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Bacterial and fungal flora in faecal samples from rooks (*Corvus frugilegus*) in the City of Zagreb, Croatia

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ABSTRACT

The objective of this study was to determine the occurrence of bacteria and fungi in populations of urban rooks. We investigated the prevalence of bacteria and fungi in the faeces of rooks (*Corvus frugilegus*) in the City of Zagreb, during their breeding period in 2006. Microbiological examination of fresh faecal samples revealed the occurrence of *Escherichia coli*, *Bacillus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Agrobacterium radiobacter*, *and Acinetobacter* spp. One rook was positive for *Campylobacter jejuni*, as confirmed by polymerase chain reaction (PCR). The fungal species *Mucor* spp., *Cladosporium* spp., *Rhodotorula rubra, Aspergillus (A.) fumigatus* and *A. flavus, Alternaria* sp., *Candida* spp., and *Penicillium* spp. were also isolated.

Key words: rook, *Corvus frugilegus,* bacteria, fungi

Introduction

The rook (*Corvus frugilegus*) is a breeding, resident bird in Croatia. Rooks feed in flocks, roost communally and breed in large colonies in tall trees, sometimes in urban areas. Their nests are bulky. made from twigs bound together with soil, and can be reused year after year. A high number of urban rooks can be found in their traditional breeding

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sites in Zagreb during early spring, when the breeding season starts (VLAHOVIĆ, 2002). They are also widely spread all over the City of Zagreb and frequently roam around residential areas, industrial zones and animal and agricultural farms, thus coming into close contact with humans and other animals. Therefore rooks can play a role as transmitters of microorganisms to other birds and to humans. According to LITERÁK et al. (2007), migratory birds such as rooks, in close contact with humans and domestic animals, can play an important role in the dispersion of these pathogens over long distances. During the day, rooks leave their roosting sites in search of food and may fly 10-25 km from their colony, occasionally as far as 40-50 km. Due to their mobility and local migration, these birds may spread pathogenic microorganisms (HUBÁLEK, 2004). The most important public health concern associated with rooks is the accumulation of faecal samples at roosting sites. Only a few sources regarding *Escherichia coli* and *Salmonella* isolates from rooks are known and the importance of rooks regarding the long-distance transfer of these bacteria has not been studied (LITERAK et al., 2007). Rooks have been examined for the presence of pathogenic bacteria in the UK (WILSON and MACDONALD, 1967), Czech Republic (HUBÁLEK and DVOŘÁK, 1968; HUBÁLEK et al., 1995) Poland (GOLEBIOWSKI, 1988), France (BOUTTEFROY et al., 1997), Germany (GLÜNDER, 1989) and Croatia (KOVAČIĆ and LACKOVIĆ, 1985; VLAHOVIĆ et al., 2004). Studies of the spread of microorganisms in wild birds have been rare in Croatia (PAVLAK et al., 2000; HORVATEK et al., 2004; VLAHOVIĆ et al., 2004; PRUKNER-RADOVČIĆ et al., 2005). According to KALETA and TADAY (2003), the bacterium *Chlamydophila psittaci* has a wide host range, and over 469 different species of birds have been reported to be susceptible to the infection. Numerous domestic and wild animal species, including birds, have been identified as natural reservoirs of *Campylobacter* (ROSEF and KAPPERUD, 1983; JONES, 2001).

The aim of the study was to carry out preliminary monitoring of some of the pathogenic microorganisms of urban rooks, focusing on potential transmission to other animals and humans.

Materials and methods

Rooks examined. Faecal samples were taken from rook breeding sites in the area of the City of Zagreb (45°49'N 16°02'E). These locations were widely distributed in the City of Zagreb, and were all situated in public areas, chosen on the basis of previous research into traditional rook breeding sites in Zagreb (KUFRIN, 1991; VLAHOVIĆ, 2002). City districts where colonies with nests were found in 2006 are marked as Districts 1-16. Locations in the districts where sampling was conducted are presented as circles on the map of the City (Fig. 1). The coordinates of satellite colonies or nest groups situated more than 100m from each other were incorporated into the maps and submitted as reference points for the

geographical information system (GIS) (ArcView GIS 3. 1 1966. AcrView GIS 3. 1 ESRI, Environmental Systems Research Institute, Inc. USA). Colonies were counted during the last week of March and first week of April 2006. By counting active nests, the number of breeding pairs was determined according to MASON and MACDONALD (2004).

