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)

**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-
We aimed to determine the genetic characteristics of CRES \textit{S. agalactiae} from urine and vagina [3]. Multilocus sequence typing (MLST) for sequence type (ST) determination has been used to evaluate the clonal distribution or persistence of \textit{S. agalactiae} from urine and vagina [3].

\textit{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 \textit{bca}) mediates adherence to the epithelium, whereas beta antigen of the surface-associated C protein (encoding gene \textit{bac}) is involved in invasiveness and resistance to phagocytic clearance [5, 6]. Protein Rib (encoding gene \textit{rib}), which exhibits resistance to proteases, confers protective immunity and is detected in most CPS III isolates, which cause severe infections in neonates [5]. \textit{Imb} and \textit{cylE} encode human laminin binding protein and beta-hemolysin, respectively.

There are two major phenotypes of macrolide resistance in streptococci: an MLS\textsubscript{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\textsubscript{B} phenotype in streptococci can result from induced/constitutive expression of the antimicrobial resistance (AMR) determinants, \textit{erm(A)} and \textit{erm(B)}, whereas the M phenotype can be caused by the \textit{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 \textit{S. agalactiae}. It has been described in clinical isolates from Korea and is caused by antimicrobial modification mediated by \textit{Inu(B)} [10]. Members of the \textit{Inu} gene family encode nucleotidyl transferase enzymes that catalyze the adenylation of lincosycin and clindamycin. The CRES phenotype is also mediated by two genes of the \textit{Isa} gene family, namely \textit{Isa(C)} and \textit{Isa(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 \textit{S. agalactiae} was quite low (65/21,186=0.31%), it had increased in 2014–2015 in the overall frequency of this phenotype in Korea [11].

We aimed to determine the genetic characteristics of CRES \textit{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 \textit{S. agalactiae} in Korea.

**MATERIALS AND METHODS**

**Study design**

\textit{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 (bioMérieux 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 (bioMérieux Inc.). CRES \textit{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**

\textit{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.
| Strain | Specimen | Date of specimen collection (yr/month) | Sex | Age (yr) | Accession numbers |
|--------|----------|---------------------------------------|-----|----------|-------------------|
| GCH2   | Blood    | 2016/03                              | M   | 75       | WHUJ0000000000    |
| GCH4   | Blood    | 2016/05                              | F   | 83       | WHUJ0000000000    |
| GCH5   | Blood    | 2016/06                              | F   | 78       | WHUK0000000000    |
| GCH7   | Blood    | 2016/07                              | M   | 74       | WHUL0000000000    |
| GCH8   | Blood    | 2016/07                              | M   | 79       | WACQ0000000000    |
| GCH9   | Blood    | 2016/07                              | M   | 0        | VYJO0000000000    |
| GCH10  | Blood    | 2016/08                              | F   | 72       | VYJO0000000000    |
| GCH11  | Blood    | 2016/08                              | M   | 40       | VYJO0000000000    |
| GCH13  | Blood    | 2016/10                              | M   | 64       | VYJK0000000000    |
| GCH14  | Blood    | 2016/12                              | M   | 67       | VYJL0000000000    |
| GCH15  | Blood    | 2016/12                              | M   | 76       | VYJM0000000000    |
| GCH16  | Blood    | 2017/01                              | M   | 43       | VYJN0000000000    |
| GCH18  | Blood    | 2017/02                              | M   | 77       | VYJO0000000000    |
| GCH19  | Blood    | 2017/02                              | M   | 60       | VYJP0000000000    |
| GCH21  | Blood    | 2017/03                              | F   | 85       | VYJQ0000000000    |
| GCH22  | Blood    | 2017/05                              | M   | 46       | VYJR0000000000    |
| GCH25  | Blood    | 2017/06                              | M   | 64       | VYJS0000000000    |
| GCH26  | Blood    | 2017/07                              | M   | 86       | VYJT0000000000    |
| GCH28  | Blood    | 2017/08                              | M   | 54       | VYJU0000000000    |
| GCH29  | Blood    | 2017/10                              | M   | 0        | VYJV0000000000    |
| GCH30  | Blood    | 2017/10                              | F   | 74       | VYJW0000000000    |
| GCH32  | Blood    | 2017/12                              | M   | 51       | VYJQ0000000000    |
| GCH33  | Blood    | 2016/08                              | F   | 56       | VYQM0000000000    |
| GCH34  | Blood    | 2016/11                              | F   | 73       | VYQN0000000000    |
| GCH35  | Blood    | 2017/05                              | M   | 84       | VYQO0000000000    |
| GCH36  | Blood    | 2017/07                              | F   | 73       | VYQP0000000000    |
| GCH37  | CSF      | 2014/07                              | M   | 51       | VYQQ0000000000    |
| GCH38  | CSF      | 2014/10                              | M   | 0        | VYQR0000000000    |
| GCH39  | CSF      | 2014/12                              | M   | 0        | VYQS0000000000    |
| GCH40  | CSF      | 2015/08                              | F   | 0        | VYQT0000000000    |
| GCH41  | CSF      | 2015/08                              | F   | 0        | VYQU0000000000    |
| GCH42  | CSF      | 2016/06                              | M   | 50       | VYQV0000000000    |
| GCH43  | Urine    | 2017/02                              | M   | 32       | VYQW0000000000    |
| GCH44  | Urine    | 2017/02                              | F   | 56       | VYX0000000000    |
| GCH45  | Urine    | 2017/05                              | F   | 84       | VYX0000000000    |
| GCH46  | Urine    | 2017/07                              | M   | 61       | VYX0000000000    |
| GCH47  | Urine    | 2017/07                              | F   | 34       | VYRA0000000000    |
| GCH48  | Urine    | 2017/08                              | F   | 73       | VYRB0000000000    |
| GCH49  | Urine    | 2017/08                              | M   | 65       | VYRC0000000000    |
| GCH50  | Urine    | 2017/11                              | F   | 13       | VYRD0000000000    |

