ANTIMICROBIAL RESISTANCE PATTERNS OF FAECAL *E. coli* AND *Salmonella* IN WILD ANIMALS IN EASTERN WILDLIFE REGION OF SRI LANKA

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SUMMARY: Resistance to antimicrobials is a worldwide problem in both human and veterinary medicine. Exposure to antimicrobials is commonly attributed to the maintenance of resistance in bacterial populations. Commensals like *Escherichia coli* (*E. coli*) can easily acquire and transfer resistance genes. The present study was conducted to identify antimicrobial resistance (AMR) profiles of *E. coli* and *Salmonella* isolated from faecal samples of wild animals. During the period of December 2015 to June 2016, samples were collected from 26 birds, 25 mammals and three reptiles within the Eastern Wildlife Health Region of Sri Lanka, which covers approximately 125,576 hectares. Isolation rates of *E. coli* and *Salmonella* from faecal samples were 37.03% (20/54) and 18.51% (10/54) respectively. Nine of the 20 *E. coli* isolates (45%) were resistant to ampicillin and 7 (35%) to trimethoprim+sulfamethoxazole and tetracycline. Two *E. coli* isolates were resistant to more than 6 antimicrobials tested. All 20 *E. coli* isolates were susceptible to amikacin and imipenem and 19 (95%) were susceptible to ceftriaxone and gentamicin. Out of the 10 *Salmonella* isolates, four were resistant to ampicillin and tetracycline, while 2 were resistant to trimethoprim+sulfamethoxazole. Ten percent resistance was observed against each nalidixic acid, streptomycin, and ciprofloxacin. Multiple Drug Resistant (MDR, i.e. not susceptible to at least one agent in at least three antimicrobial classes) *E. coli* and *Salmonella* were recovered from Jungle Cat (*Felis chaus*), Jungle Fowl (*Gallus lafayetti*), and Serpent Eagle (*Spilornis cheela*). These AMR and MDR patterns indicate a threat to wildlife and the necessity of conducting a detailed study to identify the possible sources of wildlife contamination.

INTRODUCTION

Antimicrobial resistance (AMR) is a worldwide problem in human and veterinary medicine (Paul, 1983, World Health Organization, 2011, Hajer *et al.*, 2012). Various human activities including overuse/misuse of antimicrobials, use of antimicrobials as growth promoters in animal feed and improper disposal of human and farm animal waste have led to exposure of bacteria to sub lethal levels of antimicrobials which promote the evolution of resistant bacterial strains (Poeta *et al.*, 2005). Hitherto the bacteria harboring AMR determinants have been isolated from a variety of environmental sources, including domestic sewage, drinking water, rivers and natural lakes, etc., in many countries (Kasper and Burgess, 1990, Poeta *et al.*, 2005).

The epidemiological surveillance of transmission of AMR is usually conducted using the

indicator bacteria of the intestinal flora such as *Escherichia coli* (*E. coli*) and *Salmonella* sp. (Guerra *et al.*, 2003, Clesceri *et al.*, 1993). As AMR is a global health emergency, more investigations are ongoing to evaluate the prevalence of AMR among pathogens/commensals in humans, domestic animals and wildlife in many countries. Even in Sri Lanka several studies have reported alarming trends of AMR in *E. coli* and *Salmonella* in humans and domestic animals (Asanthi *et al.*, 2014, Chandrasiri *et al.*, 2010, Kottawatta *et al.*, 2017, Liyanapathirana *et al.*, 2016, Pathirage *et al.*, 2014).

However, limited research has been conducted in Sri Lanka to reveal the level of AMR in wild ecosystems and the information about the resistance levels in bacteria isolated from wild animals are scarce. The objectives of this study were

to get basic idea about pattern of AMR in *E. coli* and *Salmonella* in wild animals against antimicrobials which are commonly used as therapeutic agents in human and veterinary practices in Sri Lanka, and to identify the current status of wildlife associated AMR in a selected area of Sri Lanka in order to get a basic idea about possible antimicrobials that can be used as effective therapeutic agents in Sri Lankan wildlife.

MATERIALS AND METHODS

Study area and sampling

This study was conducted using voided faeces and rectal or cloacal swabs collected from sick and displaced wild animals that were brought to the Eastern Wildlife Health Center of the Department of Wildlife Conservation in Ampara, Sri Lanka, from December 2015 to June 2016. A single sample was taken from each animal with a sterile swab before any treatment or feed was given. A total of 54 samples were collected, 26 from birds, 25 from mammals and 3 from reptiles. The samples were stored and transported to the laboratory on ice (4°C) within 24 to 48 hours of collection. All the laboratory procedures were conducted in the Food Microbiology Laboratory (FML), Department of Veterinary Public Health and Pharmacology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

