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Bacterial Flora of the Intestine in Normal Captive Oriental White Storks

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Abstract : A survey was conducted to examine the normal intestinal bacterial flora of captive Oriental white storks (*Ciconia boyciana*) maintained at the Korea Institute of Oriental White Stork Rehabilitation Research, Cheongwon, South Korea. From the cloaca of 44 healthy storks, 44 fecal samples were collected and cultured under aerobic and anaerobic conditions. The 16S ribosomal RNA gene sequences and the heat shock protein 60 gene were cloned and sequenced for bacterial identification. Under aerobic conditions, *Enterococcus faecalis*, *Escherichia coli*, *Bacillus* spp., *Enterococcus avium*, *Enterococcus gallinarum*, *Pseudomonas* spp., *Alcaligenes* spp., *Enterobacter* spp., *Corynebacterium* spp., and *Proteus mirabilis* were identified. Under anaerobic conditions, *E. coli*, *Clostridium tertium*, *En. faecalis*, and *P. mirabilis* were identified. *E. coli*, *En. faecalis*, or both were isolated from all samples. These results will add to the information available on this stork species and help for the interpretation of fecal culture results.

Key words **:** Oriental white stork, bacterial flora, intestine.

Introduction

The Oriental white stork (*Ciconia boyciana*) belongs to the family Ciconiidae and the order Ciconiformes. This bird has been listed as an endangered species in the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN) (Birdlife International, 2001). At present, this species of stork mainly inhabits areas surrounding the Heilong and Wusuli River basins along the border between Russia and China (13). In South Korea, this species, which used to be a permanent resident, has now become extinct. The Korea Institute of Oriental White Stork Rehabilitation Research (KIOWSRR) and Chungbuk National University are undertaking a combined effort to reintroduce them as breeding birds in this region.

During the breeding period of this species, bacterial overgrowth in feces, as a result of a primary intestinal infection or as a manifestation of systemic disease, is a common problem. Thus, bacterial culturing of fecal samples and antibiotic sensitivity testing are important for assessing and effectively treating the storks (10). Information on the normal intestinal bacterial flora of this species is necessary for interpreting the culture results. However, because of the absence of this information, it is difficult to determine the significance of the bacteria isolated from ill storks. Therefore, this study was conducted at KIOWSRR to determine the normal aerobic and anaerobic intestinal bacterial flora of captive Oriental white storks.

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Materials and Methods

This study included 44 Oriental white storks, i.e., 16 male and 28 female storks; their ages ranged between 3-6 months. All storks were housed in a 7 × 7 m outdoor cage with chain-link fencing. Each cage was covered with netting, and a pond and roost were installed on the ground of the cage. Mudfishes and chickens were provided as food in buckets once daily. As determined by experienced keepers, all storks examined in this study showed visually normal behavior and showed no clinical abnormalities on physical and laboratory examinations, including complete blood count, serum biochemistry profiles, and fecal examination.

Fecal samples were collected from the cloaca by using BBL™ CultureSwab™ Collection and Transport Systems (BD diagnostics, NJ, USA). After transportation, the samples were incubated at 37°C in Mueller-Hinton broth (BD diagnostics) under aerobic and anaerobic conditions for approximately 3 days, and bacterial growth was observed daily. Pure culturing of all the culture-positive samples was carried out repetitively on Mueller-Hinton agar at 37°C until separate colonies were isolated on the culture plates. After culturing, all the isolated bacteria were subjected to Gram staining and were examined by light microscopy for classification. For molecular typing, genomic DNA was extracted from the separated colonies by using the Dynabeads® DNA Direct™ Universal Kit (Invitrogen Life Technologies Inc., Carlsbad, CA, USA). For bacterial identification, the genes encoding 16S ribosomal RNA (16S rRNA) and heat shock protein (hsp) 60 were analyzed by using the primers 16S rRNA 27F and 16S rRNA 1492R or H1612 and H1613, respectively (7). PCR amplification was performed

with the following reaction mixture in a total volume of 50 μL: 50 mM KCl, 10 mM Tris-HCl (pH 8.3, 25°C), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 100 ng of each primer, and 5 U *Taq* polymerase (iNtRON Biotechnology, Sungnam, South Korea). PCR was performed in a TaKaRa Thermal Cycler Dice (Takara Bio Inc., Otsu, Shiga, Japan) in the following steps: initial denaturation at 94°C for 2 min 30 s; 30 cycles of denaturation, annealing, and extension at 94°C for 30 s, 53°C (for 16S rRNA gene) or 60°C (for hsp60 gene) for 30 s, and 72°C for 45 s, respectively; and final elongation at 72°C for 5 min. All the PCR products were separated by electrophoresis for 50 min at 100 V in a 2% agarose gel, which was further stained with ethidium bromide, and the PCR products were then visualized under ultraviolet light. The amplicons were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (PE Applied Biosystems, Foster City, CA, USA). The identified sequences were confirmed by comparison with gene sequences deposited in the GenBank database.

