


Low Occurrence of *Acinetobacter baumannii* in Gulls and Songbirds

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Abstract

Acinetobacter baumannii is a worldwide occurring nosocomial pathogen, the natural habitats of which remain to be defined. Recently, white stork nestlings have been described as a recurring source of *A. baumannii*. Here, we challenged the hypothesis of a general preference of *A. baumannii* for avian hosts. Taking advantage of campaigns to ring free-living birds, we collected cloacal swab samples from 741 black-headed gulls (*Chroicocephalus ridibundus*) in Poland, tracheal and cloacal swabs from 285 songbirds in Poland as well as tracheal swabs from 25 songbirds in Slovenia and screened those for the growth of *A. baumannii* on CHROMagar™ *Acinetobacter*. Of the 1,051 samples collected only two yielded *A. baumannii* isolates. Each carried one variant of the *bla*_{OXA-51-like} gene, i.e. OXA-71 and OXA-208, which have been described previously in clinical isolates of *A. baumannii*. In conclusion, our data do not support a general preference of *A. baumannii* for avian hosts.

Key words: *Acinetobacter baumannii*, seagulls, songbirds, *Erithacus rubecula*, *Chroicocephalus ridibundus*, free-living birds, nosocomial pathogen

Introduction

Acinetobacter baumannii is a Gram-negative bacterium, which causes nosocomial infections worldwide. Due to its remarkable capability to develop multidrug resistance, we are running out of treatment options in many parts of the world (Murray et al. 2015). The World Health Organization (WHO), therefore, ranked carbapenem-resistant *A. baumannii* on the top of a priority list of antibiotic-resistant bacteria for the treatment of which new antibiotics are urgently needed (Tacconelli et al. 2018).

The natural reservoirs of *A. baumannii* are poorly defined. Many members of the genus *Acinetobacter* have ubiquitously spread bacteria found in soil, water,

and other environmental sites (Afshinnekoo et al. 2015; Al Atrouni et al. 2016) but also as part of the human microbiome (Fyhrquist et al. 2014). *A. baumannii* has been isolated from different environmental sites and various animals (Eveillard et al. 2013; Rafei et al. 2015; Kittinger et al. 2017; Furlan et al. 2018). DNA-based studies suggest the rates of colonization of human head and body lice of up to around 50%, depending on the population (Kempf et al. 2012). However, an isolate from lice studied in detail was found to be phylogenetically distinct from clinical isolates and exhibited low virulence (Vallenet et al. 2008; Antunes et al. 2011). In a recent study, *A. baumannii* was isolated from white stork (*Ciconia ciconia*) nestlings in Poland at an average rate of 25% (Wilharm et al. 2017). Moreover,

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genome-based analyses revealed a multitude of distinct lineages and identified the relationship of some stork isolates to clinical strains. Given that *A. baumannii* was also isolated from poultry livestock, hatcheries, and meat (Martin and Jackel 2011; Lupo et al. 2014; Wilharm et al. 2017), we speculated about a general preference of *A. baumannii* for avian hosts. To challenge this hypothesis, we performed a culture-based screening of different free-living birds, the results of which are presented here.

Experimental

Materials and Methods

Capture and sampling of songbirds in Poland.

In total, 285 wild bird individuals were captured in mist nets during their spring and autumn migration 2016 in the Dąbkowice area (West Pomeranian province; Fig. 1). Ornithologists from the Bird Migration Research Station (Gdańsk, Poland) conduct a scientific study about birds' migration through the Polish Baltic coast under the name "Operation Baltic" every year, in spring and autumn. All captured birds are ringed, measured and assessed by inspection. Thanks to coopera-

tion with ornithologists from the Operation Baltic, we could collect cloacal and tracheal swabs from 145 bird individuals between April 10 and 16 on spring migration, and from 140 individuals between October 9 and 12 on autumn migration. We collected cloacal and tracheal swabs from all captured birds except those not approved by ornithologists based on the assessment of their general condition. Swab samples were immediately taken from birds after their examination, transferred to Amies transport medium (COPAN 110C, Hain Lifescience, Germany), and stored at 4°C until further analysis (sample processing within seven days). The Bird Migration Research Station was granted legal permission for collection by the General Directorate for Environmental Protection (DZP-WG.6401.03.36.2015.kk and DZP-WG.6401.03.98.2016.km) and by the Maritime Office (OW-A-510/87/17/ds).