Isolation and identification of E. coli

The methods described in Carson *et al.* (2001) and Quinn *et al.* (1999) were used for isolation and identification of *E. coli*. Briefly, the tube containing swab was filled with 3 ml of buffered peptone water, mixed well, and incubated at 37°C for 18 to 24 hours. A loop full of the incubated buffered peptone water solution was streaked on a MacConkey agar plate and incubated under 37°C for 24 hours. Selected one pink color presumptive *E. coli* colony on MacConkey agar plate was streaked on nutrient agar plate and incubated at 37°C for 18 to 24 hours to obtain a pure culture. All pure presumptive *E. coli* isolates were subjected to biochemical tests for confirmation as described in Quinn *et al.* (1999). Parallel analysis of the *E. coli* reference strain, ATCC 25922 was performed as the positive control of all procedures.

Isolation and identification of Salmonella

The methods described by World Health Organization (WHO) global foodborne infections network (WHO global foodborne infections

network, 2010), International Standard (ISO) 6579-1 (ISO, 2017) and Quinn *et al.*, (1999) were used for isolation and identification of *Salmonella* from the samples collected. Briefly, each swab was immersed in 3 ml of Buffered Peptone Water (BPW) and incubated at 37°C overnight as the non-selective pre enrichment step. Following day, 100 µl from the pre enriched sample was pipetted out and added to 10 ml of Rappaport Vassiliadis broth (RV broth) and incubated at 37°C for 24 hours for selective enrichment. Following the incubation, a loop-full of RV broth was streaked onto xylose lysine deoxycholate (XLD) plate and incubated for 18 hours at 37°C. A presumptive colony of *Salmonella* (a colony with rabbit eye appearance) was selected, streaked onto nutrient agar plate and incubated at 37°C for 18 to 24 hours. Confirmation of *Salmonella* species was done by biochemical tests as described previously (WHO global foodborne infections network, 2010 and Quinn *et al.* (1999).

Testing for antimicrobial resistance

The AMR profile of each confirmed isolate was assessed by the disk diffusion method using Mueller Hinton (MH) agar and antibiotic disks from Oxoid (Oxoid Limited, UK) following the procedures described in Clinical Laboratory Standard Institute (CLSI, 2013). The isolates were tested for 12 antimicrobials: amikacin, streptomycin, gentamicin, imipenem, cefatoxime, cefatazidime, ceftriaxone, ampicillin, nalidixic acid, ciprofloxacin, trimethoprim+sulfamethoxazole, and tetracycline, which are currently used in human and veterinary practices in Sri Lanka. The diameters (in millimeters) of the inhibitory zones were measured using a Vernier caliper based on the guideline given by the CLSI, 2013. Each isolate was designated as Resistant (R), Intermediately Susceptible (I) or Susceptible (S) based on the clinical break points given in CLSI, 2013. Isolates not susceptible to at least one agent in at least three antimicrobial classes were defined as MDR (Sweeney *et al.*, 2018). Data were analyzed and graphs were plotted using Microsoft Excel.

RESULTS

Isolation rates of E. coli and Salmonella and their AMR patterns

E. coli isolates were recovered from 20 wild animal faecal samples (20/54, 37 %); 11 (42%) were from birds and 9 (36%) from mammals. No *E. coli* was recovered from reptiles sampled in this study.

Salmonella was detected in 10 (10/54=19 %) samples. Of these 6 (23%) were from birds, 3 (12 %) were from mammals and 1 (33%) was from a reptile.

Forty five percent (9/20) of *E. coli* isolates were resistant to ampicillin while 35% (7/20) were resistant to each of trimethoprim+sulfamethoxazole

and tetracycline. Twenty five percent (5/20) of the isolates were resistant to streptomycin whereas 15% (3/20) resistance was detected against ciprofloxacin and nalidixic acid. Five percent (1/20) of the isolates exhibited resistance to each antimicrobial cefatoxime, ceftriaxone, and gentamycin (Figure 1).

Figure 1: Antibiogram of the *E. coli* isolated from faecal samples of wild animals

Out of the 10 *Salmonella* isolates subjected to disk diffusion test, 4 (40%) isolates were resistant to each ampicillin and tetracycline. Two (20%) isolates were resistant to trimethoprim+

sulfamethoxazole while 10% of resistance was detected against each nalidixic acid, streptomycin, and ciprofloxacin (Figure 2).

