Use of Wildlife Rehabilitation Centres in Pathogen Surveillance: A Case Study in White Storks (*Ciconia Ciconia*)

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Running title: Pathogen surveillance in nestling and injured white storks.

16 **Abstract**

17 More than 70% of new human pathogens are zoonotic and many originate from the
18 wildlife reservoir. Wildlife rehabilitation centres (WRC) are an easily accessible source
19 for sample and data collection for preventive surveillance, but data collected this way
20 may be biased. We use white storks (*Ciconia ciconia*) as a model to compare pathogen
21 prevalence obtained in the field and WRC. We address factors that may affect disease
22 prevalence data like origin, the age group and the "diseased" state of WRC admissions.
23 In this study we compared prevalence of *E. coli* and *Salmonella* spp. in the digestive
24 tract; antibodies against West Nile virus, avian influenza and Newcastle disease virus,
25 and antimicrobial resistance patterns of *E. coli* between nestling and adult wild storks
26 established in different habitats (n=90) and storks admitted to two different WRC
27 (n=30) in the same region.

28 When age groups and colonies of origin were disregarded, the mean enterobacteria
29 (*E.coli, Salmonella*) and viral antibody prevalence of the wild population (n= 90) were
30 similar to prevalence observed in the individuals admitted to WRC (n= 30). However, in
31 fledgling juvenile storks admitted to WRC, the prevalence of *Salmonella* spp. (13.3%),
32 *E. coli* showing resistance to cefotaxime (37.9%) and against two antimicrobials at once
33 (41.4%) were more similar to the prevalence in stork nestlings from landfill-associated
34 colonies (7.9%, 37.1% and 48.6%, respectively for prevalence of *Salmonella* spp. and
35 *E.coli* displaying, cefotaxime resistance and resistance against two antimicrobials), and
36 significantly higher than in colonies located in natural habitats (0%; 10.5% and 15.8%,
37 respectively).

38 Thus, pathogen surveillance in individuals from an abundant species admitted to WRC
39 is useful to monitor overall mean prevalence, but for certain pathogens may not be
40 sufficient to detect differences between local populations. In addition, the ecology of the

tested species and the specific temporal, spatial and age group distribution of WRC admissions have to be taken into account.

Keywords: Antimicrobial resistance; *Enterobacteriaceae*; landfills; white stork; surveillance; wildlife rehabilitation centre.

Introduction

Most human pathogens are zoonotic and many originate from a wildlife reservoir (Jones et al., 2008). Especially birds are frequently considered reservoirs of pathogens for domestic animals and humans. Avian Influenza viruses and *Flaviviruses* such as West Nile and Usutu virus are maintained in avian reservoirs (Jones et al., 2008). Urban birds may carry zoonotic bacteria (Palomo et al., 2013; Vazquez et al., 2010). Due to their ability to travel long distances and forage in humanized environments birds may recirculate pathogens into the human or domestic animal populations. Consequently, surveillance and monitoring of pathogens in avian wildlife has become an important preventive tool (Stallknecht et al., 2007). Wildlife rehabilitation centres (WRC) can be a valuable resource for surveillance and monitoring of the prevalence of certain pathogens in wildlife. Stitt et al. (2007) documented that in comparison with other surveillance methods WRC received a wider variety of taxa from a greater geographical area. Sick and injured wildlife received at the WRC might be more likely to suffer from diseases, facilitating pathogen emergence detection (Wendell et al., 2002; Kelly and Sleeman, 2003; Wobeser, 2006, Nemeth et al., 2008). A recent nation-wide study in Australia using data on wildlife diseases from WRC associated with zoos, concluded that this information could improve the capacity for the detection of emerging pathogens in national surveillance programs (Cox-Witton et al., 2014).

