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Evidence of carriage of antibiotic resistant salmonella species of public health and veterinary significance in the intestines of house crows(*CorvusSplendens*) in Tanzania

ABSTRACT

Background

The Indian house crow, *Corvussplendens*(Vieillot) was introduced in Zanzibar in Tanzaniaby the British and immigrants from India in 1897to help clean the town. The crow isresponsible for polluting the environment, water sources and human surroundings by their droppings and the rubbishthey carry. This behavior has led to concern that, the crows may be responsible for the spread of certain pathogens including *Salmonella* and their persistence in the environment.Given the zoonotic potential of *Salmonella*, the main aim of this study was to investigate the occurrence of *Salmonella*infections in Indian house crowand, in particular, to determine if the isolates were similar to those associated with disease in livestock or humans.

Methods

Indian house crowscrossing the Mabibocompound of the National Institute for Medical Research (NIMR) in Dar es Salaam city in Tanzania were lured with meat and blood baits to land into the Crow live-trap. A total of 100 house crows were captured. The birds were humanely sacrificed and small and large intestines were obtained by using aseptic techniques for microbiological investigations. Preliminary identification of salmonella was based on colonial characteristics on selective media and microscopic examination of smears following Gram staining.Confirmation ofSalmonella species was done by biochemical testsincluding fermentationof glucose, lactose and sucrose, hydrogen sulfideproduction, urease activity, phenylalanine deamination,lysine decarboxylation, citrate, methyl red andindole tests. Antibiotic susceptibility testing was done by using the disc diffusion method onMueller Hinton agar. Results were recorded as susceptible or resistant based on the diameter of the zone of inhibition for each antimicrobial disc used according to the guidelines

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provided by manufacturers.

Results and discussion

Eight isolates were identified by standard microbiological techniques as *Salmonella* spp. (6% suggestive of*Salmonella gallinarum*and 2% suggestive of *S. typhi*). All isolates were found to be susceptible to ciprofloxacinbut resistant to amoxicillin. Lower levels of susceptibility were noted for chloramphenicol and ceftriaxone. Our results demonstrate the presence of antibiotic resistant *Salmonella* spp. in the Indian house crows' population and provide an assessment of potential public and poultry health risks to the high-density human and poultry populations within the coastal area.

Conclusion

The reduced antibiotic susceptibility of *S.typhi* and *S.gallinarum*has veterinary, clinical and public health consequences associated with transmission of antimicrobial drug resistant Salmonella in poultry and humans.The risk of transmission of antibiotic resistant Salmonellatohumans and poultryprovides further rationalefor the public action on eradicating the house crows.

Keywords: *Salmonella*, antibiotic resistance, susceptibility, Tanzania

1. INTRODUCTION

The Indian house crow, *Corvussplendens*(Vieillot), is one of the world's most invasive bird species, causing disturbance to more than 25 countries throughout the Indian Ocean, Arabian Peninsula and South East Asia[1].Bijlsma and Meininger reported that the crow originated from Asia, but adaptively colonized many other parts of the world ranging from Malaysia to South Africa[2, 3]. It was first reported on the island of Zanzibar in East Africa in 1897 whereby it was introduced by the British and immigrants from India to help clean the town [4].The Indian house crow presents greatecological flexibility and by the 1950s,it had spread to the large part of the East African coast and itcontinues to expand its range.This bird species has been causing problems to the natural biodiversity of the regions, as well as impacting upon human health, tourism, infrastructure, and general development [1]. The crows have been found to be responsible for the reduction of small reptiles and amphibians and

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indigenous crows[5]. In addition they kill poultry, prey on the chicks and eggs of native birds and harass other birds, animals and insects [5].

