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Clonal Distribution of Clindamycin-Resistant Erythromycin-Susceptible (CRES) *Streptococcus agalactiae* in Korea Based on Whole Genome Sequences

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Background: The clindamycin-resistant erythromycin-susceptible (CRES) phenotype is rare in *Streptococcus agalactiae* (group B streptococci). We aimed to determine the molecular characteristics of CRES *S. agalactiae* using whole genome sequencing (WGS).

Methods: Sixty-six *S. agalactiae* isolates obtained from blood (N=26), cerebrospinal fluid (N=10), urine (N=17), and vaginal discharge (N=13) between 2010 and 2017 in Korea were subjected to WGS. Based on the WGS data, we analyzed antimicrobial resistance (AMR) determinants, sequence types (STs), capsular polysaccharide (CPS) genotypes, and virulence gene profiles, and constructed a phylogenetic tree. We included the clindamycin-susceptible erythromycin-resistant (CSER) phenotype for comparison.

Results: We identified seven CRES *S. agalactiae* isolates from urine (N=5) and vaginal discharge (N=2) collected between 2010 and 2011. All CRES isolates harbored AMR determinants of *lnu*(B), *lsa*(E), and *aac*(6')-*aph*(2"), revealed ST19 and CPS genotype III, and had a virulence gene profile of *rib-lmb-cylE*. Phylogenetic tree analysis revealed that all CRES isolates belonged to the same cluster, suggesting a clonal distribution. In contrast, seven CSER isolates showed a diverse distribution and clustered separately from the CRES isolates.

Conclusions: CRES isolates collected between 2010 and 2011 showed a unique cluster with ST19 and CPS genotype III in Korea. This is the first report on WGS-based characteristics of *S. agalactiae* in Korea.

Key Words: *Streptococcus agalactiae*, Group B streptococci, Antimicrobial resistance, Whole genome sequencing, Sequence types, Clonal distribution, CRES (clindamycin-resistant erythromycin-susceptible)

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INTRODUCTION

Streptococcus agalactiae (group B β-hemolytic streptococci) can cause several invasive infections, including sepsis, infective endocarditis, septic arthritis, and meningitis, especially in neonates and the elderly [1, 2]. *S. agalactiae* infections are classified as invasive (blood, cerebrospinal fluid, joint fluid, pleural effusion, ascites, and closed pus) or non-invasive (urine and vagi-

nal discharge) [3]. Multilocus sequence typing (MLST) for sequence type (ST) determination has been used to evaluate the clonal distribution or persistence of *S. agalactiae* from urine and vagina [3].

S. agalactiae possesses numerous virulence factors, including capsular polysaccharide (CPS), alpha and beta antigens of the surface-associated C protein, and the surface protein Rib. CPS is the most important virulence factor and is used for strain typing [4]. Alpha antigen of the surface-associated C protein (encoding gene *bca*) mediates adherence to the epithelium, whereas beta antigen of the surface-associated C protein (encoding gene *bac*) is involved in invasiveness and resistance to phagocytic clearance [5, 6]. Protein Rib (encoding gene *rib*), which exhibits resistance to proteases, confers protective immunity and is detected in most CPS III isolates, which cause severe infections in neonates [5]. *lmb* and *cylE* encode human laminin binding protein and beta-hemolysin, respectively.

There are two major phenotypes of macrolide resistance in streptococci: an MLS_B phenotype is resistant to macrolides, lincosamides, and streptogramin B, and an M phenotype is resistant to macrolides, but not to lincosamides and streptogramin B [7]. The MLS_B phenotype in streptococci can result from induced/constitutive expression of the antimicrobial resistance (AMR) determinants, *erm*(A) and *erm*(B), whereas the M phenotype can be caused by the *mef*(A) determinant [7]. In addition, an L phenotype exists, which is resistant to lincosamides, but not to macrolides [8]. The clindamycin-resistant erythromycin-susceptible (CRES) phenotype corresponds to the L phenotype [9].

The CRES phenotype (L phenotype) is rare in *S. agalactiae*. It has been described in clinical isolates from Korea and is caused by antimicrobial modification mediated by *lnu*(B) [10]. Members of the *lnu* gene family encode nucleotidyl transferase enzymes that catalyze the adenylation of lincomycin and clindamycin. The CRES phenotype is also mediated by two genes of the *lsa* gene family, namely *lsa*(C) and *lsa*(E), which encode ATP-binding proteins that have been classified as class 2 ATP-binding cassette transporters (antibiotics efflux pumps) [11]. While the overall frequency of this phenotype in *S. agalactiae* was quite low (65/21,186=0.31%), it had increased in 2014–2015 in the USA [11].

We aimed to determine the genetic characteristics of CRES *S. agalactiae* in Korea based on whole genome sequencing (WGS). To the best of our knowledge, this is the first report on WGS-based characteristics of *S. agalactiae* in Korea.

