Research Journal of Microbiology 6 (2): 132-139, 2011
ISSN 1816-4935 / DOI: 10.3923/jm.2011.132.139

Detection of *Escherichia coli* O157:H7 in Wildlife from Disturbed Habitats in Sarawak, Malaysia

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ABSTRACT

This study was carried out to assess the occurrence of *Escherichia coli* and to detect the pathogenic strain *Escherichia coli* O157:H7 in birds, bats and rodents from disturbed habitats comprising of two urban forests and an oil palm plantation habitats located along the Rejang Basin, Sibu in the state of Sarawak, using both standard microbiological and molecular techniques. A total of 105 bird hosts, 44 of rodent hosts and 84 bat hosts represented 48 species of birds, one species of rodent and ten species of bats were screened for the presence of *Escherichia coli*. The representative isolates were cultured on a highly selective agar, Cefaxime-tellurite sorbitol MacConkey agar for the detection of *Escherichia coli* O157:H7. From the microbiological analysis, the overall occurrences of *Escherichia coli* in the hosts were 43% in rodents, 18% in birds and 11% in bats. The isolates were tested for the presence of pathogenic *Escherichia coli* O157:H7 strain by multiplex PCR method targeting the *slt-I*, *slt-II*, *rfbE* and $fliC_{H7}$ genes. The *slt-I*, *slt-II*, *rfbE* genes were not detected in any of the *E. coli* isolates. This study indicated that bats, birds, or rodents from these habitats in Sarawak did not serve as an important reservoir of *Escherichia coli* O157:H7 and thus were of no risk in the epidemiologic cycle of emerging enteric bacterial zoonoses in the state of Sarawak, Malaysia.

Key words: *E. coli*, occurrence, multiplex PCR, disturbed habitats, Sarawak

INTRODUCTION

Wildlife is well-known to be involved in most of the zoonotic diseases (Kruse *et al*., 2004). The zoonotic agents, typically various bacteria, carried by wildlife, serves as the major reservoirs for microbial transmission to both human and domestic animals. Outbreaks of zoonoses have recently received increased attention worldwide due to their major impact on human health, agriculture production, wildlife-based economies and wildlife conservation (Chomel *et al*., 2007). Wild animals are thought to be the source of more than 70% of all emerging infections (Kuiken *et al*., 2005). According to World Health Organization (2008), millions of people are infected by zoonotic diseases every year.

Enterobacteriaceae are among the best-characterized group of microflora in the gastro-intestinal tract of wildlife. The *Enterobacteriaceae* and *Escherichia coli* (*E. coli*) are among the most common

members and harmless commensal strains found in the gastro-intestinal tract of humans and animals (Boyd, 1995). However, certain *E. coli* strain carried the toxin gene, for instance, *E. coli* serotype O157:H7 which cause haemorrhagic colitis and haemolytic uremic syndrome (Griffin and Mead, 1998). *E. coli* O157:H7 was first recognized as a human pathogen since 1982 with the outbreaks of hemorrhagic colitis occurred by consumption of undercooked meat in Oregon and Michigan (Riley *et al*., 1983). *E. coli* O157:H7 belongs to Shiga toxin-producing *E. coli* (STEC) represent the only pathogenic group of the *E. coli* that has a definite zoonotic origin with cattle being identified as a major reservoir for this organism (Caprioli *et al.,* 2005). However, it has been reported to be isolated from wild birds (Wallace *et al*., 1997; Foster *et al*., 2006) and rodents (Nielsen *et al*., 2004) which implicated wild animals also served as potentials vectors for the dissemination of *E. coli* O157:H7.

The surveillance of the epidemiology of pathogenic enterobacteria in wildlife is important due to the increasing activity of translocation of wild animals which increase the possibility of disseminating the pathogenic bacteria. In addition, the study of diseases spreading and epidemiology in human population are crucial for the development of prevention steps and control. The surveillance of pathogenic enterobacteria in different ecological niche is important as the development affects the diversity and distribution of microorganisms in the animal hosts (Gordon and Fitzgibbon, 1999). When the habitat is disturbed, this will influenced the animal hosts' community structure and living behavior which indirectly affects the enteric community structure (Gordon and Cowling, 2003). Therefore, this study was conducted to compare the occurrence of *E. coli* and the detection of *E. coli* O157:H7 in wildlife from the two urban forests and an oil palm plantation sites located along the Rejang Basin, Sibu, in the state of Sarawak, Malaysia which is one of the world's richest and most diverse ecosystem. The two urban forests and oil palm plantation represented disturbed habitats.

