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Prevalence of Siderophore Genes in Faecal *Escherichia coli* Isolates From Different Captive Animal Species

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Abstract

Faecal matter collected from 66 healthy wild captive animal species of two different sites of Rajasthan (Bikaner zoo and Machia biological park, Jodhpur) was examined for the presence of *E. coli* which carries a broad range of virulence associated genes responsible for intestinal and extraintestinal infections. The objectives of this study were to isolate and characterize *E. coli* from different wild captive animal species and to determine their siderophore gene profiles by PCR. A total of 66 faecal samples were analysed for the presence of *E. coli* and 59 isolates were positively identified as *E. coli*. 16S rRNA sequence data were used to confirm the identity of the isolates. PCR were performed to amplify *fyuA* and *iroN* genes fragment which are involved in iron acquisition and thus contribute to bacterial virulence. we reported high prevalence of *fyu*A (89.83%) and comparatively low prevalence of *iroN* (40.67%) in both Bikaner and Jodhpur regions.

Keywords – E. coli, fyuA*, gene, *iroN

Introduction

Wildlife is the term used for animals which are either free roaming like in forests or maintained in captivity like in zoos (mammals, birds, fish, reptiles, and amphibians), whereas the term zoonosis is applied for infectious disease transmittable between animals and humans. The total number of zoonoses is still unknown, but according to Taylor *et al.*, who in 2001 catalogued 1,415 known human pathogens, 62% were of zoonotic origin. The list of human pathogens which are found to be of animal origin is continuously increasing with time. Wild animals are likely to be involved in the epidemiology of most zoonoses and serve as major pool for spilling of zoonotic agents to other animals and humans (Kruse *et al.*,2004).

One of the major problem with animals is that they shed a variety of pathogens without showing any clinical symptoms. Therefore, animals who are even well maintained, or appear to be healthy and physiologically normal, may act as a reservoir of pathogenic organisms, and thus can act as a carrier of infectious disease. Although all kind of animals are at risk for shedding infectious agents, but the major concerns lies with poultry and wild animals maintained in captivity because humans are in direct contact with such animals and so there is a high risk of transmission of these infectious agents between them.

E. coli is one such notorious bacteria which can spread through faecal matter contaminated with them. Research and Data available in the past has shown that it is responsible for causing numerous outbreaks of diseases at petting zoos, or similar public animal contact places (Bender *et al.*,2004)

Most of the *E. coli* found are harmless, safe or non-irritating commensals of the gastrointestinal tract of living beings. However, currently it has been noticed that evolution has favoured the occurrence of such sub-sets of *E. coli* which has acquired virulence properties rendering them to occupy position in harmful pathogenic categories of bacteria. The virulence factors may be plasmid encoded or they may be coded by elements which were once mobile but now integrated and evolved and became the stable part of the main genome. Many *E. coli* are responsible for causing several intestinal and extraintestinal diseases by secreting virulence factors which affects and interfere with cellular processes (Kaper *et al.*,2004). The virulence factors include adhesins, toxins or iron acquisition systems encoded by siderophore genes. All the genes involved in production of adhesin, toxins and iron acquisition systems work in a co-ordinate manner to make a bacterium potentially virulent. During invasion of host, the *E. coli* needs to obtain essential nutrients and multiply successfully to cause an effective infection. One essential nutrient for bacterial growth is iron which has a limited availability because of its strong affinity by protein sequestering iron like ferritin and transferrin, low solubility of iron in form of Fe^{+3} marks another limitation. Therefore, iron ions stored in such a way have limited access to bacteria (Valdebenito *et al.*, 2006). Iron works as an electron carrier, and play a very important role in regulating basic cellular processes of cellular respiration, DNA duplication, and oxygen transport. It also works as a prosthetic group in a protein structure to maintain its function and is thus very necessary for life and proper functioning of all living beings, including vertebrates, invertebrates, and prokaryotic organisms such as bacteria (Ganz, 2009).

Bacteria needs access to iron, to regulate its metabolic activities and virulence properties (Skaar, 2010) but as only a small amount of iron in nature is available to bacteria, it must develop alternative strategies to fulfill its requirements (Casset *et al.*,2013). Virulent *E. coli* strains have developed various strategies for acquiring iron, the most common of which involves the secretion of siderophores (Holden *et al.*, 2015) which are low-molecular-weight, specific ferric ion-binding chelators produced by many members of the family Enterobacteriaceae.