Figure 2: Antibiogram of the *Salmonella* isolated from faecal samples of wild animals

Multiple drug resistant *E. coli*

One *E. coli* isolate from a Jungle Cat (*Felis chaus*) was resistant to 9 antimicrobials namely, ampicillin, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, trimethoprim+sulphamethoxazole, tetracyclin, and streptomycin. This isolate was sensitive only for amikacin, imipenem, and nalidixic acid. Another *E. coli* isolate from a Jungle Fowl (*Gallus lageyettii*) was resistant to 6 antimicrobials namely ampicillin, ciprofloxacin, nalidixic acid, trimethoprim+sulphamethoxazole, tetracyclin, and

streptomycin. Two *E. coli* isolates from Serpent Eagles (*Spilornis cheela*) were resistant to five antimicrobials whereas an *E. coli* isolate from Spotted Deer (*Axis axis*) was resistant to four antimicrobials. Table 1 shows MDR *E. coli* isolates recovered from different species of animals. Ampicillin, ciprofloxacin, nalidixic acid, trimethoprim+sulfamethoxazole, streptomycin, and tetracycline were identified as the antimicrobials against which the tested indicator bacteria were mostly (25%, 5/20) resistant.

Table 1: Profile of multiple drug resistant isolates of *E. coli* from different types of animals

Antimicrobial agent	Jungle Fowl	Serpent Eagle 1	Serpent Eagle 2	Jungle Cat	Spotted Deer
Amikacin	S	S	S	S	S
Streptomycin	R	S	R	R	R
Gentamycin	S	S	S	R	S
Imipenem	S	S	S	S	S
Cefatazidime	S	S	S	R	S
Cefotaxime	S	S	S	R	S
Ceftriaxone	S	S	S	R	S
Ampicillin	R	R	R	R	R
Ciprofloxazine	R	R	S	R	S
Nalidixic acid	R	R	R	I	S
Trimethoprim+sulfamethoxazole	R	R	R	R	R
Tetrcycline	R	R	R	R	R
No R/total No. of antibiotics tested	6/12	5/12	5/12	8/12	4/12

R = Resistant, I = Intermediate, S = Susceptible

Multiple drug resistant *Salmonella*

One *Salmonella* isolate from a Serpent Eagle was resistant to six antimicrobials namely ampicillin, streptomycin, ciprofloxacin, trimethoprim+sulfamethoxazole, nalidixic acid and tetracycline showing multiple drug resistance (Table 2). Both *E.*

coli and *Salmonella* isolates from this particular wild bird were resistant to ampicillin, ciprofloxacin, nalidixic acid, trimethoprim+ sulfamethoxazole and tetracycline showing similar resistant patterns against tested antimicrobials.

Table 2: Profile of multi resistant isolates of *Salmonella* from two different types of animals

Antimicrobial agent	Serpent Eagle	Jungle Cat
Amikacin	S	S
Streptomycin	R	I
Gentamycin	S	S
Imipenem	S	S
Cefatazidime	S	S
Cefotaxime	S	S
Ceftriaxone	S	S
Ampicillin	R	R
Ciprofloxazine	R	I
Nalidixic acid	R	R
Trimethoprim+sulfamethoxazole	R	R
Tetracycline	R	R
No. R/ Total no. of antibiotics tested	6/12	4/12

R = Resistant, I = Intermediate, S = Susceptible

DISCUSSION

The current study attempted to isolate two indicator bacteria, namely *E. coli* and *Salmonella* from different wild animals, and to identify their AMR profiles and resistance level to antimicrobials which are commonly use as therapeutic agents in human and veterinary practices in Sri Lanka. The isolation rate of *E. coli* from wild animal faeces was lower in the present study compared to studies conducted on faecal *E. coli* of humans and domestic animals. This was reported in previous studies too (Gordon and Cowling, 2003, Tenaillon *et al.*, 2010). Although the load of *E. coli* in human gut microbiota is around 90%, it is usually around 56% in wild animals (Gordon and Cowling, 2003, Tenaillon *et al.*, 2010). Some studies on reptiles showed 68.4% isolation rates, but these animals were from private owners, in captivity or captured for monitoring (Ramos *et al.*, 2009). Further, the prevalence of *E. coli* in birds and reptiles can be around 23% and 10% respectively (Gordon and Cowlin, 2003). The differences in prevalence rates in different groups of animals are greatly influenced by the host characteristics such as recovery and density of *E. coli* in the gut (Tenaillon *et al.*, 2010). Moreover, the

frequency of exposure of animals to human contaminants usually determines the recovery rates of *E. coli* from animals (Iovine Rde *et al.*, 2015).

The data generated here suggests different levels of AMR in *E. coli* and *Salmonella*. These indicator bacteria showed development of resistance against antimicrobials that have been widely used for decades. In this study, resistance was observed in *E. coli* against tetracycline, ampicillin, streptomycin and trimethoprim+sulfamethoxazole which are the classes of antimicrobials that have been widely used in human and veterinary practices in Sri Lanka.