Spain has many private and public funded WRC (56 in mainland Spain) that could become a powerful tool in monitoring certain risk pathogens. The study of potential zoonotic pathogens presumably prevalent across the Spanish geography, in a widely distributed conspicuous species that is admitted frequently to WRC, such as the white stork (*Ciconia ciconia*), allows us to test this hypothesis. Almost all WRC of mainland Spain receive white storks regularly, but concentrated in the summer months, as most stork casualties are juvenile birds.

The white stork is a widely distributed species in Europe. In Spain the population has experienced a significant increase in recent decades because of the use of landfills as predictable food source (Blanco, 1996; Schulz, 1999). In addition, the Iberian Peninsula has become the wintering area for many storks from northern Europe (Tortosa et al., 2002). Juvenile white storks frequently suffer accidents and are admitted to WRC, especially during the first few months after fledging. At the same time, only a few weeks prior to fledging banding takes place in many colonies, thus creating an interesting scenario for cost effective testing of the same species in the field and after admission to WRC within a short time period. Using the white stork, and some of the pathogens to which it may be exposed as a model, our goal was to provide a better understanding of the usefulness of WRC as sampling points for disease surveillance in wild birds. More specifically, we wanted to explore if and how factors such as the colony of origin, age group and the "diseased" status among others of WRC admitted white storks affected the detection of the surveyed pathogens.

Materials and Methods

Ethical statement

In this study, all applicable international, national, and/or institutional guidelines for the care and ethical use of animals were followed. Capture, ringing, radio-tagging and sampling of adult and nestling white storks in the field and at WRC was approved and authorised under permit from the regional government, the Junta de Comunidades de Castilla – La Mancha. JCCM (avp_13_037_aut).

Study area

The study area is located in the province of Ciudad Real (39° 0′ 0″ N, 4° 0′ 0″ W) in the south-centre of the Iberian peninsula, with a mean altitude of 629m. The area has a Mediterranean climate with very hot summers and cold winters, with average annual temperatures between 13 and 14º C and an annual rainfall of 438 mm, concentrated in autumn and spring.

Study populations

We sampled free-living white storks (nestlings and adults) in four different colonies with a different degree of exposure to human residues, and juvenile (fledgling) white storks upon admission to WRC (Table 1). One of the colonies (nº 1) was located in a National Park, the second (nº 2) in an open oak forest with extensive sheep farming, while the other two colonies (nº 3, nº 4) were associated with urban waste landfills. One of these (nº 4) is directly associated with an open active landfill site, while colony nº 3 is located at a landfill site that was sealed in 2007. Satellite tracking data from two adults captured and tagged at each colony in May 2013 (data not shown) confirmed foraging in natural habitats during nestling raising for colonies one and two. Foraging in colony three included both natural habitat and an open active landfill site located at approximately 45km flight distance from the colony. Foraging in colony four was limited to the landfill.

Nine adult white storks were trapped at the nest and sampled, three at colony nº 4 and two each at colonies one to three. For the wider purpose of this study we distinguish two subgroups within the "juvenile stork" group: "nestlings" are juvenile storks yet unable to fly and thus bound to the nest dependent on provisioning by their parents. This is the group of juvenile wild storks sampled in the field. The juvenile wild storks sampled upon admission at the WRC are "fledglings", this is they have left the nest and are thus mobile and mostly forage on their own.

Between the four colonies we sampled 81 white stork nestlings of approximately 40 to 50 days of age (Table 1). Adult white storks were sampled in May, while stork nestlings were sampled in June. Fledgling juvenile storks (n=30) were sampled upon admission to two different WRC (15 each) during June-September of the same year. This is the time of the year with the highest number of admissions of storks to WRC, as the storks leave the nest (fledge) and are most vulnerable. Individuals in WRC were sampled on the day of arrival at the centre. The WRC sample also included one nestling that was sampled 48 hours after admission due to its young age and deteriorated condition.

