主論文の要旨

Molecular epidemiology of *Enterobacter cloacae* complex isolates with reduced carbapenem susceptibility recovered by blood culture

（血液培養により分離したカルバペネム感受性が低下した *Enterobacter cloacae* complex分離株の分子疫学）

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【Introduction】

Although *Enterobacter cloacae* complex (ECC) is one of the most common causes of bacteraemia, reported to lead poor clinical outcome, only limited molecular epidemiological information on invasive reduced-carbapenem-susceptible (RCS) non-carbapenemase-producing ECC isolates is available. Therefore, we conducted a study of multicentre isolates to clarify the genetic backgrounds and antimicrobial susceptibility profiles of the RCS ECC isolates from blood samples by excluding carbapenemase producers because plasmid-mediated genes for carbapenemases can spread among bacteria with different traits, making it difficult to analyse and interpret the genetic lineages of RCS ECC isolates

【Methods】

One ECC blood isolate was collected separately from each of the 42 hospitals across Japan between 2017 and 2019. The minimum inhibitory concentrations (MICs) were measured by agar dilution antimicrobial susceptibility testing. Isolates for which imipenem MICs of ≥0.5 μg/mL were assigned the RCS group and those for which imipenem MICs of ≤0.125 μg/mL were assigned the carbapenem-susceptible (CS) group. The draft whole-genome sequence of each isolate was determined using the MiSeq platform. Expression levels of genes involved in carbapenem susceptibility was evaluated by real-time qPCR for assays of *ampC*, *ompC*, *ompF* transcripts. An in-house python script was used to extract *ampC*, *ampD*, *ampR* and *ampG* gene sequences from draft genome sequences. The RCS and CS groups were compared using Fisher's exact test for categorical variables. The relative changes in gene expression were analysed using the Wilcoxon rank-sum test.

【Results】

A total of 42 ECC blood isolates representing eight geographic regions in Japan were obtained of which 21 were assigned equally by chance to each group.

The isolates were obtained from 42 patients admitted to 42 hospitals aged 53 to 97 years (median, 80 years; interquartile range, 71–88 years), of whom 45% were male and 55% were female.

All the isolates in the RCS group were resistant to cefmetazole and susceptible to amikacin, while the remaining isolates of the CS group were all susceptible to cefepime, amikacin, ciprofloxacin, and colistin. The prevalence of resistance was <25% to all the antimicrobials tested except cefmetazole and tigecycline in both groups (Table 1).

Chromosomally encoded ACT type class C β-lactamase genes were detected in all the isolates in the CS group, and 16/21 isolates (76%) in the RCS group (Table 2). Two isolates of the RCS group harboured both class C (ACT) and class A (CTX-M or TEM) β-lactamase genes. The most common subtype of the ACT gene was ACT-7, which was present in 6/21 (28%) of the RCS group and 7/21 (33%) of the CS group.

The RCS group isolates belonged to six species (*E. hormaechei*, *E. kobei*, *E. ludwigii*, *E. roggenkampii*, *E. asburiae*, and *E. bugandensis*) and the CS group isolates belonged to three species (*E. hormaechei*, *E. kobei*, and *E. ludwigii*). Only the *E. roggenkampii* isolates belonging to the RCS group harboured genes for MIR (n=2) or CMG (n=3) type class C β-lactamases.

Multilocus sequence type (MLST) analysis distinguished 36 STs among the 42 isolates, indicating high genetic diversity (Fig. 1, Table 3, Table 4). Among these 36 STs, 8 STs (1196, 1197, 1252, 1253, 1255, 1256, 1258, 1259) were newly identified in the RCS group and three new STs (1204, 1254, 1257) were identified in the CS group.

Analysis of *ampD* and *ampR* revealed an Ile259Val substitution in AmpR and Pro28His, Ile48Asp, Trp95Arg, His19Tyr, and Asp164Ala substitutions in AmpD were exclusive to the *E. hormaechei* ssp. *steigerwaltii* RCS isolates while sample 5 had truncated AmpD caused by premature stop codon at position 95 (Fig. 2).