MATERIALS AND METHODS

Study habitats and specimens collection: Bat, rodent and bird specimens were collected from a forest park, a recreational park and an oil palm plantation in Sibu, Sarawak, Malaysia during June 2008. All three sampling habitats represented disturbed habitats located about ten km from Sibu Town. Birds and fruit bats were captured using standard mist nets; while insect bats were captured using harp traps and small rodents were captured using cage traps. Identification of mammals (bats and rodents) and birds was done following Payne *et al*. (1985) and Francis (2005), respectively.

Bacteria samples collection: Anal swabs of small mammals and cloacal swabs from avian specimens were collected using wet sterilized cotton buds and immediately stored in 900 μL Phosphate Buffer Saline (PBS). Samples were kept at 4°C until processed, usually within the same day. All of the samples were cultured directly on MacConkey agar (Oxoid, England), a non-selective media for isolation of *Enterobacteriaceae* and incubated aerobically at 37°C for 24 h, on the field. Isolates tentatively identified as *E. coli* were further plated on EMB agar (Oxoid, England) after taken back to the laboratory. Plates were examined for the characteristic appearance of *E. coli* (metallic green sheen) and colonies that exhibited these characteristics were stored in nutrient agar (Oxoid, England).

Bacteria identification: All isolates were tested through gram-staining and a series of standard biochemical tests. The biochemical tests included Kligler Iron Agar, IMVIC test and motility test.

E. coli reference cultures ATCC25922 was included as a positive control. The bacteria were identified and the results were compared to the Bergey's Manual of Systematic Bacteriology (Krieg *et al.*, 1984). The bacteria were further confirmed by using commercial identification kits, API 20E system (BioMerieux, France). Representative isolates were cultured on a highly selective agar Cefaxime-tellurite sorbitol MacConkey Agar (Oxoid, England) for the detection of *E. coli* O157:H7. Colonies which were non-sorbitol fermenter were further confirmed with *E. coli* O157:H7 latex agglutination kit (Oxoid, England).

DNA extraction: Bacterial total DNA of the *E. coli* isolate was extracted by using boiling extraction method as described by Gallian (2003) with minor modification. For the extraction of bacterial genomic DNA, *E. coli* isolates was cultured in 5 mL Luria-Bertani broth (BBL, United States) for overnight at 37°C with agitation at 150 rpm. Overnight broth suspensions (1 mL) were then centrifuged at 13,000 rpm for 5 min to collect the pellets. After that, the pellets were re-suspended in 100 μL of distilled water and boiled for 20 min. The lysed cells were then cooled in ice for another 20 min and centrifuged at 13,000 rpm for 3 min. The supernatant containing DNA was used as template in the multiplex PCR assay.

Multiplex PCR assay for the detection of *E. coli* O157:H7: Multiplex PCR assay for genes encoded Shiga toxin I and II, antigen O157 and antigen H7 were performed in a single tube reaction. Four set of primers synthesized commercially by FIRST BASE laboratory Sdn. Bhd. were used in this study. SLT-I, SLT-II primer sets were targeted for the Shiga toxin producing genes (*slt-I* and *slt-II*) and Rfb and $FLIC_{h7}$ primer sets were targeted for the gene involved in biosynthesis of O157 antigen and H7 antigen, respectively. *E. coli* O157:H7 reference strains EDL933 was included as a positive control. The primers used for the amplification of the Shiga-toxin genes were described by Meng *et al.* (1997) and the detection of *rfbE* gene and $fliC_{h7}$ gene were as previously reported by Gannon *et al.* (1997).