Given the population of storks and the number of WRC in Spain our sample size is relatively small. For comparison of pathogen surveillance results within a logistically feasible way we designed the study to include two replicas for each category of storks (WRC, landfill exposed, natural habitat) in a cluster sampling approach. We wanted to approach field and WRC samples from the same geographical study area. While the number of storks admitted to the two WRC under study is generally high, in the study year the WRC admitted storks included in the study (n=30) were the total of admissions. Moreover, some of our sample colonies in the study area are very much smaller than others a fact that we accepted, as we wanted to study colonies that clearly evidence the actually existing strong differences with view to nesting habitat and foraging behaviour.

136

137 *Sampling*

138 Blood and cloacal swab specimens were obtained from all sampled birds (n=120). We
139 used sterile 25G needles and 5ml syringes to obtain blood samples (5 ml) from the
140 brachial vein, that were immediately transferred to sterile tubes with lithium heparin as
141 anticoagulant. Cloacal swabs were taken using sterile cotton swabs in AMIES transport
142 medium (Deltalab, Barcelona, Spain).

143 All samples were kept at 4° C until arrival at the laboratory and processed less than 12
144 hours after collection. Body weight, tarsus and head-bill length were recorded in free-
145 living nestlings (n=81).

146 *Laboratory analysis*

147 In the blood samples we first determined the haematocrit and measured total solids in
148 the plasma fraction using a hand held refractometer. The rest of the sample was
149 centrifuged for the separation of cells and plasma that were frozen in several aliquots for
150 further analysis.

151 We used one of the plasma samples for detection of antibodies against avian influenza
152 virus (AIV), Newcastle disease (ND, avian Paramyxovirus -1) and West Nile virus
153 (WNV). For this the samples were thawed, inactivated for 30 minutes at 56º C, and
154 tested using commercial blocking ELISAs (Ingezim Influenza A 1.0. FLU.K.3,
155 Ingenasa, Madrid, España; ID Screen, Newcastle competition, and ID Screen, West Nile
156 Competition, IDVet, Montpellier, France) according to the manufacturer's instructions.

157 Cloacal swabs in AMIES transport medium (Deltalab, Barcelona, Spain) were cultured
158 for the detection of *E. coli*. and *Salmonella* spp. For isolation of *E. coli* cloacal swabs

were plated on MacConkey agar (Scharlab S.L., Barcelona, Spain) and were incubated at 37º C for 24 hours. Phenotypic pattern of antimicrobial resistance of *E. coli* strains was assessed by culture in MacConkey media supplemented with 16µg/ml gentamicin (Sigma-Aldrich Chemical, Madrid, Spain), 4µg/ml cefotaxime (Sigma-Aldrich Chemical, Madrid, Spain) or 4µg/ml enrofloxacin (Sigma-Aldrich Chemical, Madrid, Spain) respectively. After incubation at 37° C for 24 hours, colonies morphologically compatible with *E. coli* were recorded as evidence of phenotypic resistance to the tested antimicrobial.

The choice of different antimicrobials was based on the frequency of use in Spain. Gentamicin and enrofloxacin are used in livestock and pets. Cefotaxime and similar cephalosporins are administered to humans. The concentrations used are those recommended by the National Antimicrobial Resistance Monitoring System (NARMS, Food and drug administration, Centres for disease control and prevention, United States Department of Agriculture, 2010).

Isolation of *Salmonella* spp. was performed according to the standard ISO 6579 method (2002). Colonies considered *Salmonella* spp. by their morphology were collected in ultrapure water for DNA extraction and confirmation of the identification as *Salmonella* spp. by amplification of the invA gene using the protocol described by Rahn et al. (1992). Briefly, amplification was carried out in a reaction mixture of a total volume of 30 µl containing: 2 µl of DNA; 0.2 µl Enzyme (5U/ µl); 3 µl of invA-L (0.01 mM) primer; 3 µl invA-R primer (0.01 mM); 3 µl Buffer (10x); 0.6 µl dNTPs (10 mM); 0.9 µl MgCl2 (50 mM); 17.3 µl ddH2O. The reaction was performed in a Techne thermal cycler TC-512 (Techne Inc. Cambridge, UK) using the following protocol: 94 ° C/3min; 40 cycles of 95 ° C/30s, 55 ° C/30s., 72 ° C/30s; and a final cycle of 72° C/10 min. The samples were subjected to electrophoresis in a 2% agarose gel. Bands were stained

using GelRed™ (Biotium, Hayward, USA) and visualized in a UV transilluminator (UVitec Ltd. Cambridge, UK).

