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Letter to Editor

Identification of Bacterial Species in White Stork Chicks in Poland Using PCR Method and Sequencing of Bacterial 16SrRNA

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Abstract

The aim of this study was to identify the most common bacteria that may be found in the blood or on feathers of white stork (*Ciconia ciconia*) chicks and predict their pathogenic potential for birds and humans. A variety of microorganisms, which include *Staphylococcus aureus, Staphylococcus vitulinus,* and *Pseudomonas* sp. were found. Based on breeding population densities and reproductive success over the past 25 years, we found no apparent effects of bacterial infections on the white stork population in Poland.

Keywords: *Ciconia ciconia*, 16SrRNA, birds, human pathogens, infectious diseases, PCR

Introduction

Recently it has been widely discussed that bacteria have a great impact on avian life and influence on feather degradation, sexual selection and general health condition of birds [1-3]. Additionally, birds potentially transfer bacteria to humans, and therefore are of epidemiological significance [4]. On the other hand, human specific pathogens may have an influence on wildlife [5]. An ideal organism to evaluate both possibilities can be the white stork (*Ciconia ciconia*), a bird species living in close proximity to human. Storks build nests on man-made constructions such as roofs of farm buildings or electrical poles [6]. Moreover, white storks are transcontinental migrants and may have impact on intercontinental dispersal pattern of pathogens. Therefore chicks infected in breeding grounds can transport bacteria over migratory routes and finally to African wintering places [7].

Although knowledge on negative factors limiting the size of the local breeding population is essential for the effective protection and improvement of breeding success of the white stork population, only a few studies have been addressed to the parasites and pathogens of the white stork [8-10]. Hence, as a part of the conservation effort for this species, veterinary examination of some chicks was carried out. It is important because of the large impact on the world population, since about 25% individuals of the species occur in Poland [6]. In this paper we describe the incidence of bacteria species in chicks of the white stork (*Ciconia ciconia*).

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Additionally, we discuss the potential epidemiological role of white storks in transfer of bacteria to humans and the other way round.

Materials and Methods

Field Procedures and Animals

Field work was conducted during two breeding seasons (2005 and 2006) in the Wielkopolska region, which holds the typical breeding densities of the white storks in Poland, i.e. about 10 breeding pairs / 100 km^2 [11].

Chicks were bled from the brachial vein, and put into plastic tubes and transported to the laboratory. A total of 42 nestlings were sampled on average at 35 days old, thus a few days prior to fledging. The possibly occurring parasites were detected by visual methods in 22 blood drops. After that blood smears were prepared, which were air-dried immediately in the field. Additionally one secondary feather was taken for bacteriological analysis randomly from 20 chicks. All three different substrates (blood in plastic tubes, blood smears and feathers) were used for bacteriological studies (details below). Based on field controls of the nests, we found that all chicks lived until the time of fledging, what suggests that the procedure of blood collection was not a strongly invasive method [9].

Isolation of DNA

Isolation of DNA from Stork Blood Samples

Total DNA from 22 stork blood samples of 200 µl was isolated using QIAamp DNA Blood Midi Kit (Qiagen). The DNA concentration (225 ng x μl^{-1}) and its integrity were estimated by agarose gel electrophoresis (with 1% agarose, 1 x Tris-acetate-EDTA, 1 ng of ethidium bromide x ml^{-1}).

Isolation of DNA from Stork Feathers

20 hollow shafts from stork feathers were cut. They were incubated overnight in 56°C with proteinase K (50 µg x ml^{-1}). Total DNA was extracted using conventional technique of fenol-chloroform extraction and then ethanol precipitation in the presence of potassium acetate. Finally, the solution was suspended in free-endonuclease sterile water. The DNA concentration (225 ng x μl^{-1}) and its integrity were estimated by agarose gel electrophoresis (with 1% agarose, 1 x Tris-acetate-EDTA, 1 ng of ethidium bromide x ml^{-1}).

PCR Amplification of Bacterial 16SrRNA Gene

DNA isolated from samples was used as a template for PCR (polymerase chain reaction) to amplify bacterial 16SrRNA gene. The forward primer S-D-Bact-0008-a-S-20 (5' AGA GTT TGA TCC TGG CTC AG 3'), which targets the domain *Bacteria*, and the reverse primer S-*-Univ-1492-b-A-21 (5' ACG GCT ACC TTG TTA CGA CTT 3'), which targets all living organisms, were used [12]. Reaction tubes contained 2,25 µg (10 µl) of stork's blood DNA, 1.5 U of *Taq* DNA polymerase (DyNAzyme II DNA Polymerase, F-501S, Finnzymes), 1xBuffer for DyNAzyme (F-511, Finnzymes), 200 µM each deoxyribonucleotide triphosphate, and 1µM each primer in a final volume of 50 µl.

