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Full Length Research Paper

Detection of virulence genes of *Escherichia coli* and *Salmonella* spp. from fecal samples of Kafue lechwe (*Kobus leche kafuensis*) and pastoral cattle in the interface areas of Zambia

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Diarrhea of unknown etiology causes death in human beings, especially children with severe acute malnutrition in Africa including Zambia. Transmission of pathogenic bacteria among wild animals, livestock and people is one of the suspected causes of diseases. In this study, we looked for virulence genes specific to *Escherichia coli* and *Salmonella* species, that is, genes encoding enterotoxins (LT and ST1), EAST1, shiga toxin and invasion gene (*invA*, *invB* and ipaH) in fecal specimens of Kafue lechwe (*Kobus leche kafuensis*) antelopes and pastoral cattle in Zambia. The EAST1 gene was detected more frequently in Kafue lechwe (83.3%) than in cattle (33.3%) specimens ($p<0.01$). In contrast, the *stx-2* positive samples in Kafue lechwe (20.0%) had significantly lower percentage than that in cattle (55.1%, $p<0.01$). There was no significant difference between lechwe antelope and cattle in the distribution of *stx-1* gene. *Salmonella*-specific *invA* gene was detected in only one sample from cattle. Genes encoding enterotoxins, that is, LT and ST1, and genes *tdh*, *ipaB*, *ipaH* and *virB* were absent in all the samples. Our results demonstrate the potential of lechwe faecal materials as a possible vehicle for transmission of diarrheagenic pathogens in the interface areas of Kafue Basin, Zambia. Kafue lechwe seems to be a wild reservoir of enteroaggregative *E. coli* (EAEC). Wild animals such as Kafue lechwe can bring enteropathogenic bacteria to cattle and human beings in interface areas.

Key words: Enteroaggregative *Escherichia coli* (EAEC), enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella*, EAST1, *stx-1/stx-2*, *invA*.

INTRODUCTION

The consumption of food contaminated with pathogenic strains of diarrheagenic *Escherichia coli* and *Salmonella* spp. is capable of causing human illness by several different mechanisms. Infections with these types of bacteria pose a serious threat to public health with outbreaks arising from food and water contaminated with human or animal feces or sewage. In the Kafue Basin of

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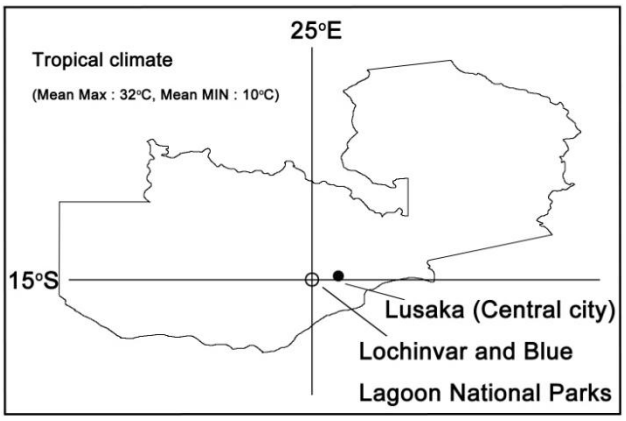

Figure 1. Map of Zambia. Lochinvar and Blue Lagoon National Parks lie about 120 km west of Lusaka on the Kafue Flats, a wide flood plain on the Kafue River. Altitude = ~1200 m.

Zambia (southeast Africa), human diarrhea is one of the most popular diseases of children and adults. To better understand bacterial pollution among domestic animals, wildlife and human, attempts have been made in the recent past to isolate enterohemorrhagic, enteropathogenic, enterotoxigenic and enteroaggregative *E. coli* (EHEC, EPEC, ETEC and EAEC, respectively) from the pastoral cattle in the interface areas of wildlife and livestock of the Kafue Basin (Mubita et al., 2008).

Pathogens such as pathogenic *E. coli* and *Salmonella* spp. have emerged and spread worldwide from reservoirs in healthy food animals (Freidl et al., 2011; Lecis et al., 2011; Obwegeser et al., 2012). *E. coli* and *Salmonella* spp. are members of the *Enterobacteriaceae* family which are widely distributed in the environment, as saprophytes or pathogens in the intestine of humans and animals (Milnes et al., 2008). *E. coli* such as EHEC and *Salmonella* spp. are today, the major causative agents of food-borne disease in human (Schmidt and Hensel, 2004).