(Continued to the next page)
### 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       | VYRR00000000      |
| 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: TACGGTTACCTTGCTTGAC) 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 Chun-Lab (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
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                                | ert(B), lnu(B), Isa(E), mre(A)         | tet(O)                      | ant(6)-la, ant(6)-la, 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                                | ert(A), mre(A)                          | tet(M)                      |                                | 335| III          | rib-lmb-cylE          |
| GCH10   | CES                                 | mre(A)                                 | tet(M)                      |                                | 23 | la           | lmb-cylE              |
| GCH11   | CSES                                | mre(A)                                 | tet(M)                      |                                | 23 | la           | lmb-cylE              |
| GCH13   | CSES                                | mre(A)                                 |                             |                                | 1,371| VIII      | rib-lmb-cylE          |
| GCH14   | CRER                                | ert(A), mre(A)                          | tet(M)                      |                                | 335| III          | rib-lmb-cylE          |
| GCH15   | CSES                                | mre(A)                                 |                             |                                | 2  | VIII         | rib-cylE              |
| GCH16   | CSES                                | ert(B)*, lnu(B), Isa(E), mre(A)         | tet(M)                      | aac(6')-aph(2''), ant(6)-la*, ant(6)-la, aph(3')-III | 19 | III          | rib-lmb-cylE          |
| GCH18   | CSES                                | Isa(C), mre(A)                          | tet(M)                      |                                | 23 | la           | lmb-cylE              |
| GCH19   | CREI                                | ert(B)*, lnu(B), Isa(E), mre(A)         | tet(M)                      | aac(6')-aph(2''), ant(6)-la*, ant(6)-la, aph(3')-III | 19 | III          | rib-lmb-cylE          |
| GCH21   | CREI                                | Isa(C), mre(A)                          | tet(M)                      |                                | 23 | la           | 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                                | ert(B), mre(A)                          | tet(M)                      |                                | 1  | V            | lmb-cylE              |
| GCH32   | CSES                                | mre(A)                                 |                             |                                | 2  | VIII         | rib-lmb-cylE          |
| GCH33   | CREI                                | ert(B), mre(A)                          | tet(M)                      |                                | 335| III          | rib-lmb-cylE          |
| GCH34   | CSES                                | mre(A)                                 | tet(M)                      |                                | 24 | la           | 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 | la           | lmb-cylE              |
| GCH38   | CREI                                | ert(B)*, lnu(B), Isa(E), mre(A)         | tet(M)                      | aac(6')-aph(2''), ant(6)-la*, ant(6)-la, aph(3')-III | 1,369| III | bca-lmb-cylE     |
| GCH39   | CSES                                | mre(A)                                 | tet(M)                      |                                | 17 | III          | rib-cylE              |
| GCH40   | CRER                                | ert(A), mre(A)                          | tet(M)                      |                                | 335| III          | rib-lmb-cylE          |
| GCH41   | CRER                                | ert(B), mre(A)                          | tet(O)                      | ant(6)-la, ant(6)-la, aph(3')-III | 17 | III          | rib-cylE              |
| GCH42   | CSES                                | mre(A)                                 |                             |                                | 2  | VIII         | rib-cylE              |
| GCH43   | CREI                                | lnu(B), Isa(E), mre(A)                  | tet(M)                      | aac(6')-aph(2''), ant(6)-la*, ant(6)-la, 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                                | ert(A), lnu(B), Isa(E), mre(A)          | tet(M)                      | aac(6')-aph(2''), ant(6)-la*, ant(6)-la, aph(3')-III | 19 | V            | cyle                  |
