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Antimicrobial Resistance and Multilocus Sequence Typing of Vancomycin-Resistant *Enterococcus faecium* Isolated from the Chungcheong Area

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Background: *Enterococcus faecium* has emerged as an important nosocomial pathogen worldwide, and this trend has been associated with the dissemination of a genetic lineage designated clonal complex 17 (CC17). In the present study, characterization of the glycopeptide resistance mechanism, genetic relatedness, and pathogenicity in isolates of vancomycin-resistant *E. faecium* in the Chungcheong area were investigated.
Methods: A total of 37 consecutive, non-duplicate, vancomycin-resistant *E. faecium* were isolated at three university hospitals in the Chungcheong area. The mechanism of glycopeptide resistance and pathogenicity factors were studied using PCR, and the genetic relatedness was determined via multilocus sequence type and esp repeat profile analysis. Additionally, the quinolone resistance-determining regions of *parC* and *gyrA* were sequenced to identify mutations involved in ciprofloxacin resistance.
Results: Two genotypes of VRE were confirmed: VanA-phenotype vanA genotype VRE (25 isolates) and VanB-phenotype vanA genotype VRE (12 isolates). MLST analysis revealed five sequence types. A significant result was that ST414 and CNS4 (4-1-1-1-1-1-1) were considered as belonging to CC17. The *esp* and *hyl* genes were found in 100% and 86.4% of the isolates, respectively. A total of 37 isolates showed genetic mutations in *parC* and *gyrA*.
Conclusion: All isolated strains in the present study belonged to one of the CC17 genotypes including ST414 and CNS4 (4-1-1-1-1-1-1), which were not previously detected in Korea. The combination of MLST and the *esp* gene repeat profiles can be useful for genetic characterization of VREF isolates with regard to the evolutionary process and epidemiology of the clones. **(Korean J Clin Microbiol 2011;14:60-66)**

Key Words: Vancomycin-resistant *E. faecium*, MLST, Clonal complex 17

INTRODUCTION

Vancomycin-resistant enterococci (VRE) were isolated in Europe for the first time in 1986. Since then, they have been spread around the world and became an important nosocomial pathogen [1]. In Korea, VRE were first reported in 1992 and the number of isolates was limited so far. But with increasing use of oral vancomycin, detection rate of VRE has been tremendously increased since 1998 [2]. According to the survey by Korean National Surveillance Antimicrobial Resistance (KONSAR), vancomycin resistance rate of *Enterococcus faecium* isolated in domestic hospitals was reported as 16% in 2004 [3].

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Recently VRE which are high-resistant to ampicillin, aminoglycoside and glycopeptide are prevalent. Among those, clonal complex 17 (CC17) adapting to prolonged exposure to hospital setting is detected worldwide. The characteristics of CC17 are its resistance to ampicillin and quinolone, and variant *esp* gene as *E. faecium* pathogenicity island (PAI) [4,5]. It has been reported that the organisms isolated in Korea belong to CC17 [6,7]. Thus study on epidemiology and genetic characteristics of CC17 is needed. For this purpose multilocus sequence typing (MLST) is designed for tracing the origin of prevalent organisms or phylogeny of each organism. MLST can give an accurate prediction about epidemiologic origin and evolutional background of same organism without errors between interpreters and laboratories.

In this study, resistance to antibiotics and the mechanism of resistance were investigated on 37 isolates of vancomycin-resistant *Enterococcus faecium* (VREF) collected in 3 university hospitals in Chungcheong area. And epidemiologic study on *esp* and *hyl* regarded as pathogenic genes was performed with

MLST. In addition, mutations of quinolone resistance determining region (QRDR) involved in resistance of CC17 to quinolone and the association between genetic alteration and resistance to antibiotics were studied.

MATERIALS AND METHODS

1. Bacterial strains and susceptibility tests

Between September and December of 2009, a total of 37 consecutive, non-duplicate, VREF were isolated at three university hospitals located in Chungcheong area of Korea. The isolates were confirmed to be *E. faecium* by biochemical profiling using conventional methods and Vitek 2 identification system (BioMérieux, Hazelwood, MO, USA).

