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Long-term carriage and rapid transmission of extended spectrum beta-lactamase-producing *E. coli* within a flock of Mallards in the absence of antibiotic selection

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Summary

Wild birds have been suggested as transmitters and reservoirs for antibiotic resistant bacteria. We performed an experimental study investigating carriage time and interindividual transmission of extended spectrum beta-lactamase- (ESBL-)producing *Escherichia coli* in Mallards (*Anas platyrhynchos)* to assess if the birds carry the bacteria long enough to transfer them geographically during migration. Mallards were inoculated intraoesophageally with four different strains of ESBL-producing *E. coli* and kept together in a flock. The ESBL-strains belonged to sequence types previously shown to spread between birds and humans. Culturing from faecal samples showed presence of ESBL-producing *E. coli* the entire 29 day experimental period. An extensive and rapid transmission of the different ESBL-strains between individuals (including non-inoculated controls) was observed. In necropsy samples, we detected ESBL-strains in the cecum even in faeces-negative birds, indicating that this part of the intestine could function as a reservoir of resistant bacteria. We demonstrate that birds can carry ESBL-producing *E. coli* for long enough times to travel far during migration and the extensive interindividual transmission suggests spread between individuals in a dense bird population as a mechanism that allow persistence of resistant bacteria.

Introduction

Antibiotic resistance is a large and growing threat to the health care and Enterobacteriaceae resistant to the common therapy option cephalosporins through production of Extended Spectrum Beta-Lactamases (ESBLs) represent a continuous clinical problem (Coque *et al*., 2008). Today, ESBL-producing bacteria are also regularly isolated from soil, crops, water and wildlife (Kummerer, 2004; Literak *et al*., 2010a; Hernandez *et al*., 2012; Ben Said *et al*., 2015). There is a growing awareness that the natural environment is important in the emergence and spread of antibiotic resistance (Guenther *et al*., 2011; Wellington *et al*., 2013; Dorado-Garcia *et al*., 2018).

Several environmental studies during the last decade have focused on the prevalence of antibiotic resistance in Enterobacteriaceae isolated from wild birds. Birds are interesting environmental indicators as they often live close to human settings and are exposed to our bacteria and antibiotic residues through, for example, garbage dumps, sewage treatment plants and agricultural areas. Furthermore, many bird species migrate between continents every year, connecting areas with high prevalence of antibiotic resistance to areas where antibiotic resistance is scarce. In addition, many bird species form flocks with close interindividual contacts that may promote transmission of microorganisms between individual birds. Therefore, birds have been suggested both as transmitters and as reservoirs in the global spread of antibiotic resistance genes. Transfer of *E. coli* between humans and wild birds often occurs, as indicated by the finding that levels of *E. coli* are higher in wild birds living close to humans compared with birds with limited human contact (Gordon and Cowling, 2003). Further, similar *E. coli* sequence types (STs) or clonal groups are often isolated from wild birds, humans and domesticated

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animals (Bonnedahl *et al*., 2009; Bonnedahl *et al*., 2010; Guenther *et al*., 2010; Hernandez *et al*., 2010; Simoes *et al*., 2010). In several bird species living close to humans or feeding from human related products, resistant *E. coli* have been found, for example, in Mallards *Anas platyrhynchos* (Literak *et al*., 2010b), Canada geese *Branta canadensis* (Cole *et al*., 2005; Middleton and Ambrose, 2005), feral pigeons *Columba livia* (Radimersky *et al*., 2010) and red-billed choughs *Pyrrhocorax pyrrhocorax* (Blanco *et al*., 2009). Faecal carriage rates of ESBL-producing *E. coli* up to 54% of sampled birds have been found (Baez *et al*., 2015). The mallard is an especially interesting species as it often forages in waterways downstream of sewage treatment plants, where it can be exposed to both antibiotic resistant bacteria and antibiotic residues originating from the sewage water; and it has been shown that 47% of faecal samples from Swedish urban Mallards grew ESBL-producing *E. coli (*Hessman *et al*., 2018*).* The Mallard is a partial migrant and winter conditions can affect the migration (Sauter *et al*., 2010). Some Mallards perform a full winter migration; some are sedentary, whereas some stay at stop-over sites (van Toor *et al*., 2013). A recent Polish study demonstrates that mallards can migrate up to 382 km in 1 day (Smietanka *et al*., 2016), and ring and recovery data show that the mallard can migrate about 1700 km in 1 month [birds reported to the Swedish Bird Ringing Centre (http://www.nrm.se/english/researchandcollections/environmentalresearchandmonitoring/birdringingcentre.214_en.html, data from personal communication, Thord Fransson)].