Similar to *E. coli* we studied the phenotypic pattern of antimicrobial resistance in strains of *Salmonella* spp., by plating colonies on XLD agar supplemented with the same antibiotics and concentrations used in the evaluation of *E. coli* antimicrobial resistance. The plates were incubated at 37° C and examined for growth of colonies compatible with *Salmonella* spp.24 hours later.

Statistical analysis

We calculated the body condition of white stork nestlings and adults sampled in the field according to the scaled mass index proposed by Peig and Green (2009). This index can be computed as: $M = M_i (L_0/L_i)^{bsma}$, where M_i and L_i are the body mass and the structural size measurement (tarsus length) of each individual respectively; bsma is the scaling exponent estimated by the standardised major axis (SMA) regression of M on L,L_0" is the arithmetic mean value for the study population; M is the predicted body mass for individual i when the structural size body measure is standardized to L_0. The scaling exponent "bsma" has been calculated indirectly by dividing the slope from an ordinary least squares (OLS) regression (bols) by the Pearson's correlation coefficient r. Normal distribution of the continuous variables (body condition index M, haematocrit, plasma total solids) was confirmed using the Shapiro Wilks test prior to further analysis. We compared body condition, haematocrit and plasma total solids between natural and landfill associated field populations using a Generalized linear model (GzLM) with the colony and the nest of origin as random factors in order to account for the expected dependency of individuals within each colony and the of sibling nestlings within each nest. We used Fisher's exact test ($p \leq 0.05$) to explore differences in the prevalence of pathogens and antibodies between adult storks and nestlings. Finally we used

209 Generalized mixed linear models (GLMM) with a binary response and a logit regression
210 and the colony and the nest of origin as random factors, to compare differences in
211 prevalence of pathogens and antibodies among natural and landfill colonies and
212 between white stork nestlings studied in the field and fledglings analysed upon
213 admission to WRC. In addition to *E. coli* prevalence in the cloaca of individuals, we
214 compared the prevalence of antimicrobial resistance of *E. coli* between the groups,
215 namely the prevalence of *E. coli* resistant to gentamicin, enrofloxacin and cefotaxime,
216 as well as the prevalence of resistance phenotypes against 2 and 3 antimicrobials at the
217 same time. We also compared the prevalence of *Salmonella* spp. and WNV antibodies.
218 All analysis were carried out using SPSS statistical software, version 19.0 (IBM®,
219 SPSS Inc., Chicago, USA).

220 **Results**

221 Overall prevalence of *E. coli* in free-living (adult and nestling) white storks was 85.6%
222 (77 out of 90) while prevalence of *Salmonella* spp. was 4.4% (4 out of 90). Prevalence
223 of *E.coli* in WRC admitted storks was 96.7% (27 out of 30) and *Salmonella* spp.
224 prevalence 13.3% (4 out of 30). Antibodies against AIV were not detected in the field.
225 An adult and a nestling from two different nests situated on one of the landfill site
226 colonies had antibodies against ND and only one adult white stork each from both
227 natural and landfill site habitats had antibodies against WNV. While *E. coli* was isolated
228 significantly more frequently in nestlings (90.1%, 73 out of 81) than in adult individuals
229 (44.4%, 4 out of 9) (Fisher's exact test, p=0.003), *Salmonella* spp. was detected more
230 frequently in adult white storks (Table 1).

