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Prevalence and genetic profiles of *Escherichia coli* from mangroves and mangrove associated foods off Goa, India

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ABSTRACT

A total of 120 samples comprising of water (45), sediment (45) and mangrove originated food (30) collected from mangrove ecosystems of Goa were screened for *Escherichia coli* employing ISO-16654 method. Seventy-one (59.16%) samples were positive for *E. coli*. The *E. coli* isolates were further characterized by serotyping, virulence gene profiling and pulsed field gel electrophoresis (PFGE). Water and sediment samples were analyzed for physico-chemical parameters. The serotypes reported were O1, O10, O13, O17, O36, O41, O50, O68, O105, O116, O141, O148, O159, O162 and rough types while, 23 strains could not be typed. The *stx*1 and *stx*2 genes were detected in 33(46.47%) and 16(22.53%) isolates, respectively. The *Xba*I restriction digestion patterns of the *stx* positive strains were diverse. Interestingly, few strains isolated from diarrheal patients and from water, sediment and food from mangrove sources were genetically similar. The study showed that the mangrove ecosystem could be a potential reservoir for pathogenic *E. coli*.

1. Introduction

Mangroves provide a unique ecological niche to different microbes which play an important role in nutrient recycling as well as various environmental activities. Mangrove forests are a large ecosystem distributed in 112 countries and territories comprising a total area of about 181,000 km^2 in over a quarter of the total coastline of the world (Sahoo and Dhal, 2009). According to State Forest Report (2013), an extent of 4445 km^2 mangrove area is present in India of which majority lies in the West or East coast estuaries (TNAU, 2013). These estuarine mangroves have been characterized for salt resistant plants growing in inter tidal areas along sheltered seacoasts and estuaries in the tropical and subtropical regions (GOA-ENVIS, 2013). Mangroves act as a sink for nutrients and provide large quantities of detritus organic matter to nearby coastal waters (Prasad and Ramanathan, 2008), which serve as an important food resource for in-shore marine biota. Therefore, the mangrove ecosystem is a protected nursery habitat for fishes, shrimps and crustaceans. However, mangroves are highly sensitive ecosystems and get easily affected by various anthropogenic factors. Human domestic sewage and industrial effluents adds organic, inorganic as well as microbial pollutants into mangrove ecosystem (Pawar, 2013).

Recent studies have reported the occurrence of pathogenic microorganisms namely, *Vibrio cholerae*, *Staphylococcus aureus*, *Salmonella*, *Shigella*, *Escherichia coli* in mangrove ecosystems (Grisi and Gorlach-Lira, 2010; Rodrigues et al., 2011). *E. coli* is a dominant bacterium in sewage, which can compete with the native microflora (Ramaiah et al., 2007). The presence of fecal indicator bacteria like *E. coli* primarily suggests sewage contamination in mangroves. The prevalence of *E. coli* in water bodies due to anthropogenic activity has been previously reported (Chandran et al., 2013). *E. coli* causes several serious consequences which range from low fever, bloody diarrhea, stomach cramps, nausea, vomiting and low fever in humans, while, some complications may lead to renal failure, anemia, dehydration, spontaneous bleeding, organ failures and death (Jafari et al., 2012).

In the State of Goa (India), the total area covered by the estuaries is approximately 12,000 ha. Out of which the mangrove forests occupy 2000 ha (GOA-ENVIS, 2013). The mangroves of Goa have been explored by locals for biota such as several fishes (*Etroplus suratensis*, *Caranx malabaricus*, *Sparus berda*), crabs (*Scylla serrata*, *Fiddler crab*) and mud Clam (*Polymesoda erosa*) as commercial food (Clemente, 2008; MSI, 2013). Rapid urbanization, population density and industrialization in Goa have drastically affected the mangrove ecosystem and in-turn the indigenous biota. Therefore, biota present at the mangrove also may get affected.

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Indigenous bacterial flora (Desai et al., 2004; De Sousa and Bhosle, 2012; Khandeparker et al., 2011) and pathogenic bacteria (Rodrigues et al., 2011; Ramaiah et al., 2007; Nagvenkar and Ramaiah, 2009) have been isolated from mangrove ecosystems of Goa. The organic/inorganic content has also been determined (Attri et al., 2011; Krishnan and Loka Bharathi, 2009; Paula et al., 2009; Krishnan et al., 2007), however, the potential effects on the local population are not known. The objective of the present study was to isolate and characterize *E. coli* strains from the mangroves and mangrove associated foods.