| GCH48   | CRER                                | ert(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                               | *isa*(C), *mef*(A), *mre*(A), *msr*(D) | tet(O)                    |                                 | 861 | III            | rib-lmb-cylE          |
| GCH50    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH51    | CRER                               | *erm*(B), *mre*(A)                    |                                 |                                 | 10  | lb             | bca-bac*-lmb-cylE     |
| GCH52    | CRES                               | *mre*(A)                              |                                 |                                 | 1   | II             | lmb-cylE              |
| GCH53    | CRES                               | *mre*(A)                              |                                 |                                 | 10  | lb             | bca-bac*-lmb-cylE     |
| GCH54    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH55    | CRES                               | *mre*(A)                              |                                 |                                 | 2   | VIII           | rib-lmb-cylE          |
| GCH57    | CRER                               | *mre*(A)                              |                                 |                                 | 654 | lb             | bca-bac*-lmb-cylE     |
| GCH58    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH59    | CRER                               | *erm*(A), *mre*(A)                    | tet(M)                    |                                 | 335 | III            | rib-lmb-cylE          |
| GCH60    | CRES                               | *mre*(A)                              |                                 |                                 | 19  | III            | rib-lmb-cylE          |
| GCH61    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH62    | CRES                               | *lnu*(B), *isa*(E), *mre*(A)          | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH63    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH64    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH65    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH66    | CRES                               | *lnu*(B), *isa*(E), *mre*(A)          | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH67    | CRES                               | *erm*(B)*+, *lnu*(B), *isa*(E), *mre*(A) | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH68    | CRES                               | *erm*(A), *mre*(A)                    | tet(M)                    |                                 | 335 | III            | rib-lmb-cylE          |
| GCH69    | CRES                               | *erm*(A), *mre*(A)                    | tet(M)                    |                                 | 335 | III            | rib-lmb-cylE          |
| GCH70    | CRES                               | *mre*(A)                              |                                 |                                 | 23  | la             | lmb-cylE              |
| GCH71    | CRES                               | *mre*(A)                              |                                 |                                 | 23  | la             | lmb-cylE              |
| GCH72    | CRES                               | *erm*(B)*+, *mre*(A)                  | tet(M)                    | *aac*(6’)-*aph*(2’)*, *ant*(6)-la*+, *ant*(6)-la, *aph*(3’)-III | 19  | III            | rib-lmb-cylE          |
| GCH73    | CSER                               | *mre*(A)                              |                                 |                                 | 1   | VI             | bca-lmb-cylE          |
| GCH74    | CSER                               | *mre*(A)                              |                                 |                                 | 19  | III            | rib-lmb-cylE          |
| GCH75    | CSER                               | *mef*(A), *mre*(A), *msr*(D)          | tet(M)                    |                                 | 23  | la             | 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  | la             | lmb-cylE              |
| GCH78    | CSER                               | *erm*(B)*+, *mef*(A), *mre*(A), *msr*(D) | tet(M)                    | *ant*(6)-la*+, *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; CRES, 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.
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; CREI, clindamycin-resistant erythromycin-intermediate; CSEI, clindamycin-susceptible erythromycin-intermediate; CIES, clindamycin-intermediate erythromycin-susceptible; OAT, Orthologous Average Nucleotide Identity Tool.
**Abbreviation:** CRES, clindamycin-resistant erythromycin-susceptible.