Enteroaggregative heat-stable toxin 1 (EAST1) is the toxin of EAEC strains, and reported to cause human diarrheal outbreaks in Japan and Chile (Nataro et al*.,* 1987; Tzipori et al., 1983; Yamamoto and Echeverria, 1996) and a followed case-control study suggested EAST1 as a putative agent of EAEC-associated diarrheal disease (Vial et al., 1988). EHEC produces two types of Shiga toxin (Stx), Stx-1 and Stx-2, which are considered to be the major virulence factors in the development of hemorrhagic colitis, central nervous system disorders and hemolytic uremic syndrome (HUS) (Paton and Paton, 1998).

Kafue lechwe (*Kobus lechwe kafuensis*) is a medium-sized, semi-aquatic animal living in large groups and is indigenous to Zambia. Kafue lechwe is found in Kafue Basin in the Southern Province of Zambia (Figure 1). The Basin is supplied by the Kafue River where the Kafue lechwe antelopes are in contact with livestock. Seasonal movement of livestock from upper to lower lands (flood plains) for the purpose of grazing during dry seasons is predominantly practiced in the Kafue Basin. Since pastoral cattle occasionally come in a close contact with wildlife, it raises the risk for bacterial pathogens transmission from cattle to wildlife or *vice versa* through contamination of grazing grounds and water sources.

In Africa, especially in developing countries, research of infectious diseases in animals is still underdeveloped. We were looking for virulence genes in fecal samples obtained from domesticated and wild animals without isolation of bacteria because this simple method is easy to perform. Therefore, we presented evidence proving that mutual infection of wild and domestic animals can occur in an interface area where wild and domestic animals live in. We hope this study will make clear the present state of pathogenic bacteria carriage among wild and domesticated animals and contribute to understanding of the risk of translocation of pathogenic bacteria between livestock and wildlife.

MATERIALS AND METHODS

Sampling sites and animals

Samples were collected from Lochinvar (410 km^2) and Blue Lagoon (420 km^2) National Parks in the interface areas of wildlife and livestock of the Kafue Basin (Figure 1). The Kafue Basin is the only known natural habitat of the Kafue lechwe.

Lechwe antelope sample size was determined by what had been authorized by Zambia Wildlife Authority (ZAWA) for harvesting. A total of 60 fecal samples from lechwe antelopes were used in the experiments. A total of 60 fecal samples, which were randomly selected from 361 freshly voided faecal samples of pastoral cattle in both Lochinvar and Blue-Lagoon National parks, was also used in the experiments.

Detection of virulence genes

After collecting fecal samples, each sample was inoculated in Brain Heart Infusion (BHI) broth, and incubated at 37°C for 24 h. After cultivation in BHI broth, samples were treated at 95°C for 10 min and virulence genes were detected by multiplex polymerase chain reaction (PCR). This technique is used to detect the presence of the multi-gene region. The reaction conditions are final volume of 5 µl Phusion® Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Vantaa, Finland), 2 µl distilled water, 2 µl primer mix (MORA-Primer Diarrheal pathogens, Takara Bio Inc., Shiga, Japan) (Table 1), and 1 µl template. Ten microliters reaction mixtures were subjected to amplification for 30 cycles (98°C denaturing for 10 s and 55°C for 5 s and 72°C for 15 s) in PIKO Thermal Cycler (Thermo Fisher Scientific). After that, PCR products were directly added to 2 µl of gel loading dye to prepare samples for electrophoresis. Samples were gently applied (2 µl per well) to agarose gel (1% agarose and 1% synergel). Electrophoresis was done at 100 V until dye markers migrated to an appropriate distance, depending on the size of DNA to be visualized. A 100 to 1517 bp DNA ladder moleculer weight marker (Quick-Load® 100 bp DNA Ladder, New England Biolabs, Ipswich, MA) was used to identify the amplified products. Gels were stained with ethidium bromide and observed under ultraviolet illumination. As a positive control, we detected appropriate fragments by using clinical isolates and *E. coli* EDL931 (*stx-1*/*stx-2* positive strain) (data not shown).

Table 1. MORA-primer diarrheal pathogens.