Collection of samples. The samples were collected only during the breeding period, in 16 districts. We collected 57 faecal samples from 35 individual breeding colonies. They were obtained individually from large pieces of sterile plastic film, exposed for one night on the ground at the breeding site. Faecal samples were collected three times during the spring of 2006. Altogether, 35, 15 and 7 faecal samples were taken in February, March and April 2006, respectively. The total of 57 faecal samples was examined to determine the prevalence of the bacteria *Chlamydophila* spp., while 35 out of the 57 samples were examined to check the presence of other potential pathogens.

Detection of aerobic bacteria and fungi. Bacteriological and mycological examinations were carried out using standard methods for aerobic bacteria and fungi (BROWN, 2005). For the detection of aerobic bacteria, all samples were directly streaked on Nutrient agar (Becton, Dickinson and Company, SAD) and Brilliantgreen - phenolrot - lactose - saccharose agar (BPLS; Merck, Germany). Nutrient agar and BPLS-Agar plates were incubated aerobically at 37 °C for 24 hours and at 24 °C for another 24 hours. Typical bacterial colonies were randomly selected, examined microscopically for their morphology and recultivated to obtain pure culture. The identification of isolated bacteria was based on colony morphology, microscopical examination, and biochemical confirmation using API 20 E and API 20 NE strips (BioMerieux, France). For the detection of fungi, the sample was plated on Sabouraud Dextrose agar (BBL™; Becton, Dickinson and Company, SAD) and incubated at 24 °C for 4-10 days. Fungi were identified macroscopically based on colony morphology, and microscopically using lactophenol staining.

Detection of Campylobacter spp. All the faecal samples collected were aseptically transferred to Campylobacter Selective Enrichment Broth (Oxoid, Unipath, UK) and incubated at 42 ºC for 48 hours in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2). One loopful of the 48-hour cultures was streaked on modified Charcoal Cefoperazone Desoxycholate Agar plates (mCCDA; Oxoid, Unipath, UK) and incubated in a microaerobic atmosphere for 48 hours at 42 ºC. Suspected colonies were identified as *Campylobacter* spp. by standard microbiological and biochemical procedures - colony morphology, oxidase and catalase tests and Gram staining (BOLTON et al., 1992). The DNA to be used as target in the multiplex PCR (mPCR) was extracted from 48-hour cultures prepared in Nutrient Broth No. 2, as described by JACKSON et al. (1993). The identification of strains was obtained by multiplex PCR as described by MANFREDA et al. (2003). Following amplification, 20 µL of PCR product were electrophoresed in 1% TAE buffer on 1% agarose gel stained with ethidium bromide and visualised under UV

light. The expected PCR amplicons were at 857, 589 and 462 bp and corresponded to the genus *Campylobacter* and to the species *C. coli* and *C. jejuni*, respectively. *Campylobacter jejuni* ATCC 33560 and *C. coli* RM 2228 were used as positive controls. Our own strain of *E. coli* was used as negative control.

Detection of Chlamydophila spp. The commercially available CLEARVIEW (CW) test (Unipath Limited, Bedford, United Kingdom), approved for the direct detection of *Chlamydia* (*C.*) *trachomatis* antigen in endocervical swab specimens, was evaluated for the detection of

Cp. psittaci in the fecal swabs of rooks (FUDGE, 1997). Samples that were positive according to the CW test were tested again by standard procedure on chicken embryos. Faecal samples, homogenised in a transport medium consisting of sucrose/phosphate/glutamate (SPG) (SPENCER and JOHNSON, 1983) and centrifuged to obtain supernatant, were inoculated in the yolk sac of 6-8 day-old specific pathogen free chicken embryos (ANDERSEN et al., 1997). The eggs were incubated in a humid atmosphere at 39 °C. Embryos that died 3-9 days post inoculation were harvested, and the yolk sacs of surviving embryos used for blind passages. After 48 to 72 hours of incubation, the yolk sac and umbilical vein was stained by the Gimenez method (ANONYM., 2004).