MATERIALS AND METHODS

Study design

S. agalactiae isolates collected between 2010 and 2017 were randomly selected from the repository at Gyeongsang National University Hospital (GNUH) in Gyeongnam Province, Korea. We included a total of 66 isolates: invasive isolates from blood (N=26) and cerebrospinal fluid (CSF) (N=10) as well as non-invasive isolates from urine (N=17) and vaginal discharge (N=13); repeated isolates from the same patients were excluded. Bacterial identification was conducted using a Vitek-2 automated identification system (bioMerieux Inc., Marcy l'Etoile, France). All isolates were stored at –70°C to –80°C before being processed for further evaluation.

Patients' sex and age were obtained from the electronic medical records. In total, 66 patients with a median age of 50.5 years (range, 0–86 years), including 12 children, 54 adults, and 32 males (48.5%), were enrolled. The study protocol was approved by the Institutional Review Board of GNUH (approval number: GNUH 2016-03-010). Informed consent was waived because of the retrospective nature of the study.

Antimicrobial susceptibility testing (AST)

AST was conducted using 11 antimicrobial agents, including β-lactam, tetracycline, macrolide/lincosamide (ML), and fluoroquinolone, to evaluate AMR levels by the broth microdilution method using a Vitek-2 System and an ST-01 test kit (bioMerieux Inc.). CRES *S. agalactiae* was defined as having a minimum inhibitory concentration of >1 μg/mL for clindamycin and of <0.25 μg/mL for erythromycin. Seven isolates showed the CRES phenotype. We also included seven isolates showing the clindamycin-susceptible erythromycin-resistant (CSER) phenotype for comparison. In addition, we included 13 isolates that were clindamycin-resistant erythromycin-resistant, and 29 isolates that were clindamycin-susceptible erythromycin-susceptible. The seven CRES isolates were recovered from urine (N=5) and vaginal discharge (N=2) during a limited period (March 2010 to August 2011).

Bacterial identification and AST had been performed previously by routine microbiological procedures, whereas WGS and bioinformatics analysis had been conducted for this study.

WGS

S. agalactiae isolates were grown at 35°C in Todd-Hewitt broth (Becton Dickinson, Sparks, MD, USA) for 16–18 hours. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit

Table 1. Specimen, date of collection, and accession numbers of draft genome sequences of 66 isolates of *Streptococcus agalactiae*

Strain	Specimen	Date of specimen collection (yr/month)	Sex	Age (yr)	Accession numbers
GCH2	Blood	2016/03	M	75	WHUI00000000
GCH4	Blood	2016/05	F	83	WHUJ00000000
GCH5	Blood	2016/06	F	78	WHUK00000000
GCH7	Blood	2016/07	M	74	WHUL00000000
GCH8	Blood	2016/07	M	79	WACQ00000000
GCH9	Blood	2016/07	M	0	VYJX00000000
GCH10	Blood	2016/08	F	72	VYJI00000000
GCH11	Blood	2016/08	M	40	VYJJ00000000
GCH13	Blood	2016/10	M	64	VYJK00000000
GCH14	Blood	2016/12	M	67	VYJL00000000
GCH15	Blood	2016/12	M	76	VYJM00000000
GCH16	Blood	2017/01	M	43	VYJN00000000
GCH18	Blood	2017/02	M	77	VYJO00000000
GCH19	Blood	2017/02	M	60	VYJP00000000
GCH21	Blood	2017/03	F	85	VYJQ00000000
GCH22	Blood	2017/05	M	46	VYJR00000000
GCH25	Blood	2017/06	M	64	VYJS00000000
GCH26	Blood	2017/07	M	86	VYJT00000000
GCH28	Blood	2017/08	M	54	VYJU00000000
GCH29	Blood	2017/10	M	0	VYJV00000000
GCH30	Blood	2017/10	F	74	VYJW00000000
GCH32	Blood	2017/12	M	51	VYQL00000000
GCH33	Blood	2016/08	F	56	VYQM00000000
GCH34	Blood	2016/11	F	73	VYQN00000000
GCH35	Blood	2017/05	M	84	VYQO00000000
GCH36	Blood	2017/07	F	73	VYQP00000000
GCH37	CSF	2014/07	M	51	VYQQ00000000
GCH38	CSF	2014/10	M	0	VYQR00000000
GCH39	CSF	2014/12	M	0	VYQS00000000
GCH40	CSF	2015/08	F	0	VYQT00000000
GCH41	CSF	2015/08	F	0	VYQU00000000
GCH42	CSF	2016/06	M	50	VYQV00000000
GCH43	Urine	2017/02	M	32	VYQW00000000
GCH44	Urine	2017/02	F	56	VYQX00000000
GCH45	Urine	2017/05	F	84	VYQY00000000
GCH46	Urine	2017/07	M	61	VYQZ00000000
GCH47	Urine	2017/07	F	34	VYRA00000000
GCH48	Urine	2017/08	F	73	VYRB00000000
GCH49	Urine	2017/08	M	65	VYRC00000000
GCH50	Urine	2017/11	F	13	VYRD00000000

(*Continued to the next page*)