Target gene examined	Target gene or protein	Amplicon (bp)
Pathogens		
Salmonella spp.	*inv A*	422
Mora-Prmer Diarrheal pathogens (AMR Co. Gifu, Japan)		
Enteroinvasive *E. coli* / *Shigella*	*Inv B (virB)*	127
Enterotoxigenic *E. coli*	*LT*	296
Enterohemorrhagic *E. coli* / *Shigella*	*Shiga 1 (stx-1)*	376
Enteroinvasive *E. coli* / *Shigella*	*ipa B*	137
Enterohemorrhagic *E. coli* / *Shigella*	*Shiga 2 (stx-2)*	272
Enterotoxigenic *E. coli* (ETEC)	*ST1*	171
Enteroinvasive *E. coli* / Shigella	*ipa H*	117
Enteroaggregative *E. coli*	*EAST 1*	173

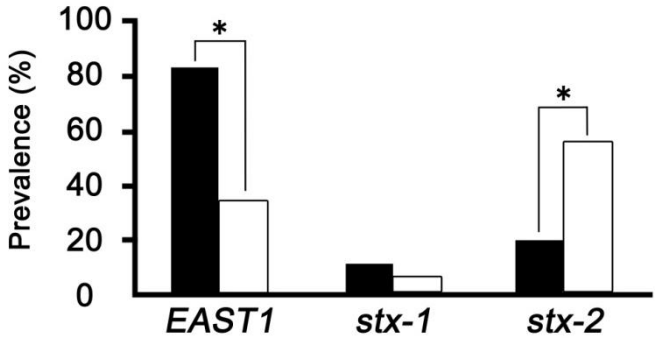

Figure 2. Comparison of pathogenic genes in cattle and Kafue Lechwe. Kafue Lechwes (black column) possessed EAST-1 gene than cattles (white column) (p<0.01). By contrast, *stx-2* gene is more frequently detected in cattle than lechwe (p<0.01). Prevalence of *stx-1* gene between cattles and lechwes is not significant.

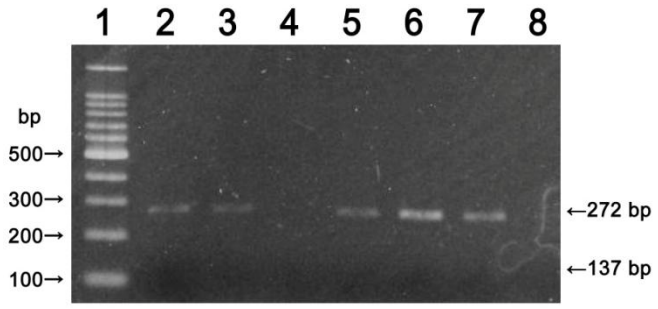

Figure 3. Typical pattern of *stx-2* positive in fecal sample of cattles in PCR. Lane 1 shows molecular marker, which indicates 100 to 1000 bp. Products of PCR for*Stx-2* gene (272 bp) are detected at lane 2, 3, 5 and 7, although 137 bp of *ipaB* gene is negative.

Statistical analysis

Chi-squared test was used to evaluate significant differences. *P*-values of less than 0.05 were considered significant.

RESULTS

Virulence genes of pathogenic *E. coli* strains were detected in the present study. Figure 2 shows the prevalence of EAST1, *stx-1* and *stx-2* in fecal samples from Kafue lechwe and cattle. The EAST1 gene was frequently detected in fecal samples from both Kafue lechwe and cattle. The positive rate in Kafue lechwes (50/60, 83.3%) was significantly higher than that in cattles (20/60, 33.3 %, p<0.01). Two major *stx* genes are classified accordingly as *stx-1* and *stx-2* in EHEC group. We detected these genes in fecal samples from Kafue lechwe and cattle. In the detection of *stx-1,* there was no significant difference between Kafue lechwes (4/60, 6.7%) and cattles (7/60, 11.7%). The *stx-2* was detected in Kafue lechwes (12/60, 20.0%) and cattles (33/60, 55.1 %), respectively. The positive percent of *stx-2* in cattles was significantly higher than that in Kafue lechwes (p<0.01). A typical electrophoresis pattern of PCR products of *stx-2* (amplicon: 272 bp) are shown in Figure 3. PCR of S*almonella invA* was detected in only one sample from cattle. In this survey, LT gene, ST-1 gene*, tdh, ipaB, ipaH* and *virB* were negative in all samples*.*

In EAST1 gene, the difference of prevalence between lechwe and cattle was significant (p<0.01, Figure 1 and Figure 4). On the other hand, there was no significant difference in multiple combinations, such as EAST1+*stx-1*, EAST1+*stx-2* and EAST1+*stx-1*+*stx-2* (Figure 3).

DISCUSSION

The prevalence of pathogenic genes can easily be detected by PCR as opposed to the traditional methods of culturing. Using the multi-PCR method, epidemiological studies can easily be conducted.