2. Materials and methods

2.1. Sampling area

Sampling was carried out across Mandovi (15°21′–15°31′N and 73°45′–73°49′E) and Zuari estuaries (15°25′N and 15°25′E) of Goa, India. The samples (water, sediment and mangrove originated biota) were collected from 15 different locations. Sampling sites were located across the areas from where mangrove associated biota are frequently harvested for human consumption (Fig. 1).

2.2. Sampling

A total of 120 samples comprising of sediments ($n = 45$), water ($n = 45$) and mangrove originated biota (fishes, crabs and mud clam) ($n = 30$) were collected in the month of October (post-monsoon). Approximately 10 gm of sediment samples were collected from 10 cm depth by Van Veen grab in sterile polythene bags. For water samples, 100 ml of water was collected in sterile screw cap tubes. All samples were transported to the laboratory in chilled conditions and processed for total viable counts and screened for *E. coli*.

Also, five *E. coli* strains isolated from diarrheal patients having a history of consumption of mangrove originated food were acquired from Goa Medical College, Goa, India.

2.3. Physico-chemical characteristics of water

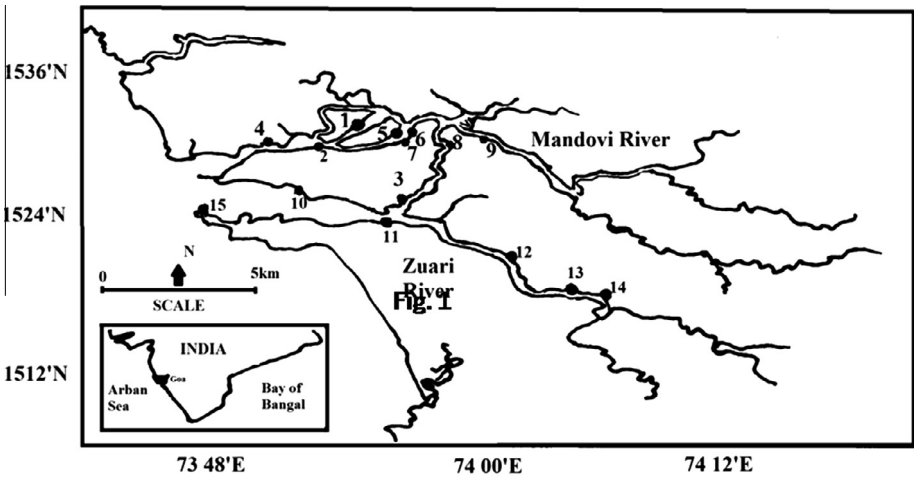
Physio-chemical parameters: temperature, pH and salinity were determined in the field during sampling using a multi-meter (Cole-Parmer). Total dissolved solids (TDS) and dissolved oxygen (DO) were determined by standard methods as described by Trivedi and Goel (1986). Bacterial load was determined by total plate count, while, the load of enteric bacteria was determined on McConkey's agar.

2.4. Isolation of E. coli

E. coli was isolated as described in ISO-16654 method. Approximately 5 ml of water or 5 gm of sediment sample was inoculated in 45 ml of McConkey's broth and incubated at 18 h at 37 °C for enrichment. A loopful of enriched broth was streaked on Eosin methylene blue agar plates and incubated at 37 °C for 24 h. Dark purple colonies with a metallic sheen were isolated as *E. coli*. These presumptive isolates were stored in nutrient broth. These isolates were further confirmed to be *E. coli* stains by Gram staining, sugar fermentation and IMViC test.