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Table 1. Continued

Strain	Specimen	Date of specimen collection (yr/month)	Sex	Age (yr)	Accession numbers
GCH51	Urine	2017/12	M	53	VYRE00000000
GCH53	Vaginal discharge	2016/04	F	34	VYRF00000000
GCH54	Vaginal discharge	2016/05	F	33	VYRG00000000
GCH55	Vaginal discharge	2016/10	F	33	VYRH00000000
GCH56	Vaginal discharge	2017/01	F	22	VYRI00000000
GCH57	Vaginal discharge	2017/01	F	33	VYRJ00000000
GCH58	Vaginal discharge	2017/02	F	37	VYRK00000000
GCH59	Vaginal discharge	2017/04	F	39	VYRL00000000
GCH60	Vaginal discharge	2017/07	F	33	VYRM00000000
GCH61	Urine	2010/03	M	51	VYRN00000000
GCH62	Vaginal discharge	2010/03	F	38	VYRO00000000
GCH63	Urine	2010/04	M	44	VYRP00000000
GCH64	Urine	2010/10	F	83	VYRQ00000000
GCH65	Urine	2010/12	F	49	VYRR00000000
GCH66	Vaginal discharge	2010/12	F	42	VYRS00000000
GCH67	Urine	2011/08	F	55	VYRT00000000
GCH68	CSF	2011/04	M	0	VYRU00000000
GCH70	CSF	2012/01	F	0	VYRW00000000
GCH71	CSF	2012/03	M	0	VYRX00000000
GCH72	CSF	2012/08	F	0	VYRY00000000
GCH73	Vaginal discharge	2014/07	F	33	WHUM00000000
GCH74	Vaginal discharge	2014/08	F	40	WHUN00000000
GCH75	Vaginal discharge	2016/02	F	25	WHUO00000000
GCH76	Urine	2015/11	F	79	WHUP00000000
GCH77	Urine	2016/02	M	60	WHUQ00000000
GCH78	Urine	2014/10	M	0	WHUR00000000

Abbreviations: CSF, cerebrospinal fluid; M, male; F, female.

(Qiagen, Hilden, Germany) after pretreatment with lysozyme (Thermo Fisher Scientific, Waltham, MA, USA) and proteinase K (Qiagen) [12]. *S. agalactiae* isolates were identified by 16S rRNA gene sequencing with amplifying/sequencing primer set (27F: AGAGTTTGATCMTGGCTCAG and 1485R: TACGGTTACCTTGTTACGAC) developed in-house using an ABI 3730 DNA sequencing instrument (Applied Biosystems, Foster City, CA, USA). The sequencing library was prepared using a TruSeq DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) for the Illumina MiSeq system. Draft genome sequences of the isolates were determined based on 300-bp paired-end reads. Illumina sequencing data were assembled using SPAdes 3.13.0 (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). For gene-finding and functional annotation, we used the whole genome analysis pipeline of ChunLab (Seoul, Korea). Protein-coding DNA sequences were predicted using Prodigal 2.6.2 (https://github.com/hyattpd/Prodigal) [13].

AMR genotyping

AMR genotyping was conducted based on the contig sequences obtained using ResFinder version 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) managed by the Center for Genomic Epidemiology [14]. This tool can detect genes conferring resistance to β-lactams, macrolide, lincosamide, tetracycline, quinolone, oxazolidinone, sulfonamide/trimethoprim, glycopeptide, aminoglycoside, phenicol, fosfomycin, nitroimidazole, rifampicin, fusidic acid, and colistin. AMR genotypes were determined based

on an identity threshold >90% and a minimum length of 60% as compared with the reference sequence in the database.

MLST

MLST (allelic profile: *adhP–pheS–atr–glnA–sdhA–glcK–tkt*) was conducted based on the contig sequences using the MLST server (https://cge.cbs.dtu.dk/services/MLST/) managed by the Center for Genomic Epidemiology [15]. The STs were grouped into clonal complexes (CCs), whereby related STs were classified as single-locus variants differing in only one housekeeping gene. An expansion of the goeBURST algorithm implemented in PHYLOViZ (http://www.phyloviz.net/) was used to produce a minimum-spanning tree representing possible relationships among the STs [16].

CPS genotyping

CPS genotyping and detection of the *S. agalactiae*-specific *dltS* gene were conducted based on the contig sequences by PCR simulation [17] in the online application, Serial Cloner (http://serialbasics.free.fr/Serial_Cloner.html). The CPS genotypes included Ia, Ib, II, III, IV, V, VI, VII, and VIII.

Virulence gene profiling

The presence of five virulence genes (*bca-rib-bac-lmb-cylE*) was determined based on the contig sequences by PCR simulation in Serial Cloner [18-20]. Sequence identity of the virulence genes in all simulation PCR-positive strains was confirmed using the basic local alignment search tool (BLAST) (http://blast.ddbj.nig.ac.jp/blastn?lang=ja).