Capture and sampling of gull adult birds and nestlings in Poland. Altogether, 741 black-headed gulls (*Chroicocephalus ridibundus*) from 16 breeding colonies in Poland were captured in 2017 and 2018 (Fig. 1). The number of breeding pairs in individual colonies varied between 100 and 3,000. All individuals were captured between 25 April and 15 June, while brooding or feeding nestlings. Cloacal swabs were collected from 187 individuals in 2017 and 554 individuals in 2018.



Fig. 1. Map of Poland illustrating the sampling places. White square: bird migration research station at Bukowo. White dots: localization of sampled black-headed gull colonies.

Adult birds were older than two years and nestlings were between 7 and 21 days old. Ornithological nets, which were set directly on nests were used to capture black-headed gulls. This procedure did not result in any loss of eggs or nestlings. Legal permissions were granted by the General Directorate for Environmental Protection (DZP-WG.6401.03.97.2017.jro and DZP-WG.6401.03.2.2018.jro) and the Ministry of the Environment (DLP-VIII-6713-21/29762/14/RN). Authority permission ID numbers are as follows: P. Indykiewicz – 116/2017 and 120/2018; M. Ledwoń – 194/2017 and 201/2018; P. Minias – 228/2017 and 235/2018, and J. Nowakowski – 245/2017 and 252/2018.

Capture and sampling of birds in Slovenia. The ringing campaign in the framework of EURING took place in Maribor and surroundings between September 18 and December 10, 2013, as previously described (Škraban et al. 2017).

Isolation and identification of *A. baumannii*. Swab samples were taken from the choana and rectum, respectively, using the COPAN Amies agar gel medium transport swabs (COPAN 110C, Hain Lifescience, Germany) and stored at 4°C until further analysis. CHROMagar™ *Acinetobacter* (CHROMagar, France) agar plates were prepared as described by the manufacturer without the use of the CHROMagar™ MDR supplement. Swab samples were spread on these agar plates and incubated for 24 hours at 37°C. Reddish colonies tentatively identified as *A. baumannii* were subjected to colony PCR to detect the *bla*_{OXA-51-like} β-lactamase gene intrinsic to *A. baumannii* (Turton et al. 2006) and for species identification using partial *rpoB* sequencing (Nemec et al. 2009). To this end, a loop-full of each colony to test was resuspended in 50 µl of sterile water. Then, the suspension was incubated at 95°C for 5 minutes and centrifuged for 1 minute at 10,000 g, and 1 µl of the supernatant was taken as a DNA template for every 10 µl of PCR reaction. Full sequencing of the amplified *bla*_{OXA-51-like} gene was accomplished as described (Zander et al. 2012) applying modified Sanger sequencing with BigDye v3.1 and ABI 3500dx genetic analyzer. Partial *rpoB* sequences of 861 base pairs in length obtained by the modified Sanger sequencing as above were subjected to nucleotide BLAST analysis for taxonomic classification. Finally, isolates with *rpoB* sequence identity above 98% to the type strain of *A. baumannii* in combination with the determination of the *bla*_{OXA-51-like} gene were considered to belong to the species *A. baumannii*.

Antimicrobial susceptibility testing. Antimicrobial susceptibilities of *A. baumannii* isolates were investigated by Etest (bioMérieux, Nuertingen, Germany) and by the automated system VITEK 2 (card AST-N248; bioMérieux) with interpretation according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST v10.0).

Results

In the course of a previously published study on the cultivable microbiota from the choana of free-living birds captured in Slovenia between September and December 2013 (Škraban et al. 2017), 25 choana samples were screened for the presence of *A. baumannii* as previously established for studies on white stork nestlings (Wilharm et al. 2017). None of the samples yielded *A. baumannii* isolates.

