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Original Research Article

ANTIMICROBIAL RESISTANCE INCLUDING EXTENDED SPECTRUM BETA LACTAMASES (ESBL) AND CARBAPENEMASE IN FEACAL *E. COLI* ISOLATES FROM BATS

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SUMMARY: Antimicrobial resistance (AMR) is one of the major causes of rising human mortality in hospitals. Although mitigation of AMR is challenging, one health approach has shown success. Bats, a key component in the ecosystem, contribute to about 20% of the world's entire mammalian population with > 1200 species. Bats found both in rural and urban environments coexisting with the human population play a significant role in agriculture. Although AMR with significant importance has been reported in bats in some countries, a dearth of literature including dissemination of phenotypic antimicrobial resistance in bats Sri Lanka. Therefore, this study was undertaken to analyse the phenotypic antimicrobial resistance of *E. coli* isolated from bats in the Kandy suburbs in Sri Lanka in June – December 2022 when the worst economic crisis prevailed in the country. The fresh faecal samples were collected from three identified sites in Kandy suburbs. Faecal samples were cultured in MacConkey agar, sub-cultured in blood agar and the identification of *E. coli* was carried out by conventional methods. A total of 251 *E. coli* isolates were screened for antimicrobial susceptibility testing by the disk diffusion test. Cefotaxime and meropenem disks were used to determine ESBL and carbapenem resistance respectively. Resistance to carbapenem was observed in *E. coli* at 8.9%, 7.5% and 7.3% and to extended spectrum beta lactamases (ESBL) at 26.9%, 38.9% and 39.8 % in the three sites where samples were collected. Over 50% of frequency was observed for trimethoprim, ampicillin and sulfa trimethoprim combination. The CTX-M was found and NDM-1 was not found by PCR similar to the finding reported previously. Surveillance of AMR in multiple sectors for baseline information on each species in the ecosystem is vital for the interpretation of resistant frequencies. However, the role of bats in the dissemination of AMR determinants needs to be further investigated.

KEYWORDS: AMR, ESBL, Carbapenemase, bats

INTRODUCTION

Antimicrobial resistance (AMR) is an overwhelming topic of discussion in human and veterinary medicine. It is also considered as a global one health issue in the near future (Devnath *et al*., 2022). Importantly, annual deaths occurring in humans due to antimicrobial resistance (AMR) have exceeded 1.27 million worldwide (WHO, 2022). AMR occurs as a result of evolution in the microbial population or survival response to harsh changes in the natural environment. However, continuous research in the field of microbiology has shown that the indiscriminate use of antimicrobial agents in humans and livestock acts as the main factor for the rapid expression of AMR that arises due to evolution

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(WHO, 2021). Spillover from humans, livestock and companion animals may be the source of dissemination of antimicrobial resistance in wildlife and the environment. The transmission of zoonotic diseases from wildlife species to humans has been investigated and implementing strategies to prevent it is required (McDougall *et al*., 2021a). However, reverse zoonosis or anthropozoonosis has also been reported with many pathogens and the acquisition of antimicrobial resistance in *E. coli* in bats has been reported in many countries in the world (Benavides *et al*., 2022).

Bats contribute to about 20% of the world's entire mammalian population with > 1200 species (Banskar *et al*., 2016). Some species are yet to be confirmed and recently a new species known as *Miniopterus phillipsi* was discovered in Sri Lanka and India (Kusuminda *et al*., 2022). This is in addition to the 30 species (Weerakoon and Wijesundara, 2012- ICUN Red List). Representing 7 families (Emballonuridae, Hipposideridae, Megadermatidae, Molossidae, Pteropodidae, Rhinolophidae and Vespertillionidae) currently known to inhabit in Sri Lanka. They are capable of occupying a wide range of ecological niches (Bai *et al*., 2017). Bats consume a highly diverse range of diets as herbivorous, carnivorous and omnivorous (Bai *et al*., 2017; Cláudio *et al*., 2018). In addition, bats are shown a longer life-span than many other non-flying mammalian species of similar size, with few exceeding thirty years of life span. Furthermore, bats may travel long distances for food, as far as 1000 km or more (Allocati *et al*., 2016). This special mammals are already known to be carriers of viral (Nipha virus), bacterial (Salmonella species) and fungal (*Histoplasma capsulatum*) pathogens causing infections in humans (Syaripuddin *et al*., 2015). However, studies on bacterial infections associated with bats found to be rare in the literature.