Antimicrobial susceptibilities were determined by the disk diffusion technique in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [8]. The following antibiotics were tested: ampicillin, vancomycin, teicoplanin, erythromycin, tetracycline, levofloxacin, norfloxacin, ciprofloxacin, nitrofurantoin, quinupristin-dalfopristin, and linezolid (BBL, Cockeysville, MD, USA). MICs were determined by the CLSI agar dilution method. Antibiotics were obtained as follows: vancomycin, teicoplanin, ampicilin from Sigma-Aldrich (St. Louis, MO, USA), ciprofloxacin from Fluka (Buchs, Switzerland). *E. faecium* BM4147 used as a control strain.

2. Detection of *vanA* and *vanB* genes

All vancomycin-resistant *E. faecium* isolates were subjects to PCR assays for the detection of *vanA* and *vanB* genes. PCR was conducted with 50 ng of template DNA (genomic DNA), 2.5 μL of 10×Taq buffer, 0.5 μL of 10 mM dNTP mix, 20 pmol of each primer, and 0.7 U of Taq DNA polymerase (Solgent, Daejeon, Korea) in a total volume of 25 μL. Primers for the detection of *vanA* and *vanB* gene were utilized as follows: *vanA*-F (5'-GGGAAAACGACAATTGC-3') and *vanA*-R (5'-GTACAATGCGGCCGTTA-3') and *vanB*-F (5'-ATGGGAAGCCGATAGTC-3') and *vanB*-R (5'-GATTTCGTTCCTCGACC-3') [9]. All of the *vanA* and *vanB* genes were amplified via the pre-denaturation of the reaction mixture for 4 min at 95°C; this was followed by 30 cycles at 94°C for 1 min, 45°C for 45 sec, and 72°C for 1 min, and a final elongation for 7 min at 72°C; these reactions were conducted in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Corp., Norwalk, CT, USA). The amplified products were separated via electrophoresis on 1% agarose gels containing ethidium bromide, and visualized using a BioDoc-14 Imaging system (UVP, Cambridge, UK). The amplicons were purified with a PCR purification kit (Solgent) and were sequenced using a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730XL DNA analyzer (PE Applied Biosystems).

3. MLST

MLST was performed according to the scheme described previously [10]. Internal fragments of seven housekeeping genes (*adk, atpA, ddl, gdh, gyd, purK* and *pstS*) were amplified by PCR and directly sequenced. The allele number for each gene assigned based on the *E. faecium* MLST database (http://www.mlst.net). The combination of the allelic sequences for the seven genes yielded the allelic profile for each isolate.

4. Detection of the *esp* and *hyl* genes

The presence of the *esp* and *hyl* gene in isolates was determined by PCR using primers; *esp*-F (5'-GGTCACAAAGCCCAACTTGT-3') and *esp*-R (5'-ACGTCGAAAGTTCGATTTCC-3') [6] and *hyl*-F (5'-ACAGAAGAGCTGCAGGAAATG-3') and *hyl*-R (5'-GACTGACGTCCAAGTTTCCAA-3') [11]. To determine repeat number variations of *esp* A and C repeats, two different primer combinations were used; *esp*fs7F (5'-CGACCGATTTAGCAGTAAC-3') - *esp*fm5R (5'-CAGCTGCGCTAACATCTAC-3') and *esp*fm5F (5'-AAAGAAGATTTACCAAAAGATACTAAG-3') - *esp*fs3R (5'-TTCGGCGCTTTTTATC-3') respectively [12]. All PCR conditions were similar to those for MLST. PCR products were confirmed by sequencing.

5. Sequencing the QRDRs of *parC* and *gyrA*

The QRDRs of the *E. faecium parC* and *gyrA* genes were amplified and sequenced.

Primers for the detection of *parC* and *gyrA* gene were utilized as follows: *parC*-F (5'-TTCCCGTGCATTTCGATCAGTACTTC-3') and *parC*-R (5'-CGTATGACAAAGGATTCGGTAAATC-3') and *gyrA*-F (CGGGATGAACGAATTGGGTGTGA-3') and *gyrA*-R (5'-AATTTTACTCATACGTGCTTCGG-3') [13]. PCR conditions included an initial denaturation at 95°C for 15 min, followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, followed by an extension at 72°C for 7 min.