Of the 145 and 140 songbirds captured along the Baltic Sea during spring and autumn, respectively, in 2016, the European robin *Erithacus rubecula* was the dominating species (n=210), followed by blackbird *Turdus merula* (n=37), and blackcap *Sylvia atricapilla* (n=11) (Table I). Choana and cloacal swabs were all found to be negative for *A. baumannii*.

The cloacal swabs sampled from black-headed gulls in 2017 (n=187) were all negative for *A. baumannii*. Among the individuals sampled cloacally in 2018 (n=554), two were positive for *A. baumannii* (from breeding colonies Koronowo and Przykona, respectively) (Table II). Sequencing of the *bla*_{OXA-51-like} gene intrinsic to *A. baumannii* revealed that the two isolates carried the OXA-71 and OXA-208 variant, respectively, of the class D β-lactamase OXA-51 family. Both isolates were susceptible to meropenem, imipenem colistin, tigecycline, gentamicin, tobramycin, amikacin, ciprofloxacin, and sulfamethoxazole-trimethoprim. Natural intrinsic resistance to chloramphenicol, penicillins (piperacillin, ampicillin), and cephalosporins (cefotaxime, cefoxitin) was observed for both isolates. In addition, eight isolates of the species *Acinetobacter pittii* were obtained from gull adults (breeding colonies Koronowo and Jankowo).

Discussion

Acinetobacter baumannii has been isolated sporadically from different avian sources in the past (Ahmed et al. 2007; Muller et al. 2010; Zordan et al. 2011; Kempf et al. 2012; Rafei et al. 2015; Klotz et al. 2018). Additional studies providing evidence for the presence of *A. baumannii* in bird samples include a study on the rectal carriage of Gram-negative bacteria among 89 seabirds in rehabilitation centres in the USA. In this study, *A. baumannii* was isolated from 10% of birds, including gulls (Steele et al. 2005). However, the taxonomic classification of bacteria in that study was solely based on phenotypic traits, without the DNA-based corroboration. Even higher isolation rates of more than 30% were recently reported from 48 wild birds' fecal samples collected in Nigeria (Dahiru and Enabulele 2015). Again, we cannot be certain about the taxonomic claim

Table I
The number and age of individuals from the songbird species sampled in 2016.

Bird Species	n ^a	April 2016				n ^b	October 2016				n ^c
		Age					Age				
		<i>im.</i>	<i>ad.</i>	L	-		<i>im.</i>	<i>ad.</i>	L	-	
<i>Turdus merula</i>	37	6	1	0	0	7	18	11	1	0	30
<i>Erithacus rubecula</i>	210	94	13	0	1	108	79	23	0	0	102
<i>Sylvia atricapilla</i>	11	7	3	0	0	10	1	0	0	0	1
<i>Emberiza schoeniclus</i>	1	1	0	0	0	1	0	0	0	0	0
<i>Fringilla coelebs</i>	1	1	0	0	0	1	0	0	0	0	0
<i>Cyanistes caeruleus</i>	7	6	0	0	0	6	1	0	0	0	1
<i>Parus major</i>	3	2	0	0	0	2	0	1	0	0	1
<i>Phoenicurus phoenicurus</i>	1	1	0	0	0	1	0	0	0	0	0
<i>Parus spp.</i>	1	0	0	1	0	1	0	0	0	0	0
<i>Emberiza citrinella</i>	2	0	2	0	0	2	0	0	0	0	0
<i>Dendrocopos major</i>	2	1	1	0	0	2	0	0	0	0	0
<i>Phylloscopus trochilus</i>	6	0	0	1	0	1	3	2	0	0	5
<i>Turdus philomelos</i>	3	2	1	0	0	3	0	0	0	0	0
Total	285	121	21	2	1	145	102	37	1	0	140

n^a – total number of sampled birds; n^b – total number of sampled birds in April; n^c – total number of sampled birds in October;
im. – an immature bird, a bird in the first year of its life; *ad.* – an adult bird (after the first calendar year of life);
 L – the precise age of bird could not be determined; (-) no data

Table II
The number of black-headed gulls (adult birds and nestlings) sampled in 2017 and 2018.