Phylogenetic tree analysis

A phylogenetic tree was constructed using Orthologous Average Nucleotide Identity Tool, which measures similarity among multiple genome sequences based on the OrthoANI algorithm and BLAST calculations, on EZBioCloud (https://www.ezbiocloud.net/tools/orthoani) [21].

Statistical analysis

We used Fisher's exact probability tests (two-sided) to determine significant differences between CRES and CSER isolates using SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ indicated statistical significance.

RESULTS

We deposited the draft genome sequences of the 66 *S. agalactiae* isolates into the National Center for Biotechnology Information (NCBI) database (Table 1). The WGS of the *S. agalactiae* isolate NCTC8181 (accession number UAVB00000000) obtained from environmental milk was used as a reference genome. The

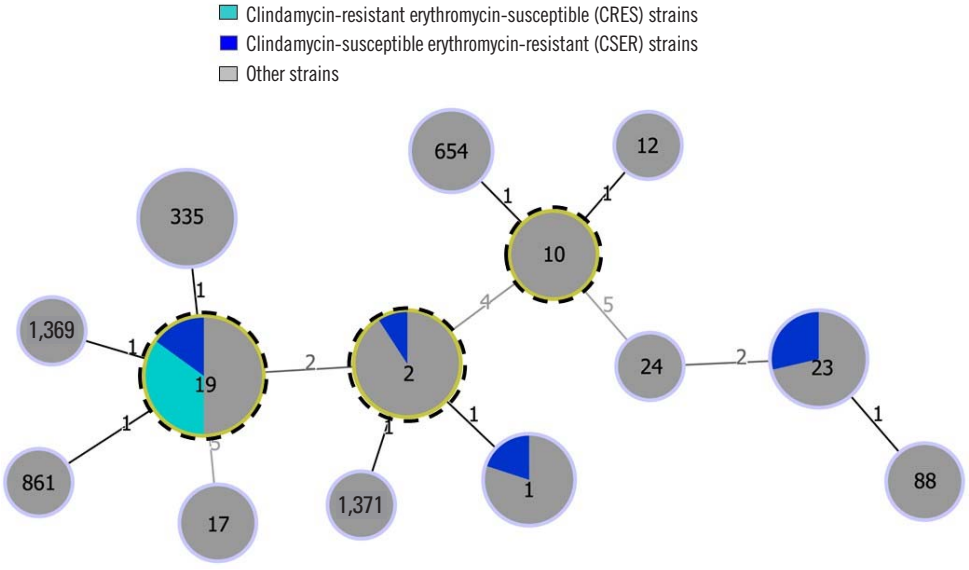

Fig. 1. goeBURST diagram of the relationships among STs of 66 *S. agalactiae* isolates. The numbers in the circles indicate the STs, and the numbers near the lines indicate the number of differing alleles between the two connected STs. Putative CCs are identified by an outer dotted frame and correspond to the STs with the highest number of single locus variants.
Abbreviations: CC, clonal complex; ST, sequence type.

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Table 2. Phenotypic and genotypic features of *S. agalactiae* for AMR, CPS, ST, and virulence

Isolate	Antimicrobial susceptibility pattern	Macrolide/lincosamide resistance gene	Tetracycline resistance gene	Aminoglycoside resistance gene	ST	CPS genotype	Virulence gene profile
GCH2	CRER	*erm*(B), *lnu*(B), *lsa*(E), *mre*(A)	*tet*(O)	*ant*(6)-Ia, *ant*(6)-Ia, *aph*(3')-III	12	Ib	*bca-bac*-lmb-cylE*
GCH4	CSES	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH5	CSES	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH7	CSES	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH8	CSES	*mre*(A)			654	Ib	*bca-bac*-lmb-cylE*
GCH9	CRER	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH10	CIES	*mre*(A)	*tet*(M)		23	Ia	*lmb-cylE*
GCH11	CSES	*mre*(A)	*tet*(M)		23	Ia	*lmb-cylE*
GCH13	CSES	*mre*(A)			1,371	VIII	*rib-lmb-cylE*
GCH14	CRER	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH15	CSES	*mre*(A)			2	VIII	*rib-cylE*
GCH16	CSES	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH18	CSES	*lsa*(C), *mre*(A)	*tet*(M)		23	Ia	*lmb-cylE*
GCH19	CREI	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH21	CREI	*lsa*(C), *mre*(A)	*tet*(M)		23	Ia	*lmb-cylE*
GCH22	CSES	*mre*(A)			88	II	*lmb-cylE*
GCH25	CSES	*mre*(A)			1	VI	*bca-lmb-cylE*
GCH26	CSES	*mre*(A)	*tet*(O)		2	VIII	*rib-lmb-cylE*
GCH28	CSES	*mre*(A)	*tet*(M)		19	III	*rib-lmb-cylE*
GCH29	CSES	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH30	CRER	*erm*(B), *mre*(A)	*tet*(M)		1	V	*lmb-cylE*
GCH32	CSES	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH33	CRER	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH34	CSES	*mre*(A)	*tet*(M)		24	Ia	*bca-lmb-cylE*
GCH35	CSES	*mre*(A)			10	Ib	*bca-bac*-lmb-cylE*
GCH36	CSES	*mre*(A)			10	Ib	*bca-bac*-lmb-cylE*
GCH37	NA	*mre*(A)			88	Ia	*lmb-cylE*
GCH38	CREI	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	1,369	III	*bca-lmb-cylE*
GCH39	CSES	*mre*(A)	*tet*(M)		17	III	*rib-cylE*
GCH40	CRER	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH41	CRER	*erm*(B), *mre*(A)	*tet*(O)	*ant*(6)-Ia, *ant*(6)-Ia, *aph*(3')-III	17	III	*rib-cylE*
GCH42	CSES	*mre*(A)			2	VIII	*rib-cylE*
GCH43	CREI	*lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *ant*(6)-Ia, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH44	CSEI	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH45	CSER	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH46	CSES	*mre*(A)			654	Ib	*bca-bac*-lmb-cylE*
GCH47	CRER	*erm*(A), *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	V	*cylE*
GCH48	CRER	*erm*(A), *mre*(A)	*tet*(M)		1	V	*lmb-cylE*