2.5. Detection of the stx genes

All the isolates obtained from mangrove associated areas and 5 *E. coli* isolates from diarrheal patients were screened for the presence of Shiga-like toxin 1 (*stx*1) and *stx*2. The PCR was performed as described by Vidal et al. (2004). DNA was extracted by snap chill method. In brief, overnight grown (5 ml) bacterial culture was centrifuged at 13,000*g* for 10 min. The pellet was then suspended in 100 µl of sterile distilled water and kept in boiling water for 10 min. The suspension was transferred immediately to −20 °C for 10 min. The treated cell suspension was centrifuged at 13,000*g* for 10 min. And the supernatant was used as a DNA template which was tested for the presence of the *stx*1 and *stx*2 genes by PCR. Primers used for the *stx*1 and *stx*2 genes were as described by Vidal et al. (2004). A reaction mixture was prepared for a total volume of 25 µl containing 10x PCR buffer, 1.5 mM of $MgCl_2$, 2 mM dNTP mixture, 0.5 µM of each primer and 50 ng of DNA template. The reaction conditions were set as initial denaturation at 94 °C for 2 min followed by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and final extension at 72 °C for 2 min. *E. coli* ATCC 8739 was used as a positive control. The amplified DNA products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide and visualized under Alpha-Imager Gel Doc system. The 348 bp and 584 bp amplicons were obtained for the *stx*1 and *stx*2 genes, respectively.



North Goa : 1. Ribandar 2. Merces 3. Madkai 4. Bettim 5. Divar 6.Old Goa
7. Bainginium 8. Kumbharjua 9. Marcel 10. Shridao
South Goa: 11.Cortalim 12. Durbhat Ferry 13. Rachol Ferry 14. Curtorim 15. Vasco

Fig. 1. Map showing sampling locations in the study area of Mandovi–Zuari mangrove ecosystem.

2.6. Serotyping

All 76 *E. coli* cultures (including 5 isolates of diarrheal cases) were referred to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India, for serotyping. The isolates were serotyped for 'O' antigen.

2.7. Pulsed-field gel electrophoresis

Genetic diversity of *E. coli* isolates carrying the *stx* genes ($n = 45$), along with 5 isolates from clinical cases were subjected to PFGE analysis according to a standard protocol developed by PulseNet for *E. coli* O157:H7 (Gautam, 1997). Briefly, agarose-embedded DNA was digested with 50 U of *Xba*I for 3 h in a water bath at 37 °C. DNA fragments were separated by electrophoresis in 0.5× Tris–borate–EDTA buffer at 14 °C for 18 h on a CHEF-II Mapper system (Bio-Rad Laboratories, USA), with a pulse time of 2.2–54.2 s at constant voltage of 6 V/cm. A PFGE Marker I (New England Biolabs) was included in each agarose run. The PFGE patterns generated were analyzed using the Phoretix 1D pro software (Total Lab, UK). The pattern clustering was performed by the unweighted-pair group algorithm and the dice correlation coefficient.

3. Results

3.1. Physico-chemical analysis

The physico-chemical parameters were almost consistent at different locations selected for the study. The water temperature ranged from 27 to 28.5 °C. The pH and dissolved oxygen ranged from 6 to 7.2 mg/l and 5.6 to 7.2 mg/l, respectively. The total dissolved solids observed were 24.6–35.1 gm/l while, salinity was found to be 17–26.4 psu (Table 1).

3.2. Total plate count

Total plate count (TPC) was performed to determine the microbial load in the mangrove region. The average TPC on nutrient agar were $66 \pm 21 \times 10^7$ cfu/gm from the sediment, while, $90 \pm 9 \times 10^5$ cfu/ml from water samples. Average load of enteric microbes on McConkey's agar was found to be $87 \pm 8 \times 10^5$ cfu/gm from sediment and $21 \pm 3 \times 10^4$ cfu/ml from water. On EMB agar, the average counts for typical *E. coli* colonies were $31 \pm 5 \times 10^2$ cfu/gm from sediment while, $11 \pm 3 \times 10^2$ cfu/ml from water samples.

3.3. Isolation and identification of E. coli

Screening of total 120 mangrove associated samples to determine occurrence of *E. coli* by ISO-16654 revealed 71(59.16%) samples to be positive. The *E. coli* isolates originated from water (57.77%), sediment (53.33%) and food (70%) samples.

Table 1
Physiochemical parameters during pre-monsoon and post-monsoon seasons.

Sample site	Temp. (°C)	pH	DO (mg/l)	TDS (gm/l)	Salinity (psu)
1	27	6.5	6.3	27.0	17.0
2	28	6.4	6.1	31.2	24.4
3	27	7.0	6.0	35.1	26.4
4	26.5	6.0	7.2	29.2	20.2
5	27	7.2	6.4	30.4	25.2
6	28.5	7.1	5.6	34.0	26.1
7	27	6.0	7.0	32.6	18.0
8	29	6.0	7.1	31.1	20.0
9	27	7.2	7.2	30.4	26.0
10	27	6.1	6.5	24.6	19.4

Temp. = temperature, DO = dissolved oxygen, TDS = total dissolved solids.