RESULTS

1. The pattern of antibiotic resistance

A total of 37 isolates of VREF were isolated from patients' specimens. All isolates showed resistance to vancomycin and ampicillin by agar dilution method. And those isolates also showed relatively high resistance to ciprofloxacin (97.3%) and teicoplanin (67.7%) (Table 1).

2. Determination of VRE genotype

PCR was performed on those 37 isolates to determine the *van* gene genotype. They were all positive for *vanA* type. But there were no detected isolates when using PCR to detect *vanB* type. Direct sequencing for those isolates confirmed the PCR results. After analyzing the pattern of antibiotic resistance and VRE genotype, we found 2 genotypes of VRE. One was VanA-phenotype *vanA* genotype VRE (25 isolates) and the other was VanB-phenotype *vanA* genotype VRE (12 isolates).

3. MLST analysis

The most common form of those 5 STs was ST192 (20 isolates) according to MLST analysis on VREF isolates. The other

Table 1. Antibiotic susceptibility profiles of VREFs

Isolates	MIC (μg/mL)			
	Ampicillin	Vancomycin	Teicoplanin	Ciprofloxacin
CNS3	>256	>256	16	64
CNS4	>256	>256	16	64
CNS5	>256	>256	128	256
CNS6	>256	>256	32	32
CNS8	256	>256	8	64
CNS9	>256	>256	128	256
CNS10	>256	>256	256	16
CNS11	>256	>256	8	32
CNS12	>256	>256	8	32
CNS13	256	>256	256	256
CNS14	256	>256	16	256
CNS15	256	>256	64	64
CNS16	>256	>256	64	32
CNS17	>256	>256	64	256
CNS18	>256	>256	64	64
CNS19	>256	>256	64	32
CNS20	>256	>256	256	64
CNS22	>256	>256	8	256
CNS23	>256	>256	128	64
CNS24	>256	>256	128	256
CNS25	>256	>256	128	2
CNS27	>256	>256	128	64
CBS2	>256	>256	128	128
CBS3	>256	>256	64	256
CBS6	>256	>256	128	256
CBS7	>256	>256	128	64
CBS8	>256	>256	128	256
CBS9	>256	>256	16	128
CBS11	>256	>256	128	256
CBS12	>256	>256	128	256
CBS14	>256	>256	64	128
EJS1	>256	>256	16	128
EJS2	>256	>256	256	128
EJS3	>256	>256	8	128
EJS4	>256	>256	64	128
EJS5	>256	>256	8	128
EJS7	256	>256	16	128

Abbreviations: CNS, isolates from CN hospital; CBS, isolates from CB hospital; EJS, isolates from EJ hospital.

forms were ST78 (12 isolates), ST17 (3 isolates), ST414 (1 isolate), and CNS4 (4-1-1-1-1-1-1) (1 isolate) in order of frequency (Table 2).

4. Detection of pathogenic factors (*esp* and *hyl* gene)

PCR was performed on 37 isolates to detect pathogenic genes, *esp* and *hyl*. All 37 isolates showed positivity for *esp* gene and 32 (86.4%) of 37 isolates for *hyl* gene. Direct sequencing of amplified *esp* and *hyl* gene products by PCR validated the sequence of the *esp* and *hyl* gene.

In addition, three different forms were noticed according to *esp* repeat profile analysis for 5 STs, 36 of 37 isolates had 2 forms and 1 isolate showed a different form (Table 2).

5. Sequencing of *parC* and *gyrA* gene

We also investigated mutations of *parC* and *gyrA* existing in QRDR. All 37 isolates had mutations in *parC* and *gyrA*. In case of *parC*, 26 isolates had Ser80→Arg and 11 isolates had Ser80 →Ile mutation. In case of *gyrA*, 19 isolates showed Ser83→Arg and 18 isolates showed Ser83→Ile mutation. 17 of 37 isolates (45.9%) had both Ser80→Arg and Ser83→Arg mutations in *parC* and *gyrA*. In addition, 9 isolates (24.3%) had Ser80→Arg and Ser83→Ile, 2 isolates (5.4%) had Ser80→Ile and Ser83→ Arg, and 9 isolates (24.3%) had Ser80→Ile and Ser83→Ile (Table 3).