231 White stork nestlings from landfill colonies (M= 3327.18, 95% CI: 3140.39-3513.97)
232 were in significantly better body condition than individuals established in natural
233 colonies (M=2962.02, 95% CI: 2830.54-3093.53; GzLM, p=0.005, β= -0.226,

234 SE=40.01). *E. coli* was present in both natural and landfill associated colonies, while
235 *Salmonella* spp. was not isolated in natural colonies but was detected in one adult (20%,
236 1 out of 5) and three nestlings (7.9%, 3 out of 38) from landfill site associated colonies
237 (GLMM, p=0.003) (Table 1). All *Salmonella* spp. strains isolated were susceptible to all
238 antimicrobials tested. Due to the small number of isolates, we could not statistically
239 analyse antimicrobial resistance patterns in salmonella. Phenotypic antimicrobial
240 resistance of *E. coli* was significantly more prevalent in landfill site colonies than in
241 natural colonies for two antimicrobials at once (48.6%, 17 out of 35, GLMM, p=0.005)
242 and marginally significantly more prevalent for cefotaxime (37.1%, 13 out of 35,
243 GLMM, p=0.053, Table 1, Figure 1).

244 For comparison of prevalences between the field and WRC we did not consider adults
245 as only juvenile (fledgling and one nestling) storks were admitted to the WRC in our
246 study period. Comparing white stork nestlings sampled in the field and fledgling white
247 storks admitted to WRC, the prevalence of pathogens and antibodies studied did not
248 differ significantly. However, if we considered storks from natural colonies and landfill
249 site associated colonies separately, and compared pathogen prevalence to that in WRC
250 admitted storks, significant differences became evident (Table 1, Figure 1). Thus
251 prevalence of *Salmonella* spp. was similar in fledgling white storks sampled in WRC
252 (13.3%, 4 out of 30) and nestlings from landfill site colonies (7.9%, 3 out of 38) while it
253 was absent in colonies in natural habitats. *E. coli* resistant to cefotaxime was detected
254 significantly more frequently in fledgling white storks at WRC (37.9%, 11 out of 29)
255 and nestlings from colonies in landfill sites (37.1%, 13 de 35) than in nestlings from
256 natural colonies (10.5%, 4 out of 38) (GLMM, p=0.003, Fig. 1). We also found
257 resistance phenotypes against two different antibiotics significantly more frequently in
258 nestlings from landfill colonies (48.6%, 17 out of 35), and fledglings from WRC

(41.4%, 12 out of 29) than in individuals from natural colonies (15.8%, 6 out of 38) (GLMM, p=0.048, Fig. 1). Antibodies against AIV and WNV were detected in one individual each admitted to WRC, while antibodies against ND, the only antibodies that had been detected in free-living nestlings were absent in WRC admitted storks (Table 1).

Discussion

In this study, we use the white stork to explore the pathogen-carrier-status information obtained in juvenile birds in the field and in WRC.

Our study evidences that at least during the breeding season significant differences in physiological condition and pathogen carrier status exist between colonies within a population. Nestlings in landfill colonies have a higher body condition index than those in natural colonies, probably due to the availability of abundant food in the vicinity, but are apparently more exposed to potential bacterial pathogens.

Carriage, of *E. coli* is similar between colonies, potentially because this *Enterobacteriaceae* is widely distributed in all types of environments and is part of the intestinal flora of white storks (Han et al., 2011). Natural colonies selected for our study are located in areas with extensive livestock farming, thus contact with livestock residues during foraging is possible. More frequent detection of *E. coli* in nestlings, could be related to a more diverse flora in the adults, longer cooling prior to culture in samples from adult birds and the comparatively smaller sample size in adult white storks.

In contrast, isolation of *Salmonella* spp. only from storks from landfill colonies may be due to the contact with human residues. In fact, the absence of *Salmonella* spp. in natural populations was previously observed in a study performed by Vlahovic et al.