In our present study, profiling of two such siderophore genes i.e. *fyuA* (encodes ferric yersiniabactin uptake receptor) and *iroN* (iron uptake) of *E. coli* was achieved in two selected captive areas of underdeveloped state of Rajasthan. *fyuA* is yersiniabactin siderophore, originally detected in Yersinia pestis. It colonizes the urinary tract and contributes to the pathogenesis of uropathogenic *E. coli* (UPEC). The *iroN* gene is considered as an ExPEC salmochelin marker and an important virulence gene in virotyping ExPEC strains (Wilson *et al.*, 2016). In this study, we screened multiple *E. coli* strains, isolated from the faecal matter of two selected captive areas of Rajasthan for the presence of siderophore genes. Our results indicate that most *E. coli* strains contain an iron transport system and that the presence of iron transport system is essential for a bacterium to become potentially virulent. This suggest that iron utilization may be important for the pathogenesis of *E. coli* in some epidemiological settings.

Methodology

Sample collection - Freshly voided faecal samples from wild captive animals were collected from Bikaner zoo during July 2018 and Machia Biological Park, Jodhpur in the Month of August 2018. A total of 66 faecal samples (appx.100 gm) of chinkara, black buck, blue bull, lion, jackal, panther and jungle cat were collected aseptically from both the selected areas of Rajasthan in sterilized collection vials. The samples were immediately transported to laboratory in insulating ice box and were inoculated in nutrient broth filled in sterilized culture tubes for overnight incubation at 37°C in shaker incubator. The details of the faecal samples collected are given in the following tables.

Table 1: No. of Faecal samples collected from captive animals from Bikaner zoo

S. no.	Species	No. of faecal Samples
1.	Chinkara (*Gazella gazelle*)	18
2.	Black buck (*Antelope cervicapra*)	18
3.	Blue bull (*Boselaphus tragocamelus*)	5
	Total	41

Table 2: No. of Faecal samples collected from captive animals from Machia biological park, Jodhpur

S. no.	Species	No. of faecal samples
1.	Chinkara (*Gazella gazelle*)	8
2.	Black buck (*Antelope cervicapra*)	10
3.	Jackal (*Canis aureus*)	1
4.	Lion (female) (*Panthera leo*)	1
5.	Lion (Cub) (*Panthera leo*)	3
6.	Panther (*Panthera pardous*)	1
7.	Jungle Cat (*Felis chaus*)	1
	Total	25

Isolation and identification of *E. coli* - The isolation and identification of *E. coli* from faecal samples was carried out by conventional methods as described by Cowan and Steel (1975) and Quinn *et al.* (1994). Each sample was inoculated on to the EMB agar by streaking and incubated aerobically at 37°C for 18 to 24 hours. Appearance of green metallic sheen on EMB agar revealed the presence of *E. coli.* Colonies with typical *E. coli* morphology were-further sub-cultured until pure isolated colonies were obtained. The obtained pure *E. coli* colonies were gram stained and biochemical identification was done using Vitek 2 automated system (BioMerieux India private LTD). The colonies were also genotypically confirmed by PCR using 16S rRNA as described by Khaled *et al.* (2010). The confirmed isolated colonies were streaked on agar slants and maintained for further use.

Detection of iron acquisition genes (Siderophores) through PCR - Polymerase chain reaction was carried out with a Veriti 96 well thermocycler to detect presence of *fyuA and iroN* genes amplified through specific primers (Table 3) and using specific conditions (Table 4). For PCR, 25 µl of PCR mixture was used, which included 7.5 µl of Nuclease free H_2O, .3uM of forward primer, .3µM of reverse primer,12.5 µl of master mix and 50ng/ul of Template DNA. The amplified DNA was visualized by Gel electrophoresis in 1% agarose gel in 1X TBE buffer.

Table 3: Primers and amplicon sizes for detection of iron acquisition genes of *E. coli*

Gene	Primer sequence	Amplicon size
fyuA	5'TGATTAACCCCGCGACGGGAA3' 5'CGCAGTAGGCACGATGTTGTA3	880 bp
iroN	5'AAGTCAAAGCAGGGGTTGCCCG3' 5'GACGCCGACATTAAGACGCAG3	665 bp