Results

The distribution of rook breeding colonies and sampling locations was shown as a map using the GIS application (Fig. 1), whereby the GIS circles represent data on the locations of all 35 colonies containing two or more pairs and five satellite colonies. The results of microbiological examination and identification of microorganisms in the area of study (Districts 1-16) are presented in Tables 1 and 2. Microbiological examination of faecal samples revealed a great number of birds positive for *E. coli, Bacillus* spp. and *Staphylococcus* spp. *E. coli* was isolated in 10 of the 16 districts and *Bacillus* spp. in 9 districts (Table 1). The fungal species *Mucor* spp., *Cladosporium* spp., *Rhodotorula rubra, Aspergillus* sp., *A. fumigatus* and *A. flavus, Alternaria* sp., *Candida* spp., and *Penicillium* spp. were also isolated (Table 2). *Campylobacter jejuni* were isolated from one of 35 faecal samples examined. The results in Table 1 show that, of 5 *Cp. psittaci* positive samples examined by CW test, none were confirmed positive by isolation.

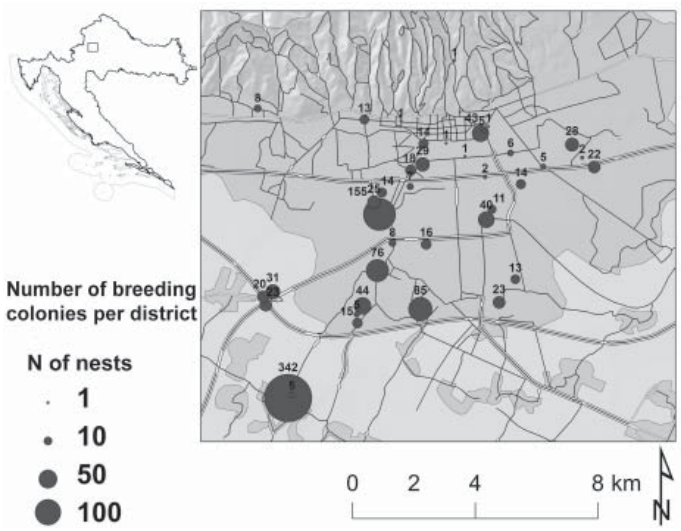

Fig. 1. The distribution of 35 rooks breeding colonies and five satellite colonies per district with number of nests per colony in 2006 in the City of Zagreb

Table 1. Districts with sampling location and number of positive rook faecal samples tested per district in the City of Zagreb in 2006

Districts	Breeding pars per		Nº colonies per districts	Investigated/Positive		Occurrence of bacteria and fungi per districts
	colony	distict		*C. psittaci* CW test	Other bacteria and fungi	
Botinec	44 5 15	64	3	6/0	3/2	*E. coli, Mucor* spp., *Altenaria* sp., *Bacillus* spp.
Dugave	13 23	36	2	4/0	2/1	*Streptococcus* spp.
Knežija	14	14	1	2/0	2/2	*E. coli, Acinetobacter* spp., *Candida* spp. *E. coli*
Trnsko	16 85	101	2	4/1	3/2	*E. coli, Staphylococcus* spp., *Bacillus* spp. *E. coli, Streptococcus* spp., *R. rubra, Cladosporum* spp.

