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Potentiellt zoonotiska tarmpatogener hos primater i Sri Lanka

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ABSTRACT

In order to understand the dynamics of zoonotic disease transmission in the animal-human interface, a One Health approach is imperative. This study investigated the occurrence of the zoonotic pathogens *Campylobacter* spp., *Salmonella* spp. and rotavirus in fecal samples from free-ranging endangered toque macaques (*Macaca sinica*) and near threatened tufted gray langurs (*Semnopithecus priam*) in Sri Lanka. During March through May of 2015 samples were opportunistically collected at five sites in Sri Lanka where these primates come into close contact with humans. Standard culturing methods were used to screen for the bacteria and an ELISA-based quick-test was used to detect presence of type A human rotavirus. Bacterial sensitivity to selected antibiotics was analysed using VetMIC^{TM} broth microdilution panels.

From the five sites, 98 samples were obtained. All samples tested negative for human type A rotavirus. All 40 samples from gray langurs were negative for *Campylobacter* spp. and *Salmonella* spp. Fifty-eight samples were collected from toque macaques, of which ten were positive for *C. jejuni*, four for *C. coli* and two for *Salmonella* Virchow. *In vitro* resistance to ampicillin, ciprofloxacin, nalidixic acid and tetracycline was detected in *C. jejuni* samples. All *C. coli* were *in vitro* resistant to ampicillin. The detected *Salmonella* Virchow were sensitive to all the antibiotics tested for.

This study has detected *C. jejuni, C. coli* and *Salmonella* Virchow in fecal samples from endangered toque macaques in Sri Lanka with close human contact. The bacteria showed varying sensitivity to antibiotics and several *C. jejuni* were multidrug resistant. The presence of these bacteria in free-ranging animals could have implications both for non-human primate conservation and public health in Sri Lanka.

SAMMANFATTNING

Ett One Health perspektiv är viktigt när epidemiologi hos zoonotiska sjukdomar undersöks – både för bevarande av hotade arter och för folkhälsan. Denna studie undersökte förekomsten av de zoonotiska patogenerna *Campylobacter* spp., *Salmonella* spp. och rotavirus i träckprover från den utrotningshotade ceylonmakaken (*Macaca sinica*) och den nära hotade grå hulmanen (*Semnopithecus priam*) i Sri Lanka. Prover samlades in opportunistiskt under mars-maj 2015 från fem platser i Sri Lanka där människor och dessa primater kommer i nära kontakt med varandra. Konventionella odlingsmetoder användes för att odla fram bakterier och ett ELISA-baserat snabbtest användes för att detektera humant rotavirus typ A. Bakteriernas känslighet för utvalda antibiotika testades med VetMIC^{TM} testpaneler.

Från de fem platserna samlades totalt 98 prover in. Alla prov var negativa för humant rotavirus typ A. Alla 40 prover från grå hulmaner var negativa för *Campylobacter* spp. och *Salmonella* spp. Totalt samlades 58 prover in från ceylonmakaker och av dessa var tio positiva för *C. jejuni,* fyra för *C. coli* och två för *Salmonella* Virchow. *In vitro* resistens mot ampicillin, ciprofloxacin, nalidixinsyra och tetracyklin påvisades hos *C. jejuni*. Alla *C. coli* var *in vitro* resistenta mot ampicillin. De två detekterade *Salmonella* Virchow var känsliga mot alla undersökta antibiotika.

Denna studie har detekterat *C. jejuni, C. coli* och *Salmonella* Virchow i träckprover från utrotningshotade ceylonmakaker i Sri Lanka med nära kontakt med människor. Bakterierna uppvisade varierande känslighet mot antibiotika och flera *C. jejuni* var multidrogresistenta. Detektionen av dessa bakterier hos vilda djur kan ha konsekvenser både för bevarande av icke-mänskliga primater och folkhälsan i Sri Lanka.

CONTENTS

INTRODUCTION

Zoonotic diseases – diseases with the ability to transmit between humans and other animals – are of increasing concern in today's globalized world. Most emerging infectious diseases (EID's) in recent years have originated in species other than humans (Pedersen and Davies, 2009, Jones et al., 2008, Wolfe et al., 2007) and the emergence and re-emergence of diseases is closely linked to anthropogenic impact on ecosystems and wild animal species (Bengis et al., 2004). Emerging diseases are not only a risk to human health, but also pose a great threat to the conservation of endangered species, since the viability of small populations can be gravely affected by the impact of disease outbreaks (World Organisation for Animal Health & International Union for the Concervation of Nature, 2014, Koendgen et al., 2008, Leendertz et al., 2006, Wallis and Lee, 1999). A fundamental understanding of infectious disease occurrence and epidemiology in wildlife is imperative to public health and the management and conservation of wildlife species (Gillespie et al., 2008, Leendertz et al., 2006).

Bacteria of *Campylobacter* spp. and *Salmonella* spp. can cause disease in several species including humans and other primates (Ivanovic, 2012, Ngotho et al., 2006, Scarcelli et al., 2005, Nizeyi et al., 2001, Ohl and Miller, 2001). Human rotavirus causes mild to severe or deadly diarrheal disease in children in resource-poor settings (Martella et al., 2010, Parashar et al., 2003, Dodet et al., 1997). The virus has been shown to be able to infect non-human primates under laboratory conditions (Chege et al., 2005, Zhao et al., 2005, Jiang et al., 2004) and seropositivity for rotavirus has been documented in free-ranging African non-human primates (Otsyula et al., 1996).

Increasing antibiotic resistance in bacteria is a global matter, and resistance is increasingly common in both *Salmonella* spp. and *Campylobacter* spp. Already nearly 50 years ago the *Swann Report* was released, where the authors addressed the emergence of *Salmonella* spp. with multiple resistance to antibiotics. The authors concluded that this emergence was due to the misuse of antibiotics as growth promoters and other non-therapeutic uses of antibiotics in animal production (Swann et al., 1969). They also warned for the spread of resistance between different bacterial species. Acquired antibiotic resistance has since then indeed spread widely and rapidly and has been detected in bacteria from various ecosystems (Radhouani et al., 2014).