Bats are scattered in the country both in rural and urban environment and urban colonies are found in a few locations. The Royal botanical garden, Kandy Lake and Polgolla dam are three locations where urban bat colonies are found in Kandy suburbs in Sri Lanka. The bats colonies at Royal Botanical Garden have been studied previously. Over 24,000 bats have been found in 20 km^2 land (density of bats 1200-3250/hectare: the highest density was West border at the river bank) of Royal Botanical garden at Peradeniya (Krystufek, 2009). Usually 30-100 bats could be found in a single tree in the botanical garden although such statistics were not found around Polgolla dam and Kandy lake (Krystufek, 2009). The high genetic diversity had been observed in different bat colonies in the country with special emphasis to the existing bat colonies at Royal Botanical Garden, Peradeniya (Karawita *et al*., 2017; Kikuchi *et al.*, 2020; Muzeniek *et al*., 2022; Perera *et al*., 2022). Therefore, millions of micro florae are excreted to the outside environment through faeces.

Gram negative bacteria are contributed a significant share in WHO priority list of antimicrobial resistance (Devnath *et al*., 2022). *E. coli* is considered as an indicator organism of antimicrobial resistance in many AMR surveillance in wild life (McDougall *et al*., 2021a). AMR in isolates from bats has been reported only few countries in Asia as Indonesia, Malaysia, Japan and India, resistance to extended beta lactamase (ESBL) has been reported previously (Devnath *et al.*, 2022). It has been identified that acquiring of antimicrobial resistant determinants may be occurred in hospital and outside environments although it has not been proven with evidences (McDougall *et al.*, 2021a; Benavides *et al.*, 2022). However, role of bats in dissemination of antimicrobial resistance from bats to human is not well identified (McDougall *et al.*, 2021b). Only minimum literature is found on role of dissemination of phenotypic antimicrobials resistance in bats, Sri Lanka. Therefore, the objective of this study was to analyze the phenotypic antimicrobial resistance of *E. coli* (in bats) from three places in Kandy suburbs in Sri Lanka. Although, presence of pathogen in bats has been studied in limited extent, antimicrobial resistance has not been evaluated in bat colonies of municipal territory in the country (Gunawardena *et al*., 2016).

MATERIAL AND METHODS

Total samples (101 feacal samples) were collected from three sites in Kandy and Kandy suburbs (Royal Botanical garden, around Polgolla Dam and in Kandy Lake) in Sri Lanka, only fresh faecal samples with watery in nature and no visible contaminations were collected in the morning hours between 6.AM to 8.30 AM and transported to the Veterinary Research Institute at 4^0C. The fresh samples were inoculated into MacConkey agar (Oxoid CM 0007) and incubated overnight at 37^0C. The grown colonies in MacConkey agar were picked up, sub-cultured on 5% sheep blood agar and randomly maximum of five individual colonies in pink coloured in MacConkey agar were selected and sub cultured (n= 433). The identification of *E. coli* was done based on colony morphology, Gram Staining, growth in triple sugar iron agar (TSI: Oxoid), growth in sulfur indole motility media (SIM: Oxoid), and biochemical test such as Urease (Oxoid) and Indole (Oxoid) test. The identified *E. coli* (n=251) were sub cultured in 5% sheep blood agar, incubated overnight at 37^0C in the incubator. At least single *E. coli* had been isolated and identified out of 98 faeacal samples in the study. A loopful of culture were inoculated in TSB (Tryptone Soya Broth: Oxoid: CM 0129) with 20% Glycerol and stored at -20^0C.