DISCUSSION

Even many articles about the mechanism of VRE resistance and its epidemiology have been published in Korea so far [2,3,6,7,14], but such studies in Chungcheong area has not been reported. VREs are classified into 6 categories depending on amino acid sequence of ligase. Among those, VanA and VanB have been frequently reported [15,16]. In general, VanA type *E. faecium* shows high resistance to vancomycin and teicoplanin. VanB type has low resistance to vancomycin and susceptibility to teicoplanin. But the presence of isolates with *vanA* which also has VanB phenotype was reported. The point mutation of sensor domain of vanS and damage to VanY and VanZ which are accessory proteins are reasons for this phenomenon and consequent susceptibility to teicoplanin [14,17]. Eom et al. reported 20 patients infected with VanB phenotype *vanA* genotype *E. faecium* in one hospital in 2001, which was the first case in Korea [14]. In this study, all 37 VRE isolates had *vanA* but no *vanB*. Antibiotic susceptibility testing revealed that 25 isolates (67.6%) were VanA-phenotype *vanA* genotype VRE which are resistant to vancomycin and teicoplanin and 12 isolates (32.4%) were VanB-phenotype *vanA* genotype VRE which are susceptible to teicoplanin. As a result, it was concluded that VanB-phenotype *vanA* genotype VRE are relatively common in this study.

MLST for molecular epidemiologic study on VRE isolates is a method to analyze the genetic diversity of multiple housekeeping genes. After analyzing MLST of VREFs isolated from 3 hospitals, we found total 5 sequence types. Among those ST192, ST78, ST17 were prevalent and ST414 (15-5-1-1-1-20-1) and CNS4 (4-1-1-1-1-1-1) existed in each different hospitals. Studies on VRE isolated in Korea showed 14 STs (ST32, ST78, ST203, ST17, ST117, ST205, ST18, ST192, ST204, ST206, ST207, ST64, ST132, ST233) in total [6,7,18]. Taking this study into consideration, ST414 found in our study is the first ST reported in Korea. In *E. faecium* MLST database (http://www.mlst.net), a total of 514 STs have been reported so far. ST414 was the first case reported in Australia in 2008 [19]. And CNS4 (4-1-1-1-1-1-1) which was never reported until now is expected to be new to MLST database. The most common form of STs in Korea is known to be ST78 [20]. But in this

Table 2. Genotypic characteristics of vancomycin-resistant *E. faecium* isolates from three Korean hospitals based on multilocus sequence typing, *esp* repeat profiles and *hyl* gene

STRAIN	ST	Allelic profile							*esp* gene repeat profile		*hyl*
		atpA	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adk*	*esp*-A	*esp*-C	
CNS3	78	15	1	1	1	1	1	1	4	8	+
CNS4	New	4	1	1	1	1	1	1	4	8	+
CNS5	192	15	1	1	1	1	7	1	4	8	+
CNS6	78	15	1	1	1	1	1	1	5	6	+
CNS8	192	15	1	1	1	1	7	1	5	6	+
CNS9	78	15	1	1	1	1	1	1	4	8	+
CNS10	78	15	1	1	1	1	1	1	5	6	+
CNS11	192	15	1	1	1	1	7	1	5	6	+
CNS12	192	15	1	1	1	1	7	1	4	8	+
CNS13	78	15	1	1	1	1	1	1	5	6	+
CNS14	192	15	1	1	1	1	7	1	4	8	+
CNS15	192	15	1	1	1	1	7	1	5	6	+
CNS16	192	15	1	1	1	1	7	1	4	8	−
CNS17	78	15	1	1	1	1	1	1	4	8	+
CNS18	192	15	1	1	1	1	7	1	5	6	+
CNS19	192	15	1	1	1	1	7	1	5	6	+
CNS20	78	15	1	1	1	1	1	1	5	6	+
CNS22	192	15	1	1	1	1	7	1	4	8	+
CNS23	192	15	1	1	1	1	7	1	5	6	+
CNS24	192	15	1	1	1	1	7	1	4	8	+
CNS25	192	15	1	1	1	1	7	1	4	8	+
CNS27	17	1	1	1	1	1	1	1	4	8	+
CBS2	17	1	1	1	1	1	1	1	4	8	+
CBS3	78	15	1	1	1	1	1	1	5	6	−
CBS6	78	15	1	1	1	1	1	1	4	8	−
CBS7	192	15	1	1	1	1	7	1	5	6	+
CBS8	192	15	1	1	1	1	7	1	4	8	+
CBS9	414	15	5	1	1	1	20	1	4	8	−
CBS11	192	15	1	1	1	1	7	1	4	8	+
CBS12	192	15	1	1	1	1	7	1	4	8	+
CBS14	78	15	1	1	1	1	1	1	5	6	+
EJS1	192	15	1	1	1	1	7	1	5	6	+
EJS2	192	15	1	1	1	1	7	1	5	6	+
EJS3	78	15	1	1	1	1	1	1	6	7	+
EJS4	78	15	1	1	1	1	1	1	4	8	−
EJS5	192	15	1	1	1	1	7	1	5	6	+
EJS7	17	1	1	1	1	1	1	1	4	8	+