The Democratic Socialist Republic of Sri Lanka is an island country off the southeast coast of India. It is considered one of the world's 25 biological hotspots (Myers et al., 2000), with many plant and animal species being endemic to the island. Sri Lanka has also been predicted to be a country with moderate to high risk of transmission of zoonotic disease from wildlife to humans (Jones et al., 2008). Deforestation in recent years has led to habitat destruction and alterations for the country's non-human primates and increasing human-to-non-human primate contact, which may pose a risk of disease transfer between the species. Previous studies on pathogens in non-human primates in Sri Lanka have covered the occurrence of parasites (Huffman et al., 2013, Ekanayake et al., 2006, Dewit et al., 1991) and seroprevalence of dengue virus (De Silva et al., 1999). Still, little is known about which bacterial and viral pathogens circulate in the free ranging non-human primates in Sri Lanka.

Aim

The primary aim of this study was to investigate the occurrence of enteric *Campylobacter* spp., *Salmonella* spp. and rotavirus in selected troops of free-ranging toque macaques (*Macaca sinica*) and tufted gray langurs (*Semnopithecus priam thersites*) in Sri Lanka. Secondly, the study aimed to assess the antibiotic sensitivity in the identified bacteria.

LITERATURE REVIEW

Primates of Sri Lanka

Alongside humans, five primate species are resident in the country of Sri Lanka: toque macaque (*Macaca sinica*), tufted gray langur (*Semnopithecus priam thersites*), gray and red slender lorises (*Loris lydekkerianus et*. *tardigradus*) and purple-faced langur (*Trachypithecus vetulus*). With the exception of the tufted gray langur and the gray slender loris, all are endemic to the island (IUCN, 2015). All Sri Lankan non-human primates show decreasing population trends due to habitat encroachment and alteration through human activities such as agriculture and biological resource use (IUCN, 2015). The country no longer has any intact forest landscapes; the total closed-canopy cover has decreased from 84 % in 1881 to about 30 % in 2005 and 112,000 Ha of dense forest has been lost between 2001 and 2014, despite large areas being protected as national parks (Hansen et al., 2013, Nahallage et al., 2008).

Due to the fragmentation of their habitat, primates are foraging on farms and in urban areas. The increasing contact between humans and other primates has lead to a human-primate conflict (Nahallage and Huffman, 2013, Nahallage et al., 2008). Interview studies by Nahallage and Huffman (2013) revealed that the majority of the interviewees perceived the non-human primate populations to have grown in recent years. Most also stated that the primates raid crops. Another study showed that the human-primate conflict in Sri Lanka is relatively temperate considering the high level of deforestation and human-primate contact (Nekaris et al., 2013). At temples and holy places, primates are allowed to roam and forage relatively undisturbed, due to religious beliefs prohibiting disturbance of the monkeys (Nahallage and Huffman, 2013). The temples have vast numbers of local and international visitors every day and food is often offered by worshipers to later be consumed by primate troops in the proximity. The troops are well habituated and often come into direct contact with humans.

Through the consumption of disposed of food, the non-human primates are exposed to the same foodborne infectious agents as humans. Suboptimal water treatment and poor sanitation makes water another important vehicle in disease transmission. In analyses of bottle, well and surface water in Sri Lanka the presence of many potentially pathogenic enteric bacteria was detected, as well as levels of total- and fecal coliforms exceeding the WHO permissible levels (Mannapperuma et al. (2013). The increasing contact between humans and non-human primates serves as an interface for zoonotic transmission of viruses, bacteria and parasites, which previously has been reported in both non-human primates in Sri Lanka as well as in other parts of the world (Kooriyama et al., 2013, Schaumburg et al., 2012, Nagel et al., 2012, Kowalewski et al., 2011, Pedersen and Davies, 2009, Koendgen et al., 2008, Rwego et al., 2008, Ekanayake et al., 2007, Goldberg et al., 2007, Ekanayake et al., 2006, Ekanayake et al., 2004).

The present study focused on screening for potentially zoonotic pathogens in fecal samples from tufted gray langurs and toque macaques, due to them being easily accessible in locations with an extensive human-to-non-human primate interface, namely at temples and historical sites.

Tufted gray langur (Semnopithecus priam thersites)

The tufted gray langur (Figure 1) is a diurnal, semi-terrestrial, mainly folivorous species found in southern India and in Sri Lanka's dry zone areas (IUCN, 2015). It is listed as near threatened and their numbers have decreased with at least 50 % in the past three generations.

Langurs are colobine monkeys and have a digestive system that entails both foregut and hindgut fermentation (Map of Life, 2015, Stewart et al., 1987). The foregut consists of two indiscrete fermentation chambers and the colon is segmented which allows for digesta being withheld for a prolonged time. This unique trait makes the colobine feeding behaviour different from more omnivorous relatives, such as macaques.

Figure 1. *A tufted gray langur in Kataragama. Photo by author.*

Figure 2. *A toque macaque carrying an infant in Polonnaruwa. Photo by author.*

Toque macaque (Macaca sinica)

The toque macaque (Figure 2) is a diurnal, mainly arboreal, frugivorous primate. It resides in various forest types in Sri Lanka, as well as in urban environments (IUCN, 2015).

The species is endangered and numbers have decreased by over 50 % in the past three generations (IUCN, 2015). Toque macaques are not only threatened by loss of habitat, but they are also exploited in the pet industry where they are captured to be used for entertainment (Nahallage and Huffman, 2013) as well as being used as target practice by the army (IUCN, 2015). The species is protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), but is the only endemic primate species to not be protected by Sri Lankan law.

Campylobacter spp.