283 (2004) in Croatia. We used the cited ISO 6579 method despite the risk of missing some
284 of the true prevalence of *Salmonella* spp., because we wanted to apply a commonly used
285 and recognised method for *Salmonella* spp. surveillance that is applied in numerous
286 reference laboratories for *Salmonella* spp. serotypes that are of interest as zoonosis.
287 Similarly, a higher prevalence of antimicrobial resistance patterns of the *E. coli* isolates
288 to cefotaxime and resistance to two different antimicrobials was observed in storks
289 exposed to human residues in the colonies associated with landfills. This has been
290 previously observed in other species such as gulls exposed to human residues (Camarda
291 et al., 2007).

292 The mean overall prevalence of *E.coli* and *Salmonella* spp. of white storks sampled in
293 the field was similar to that detected in white storks admitted to the WRC, showing that
294 data collected at the WRC reflects the situation in the field. However when considering
295 nestlings from the different habitat types separately, the prevalence of *E. coli* and
296 *Salmonella* spp. and phenotypes of antimicrobial resistance observed in storks admitted
297 to WRC were similar to those observed in nestlings sampled in colonies associated with
298 landfills and significantly higher than those observed in nestlings from colonies in
299 natural habitats. That is, our results suggest that while during the nestling period the
300 breeding habitat determines the diet and pathogen exposure of nestlings, this may
301 change rapidly after fledging. Thus, fledged young storks from either nesting habitat
302 may forage in landfills. This is further supported by satellite transmitter data that shows
303 that adult storks from natural habitats use landfills after the end of the breeding season
304 and by observation of long-distance identification rings from nestlings from natural
305 habitats at nearby landfill sites (data not shown). Unfortunately, none of the patients in
306 the WRC carried a ring or transmitter so that we were unable to determine its colony of
307 origin. We also have to take into account that an age difference of one to two months

existed between nestling white storks sampled at the colony and juveniles sampled at the WRC. During this period fledging and a change from provisioning by the parent storks to independent foraging occur, two stressful events that could have a negative effect on the condition and immune status of the fledgling white storks and increase susceptibility to certain agents. In addition, the fact that storks admitted to WRC are generally fledgling or first year birds has to be taken into account during pathogen surveillance, as for example pathogens that adult storks may become exposed to during migration and wintering may not be detected in nestling or juvenile birds.

Viral seroprevalence is more difficult to assess as it can show exposure in the past, as well as miss recently infected seroconverting individuals, but it has been shown to be a useful tool in surveillance of viral activity within a population (e.g. Alba et al., 2014). We used three avian viruses, frequently detected in wild birds across Europe and with a seasonal variation in their prevalence, all of them of surveillance interest either due to their zoonotic nature (AIV, WNV) or due to their importance for poultry (ND). AIV and ND are directly transmitted, while WNV is vector born and dependent on mosquito vector abundance and activity. WNV epidemics in the study area take place in late summer and early autumn, when bird migration is at its peak and mosquito populations are greatest, while peaks of AIV and ND prevalence are presumably associated with wintering (Perez-Ramirez et al., 2010).

Overall WNV seroprevalence, was low, but similar between storks sampled in the field and WRC. This is in contrast to the observation by Randall et al. (2012) who concluded that WNV exposure in individuals admitted to rehabilitation centres was not representative of the seroprevalence of WNV in natural populations of the same species. However, as the author stated, the low prevalence of this virus in the sampling area, working with too small an area or too many different orders of birds, may have led to

his results. In a study on WNV in Southern Spain, López et al. (2011) found a similar seroprevalence of WNV in the same species in the field and upon admission to WRC. Taking a closer look at our data we can see that in the field only adults are seropositive, consistent with exposure to the virus throughout their lives, perhaps through their migratory routes, as shown in other studies (Figuerola et al., 2007). In contrast, antibodies against WNV found in a juvenile stork admitted to a WRC could have been acquired in the field after fledging. Maternal antibodies transferred via egg yolk have been shown to be detectable for over one month (Komar, 2001; Gibbs et al., 2005).