The antimicrobial susceptibility testing was performed in disk diffusion test according to the methods describe by in EUCAST disk diffusion method for antimicrobial susceptibility testing Version 11.0 (January 2023) (www.eucast.org) and M 100: Performance standards of antimicrobial susceptibility testing,33rd edition (https://clsi.org). *E. coli* ACTCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strain in the disk diffusion test. Extended spectrum beta lactamase resistant and carbapenem resistant *E. coli* was determined by using cefotaxmine (5µg) and meropenem disks (10µg) as described in EUCAST respectively. The measured diameter for each antimicrobial was interpreted based on EUCAST clinical breakpoints for ampicillin, cefotaxime, meropenem, gentamicin, trimethoprim and ciprofloxacin. In the absence of specific clinical break point for the rest (tetracycline, streptomycin, and chloramphenicol), the clinical break point in M100: CLSI were taken into the interpretation,

data were stored and analyzed in Microsoft Excel for the calculation of percentage in the study (James S. Lewis II, PharmD, FIDSA, n.d.).

PCR test was carried out for detection of CTX-M gene is extracted DNA of the isolates which was shown phenotypic resistant to cefotaxime. PCR was done as the methods described previously (Saleem *et al.*, 2022). (Primers F; 5'-GACGATGTCACTGGCTGAGC3' and R: 5'-AGCCGCCGACGCTAATACA 3., Initial denaturation was at 94℃ for 5 minutes; annealing step at 48℃ followed by 72℃ for 1 minute each and final extension at 72℃ for 10 minutes for 35 reaction cycles. Amplified product size was 499 bp fragments in 1% agarose gel) (Saleem *et al.*, 2022). The PCR test for NDM-1 was done as previously. NDM-1 forward 5`- CAGCACACTTCCTATCTC and NDM-1 reverse 5`-GTAGTGCTCAGTGTCGGCAT., The PCR protocol was initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The final extension step was carried out at 72°C for 10 min. The PCR-amplified products were analyzed by 2% agarose gel electrophoresis (Product size was 220 bp) and stained with ethidium bromide (Liu *et al.*, 2012).

RESULTS

The *E. coli* recovery rate from sub cultured isolates originated fecal samples was low as 58% in fecal samples from bats in the study. Since five colonies were picked up, recovery percentage of *E. coli* was higher than only single colony sub cultured. Importantly, no significant differences were observed to the given antimicrobials were not significantly different in three different sampling sites in the study (Table 1 to 3).

The Highest percentage of resistance was observed for trimethoprim, ampicillin (beta lactams), and sulfamethoxazole- trimethoprim combination in the study. In addition, resistance were observed for aminoglycosides, chloramphenicol. Importantly ESBL and carbapenem resistance were also observed in the study. CTX-M was found only 34 of 93 of phenotypically resistant *E. coli* in the study. Moreover, NDM-1 was not detected in a single isolate which was shown resistance to meropenem.

Table: 1 The results of phenotypic antimicrobial resistant profile of *E. coli* isolated in bats at Royal Botanical garden, Peradeniya (n=157)

Type of antimicrobial	Number of resistant isolates	Source of clinical break point	% of resistance
Ampicillin(10µg)	88	EUCAST	56.0
Tetracycline (30µg)	24	CLSI	15.3
Cefotaxime (5µg)	61	EUCAST	38.9
Meropenem (10µg)	14	EUCAST	8.9
Sulfamethoxazole trimethoprim (23.75 µg &1.25 µg)	81	EUCAST	51.6
Trimethoprim (5µg)	91	EUCAST	58.0
Gentamicin (10µg)	48	EUCAST	30.6
Streptomycin (10µg)	43	CLSI	27.4
Ciprofloxacin (5µg)	39	EUCAST	24.8
Chloramphenicol (30µg)	8(34)*	CLSI	23.6