Campylobacter spp. are small, rapidly mobile, Gram-negative, spiral shaped microaerophilic rods. Campylobacteriosis is the number one cause of infectious bacterial gastrointestinal disease in developed countries and the most reported zoonosis in Europe (European Food Safety Authority, 2015, Silva et al., 2011, Butzler, 2004). Human campylobacteriosis is characterized by an often self-limiting gastroenteritis and sometimes colitis with abdominal cramps, diarrhoea and fever (Butzler, 2004). In many developing countries, campylobacteriosis is hyper endemic in young children, who generally acquire immunity after clearing the infection. However, the disease may lead to malnutrition (Alfredson and Korolik, 2007, Butzler, 2004). *Campylobacter jejuni* and *C. coli* are the species within the *Campylobacter* genus of greatest public health concern, causing the majority of cases in humans, with *C. jejuni* being the major causative agent (Aarestrup and Engberg, 2001). Infections with certain *C. jejuni* strains have been strongly linked to the development of Guillain-Barré syndrome, an autoimmune polyneuropathy causing acute flaccid paralysis (Nachamkin et al., 1998).

Most cases of campylobacteriosis are sporadic, but outbreaks occur. The bacteria is often foodborne, causing disease after consuming contaminated foods, most often animal products. Up to 80% of all human cases of campylobacteriosis in the EU may be linked to chicken as a reservoir of the bacteria (European Food Safety Authority, 2010) and human-to-human infections are rare (Aarestrup and Engberg, 2001). In 2014, almost 237,000 human cases were confirmed in Europe (European Food Safety Authority, 2015). The most affected age group are children <5 years of age (European Food Safety Authority, 2012b). Human stool may contain bacteria for up to seven weeks after a *Campylobacter* infection (Butzler, 2004), meaning that grossly normal appearing stool may contain infectious bacteria. Generally, asymptomatic infection is uncommon in developed countries. In an Australian study the prevalence of *Campylobacter* spp. was 0.1 % in asymptomatic individuals who were screened for gastrointestinal pathogens (Hellard et al., 2000) A prospective study reported transmission of *Campylobacter* spp. from broiler chicken to Swedish abattoir workers who did not develop symptoms of disease (Ellstrom et al. (2014).

Campylobacter spp. have previously been detected globally in both captive and free-ranging non-human primates (Table 1) (Stirling et al., 2008, Ngotho et al., 2006, Misawa et al., 2000). Asymptomatic carriers are common and clinical disease may include bloody diarrhoea and dehydration (Baskin, 2008). Rhesus macaques are used as model animals for experimental infection with *Campylobacter* spp. in lab environments, where they develop clinical illness (Gardner and Luciw, 2008, Islam et al., 2006). Taema *et al* (2008) reported *Campylobacter* spp. in 15% of gastroenteritis cases in non-human primate individuals housed at London Whipsnade Zoo. In a Brazilian study, Scarcelli *et al* (2005) found four subtypes of the bacteria being shared between humans and other primates (*Callitrix* spp.), suggesting zoonotic potential. The sampled primates were all captive animals that had chicken as a part of their diet (Scarcelli, 2015) which may indicate that also non-human primates may be infected by foodborne *Campylobacter* spp.

In Sri Lanka, a study carried out at a children's referral hospital showed that *Campylobacter* spp. represented 15 % of the bacterial isolates from gastroenteritis cases in children <12 years

of age (Patabendige et al., 2011). Between 2004-2007, human stool samples analysed at the Medical Research Institute, Colombo, Sri Lanka had a percentage of *Campylobacter*-positivity of 1.6-6.7 % per year (Cooray and Perera, 2007). Another study reported *C. jejuni* in food where *C. jejuni* was detected in 26.7% and 55 % of raw chicken and milk samples respectively (Munasinghe et al., 2002). In broiler flocks, the incidence of *Campylobacter* spp. has been shown to be over 70 % (Kottawatta et al., 2007).

Salmonella spp.

Salmonella spp. is a large bacterial genus within the family *Enterobacteriaceae.* They are Gram-negative, facultative anaerobic, non-spore-forming, and often motile rods. Globally they are found in the environment and the gastrointestinal tract of various animal species (World Health Organization, 2013).

Human salmonellosis is mostly a self-limiting disease causing acute fever, abdominal pain, diarrhoea, nausea and vomiting, but the disease may also be asymptomatic (Hellard et al., 2000). In the EU 88,715 cases of salmonellosis were reported in 2014, which makes it the second most reported zoonosis after campylobacteriosis (European Food Safety Authority, 2015). The disease is usually more severe in children, the elderly or immunocompromised individuals and may be deadly due to severe dehydration, electrolyte imbalance or sepsis – especially after infection with certain serovars such as *S.* Typhi, which causes typhoid disease. Children < 5 years of age have a higher incidence of infection than other age groups (CDC, 2015). Outbreaks may occur, but up to 80 % of *Salmonella* cases are sporadic (World Health Organization, 2013). Following infection, the stool may contain bacteria for up to seven weeks (Hohmann, 2001). A possible complication to salmonellosis is reactive arthritis that may become chronic. Antibiotic treatment is recommended in the case of life-threatening disease or to very young or elderly individuals, where fluoroquinolones, 3rd generation cephalosporins or macrolides are the drugs of choice. Salmonellosis in humans is most often caused by serovars of *S. enterica* sp. *enterica.* Within the subspecies, the bacteria are further classified into serovars depending on their O-, H-, and Vi-antigens according to the Kauffmann-White classification system (Kauffman, 1961). Over 2,500 serovars have been identified of *S. enterica* sp. *enterica* and out of these <500 have zoonotic potential (European Food Safety Authority, 2012a). *Salmonella* Enteritidis and *S.* Typhimurium are identified in the majority of human cases and the majority of infections are food-borne, often via chicken or eggs from chicken (Dunkley et al., 2009).