3.4. Detection of the stx genes

All 71 *E. coli* isolates from mangrove areas were screened for the presence of the virulence genes (*stx*1 and 2). Of the 71 isolates, 45(63.38%) were found to contain at least one virulence gene. Overall, the *stx*1 gene was found in 33(46.47%) isolates, while 16(22.53%) isolates were positive for the *stx*2 gene. The *stx*1 gene alone was found in 28 isolates while, the *stx*2 gene alone was present in 11 isolates. Five isolates contained both the *stx*1 and *stx*2 genes. Four out of five clinical *E. coli* isolates contained the *stx*1 gene while a single isolate contained the *stx*2 gene.

3.5. Serotyping

Out of 71 *E. coli* isolates from mangroves, 45 isolates could be typed while 23 could not be typed (Fig. 2). The serotypes observed were O1, O10, O105, O116, O13, O141, O148, O159, O162, O17, O36, O41, O50, O68, and rough. Among the clinical isolates, one of each isolate was serotype O50 and rough while, 3 isolates could not be typed.

3.6. Pulse field gel electrophoresis

Considering the significant difference of a single band, a dendrogram was generated showing a total of 30 PFGE patterns (pulsotypes) from a total of 51 *E. coli* isolates. Clusters formed showed grouping of isolates recovered from different sources and places (Fig. 2). Four clusters contained more than two isolates (major cluster) while, seven clusters contained at least 2 isolates (minor cluster). Twenty isolates were placed singularly all over the dendrogram. The *E. coli* isolates from mangroves clustered together with isolates from clinical cases that were isolated during the same period. Except one strain, all other strains from diarrheal cases clustered together with the isolates from mangrove areas.

4. Discussion

The mangrove ecosystem is one of the world's most productive ecosystems found as a transition zone between the sea and river hosting a wide range of flora and fauna. The mangrove area is rich in organic matter influencing a positive growth of many microbes. These sites act as a breeding and feeding ground, nursery and refuge for numerous marine dwellers, and in addition, these can serve as buffers against coastal erosion for retaining some pollutants, and are important for fisheries (Cristina et al., 2010; Cotano and Villate, 2006). However, many mangrove areas have lost their virginity with the interference of anthropogenic activities. Many industrial as well as human domestic activities are increasingly polluting the mangrove ecosystem leading to a change in the innate composition. Recent evidences suggest the occurrence of microbial pathogens such as *V. cholerae, S. aureus, Salmonella* spp., *Pseudomonas aeruginosa* (Cristina et al., 2010; Islam and Tanaka, 2004). Therefore, a question emerges whether mangroves act as a potential reservoir for pathogens?

In the state of Goa (India), the mangroves have been located across the Mandovi and Zuari rivers. The area is densely populated with human habitats and different types of industries. The waste generated gets directly disposed off in these mangrove zones. In addition, mangroves are also influenced by touristic activities. Therefore, occurrence of diverse microbial loads in such a highly disturbed ecosystem cannot be denied. On the other hand, the Mandovi and Zuari mangrove areas are rich in shrimps, fishes