The population of house crows in Tanzania mainland has increased significantly since 1980's as the species adapted well in the new environment and has no natural enemies [6]. The birds are called the world most destructive crows and are frequently observed in close contact with water sources, reserve water tanks and they keep picking unattended human food and crops. Their abundance is closely linked to human population size, due to the expanding amount of rubbish generated [7]. The crows are responsible for polluting the environment, water sources and human surroundings by their droppings and the rubbishthey carry. This behavior has led to concern that, the crows may be responsible for the spread of certain pathogens including *Salmonella*and their persistence in the environment [4].

Several studies have shown that a number of serovars of *Salmonella enterica*exist in wild birds, and it has been suggested that wild birds might be playing a significant role in the epidemiology of human and livestock salmonellosis[8]. However, little is known about the existenceof *Salmonella*strains inIndian house crowsand human and livestock associated strains.The present study was therefore aimed atinvestigating the occurrence of *Salmonella*infections in Indian house crow and, in particular, to elucidate if the isolates were similar to those associated with disease in livestock or humans.

2.0 MATERIALS AND METHODS

2.1 Indian house crow collection

Indian house crows were capturedat the Mabibo compound of the National Institute for Medical Research (NIMR) in Dar esSalaam city inTanzania. This specific site was chosen based on convenience as most of the researchers involved in this study are based there. Indian house crowscrossing the compound were lured with meat and blood baits to land into the crow live-trap. A total of 100 house crows were captured and used in this study. This sample size considered both the available funds and the study duration.

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2.2 Isolation of Salmonella spp.

The birds wereeuthanizedby an overdose of pentobarbitone sodium (50 mg/kg body weight) and then dissected immediately. While occluded by forceps small and largeintestines were aseptically removed from the abdominal cavityfor microbiological investigations. Intestinal samples from each bird were placed in a separate container. About 1cm of the opened intestine of each sampled bird was placed in bijou bottle containing 10 ml of sterile biological peptone water and incubated overnight at 37°C.A loopful of intestinal contents from biological peptone water was streaked on solid differential and selective media (MacConkey) agar(Oxoid Ltd, Basingstoke, UK)and incubated at 37°C for 24 hours. Colonies suggestive of salmonella (colorless, translucent to opaque, irregular edge colonies which are non-lactose fermenters)were sub-cultured on Salmonella-Shigella agar (Oxoid Ltd, Basingstoke, UK)in duplicate. Pure salmonella colonies from Salmonella-Shigella agar (colorless edge colonies with black centre) were then subjected to identification.

2.3 Identification of Salmonella isolates

Preliminary identification of salmonella was based on colonial characteristics on selective media and microscopic examination of Gram stained smears. Salmonellasuspected colonies were picked up using a sterile wire loop, added to a drop of normal saline and smeared on a microscopy glass slide. Gram stain was performed andgram-negative rod shaped organisms were identified as suggestive of salmonella species.Confirmation ofSalmonella species was done by biochemical testsusing theAnalytical Profile Index (API-20E) system according to manufacturer's instructions (bioMérieux, inc, Darham, USA). Nutrient agar slant and butt were utilized for determination of production of specific metabolites such as gas, acid or alkaline solution.

2.4 Antibiotic susceptibility testing

Antibiotic susceptibility testingwas done by using the disc diffusion method onMueller Hinton (MH) Agar (Oxoid Ltd, Basingstoke, UK) using *C. jejuni*NCTC 11168 for quality control purposes.Bacterial suspensions were prepared in a sterile normal saline and adjusted to a turbidity equivalent to 0.5 McFarland standards

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(1.5x10^8cells/ml). Sterile swab was used to inoculate the suspension by evenly streaking on prepared Mueller Hinton agar plate and allowed to stay for 3-5 minutes to allow drying. Sterile forceps were used to place discs on the inoculated plates and incubated at 37°C for 24 hours. The isolates were tested for susceptibility to ceftriaxone, amoxicillin, ciprofloxacin and choramphenical(Oxoid Ltd, Basingstoke, UK), which are drugs of choice for treatment of Salmonella infections. After 48 hours of incubation, the diameters of inhibition zones were measured. Interpretation of results was guided by both standardized tables supplied by the National Committee on Clinical Laboratory Standards (currently known as Clinical and Laboratory Standards Institute) (NCCLS, 2002)and manufacturer's instructions.