Table 1. Districts with sampling location and number of positive rook faecal samples tested per district in the City of Zagreb in 2006 (continued from page 85)

Districts	Breeding pars per		Nº colonies per districts	Investigaed/ Positive		Occurrence of bacteria and fungi per districts
	colony	district		*C. psittaci* CW test	Other bacteria and fungi	
Center	5 43	48	2	4/0	3/2	*E. coli*, *Staphylococcus* spp. *E. coli*, *Mucor* spp.
Horvati	25 155	180	2	6/0	4/3	*E. coli*, *Bacillus* spp., *A. fumigatus* *Bacillus* spp., *Penicillium* spp. *E. coli*, *Bacillus* spp., *R. rubra*
Trešnjevka	13	13	1	1/0	1/0	neg.
Trnje	5 7 18 29 2	61	5	5/0	3/2	*E. coli*, *Staphylococcus* spp., *Bacillus* spp. *E. coli*, *Cladosporum* spp., *A. radiobacter*
Gornji grad	14 1* 1* 1*	17	1	1/0	1/0	neg.
New Zagreb	11 40	51	2	2/0	1/1	*E. coli*, *Staphylococcus* spp., *Bacillus* spp.
Pešćenica	22 2 28 6 1*	59	4	4/0	3/2	*E. coli*, *Staphylococcus* spp. *Bacillus* spp.
Lučko	23 20 31	74	3	6/2	4/3	*Campylobacter* sp. *Bacillus* spp., *A. flavus* *Bacillus* spp.
Trstik	14	14	1	1/0	1/0	neg.
Remetinec	8 76	84	2	2/0	2/2	*Cladosporium* spp. *Bacillus* spp.
Brezovica	5 342	347	2	7/2	4/2	*E. coli*, *Streptococcus* spp., *A. fumigatus*, *Mucor* spp. *Bacillus* spp.
Črnomerec	2 1* 8	11	2	2/0	1/1	*E. coli*, *Staphylococcus* spp.
Total: 16	1174	1174	35	57/5	38/25	

*Satellite colonies

Table 2. Occurrence of bacteria and fungi in fecal samples in rooks by sampling location in the area of the City of Zagreb

Species	N° of positive/total of samples tested	%
Bacteria		
E. coli	15 /35	42.9
Bacillus spp.	12/35	34.3
Staphylococcus spp.	6/35	17.1
Streptococcus spp.	3/35	8.6
Acinetobacter spp.	1/35	2.9
A. radiobacter	1/35	2.9
Campylobacter sp.	1/35	2.9
Fungi		
Mucor spp.	3/35	8.6
Cladosporium spp.	3/35	8.6
R. rubra	2/35	5.7
A. fumigatus	2/35	5.7
A. flavus	1/35	2.9
Altenaria sp.	1/35	2.9
Candida spp.	1/35	2.9
Penicillium spp.	1/35	2.9

Discussion

In Croatia, rooks are not migratory birds, but they often change habitats within the city area, according to their daily and nightly cycles, and breeding, feeding and overwintering cycle. This supposes an easier transmission of pathogen agents between them (HUBÁLEK, 2004). One of these, *E. coli*, can be found in wild birds (SIMPSON, 2002). Previous investigations indicate that some strains of *E. coli*, such as the one marked as Avian Pathogenic *E. coli* (APEC) share significant genetic similarities with the strain of *E. coli* isolated from humans (JOHNSON et al., 2007), and that numerous new genetically modified strains can be found every day, with potential pathogenic effects on humans and other birds (DHO-MOULIN and FAIRBROTHER, 1999; SILVEIRA et al., 2002). Results of the preliminary microbiological examination of the rooks' faecal samples in the area of the City of Zagreb revealed a great number of birds positive for *E. coli*. In our investigation, serotyping or genotyping of *E. coli* was not conducted, but the high number of *E. coli* positive samples is indicative and may serve as a basis for further research. LITERÁK