(*Continued to the next page*)

Table 2. Continued

Isolate	Antimicrobial susceptibility pattern	Macrolide/lincosamide resistance gene	Tetracycline resistance gene	Aminoglycoside resistance gene	ST	CPS genotype	Virulence gene profile
GCH49	CRER	*lsa*(C), *mef*(A), *mre*(A), *msr*(D)	*tet*(O)		861	III	*rib-lmb-cylE*
GCH50	CREI	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *ant*(6)-Ia, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH51	CRER	*erm*(B), *mre*(A)			10	Ib	*bca-bac*-lmb-cylE*
GCH53	CSES	*mre*(A)	*tet*(M)		1	II	*lmb-cylE*
GCH54	CSES	*mre*(A)			10	Ib	*bca-bac*-lmb-cylE*
GCH55	CREI	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *ant*(6)-Ia, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH56	CSES	*mre*(A)			2	VIII	*rib-lmb-cylE*
GCH57	CRER	*mre*(A)			654	Ib	*bca-bac*-lmb-cylE*
GCH58	CREI	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH59	CRER	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH60	CSES	*mre*(A)	*tet*(M)		19	III	*rib-lmb-cylE*
GCH61	CRES	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH62	CRES	*lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH63	CRES	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *ant*(6)-Ia, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH64	CRES	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH65	CRES	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH66	CRES	*lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH67	CRES	*erm*(B)*, *lnu*(B), *lsa*(E), *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH68	CSES	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH70	CSES	*erm*(A), *mre*(A)	*tet*(M)		335	III	*rib-lmb-cylE*
GCH71	CSES	*mre*(A)	*tet*(M)		23	Ia	*lmb-cylE*
GCH72	CSES	*erm*(B)*, *mre*(A)	*tet*(M)	*aac*(6')-*aph*(2"), *ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*
GCH73	CSER	*mre*(A)			1	VI	*bca-lmb-cylE*
GCH74	CSER	*mre*(A)	*tet*(M)		19	III	*rib-lmb-cylE*
GCH75	CSER	*mef*(A), *mre*(A), *msr*(D)	*tet*(M)		23	Ia	*lmb-cylE*
GCH76	CSER	*erm*(A), *mre*(A)	*tet*(M)		19	V	*lmb-cylE*
GCH77	CSER	*mef*(A), *mre*(A), *msr*(D)	*tet*(M)		23	Ia	*lmb-cylE*
GCH78	CSER	*erm*(B)*, *mef*(A), *mre*(A), *msr*(D)	*tet*(M)	*ant*(6)-Ia*, *aph*(3')-III	19	III	*rib-lmb-cylE*

*Identical nucleotide length < 100% of the reference sequence in the database.
Abbreviations: AMR, antimicrobial resistance; CPS, capsular polysaccharide; CRER, clindamycin-resistant erythromycin-resistant; CSES, clindamycin-susceptible erythromycin-susceptible; CIES, clindamycin-intermediate erythromycin-susceptible; CREI, clindamycin-resistant erythromycin-intermediate; CSEI, clindamycin-susceptible erythromycin-intermediate; CSER, clindamycin-susceptible erythromycin-resistant; CRES, clindamycin-resistant erythromycin-susceptible; ST, sequence type.

