)	16 (19.8)	43 (13.0)	0.164
Fluoroquinolones	69 (16.7)	18 (22.2)	51 (15.4)	0.188
Rifaximin	**4 (1.0)**	**3 (3.7)**	**1 (0.3)**	**0.030**
Treatment				
Discontinued antimicrobial therapy	110 (26.6)	21 (25.9)	89 (26.8)	
Metronidazole	253 (61.3)	55 (67.9)	198 (59.6)	0.302
Vancomycin	149 (36.1)	28 (34.6)	121 (36.4)	0.752
Recurrence	29 (7.0)	8 (9.9)	21 (6.3)	0.329
In-hospital mortality	61 (14.8)	18 (22.2)	43 (13.0)	0.064
Pathogen characteristics				
Ribotype				**<0.001**
018	**90 (21.8)**	**46 (56.8)**	**44 (13.3)**	
014/020	64 (15.5)	2 (2.5)	62 (18.7)	
002	32 (7.7)	3 (3.7)	29 (8.7)	
012	27 (6.5)	1 (1.2)	26 (7.8)	
046	23 (5.6)	4 (4.9)	19 (5.7)	
106	23 (5.6)	2 (2.5)	21 (6.3)	
017	**23 (5.6)**	**13 (16.0)**	**10 (3.0)**	
001	22 (5.3)	0 (0)	22 (6.6)	
027	**5 (1.2)**	**5 (6.2)**	**0 (0)**	
Others	104 (25.2)	5 (6.2)	99 (29.8)	
Toxins				
***tcdA*$^+$/*tcdB*$^+$**	**368 (89.1)**	**62 (76.5)**	**306 (92.2)**	**0.011**
***tcdA*$^-$/*tcdB*$^+$**	**29 (7.0)**	**13 (16.0)**	**16 (4.8)**	**0.011**
tcdA$^+$/*tcdB*$^+$, *cdtA*$^+$/*cdtB*$^+$	16 (3.9)	6 (7.4)	10 (3.0)	0.129
Non-susceptibility to:				
Ampicillin	**270 (65.4)**	**66 (81.5)**	**204 (61.4)**	**0.001**
Cefotetan	**196 (47.5)**	**71 (87.7)**	**125 (37.7)**	**<0.001**
Clindamycin	378 (91.5)	77 (95.1)	301 (90.7)	0.293
Imipenem	380 (92.0)	77 (95.1)	303 (91.3)	0.367
Chloramphenicol	**10 (2.4)**	**6 (7.4)**	**4 (1.2)**	**0.004**
Tetracycline	80 (19.4)	17 (21.0)	63 (19.0)	0.800
Moxifloxacin	**182 (44.1)**	**75 (92.6)**	**107 (32.2)**	**<0.001**
Metronidazole	0 (0)	0 (0)	0 (0)	–
Vancomycin	0 (0)	0 (0)	0 (0)	–

NOTE: Data are n (%) unless otherwise stated.
NS, non-susceptible; S, susceptible; IQR, interquartile range; PMC, pseudomembranous colitis; WBC, white blood cell; 1/2G, first and second-generation; 3/4G, third and fourth-generation.
[a] Statistically significant characteristics (P < 0.05) are given in bold.

(44.1%; n = 182). All *C. difficile* isolates collected in this study exhibited susceptibility to traditionally used anti-CDI drugs including vancomycin and metronidazole. Antimicrobial susceptibilities of the isolates stratified according to their RTs are summarised in Table 2.

3.3. Rifaximin-non-susceptible Clostridioides difficile infection

A substantial portion (19.6%; n = 81) of the 413 *C. difficile* clinical isolates described in this study was non-susceptible to rifaximin. The most common RTs for the rifaximin-non-susceptible *C.*

Table 2
Non-susceptibility rates [*n* (%)] of *Clostridioides difficile* isolates stratified according to ribotype (RT)