Thus, as stork nestlings fledge at the age of approximately 60 (58-64) days, it is possible but not very likely that these antibodies were of maternal origin.

These results show that the age group of a particular species that is admitted to WRC has to be taken into account when assessing the information WRC samples give of the situation in the field. As an example, while exposure of white storks to WNV may be more likely in adults, storks admitted to WRC are more frequently juveniles than adults.

We only detected AIV antibodies in a juvenile white stork at a WRC. The time when we conducted our study may have influenced our findings. Previous studies show that AIV prevalence peaks in October and November (Pérez-Ramirez et al., 2010), when thousands of migratory birds come to Spain for wintering. However the peak of admissions of white storks to WRC is in summer and the bulk of these are recently fledged juvenile white storks. Nevertheless exposure of white storks to AIV appears to be low. In a previous study of our group only one of 129 faecal samples of white storks collected in the field tested positive for LPAIV and H11N9 was identified (Pérez-Ramirez et al., 2010). In Germany, out of over 600 nestlings, 103 fecal samples and 88 dead storks only three LPAIV positive faecal samples and two HPAIV H5N1 positive

dead storks were found (Müller et al., 2009). Also, antibodies against H5/H7 subtypes of AIV could not be detected in white storks from WRC in Germany (Kaleta and Kummerfeld, 2012). Antibodies against ND virus were detected in a nestling and an adult in a landfill colony. None of the storks admitted to WRC was positive. In contrast, Stenzel et al. (2008) found a seroprevalence of 20% in white storks from WRC in Poland and a long term study on white storks in WRC in Germany revealed haemagglutinating antibodies against NDV in 16 of 191 samples and carriage of virulent aPMV-1 virus in four individuals (Kaleta and Kummerfeld, 2012) . In Spain, in a study on avian Paramyxovirus surveillance in semi-free-ranging birds 17 sera from white storks tested negative for antibodies against aPMV-1, but antibodies against aPMV- 8 and aPMV-9 were found in haemagglutination inhibition tests (Esperón et al., 2014). The significant differences in pathogen prevalence between the (in this study very few) adult and nestling wild storks, and the availability in WRC of only juveniles also illustrates how WRC origin sample and data sets are often biased towards the juvenile population of a species, which on the other hand may be of advantage for the detection of pathogen emergence.

Conclusions

Sample collection from wildlife casualties admitted to WRC can be a useful method for specific pathogen surveillance if the associated bias mediated by factors such as the diseased status, age group, and spatial or temporal distribution of admissions are taken into account. These associated confounding factors are most likely strongly related to the ecology of the species sampled that should receive an important consideration in surveillance programs. In our example, juvenile white storks admitted to WRC can be used as indicators of the emergence of *Enterobacteriaceae* carrying antimicrobial resistance mechanisms in wild populations but may be less good indicators for

circulation of WNV, NDV and AIV in the field, due to the timing of the peak of admissions.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Table 1. Prevalence of *E. coli*, and *Salmonella* spp., *E.coli* with antimicrobial resistance to gentamicin (G), cefotaxime (C) and enrofloxacin (E), multiresistance agaisnt 2 (MR2) and 3 (MR3) antimicrobials, and seroprevalence of West Nile (WNV), Newcastle (ND) and Avian Influenza (AI) virus in white storks by age, habitat and colonies (National Park (1), open oak forest with extensive sheep farming (2), closed landfill (3), active landfill (4)) and WRC