2.5 Ethics statement

The trapping of Indian house crows was done using a live trap provided to NIMR by the Ministry of Natural Resources and Tourism under the crows' eradication program. The crows' eradication program has permission of the government of the United Republic of Tanzania through the Ministry of Natural Resources and Tourism to capture and humanely kill crows in Dar es Salaam city in Tanzania.Crows trapped in the program are being killed so that to reduce their population density. Permission to use the captured crows for research was provided by the Department of veterinary Medicine and Public Health in accordance with Tanzania Wildlife Research Institute (TAWIRI)*Guidelines for Conducting Wildlife Research* (2001) and in compliance with Tanzania Veterinary Act number 16 of 2003 (Veterinary regulations; Government notice no. 389 of 2005), following a mandate of the Sokoine University of Agriculture (SUA) to conduct disease investigations in wildlife in different protected and unprotected areas in Tanzania. All biological samples were collected in order to perform research on presence of*Salmonella* in house crows.

3.0 RESULTS

3.1 Occurrence of enteric Salmonella in sampled house crows

Salmonella spp. were foundin 8out of the 100intestinal samples of captured Indian house crows tested,which represents a prevalence of 8%.Using the API-20Esystem(Table 1) the isolatesexhibited biochemical profiles suggestive of two different serotypes i.e.*S.gallinarum*being the predominant*Salmonella* serotype and

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accounting for 6 (6%) of the isolates, and*S.typhi*which was found in 2 (2%) samples of Indian house crows.

Table 1 here

3.2 Antibiotic susceptibility of isolated enteric Salmonella

Table 2 shows all the 8 *Salmonella*isolates detectedwere susceptible to ciprofloxacin. More than half (75%) of isolates (6 out of 8) wereresistant to chloramphenicol, of which 5 were *S.gallinarum* and 1 was *S.typhi*. High resistance was also noted for Ceftriaxone, whereby 7 out of 8 isolates (87.5%) were resistant, of which 5 were *S.gallinarum* and 2 were *S.typhi.* All the 8 *Salmonella*isolates were resistant to amoxicillin.

Table 2 here

4.0 DISCUSSION

In this study we have isolated two serovars of Salmonella, *S. gallinarum* and *S. typhi*from the intestines of apparently healthy Indian house crows.Our results are comparable to the findings reported in 2004 by Millán*et al*., who observed that8.5% of wild birdsincluding crows were harboring Salmonellaspp [9]. Furthermore, our results correlate with those ofAgasi*et al*., who reported the isolation of *Salmonella* spp.from cloacal swabs taken from two out of 30 apparently healthy crows in Japan[10]. Contrary to these findings, other studies conducted elsewhere [11, 12] couldn't isolate salmonella from the crows.

The isolation of thetwo serovars of Salmonella (*S. gallinarum*and *S. typhi*) from the intestines of Indian house crows in this study confirms the role of these birds as potential carriers of enteric Salmonella for both humans and animals [13, 14]. *S. typhi* is the causative agent of human typhoid fever [15] while *S. gallinarum*causes fowl typhoid in birds [16].

Occurrence of *S. gallinarum*in chickens [17, 18] and *S. typhi* in humans[19-21], in the country, has been reported in previous studies. The behavior of Indian house crows of keeping in close contact and their interaction with human environment indicates their potential as vehicles of transmission of Salmonella infections in Tanzania.The risk of enteric salmonella infections originating from crows is heightened due to the fact that their population is expanding both in the country and in neighboring countries [17].