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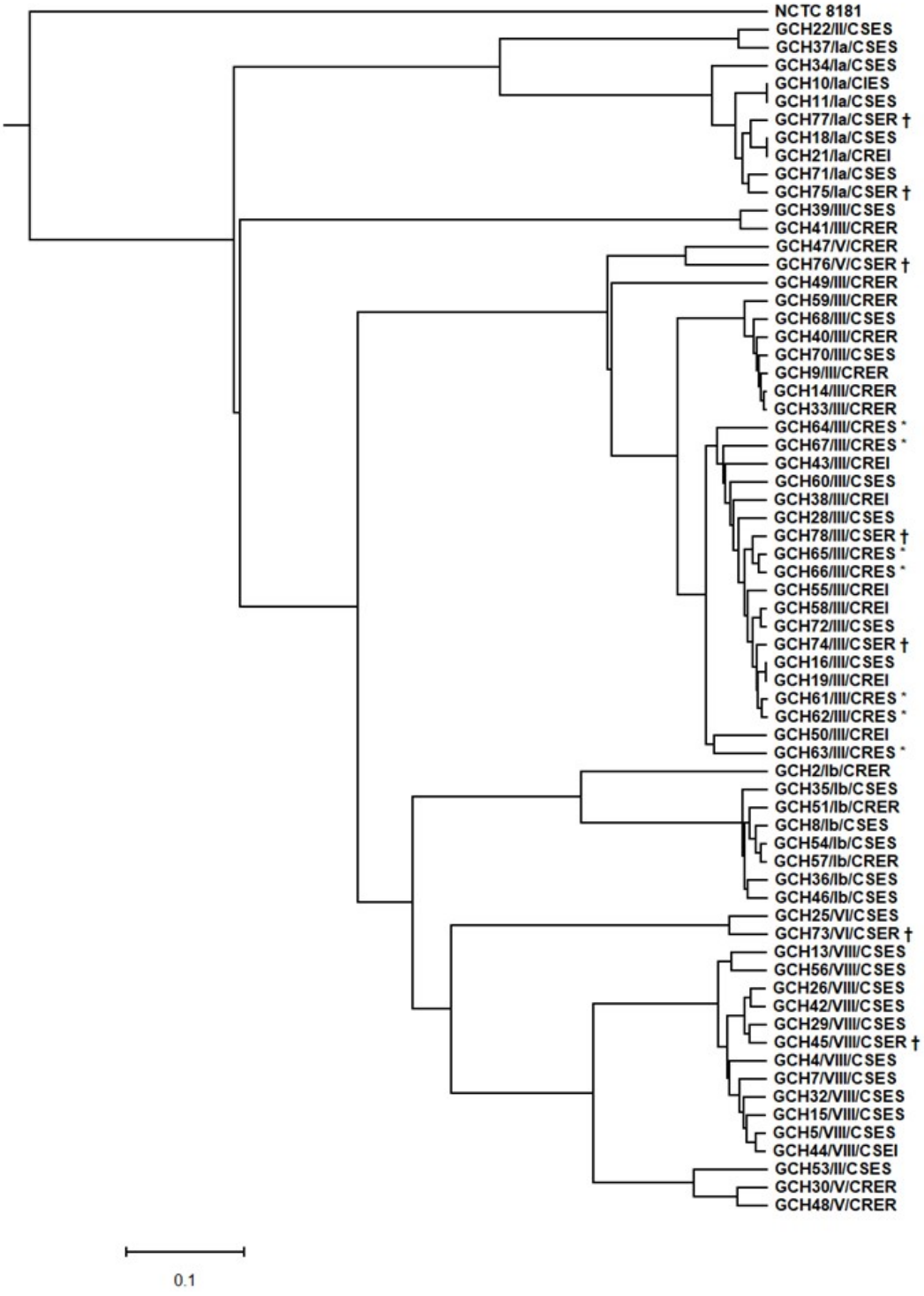

Fig. 2. Phylogenetic tree of 66 *S. agalactiae* isolates. The phylogenetic tree was constructed using OAT, based on the OrthoANI algorithm, with *S. agalactiae* strain NCTC8181 (accession number UAVB00000000) as a reference. Asterisks (*) indicate CRES isolates, daggers (†) indicate CSER isolates. There was a concordance of the group distribution on the tree with the CPS genotype distribution (Ia, Ib, III, V, and VIII).
Abbreviations: CPS, capsular polysaccharide; CRES, clindamycin-resistant erythromycin-susceptible; CSER, clindamycin-susceptible erythromycin-resistant; CRER, clindamycin-resistant erythromycin-resistant; CSES, clindamycin-susceptible erythromycin-susceptible; CSEI, clindamycin-susceptible erythromycin-intermediate; CREI, clindamycin-resistant erythromycin-intermediate; CIES, clindamycin-intermediate erythromycin-susceptible; OAT, Orthologous Average Nucleotide Identity Tool.