In this study, we detected pathogenic genes from fecal

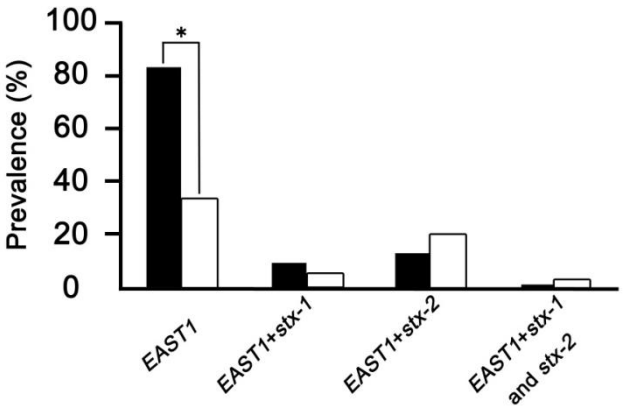

Figure 4. Prevalence of EAST1 single, or combination of *stx-1*, *2*. Lechwes (black column) which have only EAST1 gene are detected higher than that of cattles (white column). The difference between lechwes and cattles is significant ($p<0.01$). On the other hand, there are no significant differences in EAST1+*stx-1*, EAST1+*stx-2* and EAST1+*stx-1*, *stx-2*.

samples of Kafue lechwe as wild animal. Previous studies also indicated that wildlife has been an important source of infectious diseases transmissible to humans (Hang'ombe et al., 2012; Mubita et al., 2008). Therefore, it is widely acknowledged that many new human pathogens that have emerged or reemerged worldwide originated from animals or from products of animal origin. A point to be noted is that cattle and other ruminants are reported to be the most important reservoir of zoonotic EHEC (Fairbrother and Nadeau, 2006) and other foodborne pathogens (Beutin et al., 1993). Animal exhibits have been reported to be on the increase in the past decade and have also facilitated in the spread of human infections (Keen et al., 2007). In 2000 and again in 2001, enteric illness outbreaks caused by multiple pathogens occurred at a farm day camp for children in the USA. Smith et al. (2004) reported that calves were the reservoir of multiple enteric pathogens for children. Hoelzer et al. (2011) also mentioned about risk factor for transmission of non-typhoidal salmonellosis to human.

Pathogens detected in this study are associated with diarrheagenic potential and most frequently implicated in cases of epidemic and endemic diarrhea worldwide (Kaper et al., 2004). Moreover, it is reported that various animals including domestic animals can harbor pathogenic genes detected in this study (Fagan et al., 1999; Veilleux and Dubreuil, 2006).

Enteroaggregative *E. coli* (EAEC) has been isolated from endemic diarrhea cases among children in both industrialized and resource-poor countries. Some strains of EAEC produce a heat-stable enterotoxin (EAST1) different from other *E. coli* heat-stable enterotoxins. EAEC strains may be responsible for persistent diarrhea among humans, especially children, and may be responsible for growth retardation and greater susceptibility to other infections (Nataro et al., 1998). EHEC was detected in an outbreak of food poisoning in Oregon and Michigan in February through March and May through June 1982 for the first time (Riley et al., 1983). A shiga-like toxin (*stx*) produced by EHEC leads to episodes of bloody diarrhea and in addition triggers hemolytic uremic syndrome (HUS) (Banatvala et al., 2001). *Salmonella* spp. is significant in the sense that they are a threat to worldwide public health. Salmonellosis is one of the most widespread food-borne zoonoses in the world and, the problem is increasing both in industrialized and developing countries. *Salmonella* are capable of infecting both man and animals and are the major cause of diarrheal diseases all over the world. In the current study, EAST1 gene was detected more in Kafue lechwe than pastoral cattle, but *stx-2* was an opposite result. The difference in detecting *stx-1* was not observed between Kafue lechwe and pastoral cattle.

These results show that there is a considerable difference in possession of pathogenic bacteria between Kafue lechwe and cattle. This indicates that lechwes can be wild reservoir of EAEC and a vehicle for transmission of diarrheagenic *E. coli* to cattles. Since lechwes prefer to stand in water, there may be good chance to infect other animals through drinking water. And on the contrary, cattles can transmit EHEC to wild animals such as Kafue lechwe in interface area. In Africa, especially developing countries, research on infection diseases in animals is not well conducted. So, we hope this study will contribute to the knowledge on the current state of animals possessing pathogenic bacteria. Further gene analyses, including pulsed-field gel electrophoresis, are likely to bring out gene transmission between livestock and wildlife detected pathogenic genes. However, we present direct empirical evidence that a bi-model route of infection could occur at the livestock/wildlife interface areas. This result could contribute in controlling public health, such as contact between livestock and wildlife.

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