Just as in humans, *S.* Enteritidis and *S.* Typhimurium cause most cases of salmonellosis in non-human primates. Cases can be both sporadic and epizootic. Individuals can carry the bacteria asymptomatically, but the disease can also have a severe course with enteritis and colitis, resulting in bloody diarrhoea, fever, sepsis and death. The bacteria may also cause abortions, osteomyelitis and arthritis (Abee et al., 2012, Baskin, 2008). Cases of salmonellosis have been reported in both free-ranging and captive non-human primates (Table 1; (Good et al., 1969).

Salmonella spp. has been reported in samples from broiler chicken and various water sources in Sri Lanka (Jayatilleke et al., 2015, Mannapperuma et al., 2013). An outbreak of salmonellosis

after consumption of monkey meat has also been reported (Lamabadusuriya et al., 1992). One of nine infected died after consuming the flesh and internal organs of a monkey who had been found dead. Later several monkeys were found dead in the same area and the authors speculated whether the consumed monkey in fact died from salmonellosis, or if the carcass had been contaminated.

Table 1. *Examples of studies where analyzing fecal samples from non-human primates has detected Campylobacter spp. and Salmonella spp.*

Location	Species	Positive samples (%)		Reference
		Campylobacter	*Salmonella*	
Tanzania	Chimpanzee (*Pan troglodytes*)	34[1]; 88[2] *	-	Kaur *et al* (2011)
		0[1]; 53[2]	-	
Madagascar	Ring-tailed lemur (*Lemur catta)*	0	0	Villers *et al* (2008)
USA	Ring-tailed lemur (*Lemur catta*)	16	0	
Brazil	Marmoset (*Callitrix* spp.)	5	-	Scarcelli *et al* (2005)
Uganda	Mountain gorilla (*Gorilla berengei berengei*)	19	13	Nizeyi *et al* (2001)
		8	4	
Trinidad	Multiple	0	2.5	Adesiyun *et al* (1998)
Peru	Multiple	20.9	-	Tresierra-Ayala and Fernandez (1997)
	Multiple	31.9	-	

[1]Culture; [2]PCR; *56 samples originating from 29 individuals

Antibiotic resistance

Molecules with antibiotic properties are produced naturally by a vast number of microorganisms and it is thought that antibiotic substances have existed for over 500 million years. Resistance to antibiotics emerges through bacterial genes that code for a variety of protective mechanisms: changes in the target structure, production of enzymes that change the antibiotic substance and makes it harmless, active efflux pumping or changes in transport channels that limits the antibiotics' access to the bacteria. These mechanisms behind antibiotic resistance tend to have a fitness enhancing effect for bacteria also in natural environments. For example: efflux pumps used by soil bacteria to expel various toxic compounds from the cell are a common cause of multi-drug resistance in pathogens. Antibiotic resistance genes are evolutionarily old; β-lactamases are dated back to 2 billion years ago. However, since the introduction of antibiotics in human medicine and animal production, the selection pressure for bacterial strains harbouring resistance genes has massively increased (Allen et al., 2010).

Resistance genes can be acquired either by mutations or through horizontal gene transfer where genes coding for resistance mechanisms are transferred from one bacterium to another in mobile genetic elements. Soil or animal gut bacteriomes represent melting pots where genetic material is continuously interchanged. Thus, naturally occurring mechanisms in bacteria may under the right selection pressure and gene transfer between bacteria be displayed as resistance to

therapeutic drugs in pathogenic bacteria (Allen et al., 2010). Already in the Swann Report the authors highlighted the danger in antibiotic resistance being transmissible between different bacterial populations (Swann et al., 1969).

In Sri Lanka, there is currently no national policy limiting the use of antibiotics. Within animal agriculture and aquaculture the use of antibiotics as growth promoters is still practiced (SLCM, 2014, Patabendige et al., 2011) and antibiotic resistance is an increasing problem in the country. According to Patabendige *et al* (2011), compliance to existing local policies on the use of antibiotics is "*variable from satisfactory to poor*". In a report on the Sri Lankan national surveillance of antimicrobial resistance by the Sri Lankan College of Microbiology (SLCM, 2014) the overall data according to the authors indicated that "*antibiotic resistance is alarmingly frequent*". The SLCM urged for a national antibiotic policy being developed with the participation of the Ministry of Livestock and Ministry of Health, in order to promote a more rational use of antibiotic drugs in Sri Lanka.

Antibiotic resistance in Campylobacter spp.

Campylobacter spp. have the ability to acquire genetic material from other bacteria and known antibiotic resistance determinants are thought to have been transferred from Gram-positive cocci (Alfredson and Korolik, 2007). Macrolides and fluoroquinolones have traditionally been used for treating complicated, systemic, or long-lasting campylobacteriosis. Intravenous treatment with aminoglycosides is also an option for systemic infections (Aarestrup and Engberg, 2001). However, resistance to these drugs, particularly fluoroquinolones, has increased dramatically in recent years, with *C. coli* showing a higher degree and prevalence of resistance than *C. jejuni* (Aarestrup and Engberg, 2001). Resistance to macrolides and fluoroquinolones have been shown to be due to chromosomal mutations changing the drug-sensitive target (Aarestrup and Engberg, 2001).

The emergence of antibiotic resistant *Campylobacter* strains has been clearly associated to global use of antibiotics in animal production (Alfredson and Korolik, 2007, Aarestrup and Engberg, 2001). The increase in resistance correlates strongly to the varying use of antibiotics in animal production in different countries. For example, in Australia the use of fluoroquinolones in animal production is banned and the resistance in *C. jejuni* and *C. coli* low. In Denmark, resistance to antibiotics in bacterial isolates have decreased since certain antibiotics have been banned from use in food production. In contrast, in countries such as Spain, Taiwan and Germany, resistance is prevalent – which correlates to the use of the drug in animal production (Alfredson and Korolik, 2007). In the Netherlands, the introduction of fluoroquinolones for veterinary use in food production led to a concurrent increase in *Campylobacter* resistance against the drug where the fluoroquinolone resistance in isolates from broilers increased from 0 to 29% in 10 years (Aarestrup and Engberg, 2001, Endtz et al., 1991).