Abbreviations: CNS, isolates from CN hospital; CBS, isolates from CB hospital; EJS, isolates from EJ hospital.

Table 3. *parC* and *gyrA* mutation in 37 *E. faecium* isolates corresponding to ciprofloxacin MIC

Amino acid mutation in gene		No. of isolates with ciprofloxacin MIC (μg/mL)								Total no. of islates
parC (80)	*gyrA* (83)	2	4	8	16	32	64	128	256	37
R	R				1	3	6	4	3	17
R	I					2	2	1	4	9
I	R							1	1	2
I	I	1					1	3	4	9

Abbreviations: R, arginine; I, isoleucine.

study, ST192 was the most common form constituting 54%. And ST203 and ST205 commonly distributed in Korea, were not present in this study.

ST192, ST78, ST17 commonly distributed over 3 hospitals and ST414, CNS4 (4-1-1-1-1-1-1) found in this study belonged to CC17. CC17 spreading around the world has resistance to ampicillin and quinolone, presence of putative pathogenicity island including *esp* gene, and putative virulence gene *hyl* gene. *Esp* gene, one of main pathogenic factors of *E. faecium*, is a surface protein acting as adhesion factor and is involved in biofilm synthesis [21,22]. In addition, the fact that it can be transmitted with *vanA* resistance gene is believed to make contribution to wide spread of pathogenic VRE [23]. Currently there have been many reports about another pathogenic gene, *hyl* gene in addition to *esp* gene [24-26]. *Hyl* gene is translated into hyaluronidase protein acting as a pathogenic factor in *Streptococcus pyogenens*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* [26], and commonly found in spreading disease causing organisms [11]. Especially in recent study in Spain *E. faecium* with *hyl* gene was reported to belong to CC17 polyclonal subcluster, which proved the relationship between CC17 and *hyl* gene [24]. In this study, it was found all 37 isolates had *esp* gene, and most of isolates (32 isolates, 86.5%) except 5 (CNS16, CBS3, CBS6, CBS9, EJS4) had *hyl* gene. Camargo et al. [27] reported 56.5% of VREFs (13/23) isolated in Brazil had *esp* gene, 17.3% (4/23) had both *hyl* gene and *esp* gene. And Saudi Arabian study in 2008 showed 30 of 33 isolates of CC17 has either *esp* gene or *hyl* gene and concluded that 91% of CC17 isolates had pathogenic factors [11].

Esp repeat profile analysis along with PFGE or MLST is used for analyzing outbreaks of VREF resistance gene [26]. Recent report in Holland in 2004 about *esp* repeat profile analysis on isolates coming from single outbreak showed same results [12]. And also it was reported that MLST with *esp* repeat profile analysis could increase the genetic diversity of VREF isolates in recent study in Korea. For example, ST78 isolates from MLST can be reclassified into 8 subgroups according to *esp* repeat profile analysis. Consequently it was possible to determine genotype of spreading pathogenic VREFs in more detail [6]. In this study, 36 of 37 isolates showed 2 subtypes and one was a different form according to *esp* repeat profile analysis (Table 2). It is thought that genetically similar VREs are prevalent in this study.