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NUBL-9601_erm_B_    ATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACTCAACCAA 60
GCH61_erm_B_        ATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACTCAACCAA 60
KMP104_erm_B_       ATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACTCAACCAA 60
                    ************************************************************

NUBL-9601_erm_B_    ATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAA 120
GCH61_erm_B_        ATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAA 120
KMP104_erm_B_       ATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAA 120
                    ************************************************************

NUBL-9601_erm_B_    GGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGAC 180
GCH61_erm_B_        GGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGAC 180
KMP104_erm_B_       GGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGAC 180
                    ************************************************************

NUBL-9601_erm_B_    AGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTAATT 240
GCH61_erm_B_        AGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTAATT 240
KMP104_erm_B_       AGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAACATTCGTGTCACTTTAATT 240
                    ****************************************** * ****************

NUBL-9601_erm_B_    CACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGT 300
GCH61_erm_B_        CACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGT 300
KMP104_erm_B_       CACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAAT 300
                    ************************************************************ *

NUBL-9601_erm_B_    ATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCT 360
GCH61_erm_B_        ATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCT 360
KMP104_erm_B_       ATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCT 360
                    ************************************************************

NUBL-9601_erm_B_    GACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACA 420
GCH61_erm_B_        GACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACA 420
KMP104_erm_B_       GACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACA 420
                    ************************************************************

NUBL-9601_erm_B_    CTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAA 480
GCH61_erm_B_        CTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAA 480
KMP104_erm_B_       CTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAA 480
                    ************************************************************

NUBL-9601_erm_B_    TGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACA 540
GCH61_erm_B_        TGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACA 540
KMP104_erm_B_       TGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACA 540
                    ************************************************************

NUBL-9601_erm_B_    GATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGA 600
GCH61_erm_B_        GATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGA 600
KMP104_erm_B_       GATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGA 600
                    ************************************************************

NUBL-9601_erm_B_    GAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCGGTTCTGTTGCAAAGTTTT 660
GCH61_erm_B_        GAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCGGTTCTGTTGCAAAGTTTT 660
KMP104_erm_B_       GAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAAT------GAAACACGCC 654
                    ****************************************  *     * **

NUBL-9601_erm_B_    AAATCTACTA--TCAAATAAGGTAGAATAATAGAAAAAGATAGC--AGGAGGAATGACGA 716
GCH61_erm_B_        AAATCTACTA--TCAAATAAGGTAGAATAATAGAAAAAGATAGC--AGGAGGAATGACGA 716
KMP104_erm_B_       AAAGTAAACAATTTAAGTACCGTT-ACTTATGAGCAAGTATTGTCTATTTTTAATAGTTA 713
                    ***   *  *  * ** **  **  * * **    **  ** *   *    ***    *

NUBL-9601_erm_B_    TGAATCATTTTAAAGGA--AAGCA- 738
GCH61_erm_B_        TGAATCATTTTAAAGGA--AAGCA- 738
KMP104_erm_B_       TCTATTATTTAACGGGAGGAAATAA 738
                    *  ** **** *  ***  **  *
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Fig. 3. Comparison of sequences of GCH61, NUBL-9601, and KMP104. Sequence of *erm*(B) from isolate GCH61 (accession number VYRN00000000) with the CRES phenotype contained C222T (N74N), T224C (I75T), and A299G (N100S) nucleotide (amino acid) substitutions, in addition to the insertion of an IS*1216E* element at nucleotide position 642, which resulted in the deletion of a segment spanning nucleotides 642–738 (97 bp). This sequence was identical to that from the CRES isolate NUBL-9601 (accession number LC430933). The sequence of *erm*(B) from the isolate KMP104 was used as a reference (RefSeq accession number DQ355148).
Abbreviation: CRES, clindamycin-resistant erythromycin-susceptible.

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number of contigs ranged from eight (for isolate GCH73) to 90 (for isolate GCH63).

The goeBURST diagram is shown in Fig. 1. All seven CRES isolates belonged to ST19 (CC19), suggesting a clonal distribution of the CRES isolates (Table 2), whereas the seven CSER isolates belonged to ST19 (CC19) (N=3), ST2/ST1 (CC2) (N=2), or ST23 (N=2).

All CRES isolates showed the *lnu*(B)-*lsa*(E) ML resistance genotype (Table 2). However, none of the CSER isolates possessed the *lnu*(B)-*lsa*(E) genotype (P=0.001). All CRES isolates showed the *aac*(6')-*aph*(2") aminoglycoside resistance genotype, whereas none of the CSER isolates did. All isolates did not show AMR genes for β-lactams, quinolone, oxazolidinone, sulfonamide/trimethoprim, glycopeptide, phenicol (except for *cat*[pC194] in isolate GCH2), fosfomycin, nitroimidazole, rifampicin, fusidic acid, and colistin.

All isolates possessed the *dltS* gene specific to *S. agalactiae*, validating species identification. All CRES isolates were CPS III, whereas the CSER isolates possessed diverse CPS types (Table 2).

All CRES isolates exhibited the *rib-lmb-cylE* profile (Table 2). The CSER isolates had a diverse virulence gene profile. There was a significant difference in the frequency of *lnu*(B)-*lsa*(E) or *lsa*(C) between invasive (N=6/36, 16.7%) and non-invasive (N=13/30, 43.3%, P=0.017) isolates. There was no difference in the frequency of *lnu*(B)-*lsa*(E) or *lsa*(C) between urine (N=9/17, 52.9%) and vaginal discharge (N=4/13, 30.8%, P=0.200).