Even though the prevalence of resistant bacteria in birds has been studied before, the residence time of resistant bacteria in wild birds is unknown, and we do not know how rapidly resistant bacteria are transmitted between carriers and non-carriers. Both of these factors are important parameters that need to be determined to allow an assessment of the role that birds might play as reservoirs and in the spread of antibiotic resistance between different environments, including human populations. To our knowledge, there is only one study performed on carriage time of resistant bacteria in birds, analysing persistence and spread of ESBL-producing bacteria in chickens, that is, a domesticated bird (Le Devendec *et al*., 2011).

Here, we used Mallards as a model species. This model has previously been successfully used in influenza virus studies (Järhult *et al*., 2011) as an experimentally amenable and relevant model. In this study, we aimed to answer two questions: (i) Is the residence time of ESBL-producing *E. coli* in birds long enough to allow for migration, despite absence of an antibiotic selection pressure? (ii) Are resistant bacterial strains transmitted between individual birds, potentially enabling further persistence of resistance in the population?

Experimental procedures

E. coli *used for inoculation*

To inoculate the Mallards, four ESBL-producing strains (SP4, SP132, SP163 and SP165) with different resistance profiles to other antibiotics and belonging to ST-types shown previously to colonize both humans and birds or other wildlife, were chosen (Table 1). The strains used for inoculation were previously isolated from Yellow-legged gulls (*Larus michaelis)* in Spain (Stedt *et al*., 2015). In preparation for inoculation, the ESBL-producing *E. coli* strains were grown overnight (O/N) at 37 °C in Luria Broth (LB) media, supplemented with 10 μg ml^{-1} of cefotaxime to maintain the respective ESBL-plasmids. The bacterial cultures were concentrated 10 times by centrifugation and resuspended in LB to reach 10^{10} cfu ml^{-1} as determined by viable count.

Bioinformatics of the bacterial strains

The ESBL-producing strains were characterized by whole genome sequencing using the Illumina MiSeq technology. Total bacterial DNA was prepared using the MasterPure DNA Purification Kit (Epicentre an Illumina company) and used as input DNA for library construction using Nextera XT DNA Library preparation kit (Illumina, January 2015) according to the manufacturer´s instructions. Sequencing was performed with an Illumina MiSeq instrument producing 300-bp paired-end reads. Sequencing data was aligned and analysed using the CLC Genomic Workbench program v10 (CLC bio).

Multi locus sequence typing (MLST) results were extracted from the whole genome sequencing data using the seven standard housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) and sequence types (STs) were determined via the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli/#) (Wirth *et al*., 2006). Resistance genes and virulence genes were searched for using the ResFinder (Zankari *et al*. 2012), VirulenceFinder (Joensen *et al*. (2014) and PathogenFinder (Cosentino *et al*., 2013) software available at the website of Centre for Genomic Epidemiology (www.genomicepidemiology.org).

Mallards

The Mallards were purchased from a commercial breeding farm in Sweden and housed at the animal facility at the National Veterinary Institute in Uppsala, Sweden, according to guidelines for laboratory animals by the Swedish Board of Agriculture. They were fed non-medicated, clean (but not sterilized) feed. Ethical approval for the study was obtained from the Uppsala ethical committee on animal experiments (C201/11). All Mallards were screened on arrival at the Animal House by culturing for

Table 1. Resistance patterns and identified sequence characteristics of the bacterial strains.

Antibiotic/Strain	SP4	SP132	SP163	SP165
Resistance genes identified	*aadA5, bla*$_{TEM-1}$, *bla*$_{CTX-M-14}$, *dfrA17, strAB, sul1, sul2, tet(B)*	*bla*$_{CTX-M-1}$	*bla*$_{CTX-M-14}$, *tet(A)*	*aph(3′)-1c, bla*$_{CTX-M-14}$, *strAB, sul2, tet(B)*
Plasmid origins identified	IncFIB, IncFIC, IncI1, IncQ1	IncI1	IncFIB, IncFIC, IncB/O/K/Z	IncFIB, IncFIC, IncB/O/K/Z
Nalidixic acid	**256**	**4**	**256**	**256**
Ciprofloxacin	12	0.008	12	6
Fosfomycin	1	2	1.5	3
Sulfamethoxazole	**1024**	**64**	**1024**	**1024**
Streptomycin	**48**	**6**	**8**	**64**
Tetracycline	192	4	64	192
Tigecycline	0.094	0.125	0.19	0.094
Ampicillin	256	256	256	256
Mecillinam	1	0.19	0.19	0.5
Cefotaxime	32	32	32	32
Ertapenem	0.004	0.006	0.008	0.006
Chloramphenicol	3	4	4	2
Nitrofurantoin	24	24	48	32
Virulence genes identified	*iroN, iss, mchF*	*gad*	*cba, cma, gad, iss, iroN, lpfA, mchF*	*ireA, iroN, iss, mchF, tsh*
Sequence type (reference(s))	**10** (Manges and Johnson, 2012; Olesen *et al*., 2012)	**409** (Manges and Johnson, 2012; Hasman *et al*., 2014; Campos *et al*., 2015)	**359** (Maluta *et al*., 2014)	**93** (Bortolaia *et al*., 2011; Dierikx *et al*., 2013)