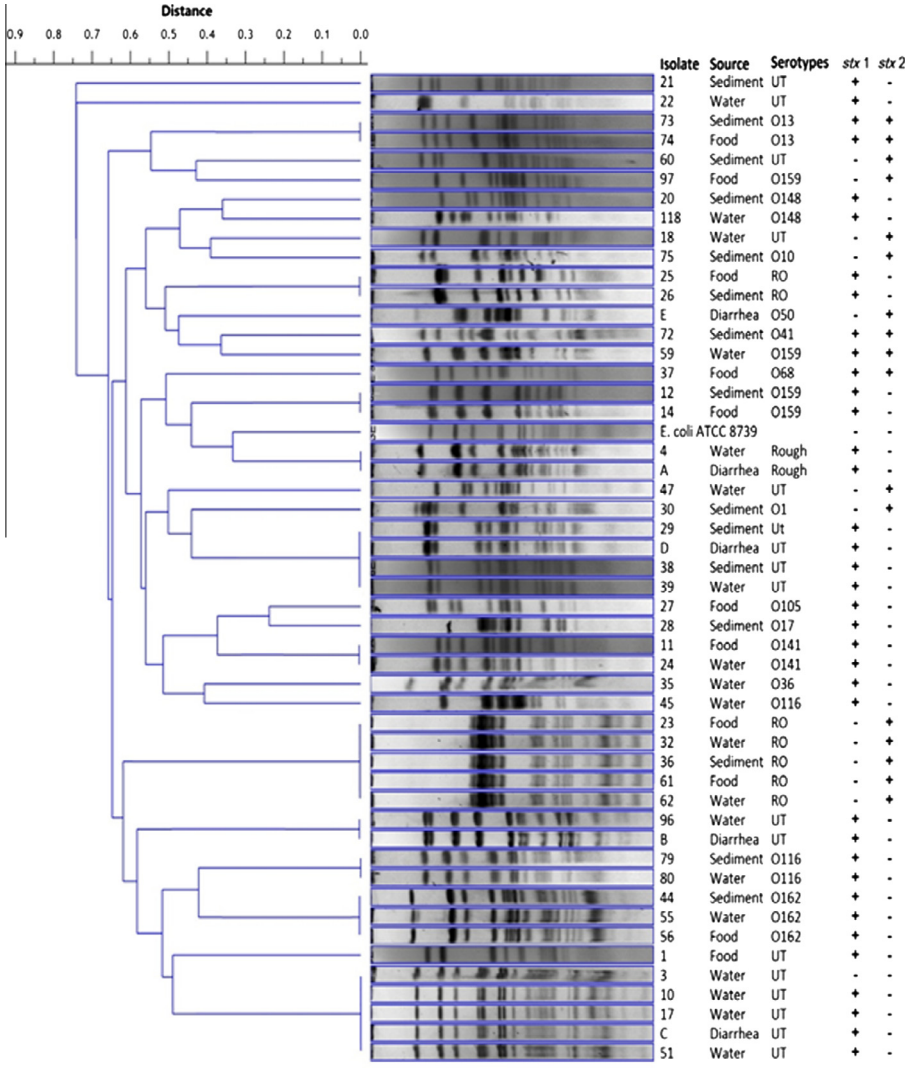


Fig. 2. Pulsed field gel electrophoresis (*Xba*I) pattern of *E. coli* isolates obtained from mangrove regions in Goa. High similarity was observed among the isolates obtained from different samples within same area. PFGE pattern clustered similar serotypes. Clonal similarity was observed among the isolates from different sample but within the same area raises the possibility of cross-contamination occurring from mangrove to associated biota (food).

and crustaceans. Such biota has been harvested by the locals and sold as food. Therefore, assessing the area for microbial pathogens is of particular interest. Fecal contamination is considered to be the main contributor of enteric pathogens to natural water resources. Infections originating from such sources, especially diarrhea are highly endemic in India, and are also a major public health concern among children in other developing countries (Abhirosh et al., 2011). *E. coli* is the main pathogen that is accountable for such illnesses. In Goa, approximately 12,000 diarrheal cases are reported per year, of which 10–15% can be traced to consumption of shrimps originating from mangroves (personal communication Dr. U. Betodkar, Epidemiologist, Goa State). The biota originating from the mangroves is consumed by locals as well as tourists with or without cooking and therefore, is hazardous to public health. However, due to lack of epidemiological focus, no investigations have been done to confirm the source. The present study revealed the presence of *E. coli* in the mangrove ecosystem of Mandovi and Zuari and contamination of foods harvested from the areas. Previous studies have reported the contamination of the mangrove originated biota with human pathogens (Degnan et al., 1994; Ellender et al., 1995; Ristori et al., 2007; Vieira et al., 2004) in other countries. Presence of *E. coli* in water-bodies is not new and has been reported from freshwater beaches (Walk et al., 2007), the tropical estuary (Chandran et al., 2008) and salt water lakes (Chandran et al., 2013). However, it was interesting to note the presence of *E. coli* in mangroves at estuaries in suboptimal conditions (bacterial pathogens). Studies performed in the last decades revealed that *E. coli* could become "naturalized" to soil, sand, sediments, and algae in tropical, subtropical, and temperate environments (Ishii and Sadowsky, 2008). In addition, studies on the survival of the different types of *E. coli* in soil, manure and water have been linked to the genetic content of the pathogen exhibiting dual growth nature as a pathogen as well as a commensal (Van-Elsas et al., 2011). Such naturalization or adaptation of *E. coli* with respect to the surrounding environment is of concern.