The phylogenetic tree revealed that all CRES isolates belonged to the same group, whereas CSER isolates belonged to diverse groups, corroborating the clonal distribution of the CRES isolates (Fig. 2). The group distribution on the tree was in accordance with the CPS genotype distribution (e.g., Ia, Ib, III, V, and VIII).

The *erm*(B) sequence of the CRES isolate GCH61 in our study was compared with the previously registered sequence (738 bp) of *S. agalactiae* isolate KMP104 (RefSeq accession number DQ355148). This sequence (accession number LC512876) contained C222T (N74N), T224C (I75T), and A299G (N100S) nucleotide (amino acid) substitutions in addition to the insertion of an IS*1216E* element at nucleotide position 642, which resulted in the deletion of a segment spanning nucleotides 642–738 (97 bp) (Fig. 3).

DISCUSSION

Our study revealed that CRES isolates have unique features compared with CSER isolates, including their AMR genotype [*lnu*(B)-*lsa*(E) with *aac*(6')-*aph*(2")], ST19/CC19, CPS type III, virulence gene profile of *rib-lmb-cylE*, and in terms of cluster on the phylogenetic tree.

We searched for the presence of CRES *S. agalactiae* isolates in the Isolates Database on the MLST website (https://pubmlst.org/bigsdb?db=pubmlst_sagalactiae_isolates&page=query). Interestingly, only one CRES isolate was previously recovered from a 61-year-old female patient with bacteremia in Kangwon Province in the north of Korea in 2010 [22]. Another CRES isolate of CPS genotype III was isolated from a patient with bacteremia in Bergen, Norway, in 2010 [23]. Three CRES isolates of serotype III were recovered from clinical specimens in Seoul during 2010–2013 [9, 24]. These findings are in line with our observation that all CRES isolates in Gyeongnam Province in the south of Korea were recovered between March 2010 and August 2011. Thus, the CRES isolates appeared to be epidemic in Korea and other countries during this limited period.

In line with a previous study [11], we found a significant difference in the distribution of AMR genotype of *lnu*(B)-*lsa*(E) between the CRES and CSER isolates. For all CRES isolates, the *lnu*(B) locus was adjacent to the *lsa*(E) locus within the same contigs, with a short 53-bp distance between these two loci. Therefore, in our study, the *lnu*(B)-*lsa*(E) gene combination seems to mainly contribute to the CRES phenotype. Further observation of the dynamic changes in the CRES phenotype and the corresponding gene transfer is needed.

The *erm*(B) confers constitutive resistance (cMLS$_B$) through a conformational change in 23S ribosomal RNA methyltransferase [8]. Deletion of a segment in *erm*(B) sequence in CRES suggests loss of function of the *erm*(B) protein in this isolate, resulting in an erythromycin-susceptible phenotype. Interestingly, three CRES isolates (NUBL-9601, NUBL-9602, and NUBL-9603) isolated at a hospital in Seoul during 2010–2013 had the identical sequences (accession numbers LC430933, LC430934, and LC430935) (3) [9, 10]. Furthermore, we observed the IS*1216E* insertion in *erm*(B) (accession numbers LC512881, LC512882, LC512883, LC512885, and LC512886) in five isolates (GCH16, GCH19, GCH38, GCH55, and GCH58, respectively) of this study. Thus, IS*1216E* seems to be common among CRES isolates in Korea.

Four CRES isolates (GCH63, GCH64, GCH65, and GCH67) in our study possessed truncated variant sequences of *erm*(B) (624 and 678 bps) due to insertion of a TAA stop codon into the open reading frame (accession numbers LC512877, LC512878, LC512879, and LC512880), suggesting that an immature *erm*(B) protein leads to the erythromycin-susceptible phenotype. Three other phenotypes (GCH50, GCH72, and GCH78) also had trun-

cated variant sequences (660, 678, and 510 bps, respectively) (accession numbers LC512884, LC512887, and LC512888, respectively) harboring the erythromycin-susceptible phenotype. Therefore, we may explain the mechanism of the presence of *erm*(B)-gene with the erythromycin-susceptible phenotype in CRES *S. agalactiae*.

This study has several limitations. First, clinical data, such as antibiotic treatment, outcome, and complications, does not suffice to demonstrate the relationship with the AMR genotype or virulence gene profile. Second, we cannot explain why the clonal outbreak occurred only during a limited period and is absent nowadays. Third, we did not determine translational activities and enzymatic functions of the IS*1216E* insertion-*erm*(B) and the truncated variant sequences.

In conclusion, CRES isolates were obtained during a limited period (2010–2011) and showed a genetic cluster having ST19 and CPS III in Korea as revealed by WGS. This rare AMR phenotype should be meticulously monitored, and the therapeutic efficacy of optimal antibiotics should be further evaluated.

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AUTHOR CONTRIBUTIONS

TT and SK contributed to the study concept and design, and prepared and revised the manuscript. D-HL collected the bacterial strains and patient information. TM analyzed bioinformatics data and interpreted the acquired results. SL analyzed WGS data and submitted the draft genome sequences to the NCBI database.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article were reported.

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