Currently the macrolide drug erythromycin is the drug of choice in treatment of clinical campylobacteriosis, since the prevalence of resistance is still relatively low (Gibreel and Taylor, 2006). However, indications are strong, especially in developing countries, that resistance is

evolving rapidly also to this drug, particularly in *C. coli* cultured from pigs. It is likely that this is due to the use of the drug tylosine – another macrolide – both therapeutically and as a growth promoter (Aarestrup and Engberg, 2001). In Sri Lanka, multiple resistance in *Campylobacter* spp. from human samples has been reported (Cooray and Perera, 2007).

Antibiotic resistance in Salmonella spp.

Emerging antibiotic resistance in *Salmonella* spp. in livestock was reported already in the Swann report (Swann et al., 1969). The authors urged for restrictions on the use of antibiotics in animal agriculture to slow the emergence of resistance to clinically relevant antibiotics. Antibiotic resistance in *Salmonella* spp. in non-human primates was reported in the same year (Good et al., 1969). Resistance to antibiotics in non-typhoidal *Salmonella* spp. increased in human clinical samples in the USA during the 1990's and since then there have been several reports of resistance to enrofloxacin, nalidixic acid, ciprofloxacin and extended spectrum cephalosporins (Hohmann, 2001). These are drugs that are used to treat life-threatening salmonellosis, which makes the development of resistance highly concerning. The prevalence of resistant strains is increasing, particularly in Asia. The isolation rate of non-typhoidal *Salmonella* spp. is relatively low in Sri Lanka (Jayatilleke et al., 2015), but cases of *Salmonella* spp. resistant to fluoroquinolones and chloramphenicol have been reported (Karunanayake and Atukorala, 2004, Fernando, 1993) and in 1999 43 % of all human isolates were resistant to chloramphenicol (Karunanayake and Atukorala, 2004).

Rotavirus

The rotavirus is a dsRNA virus with a genome consisting of 11 segments in a double-shelled capsid. Antigen proteins classify the viruses into groups, of which group A is of highest public health concern (Martella et al., 2010, Haffejee, 1995). There are also indications that group A rotaviruses have zoonotic potential (Martella et al., 2010). Globally, rotavirus infections cause gastrointestinal disease of varying severity and leads to the death of about 440,000 children under the age of 5 every year. Eighty-two per cent of deaths due to rotavirus infections are children in resource-poor countries, making the disease an important contributor to childhood mortality in these countries (Parashar et al., 2003). However, globally, children in developed countries who come from financially affluent backgrounds have higher rotavirus diarrhoea prevalence than children in developing countries. Infections can also be asymptomatic, most often in individuals < 6 months or > 6 years of age (Haffejee, 1995).

Primates can be infected by simian rotaviruses (Wang et al., 2007), which are closely related to human rotaviruses. However, under laboratory conditions, vervet monkeys (*Chlorocebus pygerythrus*), macaques and olive baboons (*Papio anubis*) have been infected with human viruses (Gardner and Luciw, 2008, Chege et al., 2005, Zhao et al., 2005). Otsyula *et al* (1996) showed that the seropositivity for anti-rota antibodies in wild caught African non-human primates was not significantly different from primates bred in captivity. Therefore, the authors concluded that rotaviruses are likely endemic in both wild and captive non-human African primates. In Brazil, Souza *et al* (2012) screened fecal samples from several free-ranging primates for enteric viruses including rotavirus type A, but found no positive samples.

MATERIALS AND METHODS

Study animals and collection sites

The toque macaque and tufted gray langur were selected as study species since several troops reside in close proximity to human populations in Sri Lanka, leading to a potential for diseases being transmitted between humans and the other primates. Samples were collected at five locations (Fig 2; Table 2) in Sri Lanka during March-May 2015. All samples were collected from urban primate troops that were resident on or near temple sites or archaeological sites frequented by local and international visitors who worship or sightsee.

Sampling

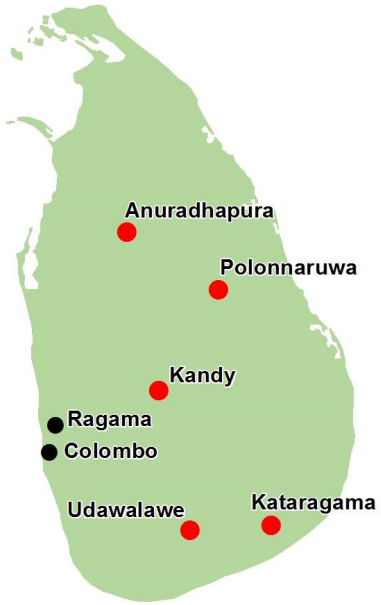

Figure 2. *The five sites selected for collection of fecal samples screening for Campylobacter* spp. *and Salmonella* spp. *in toque macaques and tufted gray langurs: Anuradhapura, Polonnaruwa, Kandy, Kataragama and Udawalawe.*
Illustration by Sara Hägglund

Primate troops were observed at a distance and fecal specimens were sampled opportunistically from the ground, when possible immediately after defection. Freshness of the specimen was evaluated on the basis of moistness and consistency. The presence of macroscopically visible blood or mucus was also recorded for each specimen.

Fecal specimens that were found fresh in the presence of a troop of the target species were assumed to originate from that troop. To minimize risk of double sampling of the same individual, no specimens within two meters of each other were sampled, unless they were directly observed defecations from different individuals. When a specimen was not fresh (dry feces), it was not sampled. If no troop had been observed in the proximity, specimens were not sampled, regardless of freshness.