Sequence of nucleotides 642–738 (97 bp). This sequence was identical to that from the CRES isolate NUBL-9601 (accession number LC430933). The tuitions, in addition to the insertion of an IS element at nucleotide position 642, which resulted in the deletion of a segment spanning 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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**Fig. 3.** Comparison of sequences of GCH61, NUBL-9601, and KMP104. Sequence of **erm** from isolate GCH61 (accession number VYRN00000000) with the CRES phenotype contained C222T (N74N), T224C (I75T), and A299G (N100S) nucleotide (amino acid) changes from isolate GCH61 (accession number VYRN00000000) with the CRES phenotype.

*B* from the isolate KMP104 was used as a reference (RefSeq accession number DQ355148).
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 \textit{lnu}(B)-\textit{lsa}(E) ML resistance genotype (Table 2). However, none of the CSER isolates possessed the \textit{lnu}(B)-\textit{lsa}(E) genotype (P=0.001). All CRES isolates showed the aac(6\textsuperscript{'})-aph(2\textsuperscript{''}) amino
glycocide resistance genotype, whereas none of the CSER isolates did. All isolates did not show AMR genes for \(\beta\)-lactams, quinolone, oxazolidinone, sulfonamide/tri
methoprim, glycopeptide, phenicol (except for \textit{cat}(pC194) in isolate GCH2), fosfomycin, nitroimidazole, rifampicin, fusidic acid, and colistin.

All isolates possessed the \textit{dltS} gene specific to \textit{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 \textit{rib-lmb-cylE} profile (Table 2). The CSER isolates had a diverse virulence gene profile. There was a significant difference in the frequency of \textit{lnu}(B)-\textit{lsa}(E) or \textit{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 \textit{lnu}(B)-\textit{lsa}(E) or \textit{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 \textit{erm}(B) sequence of the CRES isolate GCH61 in our study was compared with the previously registered sequence (738 bp) of \textit{S. agalactiae} isolate KMP104 (RefSeq accession number DQ355148). This sequence (accession number LC512876) contained C222T (N74N), T224C (I75I), and A299G (N100S) nucleotide (amino acid) substitutions in addition to the insertion of an \textit{IS}1216\textit{E} 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 [\textit{lnu}(B)-\textit{lsa}(E) with \textit{aac}(6\textsuperscript{'})-\textit{aph}(2\textsuperscript{''})], ST19/CC19, CPS type III, virulence gene profile of \textit{rib-lmb-cylE}, and in terms of cluster on the phylogenetic tree.

We searched for the presence of CRES \textit{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 \textit{lnu}(B)-\textit{lsa}(E) between the CRES and CSER isolates. For all CRES isolates, the \textit{lnu}(B) locus was adjacent to the \textit{lsa}(E) locus within the same contigs, with a short 53-bp distance between these two loci. Therefore, in our study, the \textit{lnu}(B)-\textit{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 \textit{erm}(B) confers constitutive resistance (cMLS\textsubscript{B}) through a conformational change in 23S ribosomal RNA methyltransferase [8]. Deletion of a segment in \textit{erm}(B) sequence in CRES suggests loss of function of the \textit{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 \textit{IS}1216\textit{E} insertion in \textit{erm}(B) (accession numbers LC512881, LC512882, LC512883, LC512885, and LC512886) in five isolates (GCH16, GCH19, GCH38, GCH55, and GCH58, respectively) of this study. Thus, \textit{IS}1216\textit{E} 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 \textit{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 \textit{erm}(B) protein leads to the erythromycin-susceptible phenotype. Three other phenotypes (GCH50, GCH72, and GCH78) also had trun-
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