Results

Table 1 shows the species of bacteria isolated in this study. All fecal samples were culture positive, and 87 bacteria of 12 different species were isolated from the 44 fecal samples. Under aerobic conditions, 67 organisms were isolated including *Escherichia coli*, *Pseudomonas* spp., *Alcaligenes* spp., *Enterobacter* spp., *Proteus mirabilis, Enterococcus faecalis*, *Bacillus* spp., *Enterococcus avium*, *Enterococcus gallinarum*, *Corynebacterium* spp., and *Staphylococcus* spp. Under anaerobic conditions, 20 organisms were isolated including *E. coli*, *En. faecalis*, *Clostridium tertium*, and *P. mirabilis*. Under both conditions, *E. coli* (43%, 37/87) and *En. faecalis* (32%, 28/87) were the most common isolates. All the isolated bacterial species were either aerobes or facultative anaerobes, except *C. tertium* (1/87). Of the isolates, 52% (45/87) were gram-positive organisms, and 64% (28/44) of the fecal samples showed a mixed population of organisms. *E. coli* and *En. faecalis* in combination (43%, 12/28) were the most commonly observed organisms in all the samples. The former was observed in 70% (31/44) of the storks and the latter in 52% (23/44).

Table 1. Bacterial species isolated from the feces of healthy Oriental white storks

Bacterial isolates	Gram	Aerobic (n = 67)	Anaerobic (n = 20)	Total (n = 87)
Escherichia coli	-	24	13	37
Enterococcus faecalis	+	24	5	29
Bacillus spp.	+	6	-	6
Enterococcus avium	+	1	-	1
Enterococcus gallinarum	+	1	-	1
Pseudomonas spp.	-	1	-	1
Alcaligenes spp.	-	1	-	1
Enterobacter spp.	-	1	-	1
Corynebacterium spp.	+	6	-	6
Staphylococcus spp.	+	1	-	1
Proteus mirabilis	-	1	1	2
Closteridium tertium	+	-	1	1

Discussion

The normal intestinal bacterial flora in healthy, captive Oriental white storks was identified in this study . Since this information is essential for interpreting fecal culture results, attempts have been made to characterize the normal intestinal flora in domesticated and pet bird species (1,2,5,8,9). In passerines and psittacines, gram-positive bacteria are considered as the normal flora of the intestinal tract, while gram-negative bacteria are considered transient residents or pathogens (5). Clinical abnormalities are found in over 90% of the captive and wild psittacines that have gram-negative bacteria in the intestine (5,8). In contrast, in pigeons and raptors, gram-negative bacteria are most common in the intestinal tract although these organisms are considered to be facultative pathogens in pigeons (3,11). In raptors, enterococci are the second most common intestinal bacteria. In this study, we found that the ratios of gram-positive and gram-negative bacteria are approximately equal in *C. boyciana*; in particular, *E. coli* and *En. faecalis* are the most common and the second most common isolates, respectively. This indicates that the intestinal bacterial flora of raptors and storks is similar. In addition, all the birds tested in this study had no history of illness and were clinically healthy even 8 months after sampling. Thus, we deduced that the bacteria identified are permanent residential bacteria in healthy Oriental white storks.

From 12 of the 44 storks, we also isolated *Bacillus* spp. and *Corynebacterium* spp., which are the permanent intestinal residential organisms in passerines and psittacines (5). Both these bacterial species were isolated along with either *E. coli* or *En. faecalis*, but they were not simultaneously isolated from the same bird. In the outdoor cage, the storks preyed on insects and worms, which suggests that the stork is a carnivorous, vermivorous, and piscivorous species. Although both *Bacillus* spp. and *Corynebacterium* spp. formed a small part of the isolates, this species of stork seems to have a complex bacterial flora, which is a combination of the bacterial flora of carnivorous and vermivorous bird species.

In this study, several gram-positive and gram-negative bacteria including *En. avium*, *En. gallinarum*, *Pseudomonas* spp., *Alcaligenes* spp., *Enterobacter* spp., *Staphylococcus* spp., *P. mirabilis*, and *Cl. tertium* were also isolated to a small extent. In birds, the intestinal bacterial flora consists of transient and permanent residents (4). On the basis of the frequency of isolation and the health status of the storks before and after the sampling, it seems that these organisms are transient residents of the intestinal tract of this stork. Their pathogenicity in storks has not been documented as yet; however, these organisms are reported to be facultative intestinal pathogens in birds (4,6,12). Thus, it is essential to screen the overgrowth of these organ-

isms in storks to prevent opportunistic infections in immunocompromised storks or those under stress.

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정상적인 사육 황새의 장내 세균총

한재익 · 장혜진 · 이숙진 · 강효민 · 김수경* · 박시룡* · 나기정[1]

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요 약 : 한국 황새복원센터에서 사육중인 황새의 정상 장내 세균총을 검사하기 위한 조사를 실시하였다. 44마리의 건강한 황새의 총배설강으로부터 44개의 분변시료를 채취하여 호기 및 혐기조건에서 배양하였다. 배양된 미생물의 16S ribosomal RNA 및 heat shock protein 60 유전자의 염기서열을 조사하였다. 호기조건 하에서는 *Enterococcus faecalis, Escherichia coli, Bacillus spp., Enterococcus avium, Enterococcus gallinarum, Pseudomonas spp., Alcaligenes spp., Enterobacter spp., Corynebacterium spp., Proteus mirabilis*가 동정되었다. 혐기조건 하에서는 *E. coli, Clostridium tertium, En. faecalis, P. mirabilis*가 동정되었다. 모든 시료에서 *E. coli, En. faecalis* 혹은 두 가지 모두가 분리되었다. 이러한 정상 황새의 장내 미생물 정보는 향후 분변 배양 결과의 해석에 도움이 될 것으로 판단된다.

주요어 : 황새, 세균총, 장