Minimum inhibitory concentrations determined by Etest. Susceptibility differences used to distinguish the experimental strains shown in bold type. Note that SP4 had a distinct morphological appearance when cultured on EMBL-plates, thus strengthening the discriminative power between SP4/SP165.
cba: Colicin B pore forming protein, *cma*: Colicin M, *gad*: Glutamate decarboxylase, *ireA*: Siderophore receptor, *iroN*: Catecholate siderophore, *iss*: Serum survival gene, *lpfA*: long polar fimbriae, *mchF*: ABC transporter protein, *tsh*: Temperature-sensitive hemagglutinin.

E. coli resistant to cefotaxime (10 μg ml^{-1}), nalidixic acid (10 μg ml^{-1}) or streptomycin (50 μg ml^{-1}). No *E. coli* resistant to any of the tested antibiotics were found prior to the inoculation.

Experimental design

Fifteen Mallards were divided into five groups with three birds in each group. Four groups were inoculated with 10^{10} cfu of either of SP4, SP132, SP163 and SP165 respectively, and one group was not inoculated and served as a control. The inoculation was performed using a blunt syringe that was inserted down into the oesophagus. During the experiment, all Mallards were kept together and roamed freely in an experiment room of approximately 12 m^2. They had access to a 1 m^2 pool containing approximately 170 l of water, and feed and water *ad libitum.* The water in the pool was changed daily and the pool was thoroughly cleaned. In the rest of the experimental room, faecal matter and dirty or wet bedding was removed daily.

Sampling

Faecal samples from individual birds were obtained by putting the birds in individual, clean cardboard boxes and collecting faeces when they had defecated in the box. We transferred approximately 0.5 g of faeces into 1 ml of sterile phosphate-buffered saline (PBS) with 20% of dimethyl sulfoxide (DMSO) as freeze-protectant. The samples were stored at −80 °C until analysis. Sampling was performed before inoculation and at days 1, 11, 26 and 29 days post inoculation (dpi).

Necropsies

All birds were sacrificed at 29 dpi. A minor necropsy was performed immediately after intravenous injection of an overdose of pentobarbital. Samples of intestinal content were obtained by cutting the intestine and gently pressing both ends to extract the contents. The samples were taken from the midpoint of the small intestine (jejuno-ileal junction, by Meckel's diverticulum), the midpoint of one caecum and the midpoint of the colon. The intestinal contents were then sampled and processed in the same fashion as the faecal samples.

Selective culturing of ESBL-producing bacteria

Faecal samples of 100 μl were plated on Eosin Methylene-Blue 1% Lactose (EMBL) plates and EMBL plates supplemented with 10 μg ml^{-1} cefotaxime (EMBL Ctx), incubated O/N at 37 °C and the next day the numbers of colonies were counted. Up to 50 individual big brown-red or green colonies per sample were further screened on individual selective EMBL plates containing

nalidixic acid (10 μg ml^{-1}), fosfomycin (8 μg ml^{-1}), sulfamethoxazole (1000 μg ml^{-1}), streptomycin (50 ng ml^{-1}) and cefotaxime (10 μg ml^{-1}) respectively. The original strains SP4, SP132, SP163 and SP165 were also patched on all different plates as controls. By comparing the results with the resistance patterns shown in Table 1 and phenotypic differences in colony morphology, each colony from the EMBL Ctx plate was attributed to one of the inoculated strains.

