別紙様式第４

学　位　論　文　要　旨

博士課程 (甲)・乙	第　　号	氏　名	Eddy Sukmawinata

[論文題名]

Dissertation title:

Molecular Biological Study of Antimicrobial Resistant in Commensal Fecal Bacteria Isolated From Healthy Thoroughbred Racehorses

(サラブレット競走馬の糞便由来耐性菌の分子生物学的研究)

Journal paper:

1. Antimicrobial resistant *Enterococcus faecium*, *Enterococcus faecalis*, and other *Enterococcus* species isolated from foal feces in Japan
   (国内の仔馬の糞便から分離された薬剤耐性腸球菌)
   Journal of Equine Veterinary Science, 63:51-54, 2018, DOI: https://doi.org/10.1016/j.jevs.2018.01.005
2. Extended-spectrum β-Lactamase-producing *Escherichia coli* isolated from healthy Thoroughbred racehorse in Japan
   (国内のサラブレット競走馬から分離された基質特異性拡張型 β ラクタマーゼ産生大腸菌)
   Journal of Equine Science, 30:47-53, 2019; DOI: https://doi.org/10.1294/jes.30.47
3. IncI1 plasmid associated with $bla_{\text{CTX-M-2}}$ transmission in ESBL-producing *Escherichia coli* isolated from healthy Thoroughbred racehorse, Japan
   (国内のサラブレット競走馬由来基質特異性 β ラクタマーゼ産生大腸菌における $bla_{\text{CTX-M-2}}$ を媒介する IncI1 プラスミド)
   Antibiotics, 9(70):1-7, 2020; DOI: https://doi.org/10.3390/ antibiotics9020070
4. Multidrug-resistant ESBL/AmpC-producing *Klebsiella pneumoniae* isolated from healthy Thoroughbred racehorses in Japan
   (国内のサラブレット競走馬由来多剤耐性ESBL/AmpC産生 *Klebsiella pneumoniae*)
   Animals, 10(369): 1-9, 2020; DOI: https://doi.org/10.3390/ ani10030369

[要　旨]

Antimicrobial resistant (AMR) is one of the major health concern worldwide. Commensal bacteria, such as *Escherichia coli* and enterococci could play roles as both acceptors and donors of AMR. The emergence of AMR *E. coli* and enterococci have been reported as the important nosocomial pathogens in human and animal health. Also, resistance to extended-spectrum cephalosporins which are critically important antimicrobials and mainly mediated by extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase (AmpC) has rapidly spread among *Enterobacteriaceae*. Horse can act as reservoirs of AMR bacteria and their presence has increased the risk of treatment failure. Moreover, risk of infection by AMR bacteria may occur in people who work in close contact with racehorses (e.g., veterinarians, caretakers, and owners). In this study, we evaluated the occurrence of AMR *E. coli* and enterococci in healthy Thoroughbred racehorses in Japan. Also, ESBL/AmpC-producing *E. coli* and *Klebsiella pneumonieae* were investigated in this work.

Feces samples were collected from 72 of healthy foals from seven stables in Hokkaido

Prefecture between June 2015 to January 2016 for the study of AMR enterococci. After that, 212 of healthy Thoroughbred racehorse feces samples were collected from Japan Racing Association (JRA) training centers between April 2017 and August 2018 for the study of AMR *E. coli* and enterococci, and ESBL/AmpC-producing bacteria. *Escherichia coli* and enterococci were isolated by using selective culture medium, and the species confirmed using MALDI TOF-MS. The phylogenetic analysis was performed to *E. coli* isolates. The screening of ESBL/AmpC-producing *E. coli* and *K. pneumonieae* were performed by using MacConkey agar supplemented with 1 μg/ml cefotaxime and presumptive isolates were confirmed for the ESBL/AmpC phenotype using an AmpC and ESβL Detection Set (D68C). *Escherichia coli,* enterococci, and ESBL-producing *E. coli* (ESBLEC) isolates were tested for susceptibility to antimicrobials by determining the minimum inhibitory concentration (MIC) of these antimicrobials, and the susceptibility test for ESBL/AmpC-producing *K. pneumoniae* (ESBL/AmpC-KP) were performed by using the disk diffusion method based on recommendations from the Clinical Laboratory Standard Institute guidelines. The transfer of ESBL/AmpC genes was studied using the conjugation assay for all ESBL/AmpC producer isolates. All donors and transconjugants were confirmed by polymerase chain reaction (PCR) for genes encoding ESBL/AmpC production. Furthermore, all ESBLEC isolates were subjected to class 1 integron detection, the PCR Based Replicon Typing (PBRT) and pulsed-field gel electrophoresis (PFGE) analysis and ESBL/AmpC-KP were subjected to multilocus sequence typing (MLST) analysis.

In foals, a total of 183 isolates of enterococci were identified as *Enterococcus faecium* 54.1%, *Enterococcus faecalis* 16.4%, and other species 29.5%. The highest resistance of *E. faecium* was to erythromycin at 55.6%, followed by enrofloxacin, kanamycin, and oxytetracycline, at 30.3%, 7.1%, and 4.0%, respectively. In contrast, *E. faecalis* isolates showed higher resistant to oxytetracycline 76.7%, kanamycin 46.7%, gentamycin 30.0%, chloramphenicol 16.7%, lincomycin 30.0%, and tylosin 30.0%. *E. faecium* highly resistant to erythromycin and enrofloxacin but lowly resistant to gentamycin and tylosin, and *E. faecalis* was highly resistant to kanamycin, oxytetracycline, and lincomycin. Antimicrobial resistant *E. faecalis* (30.0%) and *E. faecium* (4.0%) isolates showed multidrug resistant (MDR). Our study indicated that foal feces may act as a source of AMR genes for enterococci that may be transmitted to other animals, humans, and the environment.