Initial DNA denaturation was performed at 95°C for 5 min in a Uno II thermocycler (Biometra, Germany), followed by primers annealing at 48°C for 1 min and elongation at 72°C for 1 min 30 s. The cycle of denaturation, annealing and elongation was repeated 18 times and followed by final elongation at 72°C for 15 min.

The size of amplification product was 1500 base pairs (1.5 kb). PCR products were purified and concentrated with a QIAquick PCR Purification Kit (Qiagen). Their concentration (25 ng x μl^{-1}) and size were estimated by agarose gel electrophoresis.

Ligation and Transformation

The purified PCR products were ligated into pGEM-T Easy vector (Promega) as specified by the manufacturer. Competent *Escherichia coli* DH5α cells were transformed with ligation products by heat shock (45 s at 42°C). Recombinant cells were selected on Luria-Bertani medium with ampicylin, IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Colonies, which contained a plasmid with an insert, were white, because they could not produce β-galactosidase and degrade X-gal. Colonies which contained a plasmid without an insert were blue.

Plasmid Extraction and Sequencing

Plasmids with inserts were extracted from 3 ml LB cultures of white transformed Escherichia coli DH5α colonies with a QIAprep Plasmid Kit (Qiagen) according to manufacturer. Plasmids were eluted with 50 µl of water, and the products were stored at -20°C. Sizes and concentrations of plasmids were checked by 1% agarose gel electrophoresis.

Inserts from plasmids were sequenced with an automated 3130x Genetic Analyzer (Applied Biosystems) in Faculty of Biology, Adam Mickiewicz University in Poznan, using the same set of primers.

Sequences obtained were analysed by comparing to those available in GenBank database (NCBI, USA) using BLAST search.

Results

From 22 blood samples, bacterial 16SrRNA sequences were found in 10 (45.4%). After sequencing, 6 of them were identified as *Staphylococcus vitulinus* which is taxonomically the same as *Staphylococcus pulvereri* [13]. One of the samples contained 16SrRNA of *Pseudomonas* sp., and one *Gardnerella vaginalis* (Table 1).

Table 1. Cultured isolates from white stork (*Ciconia ciconia*) chicks in Wielkopolska, Poland in two different substrates.

Identification	Blood samples (n = 22)	Feathers (n = 20)
Enterococcus faecalis	-	2
Gardnerella vaginalis	1	-
Macrococcus caseolyticus	-	1
Pseudomonas sp.	1	-
Staphylococcus aureus	1	-
Staphylococcus vitulinus	6	-

In 20 secondary feathers of chicks bacterial 16SrRNA sequences were recorded only in 3 (15.0%) samples.

Two methods of sampling used for bacteriological analysis differed significantly by prevalence of bacteria found ($\chi^2 = 6.77$, df = 2, $P < 0.05$). Interestingly, also bacterial species detected in different substrates differed between each other (Table 1).

Discussion

Taking into account findings from two substrates used, we received a general view of bacterial species connected with the white stork chicks. To date Shawkey et al. [3] suggested that culture-based and culture-independent techniques may provide different results. Microbial diversity on plumage probably is of behavioral origin (transmission through parental care, faeces). On the other hand, pathogens found in blood could originate from infections or possibly also from food of the birds. Bacteria recorded in our study were previously detected in several wild and domestic bird species [14]. For example *Staphylococus* aureus was found in samples taken from bullfinch (*Phyrulla phyrulla*), some peregrine falcons (*Hierofalco peregrinus*), vulture (subfamily *Aegypiinae*), pigeons (*Columba* sp.) and chickens (*Gallus gallus domesticus*) [15]. It causes diseases like septicaemia, skeletal infections and even pneumonia in birds [16]. Bacteria from the genus Pseudomonas, were reported as aethiologic agents of respiratory tract infections of kori bustards (*Ardeotis kori*) [17]. Recorded *Enterococcus faecalis* is an inhabitant of the intestinal tract of humans and many other animals, including birds. This bacterium has been described as a commensal or opportunistic, gram positive, facultative anaerobae. However, when it inadvertently enters circulation it can cause endocarditis, as well as urinary, intra-abdominal and pelvic infections [18]. To our best knowledge, these bacteria were not recorded in the white stork to date.

Our findings generally suggest that white storks act as reservoirs and/or carriers of some bacterial species, including some pathogens for humans. Probably they are not a key problem for human health in Europe, but they could be in Africa, where people hunt these birds for their meat [6]. Moreover, people who have professional contact with birds could be potentially exposed to transmission of bacteriosis from birds.

To sum up, bacterial infections found in this study exhibit no apparent adverse effects on our studied white stork population. The Wielkopolska region local population was stable (or even locally was growing) over the last 25 years [11] and also chick productivity was on the proper level. We conclude that parasites and pathogens are not serious limiting factors for white stork population, but could be important limiting factors on an individual level [10].

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