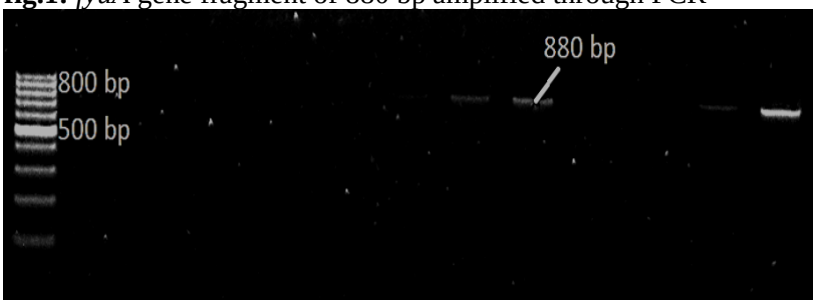
Table 4: PCR conditions for detection of iron acquisition genes (siderophores)

Gene	Initial Denaturation	Denaturation	Annealing temperature	Extension	Final Extension
fyuA	95°C (5 minute)	95°C (1 minute)	50°C (1 minute)	72°C (1 minute)	72°C (7 minute)
iroN	95°C (3 minute)	95°C (1 minute)	60°C (1 minute)	72°C (2 minute)	72°C (7 minute)

Results and discussion

A total of 59 *E. coli* isolates (89.39%) were recovered from 66 faecal samples. Thirty-eight isolates were recovered from Bikaner zoo (18 from chinkara, 16 from black buck and 4 from blue bull) and 21 isolates from Machia biological park, Jodhpur (7 from chinkara, 9 from black buck, 1 from jackal ,1 from lion female and 3 from lion cub) were recovered after phenotypic and genotypic confirmation for analysis in this study. High prevalence of *E. coli* (82%) in faecal matter was also observed by Akond *et al.* (2009). *fyu*A gene amplification through PCR using gene specific primers yielded a product of 880 bp (fig.1). The amplified products were visualized after performing agarose gel electrophoresis using 1.5% agarose gel and DNA ladder of 100 bp (fig.1).

fig.1: *fyuA* gene fragment of 880 bp amplified through PCR



Analysis of PCR results revealed that 34 of 38 (89.47%), and 19 of 21 (90.47%) of the isolates carried the *fyuA* gene in Bikaner Zoo and Machia biological park, Jodhpur respectively. In Bikaner zoo, *E. coli* from chinkara, black buck and blue bull, 94.44%, 87.50%, and 75%, respectively were shown to possess *fyuA* gene. While Jodhpur presented more positive results in which all the isolates of chinkara, black buck, jackal and lion (female) contain the gene. Only two isolates of lion (cub) were found negative for the gene (Table 5)

Table 5: No. of positive isolates and prevalence of *fyuA* gene in *E. coli* isolated from wild captive animals of Bikaner zoo and Machia biological park, Jodhpur

S.no	**Source of *E. coli***	**Total isolates**	**No. of positive isolates**	**Prevalence (%)**
Bikaner zoo				
1.	Chinkara	18	17	94.44
2.	Black buck	16	14	87.50
3.	Blue bull	4	3	75. 0
	Total	38	34	89.47
Machia biological park, Jodhpur				
1.	Chinkara	7	7	100
2.	Black buck	9	9	100
3.	Jackal	1	1	100
4.	Lion (female)	1	1	100
5.	Lion (cub)	3	1	33.33
	Total	21	19	90.47
Overall prevalence = 89.83%				

iroN gene amplification results also exhibited good observance but its prevalence was lower as compared to prevalence of *fyuA* gene. PCR amplification gave a product of 665 bp (fig.2) which were visualized after performing agarose gel electrophoresis using 1.5% agarose gel and DNA ladder of 100 bp. Twenty one of 38 (55.26%), and 3 of 21 (14.28%) isolates carried the *iroN* gene in Bikaner zoo and Machia biological park, Jodhpur respectively. In Bikaner zoo, *E. coli* from chinkara, black buck and blue bull 44.44%, 81.25%, and 0%, respectively were shown to possess *iroN* gene. While Jodhpur presented less positive results in which 28.57% of chinkara, 11.11% of black buck harboured the gene. While gene was not found in any isolates of jackal and lion (Table 6).