Each selected specimen was assigned a unique number and sampled in duplicates directly on the ground by using the Copan FecalSwab™ (Copan Diagnostics Inc.) containing a modified, liquid Cary-Blair transport medium. Specimens were sampled from the centre, or from the top of the specimen to minimize the risk of contamination with soil bacteria. All samples were immediately stored in a cooler box with ice packs keeping refrigerator temperature (approx. +5°C) as controlled by a digital thermometer. At the end of each collection day samples were stored in a refrigerator until the time of analysis.

To detect the presence of occult blood in sampled specimens the Hemoplus ® test (Sarstedt) was used. Hemoplus ® test kits were prepared in the field by placing small amounts of faeces in the test kits, which were transported and stored in room temperature until performing the test by adding reagent. The Hemoplus ® test was performed after the sample kits had completely dried, according to the manufacturers instructions.

Microbiological analyses

All bacterial and viral analyses were initiated within two days of sampling. Initial fecal culturing was performed at the laboratory of the Department of Medical Microbiology, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka. Typing and resistance profiling was performed at The National Veterinary Institute (SVA), Uppsala, Sweden.

Salmonella spp.

The detection of *Salmonella* spp. was performed using a modified NMKL (Nordisk metodikkommitté för livsmedel) no. 71 method. Samples were enriched in two steps using Buffered peptone water and Rappaport-Vassiliadis soya peptone broth. After enrichment, samples were inoculated to the selective media Xylose-Lysine-Desoxycholate and brilliant green agars and incubated overnight. When needed, multiple inoculations were thereafter performed to obtain pure cultures.

Colonies that were suspected positive based on macroscopic and microscopic appearance following Gram staining were inoculated to blue agar and incubated overnight. Lactose negative pure cultures were stored in serum broth with 15 % glycerol in -20°C, for later transport on dry ice to SVA. Before transport, culture broth was also inoculated to blood agar and incubated for 48 h at 37°C. The cultures were swabbed and transported in Amies medium (Citotest Citoswab™ Amies) in a Styrofoam box with ice packs.

Campylobacter spp.

Samples were cultured using a modified ISO-method for the detection of *Campylobacter* spp. The sample swabs were placed in 5 ml Preston broth and incubated overnight in modified atmosphere at 41.5°C using an airtight vial and CampyGen™ atmosphere generator packs (Thermo Fisher Scientific). Thereafter, broth was inoculated to Campylobacter blood free selective medium (mCCDA) agar and incubated for 44-72 h. Macro- and microscopically typical colonies were inoculated to blood agar and incubated 48-72 h. Colonies were Gram stained and examined microscopically, and tested with oxidase and catalase tests. Colonies were then stored in -20°C in serum broth with 15 % glycerol for later transport to SVA on dry ice. Before transport culture broth was also inoculated to blood agar and incubated for 72 h in modified atmosphere at 41.5°C. Colonies were swabbed with a transport swab in Amies charcoal medium (Copan, Transystem™ Amies W/CH 114C.), in a Styrofoam box with ice packs.

Species identification and antibiotic sensitivity testing

At SVA, species conformational tests were performed using Matrix-Associated Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), biochemical methods – including Hippurate hydrolysis for *Campylobacter* spp. – and serotyping with antisera to determine *Salmonella* species and serovar according to the Kauffman-White classification system (Kauffman, 1961). The strains were tested for antibiotic resistance using VetMIC™ panel analysis systems (National Veterinary Institute, Sweden). The VetMIC™

system is based on broth microdilution, testing antibiotic susceptibility by obtaining the *in vitro* minimum inhibitory concentration (MIC). The VetMIC™ panels Camp EU, CLIN GN and GN-mo (version 4) were used for the determination of MIC for selected antibiotic substances. Defined strains of *E. coli* and *C. jejuni* were used as batch quality control organisms for each run. Epidemiological cut-off (ECOFF) values for comparison with MIC-values were obtained from the European Committee on Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing). Strains with MIC exceeding the ECOFF-value for an antibiotic were considered *in vitro* resistant to the substance.

Rotaviruses

Analyses for the detection of rotavirus group A was performed using the Combi-Strip Dipstick system (Coris Bioconcept) within 48 hours of sample collection. The Cary-Blair transport medium was thoroughly vortexed and 200 µL of sample-containing medium was diluted 1:1 with dilutant of the Combi-Strip system, after which the dipstick was allowed to react with the solution. Positive and negative controls from the manufacturer were used for batch quality control. The Coris Bioconcept dipstick system has previously been validated for analysis of samples transported with the Copan FecalSwab™ (Castriciano and Leclipteux, 2009).

RESULTS

From the five sites, 98 samples were obtained, of which 58 were from toque macaques and 40 from tufted gray langurs (Table 2). Seven out of 58 toque macaque samples and 17 out of 40 gray langur samples were from observed defecations.

All samples tested negative for human type A rotavirus. *Salmonella* spp. and *Campylobacter* spp. were only detected in samples from toque macaques; all langur samples were negative for both bacteria. Out of 58 toque macaque samples, 16 were positive on culture for either *Salmonella* spp. or *Campylobacter* spp.; two samples were positive for *Salmonella enterica enterica* serovar Virchow, ten for *C. jejuni* and four for *C. coli*. All four *C. coli* and one *C. jejuni* strain were Hippurate-negative, whereas the remaining nine were Hippurate-positive.

Table 2. *Fecal samples from tufted gray langurs and toque macaques collected in five locations in Sri Lanka tested for Campylobacter* spp. *and Salmonella* spp. *by culturing*

Location	Total samples	Gray langur	Toque macaque			
				Positive samples		
	n	*n*	*n*	*Salmonella* Virchow	*C. jejuni*	*C. coli*
Anuradhapura	30	17	13		4	
Kandy	19	N/A	19	2	4	4
Polonnaruwa	21	9	12		2	
Kataragama	14	14	N/A	-	-	-
Udawalawe	14	N/A	14			
	98	40	58	2	10	4

N/A = not applicable since the species was not resident at location.