Antimicrobial	RT018 (*n* = 90; 21.8%)	RT014/020 (*n* = 64; 15.5%)	RT002 (*n* = 32; 7.7%)	RT012 (*n* = 27; 6.5%)	RT106 (*n* = 23; 5.6%)	RT017 (*n* = 23; 5.6%)	RT046 (*n* = 23; 5.6%)	RT001 (*n* = 22; 5.3%)	RT027 (*n* = 5; 1.2%)	Others RTs (*n* = 104; 25.2%)
Ampicillin	77 (85.6)	35 (54.7)	30 (93.8)	16 (59.3)	13 (56.5)	18 (78.3)	20 (87.0)	7 (31.8)	5 (100)	49 (47.1)
Cefotetan	89 (98.9)	8 (12.5)	24 (75.0)	6 (22.2)	4 (17.4)	22 (95.7)	10 (43.5)	8 (36.4)	5 (100)	20 (19.2)
Clindamycin	89 (98.9)	59 (92.2)	30 (93.8)	27 (100)	20 (87.0)	22 (95.7)	19 (82.6)	19 (86.4)	5 (100)	88 (84.6)
Imipenem	89 (98.9)	57 (89.1)	32 (100)	23 (85.2)	21 (91.3)	22 (95.7)	21 (91.3)	20 (90.9)	5 (100)	90 (86.5)
Chloramphenicol	2 (2.2)	0 (0)	0 (0)	0 (0.0)	0 (0)	4 (17.4)	2 (8.7)	1 (4.5)	1 (20.0)	0 (0)
Tetracycline	1 (1.1)	0 (0)	0 (0)	9 (33.3)	0 (0)	20 (87.0)	19 (82.6)	18 (81.8)	0 (0)	13 (12.5)
Moxifloxacin	89 (98.9)	6 (9.4)	22 (68.8)	2 (7.4)	1 (4.3)	18 (78.3)	7 (30.4)	13 (59.1)	5 (100)	19 (18.3)
Rifaximin	46 (51.1)	2 (3.1)	3 (9.4)	1 (3.7)	2 (8.7)	13 (56.5)	4 (17.4)	0 (0)	5 (100)	5 (4.8)
Metronidazole	0 (0.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Vancomycin	0 (0)	0 (0)	0 (0)	0 0.0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 3
Amino acid substitutions in the β subunit of the bacterial RNA polymerase (RpoB) in the causative *Clostridioides difficile* isolates exhibiting non-susceptibility to rifaximin

Ribotype	No. of isolates	Amino acid substitution in RpoB							Rifampicin MIC (μg/mL)							
		L487	D492	S498	H502	R505	I548	S550	0.03	0.06	0.12	0.25	0.5	1	2	>128
018	29					K	M									29
	6				N	K										6
	4	S				K										4
	4				N								1	2	1	
	1					K										1
	1			T							1					
	1							Y						1		
017	12				N	K										12
	1			T	N	K										1
027	4					K	M									4
	1					K										1
106	2								2							
046	3					K	M									3
	1				Y											1
014/020	2				K											2
002	1		E			K										1
	2								2							
012	1					K	M									1
163	1					K										1
159	1					K										1
078	1								1							
NT	1				Y											1
NT	1								1							

MIC, minimum inhibitory concentration; NT, non-typeable.

difficile isolates were RT018 (56.8%; 46/81), RT017 (16.0%; $n = 13$) and RT027 (6.2%; $n = 5$) (Table 1). Rifaximin-non-susceptible *C. difficile* isolates exhibited higher rates of non-susceptibility to most of the other drugs evaluated in this study compared with rifaximin-susceptible isolates, especially to ampicillin (81.5% vs. 61.4%; $P = 0.001$), cefotetan (87.7% vs. 37.7%; $P < 0.001$), chloramphenicol (7.4% vs. 1.2%; $P = 0.004$) and moxifloxacin (92.6% vs. 32.2%; $P < 0.001$) (Table 1). In addition, strains collected from all five patients with a previous history of pulmonary tuberculosis exhibited rifaximin non-susceptibility. Previous antimicrobial treatment using rifaximin was also more frequently identified in patients with rifaximin-non-susceptible CDI [3.7% (3/81) versus 0.3% (1/332); $P = 0.030$] (Table 1).