The condition for the multiplex PCR assay was performed as described by Hu *et al.* (1999). Each PCR was performed in a total reaction volume of 25 μL containing 1 X PCR buffer, 2.5 mM Magnesium Chloride ($MgCl_2$) (Promega Corp, USA), 0.2 mM dNTP mix (Promega Corp, USA), 0.2 μM of primers SltI-F/ SltI-R, SltII-F/ SltII-R, Rfb-F/ Rfb-R, $FLIC_{h7}$-F/ $FLIC_{h7}$-R , 1 Unit Go*Taq*® Flexi DNA polymerase (Promega Corp, USA) and 10 μL of DNA template. PCR amplification was performed using iCycler™ Thermal Cycler (Bio-Rad, USA) as follows: Initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec; annealing at 59°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 7 min.

Gel electrophoresis: A 10 μL aliquot of each amplification product was analyzed using electrophoresis on 2% agarose gels cast and ran in 1X TAE buffer. A 100 bp marker (Vivantis, Malaysia) was included in the gel. Gel was stained with ethidium bromide (0.5 μg mL^{-1}) and visualized using transmitted ultraviolet illumination and photographed using gel documentation system (AlphaDigiDoc RT).

RESULTS

A total of 105 birds (48 species), 84 bats (10 species) and 44 rodents (one species) were captured during the fieldtrips. The results of the 105 bird hosts revealed that 19 cloacal swab samples were positive for *E. coli*. Nine anal swab samples from 84 bat hosts were positive for *E. coli* and 19 anal

Table 1: Occurrence of *E. coli* in selected wildlife sampled from disturbed habitats of Sibu, Sarawak

	Animal source					
	Birds (n = 105)		Bats (n = 84)		Rodents (n = 44)	
Sampling site	No. of samples tested	No. (%) positive for *E. coli*	No. of samples	No. (%) positive for *E. coli*	No. of samples	No. (%) positive for *E. coli*
Forest park	30	8 (27)	17	0	11	4 (36)
Recreational park	19	6 (32)	39	8 (21)	10	4 (40)
Oil palm plantation	56	5 (9)	28	1 (4)	23	11 (48)

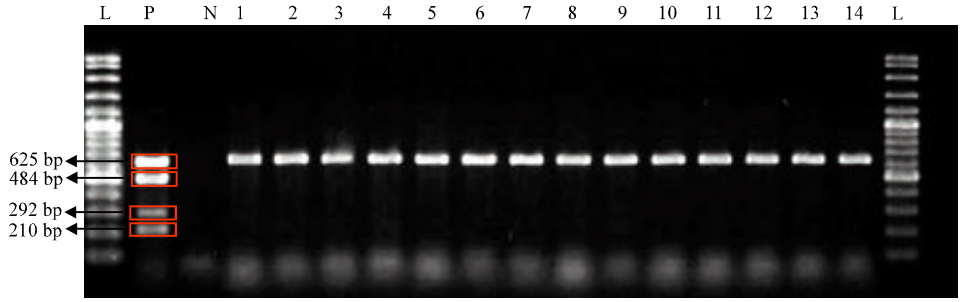

Fig. 1: Amplicon obtained by multiplex PCR for the 14 *E. coli* isolates isolated from Sibu with $flic_{h7}$ gene with expected size of 625bp fragmented by 2% agarose gel electrophoresis. Lane L: 100bp ladder, Lane P: *E. coli* O157:H7 reference strains EDL933 as Positive control, Lane N: Negative control, Lane 1: Isolate (C0813) from bat sample in recreation park, Lane 2: Isolate (TK153581) from bat sample in recreation park. Lane 3: Isolate (TK153583) from bat sample in recreation park, Lane 4: Isolate (BA025) from bird sample in recreation park, Lane 5: Isolate (A2110) from bird sample in forest park, Lane 6: Isolate (TK156080) from rodent sample in oil palm plantation, Lane 7: Isolate (PL007) from rodent sample in oil palm plantation, Lane 8 : Isolate (PL010) from rodent sample in oil palm plantation, Lane 9: Isolate (PL011) from rodent sample in oil palm plantation, Lane 10: Isolate (PL0031) from rodent sample in oil palm plantation, Lane 11: Isolate (PL033) from rodent sample in oil palm plantation, Lane 12: Isolate (PL0035) from rodent sample in oil palm plantation, Lane 13: Isolate (PL0036) from rodent sample in oil palm plantation and Lane 14: Isolate (D0113) from bird sample in oil palm plantation

swab samples out of 44 rodent hosts harbored *E. coli*. The 106 isolates of *E. coli* represented 32, 40 and 34 isolates from the forest park, recreational park and oil palm plantation, respectively.