In Thoroughbred racehorses, among of 583 enterococci isolates, *E. faecium*, *E. faecalis*, and other enterococci were identified for 48.2%, 7.4%, and 44.4% respectively. One isolate that was representing *E. faecium* (153 isolates) and *E. faecalis* (31 isolates) from each sample were selected for antimicrobial susceptibility test. The highest rate of resistance for *E. faecium* isolates was observed against enrofloxacin (57.5%), followed by streptomycin (32.0%), kanamycin (18.3%), gentamycin (5.9%), erythromycin (5.9%), and oxytetracycline (4.6%). For *E. faecium* isolates, the highest resistance was observed against streptomycin (90.3%), followed by kanamycin (41.9%), gentamycin (29.0%), lincomycin (9.7%), oxytetracycline (6.5%), erythromycin (6.5%), tylosin (6.5%), enrofloxacin (6.5%), and chloramphenicol (3.2%). The results indicated that enrofloxacin and aminoglycosides were highly resistant among tested antimicrobials.

On the other hand, a total of 417 *E. coli* isolated from Thoroughbred racehorses were

examined in this study. The highest proportion of resistance was observed for streptomycin (30.9%), and followed by ampicillin (19.4%), trimethoprim (15.8%), tetracycline (8.4%), chloramphenicol (2.6%), kanamycin (1.2%), nalidixic acid (0.5%), cefazolin (0.2%), colistin (0.2%), and gentamycin (0%). Multidrug resistant *E. coli* were detected in 7.9% isolates. The highest proportion of phylogenetic group of *E. coli* isolates was identified as group B1 (57.3%), followed by group A (23.3%), B2 (9.8%), and D (9.6%), whereas the proportion of resistance against ampicillin, streptomycin, kanamycin, chloramphenicol, and also MDR in the group B2 were significantly higher than other groups. This study described the situation of AMR *E. coli* in Thoroughbred racehorses in Japan.

In the study of ESBL/AmpC-producing bacteria, 24 ESBLECs were isolated from 23 (10.8%) Thoroughbred racehorse feces samples. The ESBLEC harboring $bla_{\text{CTX-M-2}}$ was detected in 87.5% of isolates, followed by $bla_{\text{CTX-M-1}}$ (8.3%) and $bla_{\text{TEM-116}}$ (4.2%). None of the ESBLEC isolates were also positive for AmpC β-lactamase production. ESBLEC isolates were showed co-resistance to trimethoprim/sulfamethoxazole (66.7%), streptomycin (50%), tetracycline (20.8%) and oxytetracycline (20.8%) and MDR ESBLECs were identified from 45.8%. The IncI1 plasmid was highly distributed among ESBLEC isolates where the FIB, F, HI1, Y, and L groups were also detected. All ESBLECs investigated were genotypically diverse, as shown by a variety of PFGE patterns. Conjugation assays were successful in 62.5% (15/24) of ESBLEC harboring $bla_{\text{CTX-M-2}}$ isolates. Two transconjugants were identified phenotypically to have co-resistance with tetracycline derivates and/or trimethoprim/sulfamethoxazole. Plasmid transmission to the recipient strains was only shown by the IncI1 plasmid. Additionally, class 1 integron was detected from 6 of 24 (25.0%) ESBLEC isolates.

On the other hand, 12 ESBL/AmpC-KP were isolated from 7 (3.3%) Thoroughbred racehorse feces samples and all isolates showed MDR. The highest resistance (100%) was observed against ampicillin, cefuroxime, cefotaxime, tetracycline, oxytetracycline, doxycycline and fosfomycin, followed by ceftazidime (83.3%), gentamicin (75.0%), kanamycin (66.7%), streptomycin (8.3%), and chloramphenicol (8.3%). Only 1 isolate was confirmed as an ESBL producer ($bla_{\text{CTX-M-2}}$-positive), whereas the other 11 isolates were plasmid-mediated AmpC (pAmpC) producers (bla_{CMY}-positive). On the basis of MLST analysis, the ESBL-KP isolate was identified as sequence type (ST)-133 and four different STs among AmpC-KP isolates, ST-145, ST-4830, ST-4831, and ST-4832, were found to share six of the seven loci constituting a single-locus variant. Interestingly, ESBL-KP ST-133 (JMT68b-KP) was originated from the same sample with an ESBLEC isolate (68a) and both of them were conjugated in this study. This result might describe that horizontal transmission between bacteria species in horse intestine was occurred. Our findings suggested that the IncI1 plasmid has an important role in the spread of $bla_{\text{CTX-M-2}}$ in racehorses. This information is very useful for the control of the dissemination of $bla_{\text{CTX-M-2}}$ among the racehorse population. Moreover, this is the first study to show *K. pneumoniae* carrying MDR plasmid-mediated AmpC isolated from racehorses. In conclusion, this is the first study that evaluated AMR in commensal bacteria in healthy Thoroughbred racehorses in Japan, and continuous monitoring AMR is required in order to control the spread of AMR bacteria in racehorse community.

備考　論文要旨は、和文にあっては 2，000 字程度、英文にあっては 1，200 語程度