Even among these widely used agents, resistance was seen mostly against tetracycline and ampicillin. Not only in Sri Lanka, but globally tetracycline is commonly used as a first-line antibiotic for many different species of domestic animals because of its wide spectrum, availability and cost. Tetracycline is often used before the antimicrobial susceptibility of the pathogen is determined (Rosalind *et. al.*, 1985). Resistance to ampicillin and tetracycline has been observed in *E.*

coli isolated from a variety of sources throughout the world (Erskine *et al.*, 2002, Klein and Bulte, 2003, Schlegelova *et al.*, 2002). As an example, in a cross sectional study done in Michigan by Sayah *et al.* (2005) that included *E. coli* isolates from variety of sources (human sewage, wildlife, domestic animals, farm environments, and surface water) showed resistance to tetracycline and cephalothin. The author claims that contamination of surface water from farm environments has a significant contribution in spreading AMR bacteria. Over the counter drug purchases, misuse of antimicrobials, continuation of the same antimicrobial without veterinary consultation, and inappropriate farm waste disposal may facilitate emergence of AMR bacteria in communities and eco systems in Sri Lanka.

Lunt *et al.* (2010) highlighted that unregulated drug purchase and low quality patient care also can increase the environmental contamination of antimicrobials, and this will be influenced by the rapid internet usage.

Multiple drug resistant *E. coli* strains were isolated from Jungle Cat, Jungle Fowl, Serpent Eagles and Spotted Deer. It is unlikely that these wild animals have had a direct contact with antimicrobials through antibiotic therapy. Except the Spotted Deer, others usually roam around interacting with humans and livestock, especially around the poultry farms. Antimicrobials are used as growth promoters or as therapeutics in the poultry industry and these wild animals have been reported to act as predators or to feed on poultry residues, feed residues or poultry manure. Local communities state that Jungle Cats are predators of poultry and often eat poultry house waste. Moreover, Serpent Eagles in this area are predators of small rodents, which largely depend on poultry manure and feed. Therefore, bioaccumulation of antimicrobial residues could be expected in predators such as Serpent Eagles and Jungle Cats. Findings from an influential study by Gilliver *et al.* (1999), showed a high prevalence of acquired AMR genes among fecal *E. coli* from woodland rodents in the Wirral, UK. Because there was no direct antibiotic use on this land, the investigators argued that this prevalence was not a direct result of anthropogenic disturbance. This provides evidence that proximity to human populations, rather than direct antibiotic use on the land, is sufficient to substantially affect the gut flora of local wildlife (Winokur *et al.*, 2001). An *E. coli* isolate from Spotted Deer also showed MDR (resistant to four antimicrobials). Even though the

means of Spotted Deer being exposed to antimicrobials is not fully understood, with the available data, surface water contamination with antimicrobial residues or AMR bacteria can be considered as a potential source of exposing wild fauna to antimicrobials which eventually leads to emergence of AMR among pathogens and commensals (Martinez, 2009). The worst case scenario of emergence of AMR in wildlife is the establishment of environmental reservoirs of resistant bacteria ultimately causing problems in medical treatments to wildlife and to humans in cases of zoonotic disease outbreaks (Hajer *et al.*, 2014).

This study detected one isolate of *Salmonella* from a Serpent Eagle which was resistant to six antimicrobials and another *Salmonella* isolate from Jungle Cat which was resistant to four antimicrobials (Table 2). Interestingly the resistant pattern of the *Salmonella* isolate from the Serpent Eagle was similar to that of the *E. coli* isolated from the same bird. These findings give hints on commensals of different genera acquiring similar resistant determinants. Observing resistance or intermediate resistance for the same panel of antimicrobials might suggests that Serpent Eagle and the Jungle Cat have been predating or scavenging from a similar environment. Rosalind *et al.* (1985) has hypothesized that food wastes and other forms of refuse can be sources of AMR for nonpathogenic bacteria in the intestine in the absence of known antibiotic selective pressure.

Conclusion

It is difficult to make strong conclusions with the results generated from this preliminary study with a small sample size. However, the results generated are valuable in identifying AMR profiles of investigated indicator bacteria in wildlife, speculating future empiric treatments and to take necessary precautions to suspend the transmission of AMR bacteria between wildlife, humans, and livestock. Limitations identified in the current study include; small sample size, lack of data on other antimicrobials which are not used in Sri Lanka for treatment of human and domestic animals, and not including other classes of antimicrobials frequently used as therapeutic agents in Sri Lanka.

Compared to studies from different parts of the world, *E. coli* and *Salmonella* recovered from Sri Lankan wildlife are still susceptible to many antimicrobials. Hence, still we have opportunities to

safeguard our wildlife by minimizing development of antimicrobial resistance. However, data from the current study may indicate a considerable wildlife contamination with AMR microbes and their genes, and the sources are unclear. Therefore, a detailed study including wild animals representing all the local wildlife health regions is warranted for the proper understanding of the AMR levels of indicator bacteria in wild animals in Sri Lanka.

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