3.4. Amino acid substitutions in RpoB in rifaximin-non-susceptible *Clostridioides difficile* isolates

Rifaximin MICs for the 81 *C. difficile* isolates ranged from 0.03 μg/mL to >128 μg/mL (MIC_{50}, >128 μg/mL; MIC_{90}, >128 μg/mL) (Table 3). Nine RpoB amino acid substitutions were identified in this study, with most of these amino acid substitutions being previously reported [20–22]. The R505K amino acid substitution was identified in 65 isolates, with an additional I548M substitution in 37 isolates, H502N substitution in 18 isolates, L487S substitution in 4 isolates, and S498T and H502N substitution in 1 isolate; all of them exhibited very high MICs for rifaximin (>128 μg/mL). Four isolates carried the H502N substitution alone, with these isolates exhibiting lower MICs (ranging from 0.5 μg/mL to 2 μg/mL). Finally, four isolates exhibiting MICs of 0.03 μg/mL did not show any substitution in their RpoB amino acid sequences (Table 3).

4. Discussion

National monitoring of antimicrobial resistance in clinically important pathogens is essential for preventing the dissemination of multidrug-resistant organisms; *C. difficile* has been identified by the US Centers for Disease Control and Prevention (CDC) as one such pathogen that needs urgent monitoring [23]. A new surveillance system for antimicrobial resistance (Kor-GLASS) was initiated in South Korea in 2016 using the World Health Organization's GLASS programme as a model [13]. As a part of Kor-GLASS, monitoring the antimicrobial resistance of *C. difficile* isolates was initiated at two sentinel hospitals in 2018. Isolates from clinical CDIs are collected and subjected to antimicrobial susceptibility testing for the first-line drugs used in the treatment of these infections and any drugs that could be risk factors for developing CDI under this programme. This surveillance programme include characterisation of the toxin genes as well as ribotyping of each isolate.

RT018 *C. difficile* strains have been shown to be disseminated in South Korea since 2006 and have been the most common RT since 2009 [24,25]. RT018 clones exhibit high levels of resistance to ampicillin, cefotetan, imipenem and moxifloxacin, which have been suggested as a contributing factor to their dominance [26]. In this study, RT018 was still the most common RT, and more than one-half of the RT018 isolates were non-susceptible to rifaximin. In addition, three of the four patients with CDIs who had been exposed to rifaximin within the last 3 months were infected by rifaximin-non-susceptible *C. difficile* strains, and all five patients with a history of pulmonary tuberculosis were infected with rifaximin-non-susceptible *C. difficile*. Reigadas et al. reported that a high percentage of rifaximin-non-susceptible CDI cases were detected in cirrhotic patients receiving rifaximin to prevent hepatic encephalopathy [27]. The successful clonal expansion of RT018 strains in South Korea where the incidence of pulmonary tuberculosis is still high may result in a high proportion of rifaximin-non-susceptible *C. difficile* strains.

Sequence analysis of *rpoB* from the rifaximin-non-susceptible *C. difficile* isolates identified nine amino acid substitutions. Of these, three substitutions (L487S, D492E and H502K) were novel and the remaining six substitutions (S498T, H502N, H502Y, R505K, I548M and S550Y) have been described in previous studies [20–22]. Among the 68 *C. difficile* isolates exhibiting very high MICs for rifaximin (>128 μg/mL), 65 isolates had the R505K polymorphism alone or in conjunction with one or two additional amino acid substitutions, which are believed to affect the direct interaction of *rpoB* with rifamycin [20].

A limitation of this study is that the sentinel hospitals are geographically restricted to a single country, South Korea, where the racial homogeneity is very high. Therefore, ethnic or racial diversity was not considered in this study. In addition, a lack of functional evaluation of the novel amino acid substitutions in RpoB could be another potential limitation. Further studies should be conducted to evaluate the possible effects of these polymorphisms.

In conclusion, this multicentre observational study shows that the rifaximin-non-susceptibility rate in *C. difficile* isolates was very high, with most of the non-susceptible strains belonging to RT018 and RT017, which are the most common RTs in South Korea. The success of these RTs may be facilitated by increased antimicrobial selection pressure following rifaximin treatment associated with the high incidence of pulmonary tuberculosis in these populations. A continuous surveillance programme for investigating the molecular epidemiology of *C. difficile* is required.

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Competing interests

None declared.

Ethical approval

Requirement for informed consent from the participants was waived by the local ethical committees [approval no. 3-2020-0319].

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