The differences in the relative *ampC*, *ompC*, and *ompF* genes expressions between the RCS and CS groups were statistically significant ($p<0.001$), ($p=0.005$) and ($p=0.003$) respectively. An RCS group member strain 24017 was excluded from statistical analysis of the *ampC*, *ompC*, *ompF* transcripts as no evident relative increase in the expression of the *ampC* gene was detected, and failed to identify Class A and/or D β-lactamases in this strain. Therefore, factors contributing to RCS phenotype of strain 24017 should be further explored. Reduction in the expression of either major porin channel was not detected in the RCS group member strain 23635 (sample 1), but both ACT and CTX-M-9 type β-lactamases were identified. Increased expression of the *ampC* gene and decreased expression of the *ompF* gene were exclusively identified in all the other members of the RCS group (Fig. 2).

【Discussion】

Enterobacter spp. have emerged as one of the main causes of bacteraemia following *Escherichia coli* and *Klebsiella pneumoniae*. *K. pneumoniae* ST14 and ST258 and *Pseudomonas aeruginosa* ST235 were identified as specific genetic lineages that cause invasive infections with carbapenem-resistant bacteria. However, there is limited information on the specific genetic lineages of ECC that cause invasive infections.

Genetic diversity of ECC blood isolates has been demonstrated by Wang et al. in China with 51 STs from 53 isolates. The authors did not specify the carbapenem resistance and carbapenemase production of each isolate, but the majority of the CS isolates were non-carbapenemase-producers. Tetsuka et al. demonstrated genetic divergence among three carbapenem-resistant ECC blood isolates producing no carbapenemases.

We found that the RCS non-carbapenemase-producing ECC blood isolates belonged to

distinct species, sequence types and produced distinct types of class C β-lactamases. This suggests that the selection of RCS mutant among ECC blood isolates is independent of species, sequence types and type of class C β-lactamases produced. Therefore, RCS phenotype might independently evolve from CS phenotypes of ECC representing diverse genetic backgrounds.

ECC Hoffmann cluster IV was defined as a novel species of *E. roggenkampii* by Sutton et al. in 2018. Non-carbapenemase-producing carbapenem-resistant ECC Hoffmann cluster IV blood isolates have been reported in Nagoya University hospital. An RCS ECC Hoffmann cluster IV blood isolate belonged to ST997 carrying both IncFIB and IncN plasmids that mediated genes for IMP-1 metallo-β-lactamase, CTX-M-14 extended-spectrum β lactamase, and MIR-6 type class C β-lactamase was reported in Japan. *E. roggenkampii* was recently established as a novel species. Thus, knowledge of its carbapenem susceptibility is limited. We found that the five *E. roggenkampii* blood isolates with the RCS phenotype had different sequence types, suggesting polyclonal spread.

The association between single nucleotide polymorphisms in the *ampD* gene with derepression of *ampC* leading to cephalosporin resistance has been shown. Our study showed an association of AmpC increased production and OmpF decreased production with reduced-carbapenem susceptibility in *E. hormaechei* ssp. *steigerwaltii*. Thus, replacement of larger channel OmpF with smaller channel OmpC in *E. hormaechei* ssp. *steigerwaltii* might contribute to decreased efficacy of carbapenems by maintaining their concentration in the periplasm below saturation level by facilitating activity of low level carbapenem hydrolysing β-lactamases.

Indeed, MIC ≥4 μg/mL is the criterion for resistance to diagnose clinical resistance in imipenem and meropenem, but a cut-off value of imipenem MIC ≥0.5 μg/mL was used to define the RCS group for comparative characterization of bacterial isolates for which carbapenem MICs are just below the breakpoint for “resistance” to exactly perform present study.

【Conclusion】

The results observed in this study suggested that almost all ECC isolates have the potential to develop the RCS phenotype regardless of their genotype and species. Which emphasizes the necessity of prudent antibacterial treatments and infection control measures against ECC blood isolates irrespective of their genomic STs or β-lactamase types.