The occurrence of *E. coli* in the wildlife samples was illustrated in Table 1 the overall occurrence of *E. coli* in the wildlife isolated from the three habitats in Sibu was 11, 18 and 43% for bats, birds and rodents, respectively. The occurrence of *E. coli* was the highest in rodents from the three sampling habitats and the least in bats.

All of the positive *E. coli* isolates were then further cultured on Cefaxime-tellurite Sorbitol MacConkey Agar (CT-SMAC) for the detection of *E. coli* O157:H7. Shiga toxin-producing strains of *E. coli* did not ferment sorbitol and appeared as colourless colonies. There were twelve isolates that produced colourless colony (non-sorbitol fermenter) on CT-SMAC. All the isolates did not show any agglutination for *E. coli* O157:H7 latex agglutination kit, indicating negative reaction. The remaining *E. coli* isolates (sorbitol fermenter) showed pink colonies on CT-SMAC.

To confirm if any of the positive (sorbitol fermenter) *E. coli* isolates belong to *E. coli* O157:H7, 47 representative *E. coli* isolates from the three habitats were subjected to multiplex PCR. From 47 isolates tested, none of the isolates generated *slt-I*, *slt-II* and *rfbE* gene with the expected band size of 210, 292 and 484 bp, respectively. However, gene encoded for antigen H7 of expected size of 625bp was detected in 14 *E. coli* isolates recovered from three bats hosts, three bird hosts and eight rodent hosts (Fig. 1). This result indicated that none of the *E. coli* isolates were *E. coli* O157:H7 or STEC.

DISCUSSION

Present results showed that among the animal hosts studied, the occurrence of *E. coli* was always higher in rodents, followed by birds and lastly bats, in all the three sites examined. The occurrence of *E. coli* in birds (18%) and rodents (43%) was in general agreement with a previous study in Australia, where 23 and 43% of *E. coli* were found in bird and rodents, respectively (Gordon and Cowling, 2003). The occurrence of *E. coli* in bats (11%) was generally in accordance with previous study by Adesiyun *et al.* (2009), where 13% of the bats in Trinidad and Tobago were reported positive for *E. coli*.

Earlier study by Gordon and FitzGibbon (1999) revealed that enteric bacteria were distributed according to their animal host or geographical locality where the animal isolate was collected. Factors thought to be responsible for this distribution were the living behavior of the animal hosts and the adaptation of the bacteria in the hosts. The occurrence of *E. coli* in these animal hosts may be associated with the feeding habits, diet and living behavior such as contact or exposure with livestock or human. In the present study, occurrence of *E. coli* was consistently higher in rodents in all the three sites of the disturbed habitats. However, when the occurrence was compared between the three disturbed habitats, the occurrence of *E. coli* in rodents was the highest in the oil palm plantation (48%) where permanent habitation for the workers were more compared to the recreational park and forest park. In this environment, rodents may feed on food sources originated from human wastes, garbage, food waste, or sewage effluent which may serve as sources of *E. coli*. In addition, rodents lived on land and came into contact with soil and fecal samples, which served as reservoir for enteric pathogen (Santamaria and Toranzos, 2003). The occurrence of the *Escherichia coli* in birds (32%) was lower than in rodent (43%). The presence of *Escherichia coli* in birds was attributed to its feeding habits where the bacteria could be picked from rubbish tips, sewage outfalls and consumption of contaminated food (Wallace *et al.*, 1997). It was possible that the recreation park provided more opportunity for the birds to feed thus acquiring a higher proportion of *E. coli*. Bats were found to harbor the least *E. coli* (11%) and this could be attributed to their living behavior as the least contact with human. Bats are known to live in roosts, in caves, under storey of forest or hollow trees. In addition, bats have varied feeding habit, for example, insect bats only feed on insects and fruit bats feed on fruits or nectar (Payne *et al.*, 1985), which has the least association with human and contaminated environment. Present results were in agreement with previous reported by Prem-Anand and Sripahi (2004) that feeding habits could be affected by the types and distribution of bacteria in bats.