***** Only 34 isolates were tested due to the scarcity of specific disk

Table: 2 The results of phenotypic antimicrobial resistant profile of *E. coli* isolated in bats at Kandy Lake, Kandy (n=53)

Type of antimicrobial	Number of resistant isolates	Source of clinical breakpoint	% of resistance
Ampicillin(10µg)	34	EUCAST	64.1
Tetracycline(30µg)	24	CLSI	45.3
Cefotaxime (5µg)	21	EUCAST	39.6
Meropenem (10µg)	4	EUCAST	7.5
Sulfamethoxasole trimethoprim (23.75 µg &1.25 µg)	17	EUCAST	32.1
Trimethoprim (5µg)	28	EUCAST	52.8
Gentamicin (10µg)	17	EUCAST	32.1
Streptomycin (10µg)	21	CLSI	39.6
Ciprofloxacin (5µg)	12	EUCAST	22.6
Chloramphenicol (30µg)	0*	CLSI	

*isolates were not tested for chloramphenicol

Table: 3 The results of phenotypic antimicrobial resistant profile of *E. coli* isolated in bats at Polgolla Dam (n=41) M100 performance standard of antimicrobial susceptibility testing, 30th edition, CLSI.

Type of antimicrobial	Number of resistant isolates	Source of clinical breakpoint	% of resistance
Ampicillin(10µg)	18	EUCAST	43.9
Meropenem (10µg)	3	EUCAST	7.3
Sulfamethoxazole trimethoprim (23.75 µg &1.25 µg)	12	EUCAST	29.3
Trimethoprim (5µg)	17	EUCAST	41.5
Gentamicin (30µg)	11	EUCAST	26.8
Streptomycin (30µg)	12	CLSI	29.3
Ciprofloxacin(5µg)	8	EUCAST	19.5
Chloramphenicol	1(8)	CLSI	12.5

*only 8 isolates were tested for chloramphenicol due to scarcity of specific disk at that time

DISCUSSION

Bats are an important flying animal in the ecosystem of our region of South Asia including Sri Lanka, contribution to agriculture sector is considered highly significant (Durrance-Bagale *et al*., 2021; Perera *et al*., 2022). As described previously bats live long time than other creature of similar body size in the environment which is considered as unique feature of the group of mammals in the environment (Lagunas-Rangel, 2020). In addition, immune response of bats has not fully understood, while responses against bacterial pathogen has not properly studied (Banerjee *et al*., 2020). Because of all three factors described, bats may be an indicator of antimicrobial spillover in the environment from human and livestock related activities.

Resistance to carbapenem was observed in limited and low frequencies in *E. coli* (8.9%, 7.5% & 7.3%) isolated in bat faeces, which may be considered as either an indicator of environment contamination or some unknown reason. This was the first reporting phenotypic resistance to carbapenem in bacterial isolates in the country although genotypic confirmation was not found. The finding revealed possible outcome of an environmental pollution and contamination with critically important antimicrobials which is only used in human medicine. The resistance to carbapenem in bats had been reported in Australia and Algeria previously, only few isolates were shown resistance to carbapenem both in *E. coli* and *Kelbsiealla pneumoniaea* respectively (Gharout-Sait *et al*., 2019; McDougall *et al*., 2021b, 2021a). In addition, resistance to extended spectrum beta lactamases (ESBL) was observed different frequency of 26.9%, 38.9%, and 39.8 % in the three sites where samples were collected. High frequency of ESBL had been reported in bats as 60% and 74% in Congo, Australia previously (Nowak *et al*., 2017; McDougall et al., 2021a, 2022). Only CTX-M has been identified in 13.5% of isolates in the study. The *bla* $_{\text{CTX-M}}$ had been isolated in number of occasions in the literature from bats previously (Nowak *et al*., 2017; Garcês *et al*., 2019). Although genetic diversity of ESBL has not been investigated in the our study, bla$_{\text{CTX-M-55}}$, bla$_{\text{CTX-M-15}}$, bla$_{\text{CTX-M-65}}$, bla$_{\text{CTX-M-3}}$, bla$_{\text{CTX-M-14}}$ had been reported in *E. coli* form bats previously (Benavides *et al*., 2022). Conversely, extended-spectrum beta-lactamases (bla$_{\text{CTX-M-3}}$, bla$_{\text{CTX-M-15}}$, bla$_{\text{TEM-1}}$) has been identified in bats in Poland (Nowakiewicz *et al*., 2021). Therefore, presence of ESBL resistance may be considered as the baseline information in faeces from bats although it is considered as an indicator or environment contamination. Since high genetic diversity of ESBL has been reported in *E. coli* previously, further study on genetic diversity is encouraged to understand diversity and different genotypes of ESBL in future.