			PREVALENCE n/N (%)		PREVALENCE OF ANTIMICROBIAL RESISTANCE PATTERNS n/N (%)					SEROPREVALENCE n/N (%)		
Population		Age	*E. coli*	*Salmon.*	G	C	E	MR3	MR2	WNV	ND	IA
NATURAL	1	Nestling	13/15 86.8%	0/15 0%	7/13 53.8%	2/13 15.4%	2/13 15.4%	1/13 7.7%	3/13 23.1%	0/15 0%	0/15 0%	0/15 0%
		Adult	0/2 0%	0/2 0%	0/0 0%	0/0 0%	0/0 0%	0/0 0%	0/0 0%	0/2 0%	0/2 0%	0/2 0%
	2	Nestling	25/28 89.3%	0/28 0%	5/25 20%	2/25 8%	11/25 44%	2/25 8%	3/25 12%	0/28 0%	0/28 0%	0/28 0%
		Adult	1/2 50%	0/2 0%	1/1 100%	0/1 0%	0/1 0%	0/1 0%	0/1 0%	2/2 100%	0/2 0%	0/2 0%
LANDFILL	3	Nestling	27/30 90%	2/30 6.7%	17/27 63%	8/27 29.6%	15/27 55.5%	5/27 18.5%	13/27 48.1%	0/30 0%	1/30 3,3%	0/30 0%
		Adult	1/2 50%	0/2 0%	0/1 0%	0/1 0%	0/1 0%	0/1 0%	0/1 0%	2/2 100%	1/2 50%	0/2 0%
	4	Nestling	8/8 100%	1/8 12.5%	6/8 75%	5/8 62.5%	3/8 37.5%	2/8 25%	4/8 50%	0/8 0%	0/8 0%	0/8 0%
		Adult	2/3 66.7%	1/3 33.3%	0/2 0%	0/2 0%	0/2 0%	0/2 0%	0/2 0%	0/3 0%	0/3 0%	0/3 0%
Total Free-living		Nestling & Adult	77/90 85.6%	4/90 4.4%	36/77 46.7%	17/77 22%	31/77 40.2%	10/77 13%	23/77 29.9%	4/90 4.4%	2/90 2.2%	0/90 0%
WRC		Fledg-ling	29/30 96.7%	4/30 13.3%	13/29 44.8%	11/29 37.9%	12/29 41.4%	6/29 20.7%	12/29 41.4%	1/27 3,7%	0/27 0%	1/27 3,7%

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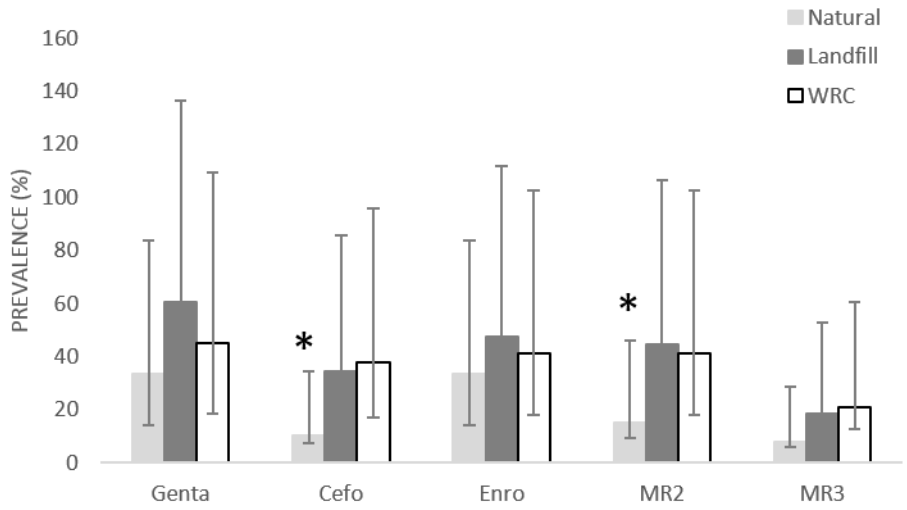

Fig. 1 Prevalence of antimicrobial resistance patterns to *E. coli* in white storks analyzed in natural and landfill colonies and WRC (G = Gentamicin, E = Enrofloxacin, C = Cefotaxime; MR = multiresistance against two (MR2) or three antibiotics (MR3). Significant differences ($p<0.05$) are marked with "*"