Occult blood occurrence

None of the samples that were positive for either bacterium were positive for macroscopically visible mucus or blood. Seven out of 58 toque macaque samples were positive for occult blood. Six out of seven were positive for either bacterium. None of the samples from tufted gray langur were positive for occult blood.

Antibiotic resistance

The *Salmonella* Virchow strains were sensitive to all antibiotics tested for. Antibiotic resistance was detected in 4 out of 10 *C. jejuni* (Table 3). One of which was resistant to ampicillin only; one to ampicillin, ciprofloxacin, nalidixic acid and tetracycline; two to ciprofloxaxin, nalidixic acid and tetracycline. All strains were susceptible to chloramphenicol, erythromycin, florfenicol, gentamicin and streptomycin. All *C. coli* were highly resistant to ampicillin but susceptible to ciprofloxacin, chloramphenicol, erythromycin, florfenicol, gentamicin, nalidixic acid, streptomycin and tetracycline. MIC-distributions for colistin and sulfamethoxazole are reported for both *Campylobacter* spp. ECOFF-values for these antibiotics are not determined for *C. jejuni* and *C. coli*, so ECOFF-values for *E. coli* was used for comparison with the determined MIC-values.

Table 3. *MIC for Campylobacter* spp. *cultured from toque macaque fecal samples. ECOFF-values are marked with a red vertical line for each antibiotic. The vertical blue line represents the ECOFF-value for E.coli*

Species	MIC distribution								
C. jejuni (n=10)	Ampicillin								
	≤0.5	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
	6		2				1	1	
	Ciprofloxacin								
	≤0.12	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**
	6	1						2	1
	Nalidixic acid								
	≤0.5	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
			1	6					3
	Tetracycline								
	≤0.5	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
	7							1	2
	Sulfamethoxazole								
	≤4	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**>512**
				1	5	3	1		
	Colistin								
	≤0.25	**0.5**	**1**	**2**	**>2**				
					10				
C. coli (n=4)	Ampicillin								
	≤0.5	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
									4
	Sulfamethoxazole								
	≤4	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**>512**
				2	2				
	Colistin								
	≤0.25	**0.5**	**1**	**2**	**>2**				
		1	3						

DISCUSSION

In a globalized world battling increasing problems with emerging zoonotic diseases and a rapidly emerging bacterial resistance to various antibiotic substances, an understanding of the characteristics and epidemiology of diseases is essential. The present study has detected the zoonotic enteric bacteria *C. jejuni, C. coli* and *S.* Virchow in free-ranging toque macaques in Sri Lanka. Due to the close contact between humans and other primates, this indicates a potential for disease transmission between humans and other primates, which could have consequences for the conservation of these endangered and endemic non-human primates as well as for public health in the country. The detected bacteria do not always cause disease in non-human primates, but have been detected as causative agents to fatal illness, which is a reason for concern (Abee et al., 2012, Baskin, 2008, Ngotho et al., 2006). In addition, campylobacteriosis and salmonellosis cause problems in the human population in Sri Lanka, as well as causing gastrointestinal disease in visiting tourists.

According to Coorey and Perera (2007), the vehicle for human campylobacteriosis in Sri Lanka is still unknown, although one study has reported *C. jejuni* in milk and chicken meat (Munasinghe et al., 2002). It can be assumed that poultry plays a role as an important vehicle for both *Salmonella* spp. and *Campylobacter* spp., as in many other countries. No reports have been found on the epidemiology of *Salmonella* spp. in the human population in Sri Lanka. There is however a report of *Salmonella* spp. being present in both surface- and bottled water in Sri Lanka (Mannapperuma et al., 2013) , which may serve as a source of infection for both humans and non-human primates.

The proportion of positive toque macaque samples for *C. jejuni* and *C. coli* in this study is similar previous findings in studies on non-human primates (Table 1). As in other studies, the prevalence of *Salmonella* spp. in the populations is likely low and can be estimated to ≤5.2 % (95 % CI) in the toque macaque population (Hanley and Lippman-Hand, 1983). In the gray langur population the prevalence for either bacterium can be estimated to be ≤7.5 % (95 % CI). With the limited sample size one must be cautious to draw conclusions about the true prevalences in both tufted gray langur and toque macaque populations. The true prevalences may in fact be similar despite the lack of detected positive langur samples in this study. However, the difference between the two species is interesting considering the differences between the macaque and langur digestive systems. The langur is a foregut fermenter, with a higher level of efficient bacteriolytic lysozyme in the stomach as compared to many other mammals (Stewart et al., 1987). The lysozyme is like that of bovines, which digests many bacteria efficiently before they can pass into the true stomach and remainder of the digestive canal. This may cause the tufted gray langurs to be less susceptible to oral infection by the investigated bacteria than toque macaques. Further studies with more samples from the two species in Sri Lanka could shed light on this phenomenon and whether the presence of a more efficient lysozyme could indeed have an effect on the gastrointestinal health of the tufted gray langurs as compared to the toque macaques sharing the same environment.

Although no human rotavirus type A was detected in the collected samples the prevalence may be ≤5.2 % in the toque macaque (95 % CI) and ≤7.5 % in the tufted grey langur (95 % CI)

populations (Hanley and Lippman-Hand, 1983). Since the virus is known to affect young individuals in many species the lack of positive samples is not unexpected (Martella et al., 2010). The likelihood of opportunistically acquiring samples specifically from infants is very low with a small sample size. A sample collection targeted exclusively for samples from young individuals would have to entail more invasive methods where individuals would be directly targeted, likely demanding direct contact and disturbance to the animals. The benefit of more invasively investigating the rotavirus status in the young in free-ranging primate troops is questionable compared to the cost of disturbing the troop and infant-mother bonds.