All of the *E. coli* isolates were screened for O157:H7 strains. The primary screening of *E. coli* O157:H7 was carried out by direct culture on CT-SMAC and further confirmed with *E. coli* O157:H7 latex agglutination kit (Oxoid, England). The results of sorbitol fermenter on CT-SMAC did not show any positive reaction. The CT-SMAC was-previously reported to be less sensitive in detection of *E. coli* O157:H7 (Karch *et al.*, 1996). Therefore, multiplex PCR were used for

simultaneous identification of *E. coli* O157:H7 and its virulence factors in a single reaction tube. Multiplex PCR has been widely used for the detection of *E. coli* O157:H7 in previous studies through the detection of Shiga-toxin genes and gene encoded for antigen O157 and antigen H7 (Hu *et al*., 1999; Radu *et al*., 2001). Results from the multiplex PCR in our present study revealed that none of the isolates harbored genes encoding for shiga toxin 1, shiga toxin 2 and the gene involved in biosynthesis of O157 antigen. These results suggested that none of the isolates tested possessed the virulence factors. However, gene encoded for H7 antigen was detected in 14 *E. coli* isolates. Although, H7 gene was present in these 14 isolates, they however lacked in the gene encoding for O157 antigen as shown by the multiplex PCR assay. Thus, this *E. coli* could be identified as non *E. coli* O157:H7 serotype. It has been revealed that the H7 gene encodes adhesive properties, which contributed to colonization of mucosal surfaces (Erdem *et al*., 2007). The presence of the gene encoded for H7 antigen in the 14 *E. coli* isolates may indicated that they possessed flagella which could assist in binding to host cells. However, the absence of the Shiga toxin producing gene suggested that these isolates were not virulent strains.

The absence of STEC or *E. coli* O157:H7 in the wildlife examined in the present study was in agreement with previous published reports. *E. coli* O157:H7 was not detected in free-ranging and captive avian and mammalian wildlife of Trinidad and Tobago (Adesiyun, 1999). The absence of *E. coli* O157:H7 was also reported from pigeons in Colorado (Pedersen *et al*., 2006). In recent study by Adesiyun *et al*. (2009), *E. coli* O157 strain was also not detected in bats in Trinidad and Tobago.

To the best of our knowledge, the present study is the first in Malaysia to assess the occurrence of the pathogenic *E. coli* from wildlife in a disturbed environment. In the present study, the absence of *E. coli* O157:H7 from wildlife sampled in the disturbed habitats, which was thought to provide opportunity for higher contact between animals and human, indicated that there is low risk for the transmission of *E. coli* serotype O157:H7 (which may cause haemorrhagic colitis and haemolytic uremic syndrome) in the environment. Therefore, the three types of wildlife studied here may not play an important role as reservoir in the transmission of the pathogenic strain of *E. coli* from animal to human or vice versa in the state of Sarawak, Malaysia.

CONCLUSION

In conclusion, *E. coli* was distributed according to their animal host or habitats of the animal host. The occurrence of *E. coli* showed that it was always higher in rodent followed by bird and the least in bats regardless of the habitats. Results from this study indicated that bats, birds, or rodents from these habitats are not important reservoir in the epidemiologic cycle of emerging enteric bacterial zoonoses in the state of Sarawak, Malaysia.

ACKNOWLEDGMENTS

This project was funded by UNIMAS grant E14006/F07/06/ZRC/03/2007(03). The authors also would like to thank Mr. Wahab Marni, Mr. Besar Ketol, Mr. Azis Ajim, Mr. Dahlan Rambli and all the staff and students from Zoology Department in the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak who assisted in the sampling trip. Samples collected were approved by the State Planning Unit under permit (13) UPN/S/G1/I/10.1 Vol.26, 37/2008 for Forest Park Entrance Permit and NCCD.907.4 (IV)-29 for sampling in Forest Park by Sarawak Forestry Department, TAP-OP/GM/0508/11 by Ta Ann Plywood Sdn Bhd for sampling permit at oil palm plantation and also Sibu Municipal Council for approval letter to sample at recreational park.

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