et al. (2007) also isolated the *E. coli* from rooks faecal samples. The study suggests that rooks can harbour *E. coli* and *Salmonella* isolates, probably reflecting the presence of such isolates in their sources of food and/or water in the environment. According to EJIDOKUN et al. (2006), indirect contact with wild birds (rooks) faeces may be a way of transmitting the pathogen isolate *E. coli* O157 from birds to human. BRITTINGHAM et al. (1988) and KOCIJAN et al. (2009) reported that *Staphylococcus* spp. and *Streptococcus* spp. are ubiquitous, of low pathogenicity, and usually found in birds' feed. Birds excrete these bacteria via the alimentary tract. The same authors assume that *E. coli* is a pathogen species and reported that the prevalence of *Streptococcus* spp. was higher in omnivorous than in granivorous species (20% versus 8%). Since *C. jejuni* was isolated, the results of our research indicate that rooks can be infected by agents that may cause a disease affecting humans. *Campylobacter* spp. was isolated from 28 rooks in Germany (GLÜNDER, 1989) indicating that nestlings are more often infected with Campylobacter than older birds. *Campylobacter* spp. may be spread from wild animal reservoirs to the environment and transmitted to other animals and humans (ROSEF and KAPPERUD, 1983; JONES, 2001). Infection with *Chlamydophila* and *Salmonella* species was not established in our research, in contrast to our earlier study investigating *Salmonella* species (VLAHOVIĆ, 2004), in which the *Salmonella enterica* serovar *Enteritidis* was isolated in rooks. The possibility of a sporadic infection of rooks by chlamydias was reported by the World Organization for Animal Health (ANONYM., 2005), but chlamydias was not isolated in this research. In accordance with our research, HUBÁLEK (1978) also reported a large number of fungus species isolated from rooks. He proved the presence of 41 species of fungi isolated from 858 samples (feathers, nests, pellets, and droppings) collected from different species of free-living birds, including rooks.

The results also showed that the rook population in Zagreb feeds at a landfill during the summer. It should be emphasized that the birds' feeding at the landfill has a possible epidemiological implication. In one of their investigations, OLEA and BAGLIONE (2008) established the positive effect of refuse on the growth of the Spanish population of reproductive rooks. They emphasised that anthropogenic food on refuse tips can affect population dynamics in birds. The number of birds in colonies declines rapidly when local refuse sites are closed, and recovers only when the supply of refuse increases. During our study, we found that the number of breeding pairs in the 2006 season increased in comparison with the known data from 1990, 1993 and 2002 (VLAHOVIĆ, 2002). The first breeding colonies in the City of Zagreb were recorded by IGALFFY (1959). Since their number was not surveyed systematically in the past, the size of the rook population in Zagreb before the 1990s remains unknown. According to studies conducted by KUFRIN (1991), there were 281 breeding pairs in 18 breeding colonies in the entire area of City of Zagreb in 1991. VLAHOVIĆ (2002) pointed out an increase in the number of breeding pairs as well as the abundance of breeding colonies settled in several Zagreb districts.

An evaluation of the abundance of the rook population and breeding pairs is the first step towards a better understanding of these birds and their life-cycle. In addition, our data on alimentary microflora may be indicative for an assessment of potential public health risks. Further microbiological investigations are needed, to define the health status of these birds, in order to estimate the real risks of the cohabitation of rooks and humans. In conclusion, the contribution of each reservoir of bacterial species identified to the incidence of human infection is still unknown and needs to be investigated.

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SAŽETAK

Cilj rada bio je odrediti pojavnost bakterija i gljivica u izmetu vrana gačaca u gradskoj sredini. Istraživana je pojavnost bakterija i gljivica u svježem izmetu vrana gačaca (*Corvus frugilegus*) na području grada Zagreba tijekom razdoblja njihova gniježđenja u 2006. godini. Mikrobiološkim pretragama, u skladu sa standardnim metodama, iz pretraženih izmeta izdvojene su bakterije sljedećih rodova: *Escherichia coli*, *Bacillus* spp. i *Staphylococcus* spp., nadalje *Streptococcus* spp., *Agrobacterium radiobacter* i *Acinetobacter* spp. U izmetu jednog gačca postupkom lančane reakcije polimerazom (PCR) dokazana je bakterija *Campylobacter jejuni*. Iz prikupljenih uzoraka izmeta također su izdvojene i gljivice: *Mucor* spp., *Cladosporium* spp., *Rhodotorula rubra*, *Aspergillus (A.) fumigatus* i *A. flavus*, *Alternaria* sp., *Candida* spp. i *Penicillium* spp.

Ključne riječi: gačac, *Corvus frugilegus*, bakterije, gljivice