Colony	X Coordinate	Y Coordinate	No. BP	No. BS 2017	No. BS 2018	Total
Koronowo	53.3341667	17.965	657	53	70	123
Bydgoszcz – Stary Port	53.1211111	18.09361111	185	16	–	16
Bydgoszcz – Przemysłowa	53.1186111	18.10527778	170	50	46	96
Skoki Duże	52.6063889	19.78916667	4500–5500	–	70	70
Jankowo	52.7827778	18.08416667	451	68	57	125
Kościeszki	52.5733333	18.33111111	354	–	56	56
Borów	52.1202778	19.56	100	–	21	21
Jeziorsko	51.7372222	18.64916667	100	–	15	15
Przykona	52.0055556	18.65777778	3000	–	33	33
Wola Rogozińska	51.9713889	19.455	3000	–	18	18
Złaków Kościelny	52.1969444	19.78916667	300	–	16	16
Jezioro Ryńskie	53.9194444	21.50861111	1900–2500	–	30	30
Sętał	53.9036111	20.48222222	425–450	–	30	30
Jezioro Nielbark	53.3613889	19.52777778	3000	–	30	30
Zbiornik Poraj	50.6411111	19.2313888	1060	–	22	22
Łęczczok	50.1436111	18.2797222	360	–	21	21
Stawy Zawadka	49.9644444	19.1163888	460	–	19	19
Total				187	554	741

No. BP – number of breeding pairs in a particular colony; No. SB – number of sampled birds in a particular year

regarding these isolates due to the lack of genetic evidence. A DNA-based study on 73 pigeon droppings revealed the presence of *A. baumannii* in 5% of the sam-

ples from France and Algeria (Morakchi et al. 2017). Moreover, isolation of *A. baumannii* was reported from poultry hatcheries (Martin and Jackel 2011; Wilharm

et al. 2017), poultry meat (Lupo et al. 2014; Carvalheira et al. 2017), and poultry livestock (Lupo et al. 2014). It has recently been demonstrated that 661 white stork nestlings studied in different regions of Poland over a period of four years were colonized at an average rate of 25% (Wilharm et al. 2017). However, systematic studies for the presence of *A. baumannii* are not available for other free-living birds leaving open the question of whether birds, in general, are favourable hosts for *A. baumannii*.

Here, for the first time, a large and diverse set of altogether 1,051 free-living birds was selectively screened for the presence of *A. baumannii* in cloacal samples (overall 1,026 samples) and tracheal samples (overall 310 samples), using the protocol established for studies on white stork nestlings (Wilharm et al. 2017). The extremely low prevalence of 0.3% in black-headed gulls and non-detection in 310 songbirds compared to 25% prevalence in white stork nestlings strongly argues against a general preference of *A. baumannii* for avian hosts. In line with this conclusion, no *A. baumannii* was isolated in a study on the aerobic cloacal and pharyngeal bacteria of 167 free-living birds in Germany (Stenkat et al. 2014). However, our study is limited by the opportunistic sampling design, affecting the species range, further causing potential biases due to specific behaviour and seasonality.

The two *A. baumannii* strains isolated from gulls were susceptible to clinically relevant antibiotics and exhibited only natural resistance to some penicillins, cephalosporins, and chloramphenicol. This is in full agreement with the resistance profiles previously described for the isolates from white stork nestlings (Wilharm et al. 2017). In conclusion, there is no evidence that these strains faced significant anthropogenic selection pressure in the past suggesting that the strains were acquired from natural habitats.

In summary, the data presented here indicate that there is no general association of *A. baumannii* with wild birds. Given the obvious risk of poultry livestock to serve as a vector for spreading of *A. baumannii* (Martin and Jackel 2011; Lupo et al. 2014; Wilharm et al. 2017), it is important to study poultry livestock as well as wild populations of chicken, geese, ducks, turkeys and related species globally to assess the risk potential associated with these populations and to complete our ecological understanding of pathogenic *Acinetobacter* species.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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