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It has been reported by several authors that infections with *S. typhi*are restricted to humans [22, 23] and that there exists no animal or environmental reservoirs for the bacterium [24]. Experimental infections with *S. typhi*have however been reported in higher primates [25].The observation that this bacterium infected crows in the present study could be an attribute of the use of an identification technique with low discriminatory ability thereby leading to misidentification. Another possibility could be that this studyhas hit upon a major discovery in Salmonella biology by identifying a new host for this particular species.

The present study revealed the highest antibiotic sensitivity level of 100% shown by the isolates to ciprofloxacin which was in agreement with the previous report of Zahrei*et al*., 2005 [26]. This signifies continual use of ciprofloxacin as a drug of choice for treatment of infections caused by these organisms. In addition, the present study also revealed the prevalence of antibiotic resistance among Salmonellaisolates.The isolated strains were highly resistant to amoxycillin, ceftriaxone and chloramphenicol. The highest resistance level of 100% was observed for amoxycillin, followed by ceftriaxone (87.5%) and lastly, chloramphenicol (75%); rendering them ineffective for the treatment of infectionscaused by these strains. The possible sources of antibiotic resistant Salmonella species could behumans and animals as overuse and misuse of antibiotics both in veterinary [27, 28] and medical fields [29], which are key attributes in development of antibiotic resistant among different microbes, have been reported in the country. In turn, Indian house crows which are ubiquitous scavengers could bean important factor in spread of antibiotic resistant Salmonellosis bothto humans and animalsover the distances they cover by their droppings. In theOne Health Initiative, the problem of antibiotic resistance in which house crows now seem to take part,collective efforts from public health and veterinary professionalsare needed for its mitigation.

The limitations of the current study included the following i) sampling of the crows was conducted in a limited area making the sample less representative of the city crow population ii) adoption of an identification technique with low discriminatory power which could lead to miss-classification of the isolates into serovars.

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5.0 Conclusion

From our results we can conclude that, the Indian house crowscould be a source of Salmonella (*S. gallinarum* and *S. typhi*) infectionsin humansand animals. The observedantibiotic resistanceamong crow derived salmonella isolates is of particular interestsince it suggests that the crows might contributeto spread the antibiotic resistant Salmonellosis in humans and animals thereby complicating treatment of such cases.Based on the findings from this study, it is advisable that people should avoid contacts with crows and maintain good hygienic practices, treat drinking water and improve sanitation so as to avoid acquiring infections originating from crows. Crow eradication programs should be strengthened so as to stem the risk of transmission of antibiotic resistant Salmonellafrom these wild birds tohuman and animals.

References

1. Ryall C.The pest status of the Indian house crow *Corvussplendens*in Mombasa and a survey of its expansion of range in coastal Kenya; In: Bennun,L. (ed. 1992). *Proceedings of the VIIth Pan African Ornithological Congress, Nairobi*, Aug 1988.
2. Bijlsma GGandMeiningerPL.Behaviour of the house crow *(Corvussplendens),* and additional notes on its distribution. Le Gerfaut.1984.74, 3-13.
3. Lever C.*Naturalised birds of the world*. T and A D Poyser. 2006, 352 .
4. Cooper JC.Health studies on the Indian house crow. Avian Pathology.1996, *25*: 381-386.
5. GISD-2010.Global Invasive Species Database of the IUCN/ISSG (Invasive Species Specialist Group of the World Conservation Union). Fact sheet on *Corvussplendens***.** Accessed online at http://www.issg/database on 12th January 2010.
6. Suliman AS, Meier GGand HaversonPJ.Eradication of the house crow from Socotra Island, Yemen in Island Invasives: eradication and management. Veitch CR, Clout MN, and Town CR. (eds). *IUCN*, Grand, Switzerland.2011.361-363.

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7. Nyari A, RyallC and PetersonA:Global invasive potential of the house crow based on ecological niche modeling. Journal of Avian Biology.2006, 37: 306- 309.