The genetic background of quinolone resistant *E. faecium* and its relation with CC17 were proved in Holland in 2005 [13]. MLST revealed that most of isolates belonged to CC17 and consequently investigation on CC17 as possible genetic aberrations causing quinolone resistance was made. All 37 isolates showed genetic mutations in *parC* and *gyrA*. In case of *parC*, Ser80→Arg or Ser80→Ile were found. In case of *gyrA*, Ser83→Arg or Ser83→Ile mutations were found. 97% of those isolates showed ciprofloxacin resistance according to antibiotic susceptibility testing. Ser80→Arg mutation of *parC* and Ser83→Arg mutation of *gyrA* were most commonly found in 17 of 37 isolates (45.9%), and these mutations are known as most frequently observed mutations in QRDR [13].

In this study, VanB-phenotype *vanA* genotype VRE (32.4%) as well as VanA-phenotype *vanA* genotype VRE (67.6%) were relatively common. In addition, epidemiologic study confirmed that CC17 known to be spreading in the world was also widely prevalent in this study. ST 414 and CNS4 (4-1-1-1-1-1-1-1) Which were not detected in Korea before were also notable findings. This study also found the relations between *hyl* gene and CC17 for the first time in Korea.

Control of VREF organisms and its epidemiologic study are thought to be important issues because CC17 seems to be naturalized in hospital settings. In this regards, identification of *esp* and *hyl* gene and MLST can be very useful for early detection of CC17 and other sequences for subsequent prevention of its naturalization.

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=국문초록=

충청지역에서 분리된 Vancomycin 내성 *Enterococcus faecium*의 항균제 내성과 Multilocus Sequence Type 분석

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배경: 장알균은 전 세계적으로 출현하고 있는 중요한 병원균으로, 특히 Clonal Complex 17 (CC17)이 유행하고 있는 실정이다. 본 연구에서는 충청지역에서 분리된 vancomycin 내성 장알균의 항균제 내성 양상과 분자역학적인 성상에 대하여 알아보고자 하였다.

방법: 2009년 9월부터 12월까지 충청지역의 3개 의과대학병원 진단검사의학과에 의뢰된 임상검체에서 분리된 장알균 중 vancomycin에 내성을 보인 37주를 대상으로 하였다. VRE의 유전형 분석과 병독성 인자의 검출을 위하여 PCR을 시행하였고, 균주 간의 분자역학적인 관계를 알아보기 위해 MLST와 *esp* repeat profile을 통하여 분석하였다. 또한 염색체상의 QRDR에 존재하는 유전자인 *parC*와 *gyrA*의 유전자 변이를 조사하기 위해 염기서열 분석을 수행하였다.

결과: 항균제 감수성 양상과 VRE 유전형 확인 결과, VanA-표현형 *vanA* 유전형 VRE가 25주, VanB-표현형 *vanA* 유전형 VRE가 12주였다. MLST 분석에서는 5개의 ST를 확인하였다. 이 중 ST414와 CNS4 (4-1-1-1-1-1-1)는 국내에서 처음으로 발견되었는데, CC17에 속하는 것을 확인하였다. *Esp*와 *hyl* 유전자의 검출은 PCR 결과, 각각 100%와 86.5%로 양성반응을 보였고, *parC*와 *gyrA*에서의 유전적 변이는 37주 모두 가지고 있었다.

결론: ST414 및 CNS4 (4-1-1-1-1-1-1)를 포함하여 본 연구에서 분리된 모든 균주가 CC17에 속하는 것을 확인하였다. CC17은 전 세계적으로 토착화되려는 양상을 보이고 있는데, 이러한 확산을 방지하기 위하여 MLST와 *esp* repeat profile 분석은 장알균의 유전적 특성뿐만 아니라, 분리된 균주의 진화 과정 및 역학적 관계를 규명하는데 유용하게 사용될 수 있다.

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