The high frequencies of phenotypic resistance were observed for none of isolates in the study. However, over 50% of frequency was observed for trimethoprim, ampicillin and sulfa trimethoprim combination. The similar findings were shown in Australia, high frequency of resistance was observed for amoxicillin and tetracycline. In addition, same study had concluded the dissemination of resistance to amoxicillin (WHO, 2022). Globally, dissemination of resistance to ampicillin, amoxicillin, amoxicillin & clavulonic acid combination, tetracycline, cefoxitin, erythromycin, streptomycin were reported (Devnath *et al*., 2022). In addition resistance to aminoglycosides (37.7%), Sulfa trimethoprim (35.8%) and tetracycline (26.4%) were also reported previously (McDougall *et al*., 2022). Although multi drugs resistance strain were found low in our study as 18%, high frequency of multidrug resistant *E. coli* has been reported in Poland among *E . coli* from bats (Nowakiewicz *et al*., 2021). Although genetic diversity of *E. coli* was not determined in our study, such diversity were found in other studies carried out globally (Nowak *et al*., 2017; Kraemer *et al*., 2019; McDougall *et al*., 2021b, 2022; Nowakiewicz *et al*., 2021; Devnath *et al*., 2022). The high genetic diversity of *E. coli* had been observed in fruit bats isolated in Congo, although naive micro flora where minimum contact with the human population (Nowak *et al*., 2017). In addition, multi-drug resistant *Enterococcus feacalis* had been isolated and identified in bats previously (Nowakiewicz *et al*., 2021).

Although carriage of antimicrobial resistance through migratory birds has been reported, role of local bats in dissemination of was not found and which has not been investigated thoroughly (Devnath *et al*., 2022). As mentioned previously, bats are commonly found mammal in Peradeniya, Kandy suburbs. The habitat of bats is being considered as continuing changing in this region due to the unorganized urbanization, deforestation in recent past (Kudagammana *et al*., 2018). In addition, only a few reports is found in the global context (Devnath *et al*., 2022). However, ecosystem may play a vital role in AMR, evolution, dissemination and persistence of AMR are being equally important to understand to mitigating such as overwhelming issue under the one health concept (Devnath *et al*., 2022). Furthermore, changes in dietary pattern may contribute changes of microflora in gastrointestinal tract (GIT) in bats, which has effect on dissemination of pathogen to the environment (Federici *et al*., 2022). Although it has not been well understood, gut micro flora of bats is believed to be preventing excretion of pathogenic organism into the environment (Li *et al*., 2022).