8. HughesLA,ShoplandS,WigleyP,BradonH,LeatherbarrowAH,WilliamsNJ,Bennett M,de PinnaE,LawsonB,CunninghamAA andChantreyJ:Characterisation of *Salmonella enterica*serotype Typhimurium isolates from wild birds in northern England from 2005 – 2006. BMC Veterinary Research. 2008, 4:4doi:10.1186/1746-6148-4-4.

9. Millán J, AdurizG, MorenoB, JusteRAand BarralM.Salmonella isolates from wild birds and mammals in the Basque Country (Spain).*Rev. sci. tech. Off. int. Epiz.,*2004, 23 (3), 905-911.

10. Agasi M, Oka C and SatoG.Isolation of *Salmonella typhimurium*var Copenhagen from crows in the city of Otaru. *Japanese Journal of Veterinary Science* 1967, 38,521-522

11. Kapperud G, Rosef O.Avian wildlife reservoir of *Campylobacter fetus* subsp. *jejuni*, *Yersinia* spp., and *Salmonella* spp. In Norway. *Appl. Environ. Microbiol.*1983, 45, 375–380.

12. Ganapathy K, SalehaAA, JaganathanM, TanCG, ChongCT, TangSC, IderisA, DareCMand BrandburyJM.Survey of Campylobacter, Salmonella and mycoplasmas in house crows (*Corvussplendens*) in Malaysia. Veterinary record. 2007, 160, 622-624.

13. Willumsen B and HoleS.Presence of thermotolerant *Campylobacter spp*. and *Salmonella spp.* from seagulls and crows and its association with human enteritis in Bodo area (Norway). *Norsk. Vet*. 1987, **99**:277-282.

14. Refsum T, HandelandK, BaggesenDL, HolstadG and KapperudG.*Salmonellae* in avian wildlife in Norway from 1969 to 2000. Appl. Environ. Microbiol. 2002, 68:5595-5599.

15. Farmakiotis D, VarugheseJ, SueP, AndrewsP, BrimmageM, DobroszyckiJC and CoyleM.Typhoid Fever in an Inner City Hospital: A 5- Year Retrospective Review. Journal of Travel Medicine.2012, 20(1): 17-21.

16. Barrow PA and FreitasNetoOC.Pullorum disease and fowl typhoid-new thoughts on old diseases**:** A review. Avian pathology.2011, 40(1): 1-13.

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17. Minga UM, Kikopa R, Minja KSGZ, Mwasha JD. The prevalence and improved serodiagnosis of fowl typhoid in Tanzania. Proceedings of the 5th Tanzania Veterinary Association Scientific Conference. 325–338, 1987.

18. Mdegela RH, Yongolo MG, Minga UM, Olsen JE. Molecular epidemiology of *Salmonella gallinarum* in chickens in Tanzania. Av. Path.29, 457-463, 2000.

19. Mtove G, Amos B, von Seidlein L, Hendriksen I, Mwambuli A, Kimera J, Mallahiyo R, Kim DR, Ochiai RL, Clemens JD, Reyburn H, Magesa S, Deen JL: Invasive salmonellosis among children admitted to a rural Tanzanian hospital and a comparison with previous studies. *PLoSOne* 5(2): e9244, 2010.

20. Crump JC, Ramadhani HO, Morrisey AB, Saganda W, Mwako MS, Yang LY, Chow SC, Morpeth SC, Reyburn H, Njau BN, Shaw AV, Diefenthal HC, Shao JF, Bartett JA, Maro VP: Invasive bacterial and fungal infections among hospitalized HIV-infected and HIV-uninfected adults and adolescents in northern Tanzania. *CID* 52(3):341-348, 2011.

21. Ley B, Thriemer K, Ame SM, Mtove GM, von Seidlein L, Amos B, Hendriksen IC, Mwambuli A, Shoo A, Kim DR, Ochiai LR, Favorov M, Clemens JD, Wilfing H, Deen JL, Ali SM. Assessment and comparativeanalysis of a rapiddiagnostictest (Tubex®) for the diagnosis of typhoid fever among hospitalizedchildren in rural Tanzania.*BMC Infect Dis*. 11:147, 2011.

22. Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, Casadesus J, Platt DJ, Olsen JE.Host adapted serotypes of *Salmonella enterica*. *EpidemiolInfect*125: 229-255, 2000.

23. Parry C, Hien TT, Dougan G, White N, Farrar J. Typhoid fever. *N Engl J Med*. 347:1770-1782, 2002.

24. Wain, J., D. House, J. Parkhill, C. Parry, and G. Dougan. Unlocking the genome of the human typhoid bacillus. *Lancet Infect. Dis.* 2:163–170, 2002.

25. Edsall G, Gaines S, Landy M, Tigertt WD, Sprinz H, Trapani RJ, Mandel AD, Benenson AS. Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with Salmonella typhosa. *J. Exp. Med.* 112:143,1960.

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26. Zahrei ST and Saeedzadeh MM.The isolation of antibiotic resistant *Salmonella* from intestine and liver of poultry in Shiraz province of Iran.*Int. J. Poult. Sci*., 4(5): 320-322, 2005.

27. Karimuribo ED, Mdegela RH, Kusiluka LJM and Kambarage DM. Assessment of drug usage and antimicrobial residues in milk on small holder farms in Morogoro, Tanzania, Bulletin of Animal Health and Production. 53: 234 – 241, 2005.

28. KatakwebaAAS, MtamboMMA, Olsen JE and MuhairwaAP.Awareness of human health risks associated with the use of antibiotics among livestock keepers and factors that contribute to selection of antibiotic resistance bacteria within livestock in Tanzania.*Livestock Research for Rural Development* 24 (10), 2012.

29. Aminov RI. The role of antibiotics and antibiotic resistance in nature. *Environ. Microbiol*. 11**:**2970-2988, 2009.

Table 1: API- Biochemical confirmation of *Salmonella* strains

Test	*Salmonella gallinarum*	*Salmonella typhi*
ONPG	-	-
ADH	-	-
LDC	+	+
ODC	+	-
CIT	+	-
H_2S	+	+
URE	-	-
TDA	-	-
IND	-	-
VP	-	-
GEL	-	-
GLU	+	+
MAN	+	-
INO	-	-
SOR	-	-
RHA	-	-
SAC	-	-
MEL	+	+
AMY	-	-
ARA	+	-

Key:*ONPG = β-galactosidase, ADH = arginine dihydrolase, LDC = lysine decarboxylase, ODC = ornithine decarboxylase, CIT = Citrate, H_2S = hydrogen sulfide, URE = urea agar,* TDA *= tryptophan deaminase, IND = indole, VP = acetoin production test, GEL = gelatinase test, GLU = glucose assimilation test, MAN = mannitolassimilation test, INO = inositol assimilation test, SOR = sorbitol assimilation test, RHA =*

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rhamnoseassimilation test, SAC = saccharose assimilation test, MEL = melibiose assimilation test, AMY = amygdaline assimilation, ARA = arabinose assimilation test

Table.2 Antibiotic susceptibility of isolated *Salmonella*strains

Antibiotic	**Concentration per disc (µg)**	**Susceptible strains (N)**	**Resistant strains (N)**
1. Ciprofloxacin	10	*S. gallinarum* = 6 *S. typhi* = 2	*S. gallinarum* = 0 *S. typhi* = 0
2. Chloramphenicol	30	*S. gallinarum* = 1 *S. typhi* = 1	*S. gallinarum* = 5 *S. typhi* = 1
3. Ceftriaxone	30	*S. gallinarum* = 1 *S. typhi* = 0	*S. gallinarum* = 5 *S. typhi* = 2
4. Amoxycillin	30	*S. gallinarum* = 0 *S. typhi* = 0	*S. gallinarum* = 6 *S. typhi* = 2