The presence of occult blood was higher in samples that were positive for either bacterium than in those that were negative on culturing. Although bloody diarrhoea may be a part of the clinical signs in both campylobacteriosis and salmonellosis (Ivanovic, 2012, Baskin, 2008), the presence of occult blood does not detect a specific disease. In addition, none of the samples where occult blood was detected were in fact from diarrhoeic specimens. The health status of the sampled individuals was not known due to non-invasive collection and the samples mostly originating from unknown individuals. One individual in the Kandy area was observed vomiting and another individual in the same troop had an appearance of being ill (ungroomed, dirty, was sleeping while the troop was foraging). It is unknown if any of the samples originated from these individuals, since they were not observed defecating. However, the two samples that were positive for *Salmonella* Virchow and four of the *C. jejuni*-positive samples originated in this troop, which may indicate that these bacteria had an effect on the health of the animals in the troop.

Standard culturing methods were used to detect bacteria in this study. The sensitivity for bacterial culturing is variable depending on the method and sampling conditions. Also, the handling of samples until start of analysis may affect the result regardless of which method is chosen, as well as how fresh specimens are at the time of sampling. It is possible that the true number of positive samples for the respective bacterium is higher than reported here. Ideally, all specimens should have been sampled directly after observed defecations to ensure freshness and minimizing the risk of overgrowth with non-target organisms or death of the target bacteria in the sample. However, selective growth media and modified atmosphere was used to supress overgrowth of competing agents. Other methods such as PCR-based methods may detect a higher number of positives, but methods detecting genetic material do not distinguish between viable and non-viable bacteria. Additionally, all specimens were sampled by swabbing; meaning a small amount of fecal material was collected. In asymptomatic individuals, normally low numbers of bacteria are shedded in the feces and sampling larger quantities may have detected more positive samples. The Copan FecalSwab™ (Copan Diagnostics Inc.) sampling system was chosen due to the fact that the transport media had been used in a similar study (Ngotho et al., 2006) as well as the system being validated with the rotavirus dipstick test (Castriciano and Leclipteux, 2009).

When analysing bacteria from wild animals for antibiotic resistance a pattern is seen: animals living in close proximity to human settlements tend to harbour a higher number of resistant bacteria (Allen et al., 2010). In the present study three out of 10 *C. jejuni* showed *in vitro*

resistance to ciprofloxacin, which is a broad-spectrum, bacteriocidal, 2nd generation fluoroquinolone that hinders bacterial cell division by inhibiting the enzyme DNA gyrase. This is one of the drugs of choice to treat campylobacteriosis in humans (CDC, 2013), but clinical resistance is a known problem. Resistance to fluoroquinolones in *Campylobacter* spp. is most often due to mutations in *gyrA* gene that is partly codes for DNA gyrase (Aarestrup and Engberg, 2001). The same three *C. jejuni* were also resistant to nalidixic acid, another quinolone. In previous resistance testing of human *Campylobacter* spp. samples in Sri Lanka, 38 % and 69 % were resistant to ciprofloxacin and nalidixic acid, respectively (Cooray and Perera, 2007). The primate bacterial strains in the present study are hence less prevalently resistant to ciprofloxacin and nalidixic acid than in previously detected human strains. Additionally, these three *C. jejuni* strains were also *in vitro* resistant to tetracycline. Unfortunately, there is no information on tetracycline resistance in human *Campylobacter* spp. strains in Sri Lanka (Cooray and Perera, 2007). Tetracycline resistance in *C. jejuni* and *C. coli* is mediated through a ribosomal protecting gene, tet(O), that may be self-transmissible on plasmids. Probably this gene was historically transferred through horizontal gene transfer from Gram-positive bacteria (Aarestrup and Engberg, 2001). The ampicillin resistance seen in both *C. jejuni* and *C. coli* strains in the present study is likely mediated by the production of β-lactamases, which is seen in a large proportion of *C. coli* and *C. jejuni* (Aarestrup and Engberg, 2001).

MIC-distributions for colistin and sulfamethoxazole are reported in this study, despite a lack of reported ECOFF-values. *Campylobacter* spp. have to the author's knowledge not been reported as intrinsically resistant to these antibiotics. All *C. jejuni* isolates displayed MIC-values for colistin that were not quantifiable by the used VetMIC™ panel. This indicates resistance against the drug in spite of the absence of established ECOFF-values, since the panels are adapted to expected MIC-values in Gram-negative bacteria. According to Biswas *et al* (2012), the susceptibility to colistin varies in *Campylobacter* spp. and the value in reporting MIC-distributions can therefore be argued for, especially considering the globally emerging bacterial resistance against this drug. Similarly, despite ECOFF-values being available for sulfamethoxazole, resistance genes and various resistance mechanisms have been described (Gibreel and Skold (1999) and these genes are not present in all isolates. When comparing the MIC-values of the analysed strains to the ECOFF-value for *E. coli* – another Gram-negative enteric bacterium – it appears likely that all the *C. jejuni* strains are resistant to colistin and that four of the isolates were also resistant to sulfamethoxazole.

Further studies investigating the characteristics of – and relationship between – the strains of *Campylobacter* spp. and *Salmonella* spp. that affect humans, domestic animals and free-ranging primates in Sri Lanka would be an interesting continuation of this study. A One Health approach would entail screening for these bacteria in humans, domestic animals, non-human primates and the environment they share. To strengthen the data, studies would ideally include analysing a larger sample size originating from several non-human primate troops, allowing for a comparison of the prevalence of the bacteria in troops with varying human contact. This could shed light on whether urban non-human primates indeed share bacterial strains with humans to a greater extent than those with less contact. The role of non-human primates in the

epidemiology of *Campylobacter* spp. and *Salmonella* spp. in Sri Lanka remains unknown. This study has shown that both bacteria species are present in free-ranging non-human primates with close human contact. Long-term studies could bring more information about the epidemiology and dynamics of these zoonotic enteric bacteria in Sri Lanka and what role the non-human primates play.

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