Results and discussion

Carriage of ESBL-producing E. coli *proceed for over 4 weeks*

To assess the carriage time and spread of ESBL-producing *E. coli* strains among Mallards in a flock, we inoculated four groups of three Mallards each with one of four different ESBL-producing *E. coli* strains originally isolated from gulls and kept three birds as uninoculated controls (Fig. 1). The bacterial strains belonged to different MLST-types (Table 1) that have all previously been identified as avian pathogenic *E. coli* (APEC) and extraintestinal pathogenic *E. coli* (ExPEC) and shown to infect both humans and birds (references in Table 1). The strains were chosen for their differential resistance phenotypes that facilitated post-inoculation screening for the respective strain. In addition, we whole genome sequenced all four strains to have a better understanding of their differences in genetic context. In accordance with differences in resistance gene content, the strains also contained individual but partly overlapping sets of virulence genes previously identified in *E. coli* of avian origin (Table 1). Before inoculation, all mallards were negative for ESBL-producing *E. coli* but already at 1 dpi, all birds, including the negative controls, were positive for excretion of ESBL-producing *E. coli* (Fig. 1). At the end of the experiment (29 dpi), only 1 of 15 birds excreted ESBL-producing *E. coli.* However, based on the large number excreting ESBL-producing *E. coli* at 26 dpi (10/15) and positivity in intestinal samples as discussed below, our findings suggest that ESBL-producing *E. coli* carriage can continue for well over 4 weeks in a Mallard flock in the absence of any antibiotic selection.

ESBL-producing E. coli *in the intestine*

To assess if ESBL-producing *E. coli* can be present in the intestinal content of the Mallards without being shed in faeces, we performed a minor necropsy and sampled intestinal content from each bird by the end of the experiment at dpi 29. ESBL-producing *E. coli* were present in four caecal samples and one colon sample. None of the birds with ESBL-producing *E. coli* in the caecum excreted ESBL-producing *E. coli* in the faeces. A possible explanation for this observation is that the caecum in Mallards has fermentative properties and limited exchange of contents with the rest of the intestine. Thus, studies that rely solely on faecal samples could underestimate the prevalence of resistant coliform bacteria in Mallards and likely also in other birds that have a similar fermentative function of their caeca. The only bird positive in colon at the necropsies was also positive in the faeces, indicating that

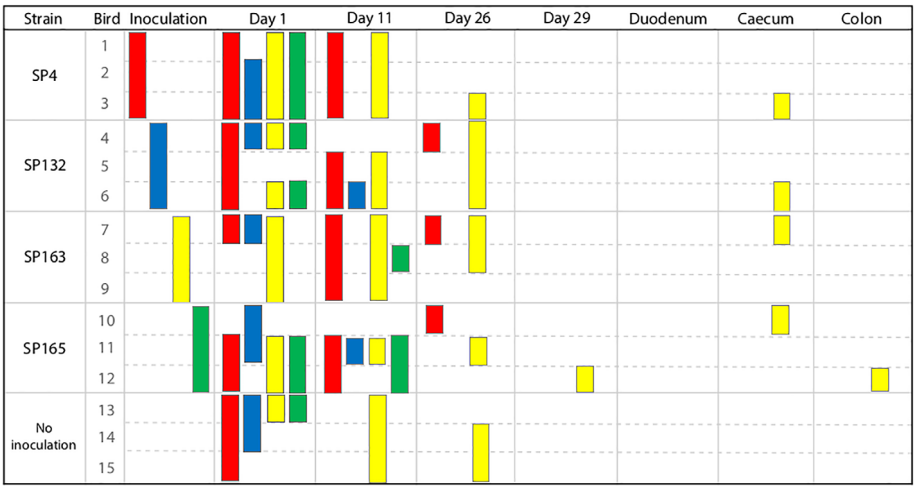

Fig. 1. Overview of inoculation strains and results from culturing of faecal and necropsy samples.
Red denotes strain SP4, blue SP132, yellow SP163 and green SP165. Each group of three inoculated birds are grouped according to the inoculated strain and the coloured bars indicate which birds were colonized with that strain at each sampling point. Multiple coloured bars in the same column mean that more than one strain was detected in the individual bird during strain specific culturing. The first column displays which strain each bird was inoculated with, the following columns display the culturing results from faecal samples at different time points in the experiment, and the final three columns display culture results from necropsy samples obtained at different positions in the intestine. Necropsies were performed at 29 dpi, at the same time as the last faecal sample was obtained.

carriage in the colon might be better reflected in faecal samples. All necropsy samples from the small intestine were negative, consistent with the fact that *E. coli* mainly colonizes the distal parts of the intestine. All ESBL-producing *E. coli* found in samples from necropsies were SP163 (yellow in Fig. 1).