According to the existing literature, bats can act as reservoir of antimicrobial resistant determinants due to the roaming and long distance flying capacity in their life style (Nowakiewicz *et al*., 2021). Furthermore, presence of AMR determinants of clinically important antimicrobials in wild inhabitants as bats are considered as an indicator of environmental pollution (Laborda *et al*., 2022). The presence of migrating and non-migrating wild animals may have a role in antimicrobial resistance, migrating animals as bats play a significant role in dissemination of antimicrobial resistance in different geographical areas including across continents globally (Laborda *et al*., 2022). However, presence of genetic resistance to antimicrobials in micro flora of bats is poorly understood (Nowakiewicz *et al*., 2021). Although acquisition of antimicrobial resistant determinants were not clear, it is assumed that human food waste, wastewater treatment plants, and aquaculture operations with antimicrobial residues may contribute globally (Kraemer *et al*., 2019). In addition, bats contribute dissemination of AMR in vector based spreading in different ecosystems (Kraemer *et al*., 2019).

Bats fly average of 9-30 km for feeding daily basis and approximately 100km daily flying also been reported (Oleksy *et al*., 2015). The home range may be affected by a number of factors such as sex, age, reproductive status, hierarchy or time of the year. It has been observed that presence of bat colonies was significantly high in spring month of the year in the Royal botanical garden at Peradeniya. The rest of the time only few bat colonies were found in the garden at the corner or close to the Mahaweli River. Therefore, seasonal variation has been observed based on the time of year although exact reason has not been determined, presence of natural food may be the contributory factor. However, significant variation in number of bat colonies has not been observed in Kandy Lake and around Polgolla dam associated reservations (by the site of Mahaweli River). Since samples collection carried out in, June, July, August and September both migratory and other bats may be found in the sampled sites. Movement for feed and disturbance to the such migration has been studied limited extent in Sri Lanka, high electrocution risk may contribute limiting movement of bats in the ecosystem (Tella *et al*., 2020). Therefore, bats may have limited movement in Kandy suburbs due to the unorganized electric power line laid in the urban area and suburbs. The environment where bats are fed or survived may be contaminated with antimicrobials through hospital waste, fecal waste and disposal of garbage in the urban and peri-urban setting. ESBL antimicrobials such as cephalosporins were found stable although carbapenem was found not stable in aqueous solutions (Viaene *et al*., 2002). However, exact way of acquiring resistance to antimicrobials in bats has not been fully understood. On the other hands, contamination of houses, clothes, vehicles and soil of human inhabits by fecal materials of bats are also reported common. Therefore, high risks of recontamination in human with AMR also need to be considered.

Furthermore, it was significant in this study that usage of antimicrobial is not the only reason for the presence of antimicrobial resistant determinants in animals. Therefore, one health approach of mitigating antimicrobial resistance may be success; than individual sectoral approach from human, livestock or companion animals. In addition, understanding baseline resistant frequencies of each wild species is also a vital factor for the interpretation of resistant profile against spillover of antimicrobial resistance. Therefore, investigation into antimicrobial resistance in wild life and environment need to be revisited with a multiple approach.

CONCLUSION

Presence of *E. coli* which was resistance to carbapenem and ESBL, other beta lactams, aminoglycosides, quinolone, tetracycline, and sulfonamides is threat to the public health an indicator of environmental pollution in the region. AMR surveillance in bats is a good indicator of contamination and pollution of antimicrobials in the environment due to the changes of their behavior in the ecosystem. Continuing AMR surveillance in the environment including wild life is encouraged in order to understand the trend of antimicrobial resistance in the environment. In addition, baseline resistant frequencies of each wild species are also an important factor for the interpretation of resistant profiles in the ecosystem. Antimicrobial resistance in *E. coli* from bats faeces may be an indicator of level of contamination in the environment in developing countries.

Ethical clearance

Research Management committee at Veterinary Research Institute has approved the research for the year 2022 considering research contents, output, outcome together with ethical clearance for the study. The permission was granted by the department of wildlife conservation form specific sites during specific period, subject to the provisions of Fauna & Flora Protection Ordinance (FFPO) of Sri Lanka (10/06/2022).

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Permission
Director General/Botanical garden, Peradeniya has approved the study and department of wild life granted the permission to carry out the study in the selected three location in Kandy within the selected period of time.

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