The association of only certain strains of *E. coli* causing disease is not fully elucidated. The inventory and precise function of virulence factors in *E. coli* are still not known, but epidemiological studies have suggested that the association between the virulence factors could increase the ability of some serotypes to cause disease in humans (Loukiadis et al., 2006; Brooks et al., 2005). Certain *E. coli* strains produce Shiga-like toxins that are encoded by the *stx* genes and damage intestinal, vascular, and renal cells (Loukiadis et al., 2006; Law, 2000). The *E. coli* isolates obtained from mangroves showed the presence of the *stx*1 and *stx*2 genes indicating their potential virulence ability. *E. coli* present in environment

can easily acquire the *stx* genes from lysogenic bacteriophages by horizontal gene transfer (Muniesa and Jofre, 2000, 2004). Mangroves have been known for their favorable environment where chances of horizontal gene transfers are high (Martin et al., 2004). Presence on the *stx* genes in *E. coli* from mangrove contaminated with sewage is therefore obvious. Also involvement of STEC among the clinical cases within the same region indicated the role of environmental *E. coli* supporting the hypothesis that virulent strains of *E. coli* may occur in the mangroves.

Serotyping data revealed high variation among the isolates showing capability of different *E. coli* strains to be prevalent and survive in these mangrove ecosystems. Several serotypes observed in this study have been reported to cause disease outbreaks in various countries. *E. coli* serotype O148 has been previously shown to cause a diarrheal outbreak in France (Espié et al., 2006). *E. coli* serotypes O1 and O17 were predominant among the urinary tract infections (Manges et al., 2008). Serotype O116 was involved in gastroenteritis outbreak in UK (Smith et al., 1997). The major serotypes found in this study, O159 has been shown to be involved in neonatal diarrhea in Spain (Blanco et al., 1992). This study thus revealed the occurrence of *E. coli* strains in the mangroves of Goa that are known for human disease outbreaks.

Further to confirm, if the *E. coli* strains observed in mangroves and associated foods are clonal, isolates were subtyped using PFGE. No predominant pulsotypes were observed. An enormous diversity was observed among the *E. coli* strains obtained from mangroves, showing ability of a wide range of strains surviving in this ecosystem. *E. coli* is more prone to undergo genetic changes (Ishii and Sadowsky, 2008; Walk et al., 2007), particularly under stressed conditions *E. coli* may undergo several mutations, recombination of genes, horizontal uptake and disposal of genes (Ishii and Sadowsky, 2008). Incidentally, *E. coli* from the mangrove water and sediment samples were also observed in the food harvested at that particular location. Presence of a clonal *E. coli* in mangrove and clinical cases suggested the contamination of mangrove areas by domestic discharge.

Though recent literature questions the validity of *E. coli* as a fecal indicator (Ishii and Sadowsky, 2008; Brennan et al., 2010), the clonal genetic pattern of isolates from mangroves and clinical cases observed in this study suggests a high probability of fecal contamination. Similar studies were carried out by Keller et al. (2013) at mangroves in Brazil, where *E. coli* strains were found in water as well as mangrove associated food for over a 14 month period indicating a history of chronic contamination. In another study on the mangroves of Brazil, Grisi and Gorlach-Lira (2010) reported the prevalence of pathogens such as *S. aureus* and *Salmonella* spp. There is a probability that *E. coli* forms a vicious chain by entering into the mangroves through domestic discharges, subsequently survives in the mangrove areas, contaminate the food and reenters humans completing the cycle.

The study showed the occurrence of potentially virulent *E. coli* strains in the mangrove region of Goa supporting the fact that mangroves may act as a reservoirs for pathogenic strains. Unknowingly, the local population consumes the food harvested from such areas. There is need for general awareness about this microbial contamination. Monitoring systems need to be established for the food being harvested and sold locally. Effective measures to control the direct disposal of the domestic waste in the mangroves and associated estuaries need to be implemented and ascertained in order to protect these so called pristine environments.

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