Rapid and extensive transmission of ESBL-producing E. coli *between birds in the flock*

At 1 dpi, all birds excreted ESBL-producing *E. coli*, even the non-inoculated controls. This rapid transmission demonstrates that resistant coliform bacteria can spread readily between individual Mallards in a flock, most likely via the *faecal-oral* route. There was also a rapid spread of all four different ESBL-strains at 1 dpi; from five birds all four strains were recovered, four birds carried three strains, one bird two strains and five birds one strain (Fig. 1). The Mallards in our study were kept in a confined space with shared sources of water and feed which could increase the chance of interindividual transmission. However, this scenario is similar to the wild behaviour of many wetland bird species. Gulls and ducks often feed in highly aggregated flocks in small water ponds and gulls breed close together in densely populated colonies. A possible contributing factor to the rapid spread is the large amounts of bacteria used for inoculation (approximately 10^{10} cfu/bird inoculated) that might give an initial increased shedding of the bacterial strains in the faeces. However, daily water change and cleaning of the pool was performed to decrease the influence of contamination of bacteria from the initial inoculation and support the hypothesis of ongoing transmission between birds throughout the experiment rather than initial contamination of the experimental room. The pronounced changes over time in which bacterial strains were recovered from the different Mallards is also a sign of ongoing transmission (Fig. 1). From our data, it seems that SP163—and to some extent SP4—are more successful colonizers as they gradually dominate the faecal/intestinal samples over time. SP163 was also the only strain found in the necropsies of the birds. This may reflect a better general colonizing ability. Strain SP4 belong to MLST cluster ST10 and SP163 to ST359, both of which are lineages that have previously been isolated both clinically from humans and from birds (Valverde *et al*., 2009; Okeke *et al*., 2010; Bortolaia *et al*., 2011) and has been attributed to the avian pathogenic *E. coli* group (APEC) (Maluta *et al*., 2014). If differences in the presence of virulence factors between the strains contributed to the differences in carriage time remains to be investigated.

Implications of long-term carriage of resistant bacteria in bird flocks

Our results demonstrate that Mallards can carry ESBL-producing *E. coli* for at least 29 days. Mallards can migrate over 600 km in only a few days (Smietanka *et al*., 2016) and about 1700 km in 1 month (according to ring and recovery data), potentially carrying resistant bacteria to new areas. Most likely, the ability to carry resistant bacteria for several weeks is not limited to Mallards and chickens. The transmission of ESBL-producing *E. coli* in the Mallard flock seen in our experiment suggests that the carriage time in flock-dwelling birds is prolonged by inter-bird transmission of bacteria via the faecal-oral route. Previous studies have also suggested that there is a regular transfer of bacterial strains between humans and birds (Bonnedahl *et al*., 2009; Bonnedahl *et al*., 2010; Guenther *et al*., 2010; Hernandez *et al*., 2010; Hernandez *et al*., 2013; Simoes *et al*., 2010). Thus, birds in general could pick up and spread resistant bacteria during migration as a consequence of human-bird interactions. Even though the *E. coli* strains used in our experiment were isolated from Gulls and not adapted to the Mallard as a host species, all of the strains could be found in the Mallards during at least the first 11 days post inoculation. This indicates that the colonization ability is widespread among wild bird *E. coli.* However, the difference seen with respect to which strain persisted the longest in the bird population suggests that strain specific traits may indeed influence the colonization time. Thus, our results could underestimate the potential carriage time as compared to if resistance was established in Mallard-adapted Enterobacteriaceae. Furthermore, in many parts of the environment such as sewage water and sludge, low levels of antibiotics are present. Earlier studies have shown that even very low concentrations of antibiotics can select for resistant bacteria (Gullberg *et al*., 2011). Thus, weak antibiotic selection pressures present in the environment could be a factor further increasing carriage of resistant bacteria in birds.

Conclusions

Mallards can carry ESBL-producing *E. coli* for at least 1 month, enough time to cover long distances. Extensive transmission of ESBL-producing strains occurs between individuals, which in dense wild bird populations could increase persistence of resistance. Detection of ESBL-producing *E. coli* in samples from the caecum in faeces-negative birds demonstrates that this part of the intestine may function as a reservoir of resistant bacteria. This needs to be taken into account in surveillance studies when extrapolating frequency of faeces positivity for resistant bacteria to true prevalence that thus may be

underestimated. Our and previous results indicate that wild birds could play a role in the global dissemination of antibiotic-resistant bacteria. Measures to alleviate the problem include limitation of dispersion of resistant bacteria and antibiotics to the environment but more research is needed to better understand and assess the risks of antibiotic resistance at the human/animal/environment interface.

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