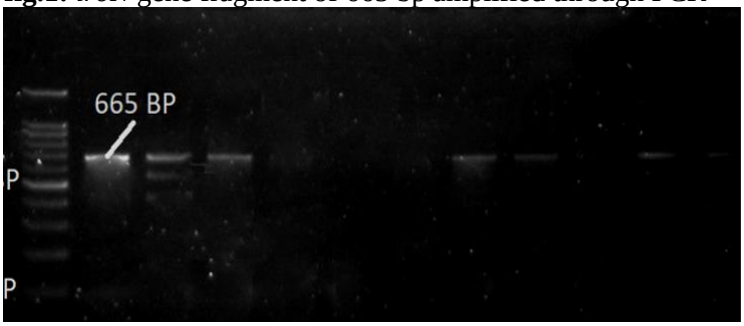
Table 6: No. of positive isolates and prevalence of *iroN* gene in *E. coli* isolated from wild captive animals of Bikaner zoo and Machia biological park, Jodhpur

S.no	**Source of *E. coli***	**Total isolates**	**No. of positive isolates**	**Prevalence (%)**
Bikaner zoo				
1.	Chinkara	18	8	44.44
2.	Black buck	16	13	81.25
3.	Blue bull	4	0	0
	Total	38	21	55.26
Machia biological park, Jodhpur				
1.	Chinkara	7	2	28.57
2.	Black buck	9	1	11.11
3.	Jackal	1	0	0

4.	Lion (female)	1	0	0
5.	Lion (cub)	3	0	0
	Total	21	19	14.28
Overall prevalence = 40.67%				

Siderophore genes have been known to record high prevalence in majority of the reports which makes it an important virulence tool for pathogenic bacteria. Due to scarcity of data pertaining to presence of siderophore genes in captive wild animals, we tried to compare our results to that of other similar sources. Results of present study agreed to Schubert *et al*. (1998) who detected high prevalence of 92% of *fyuA* gene in EAEC strains isolated from humans. *iroN* gene was also reported in 39% of the 67 urosepsis *E. coli* isolates by Johnson *et al*. (2000). *iroN* was known to be associated with many other virulence genes, but due to horizontal transmission and stratification by other genes, (Marrs *et al*., 1999) *iroN* is more likely to show its independent effect. While *fyuA* is mostly found associated with other virulence genes, which may be a reason of its very higher prevalence as compared to *iroN* (in majority of the cases reported). These results were further strengthened by Soto *et al*. (2011) as 83% prevalence of *fyuA* gene and 39% prevalence of *iroN* gene among the 30 isolates collected from 15 patients with febrile UTI (urinary tract infection) having a bacteriological recurrence during long term follow up.

fig.1: *iroN* gene fragment of 665 bp amplified through PCR



Results were also supported by Johnson *et al*. (2002). They analysed 70 meningitis – associated *E. coli* isolates from neonates and detected *fyuA* gene in 66 of isolates giving it overall frequency of 94%, while in contrast to our results, much higher frequency of 64% was detected in case of *iroN* in 45 isolates.
Wide availability of Yersiniabactin siderophore production gene in nature was also observed by Okele *et al*. (2004) in 85.7% of the EAEC reference strains. But *iroN* gene was found in none of the isolates as compared to our results. We observed less frequency of *iroN* as compared to *fyuA* gene, these results suggest that *fyuA* gene is more widely distributed in nature as compared to *iroN* gene. Much lower frequency of *iroN* was also observed in some cases, as reported by Chapman *et al*. (2006), its frequency was recorded 25% in neonatal isolates. while a very high frequency of 90% was also observed by Masters *et al.* (2011) in *E. coli* isolates collected from 3 estuarine, 4 brackish and 23 freshwater sites during the wet and dry seasons. These data indicate that although *fyuA* is mostly reported with high occurrence, but data regarding *iroN* gene is fluctuating depending upon the sources, location and environmental conditions. results of present study are also supported by studies done by Searle *et al.* (2015), Iovine *et al*. (2015) which suggested that anthropogenic actions are mainly responsible for such high occurrence of virulence genes in animals. Variable prevalence of *fyuA* and *iroN* gene was also recorded by Frommel *et al.* (2013) as follows: 47% in healthy human, 75% in human UPEC, 23% in domestic healthy pig, 33% in domestic pig UPEC, 26 % in roe deer, 9% in European hedgehog and 0% in European hare. While 37%, 55%, 32% 33% 0% and 5% of prevalence of *iroN* genes was observed in the above same cases. Prevalence of iron acquisition genes was also found better in wild bird's isolates as compared with mammal's isolates. This difference could be due to higher number of wild mammal's isolates (54 wild birds and 187 wild mammals). A little lower frequency of 70.3% of *fyuA* was observed by Munkhdelger *et al*. (2017).

Presence of *fyuA* indicates that *fyuA* could be a useful target for intervention if *fyuA* can independently be demonstrated to contribute to invasiveness rather than merely serving as a marker gene. High occurrence of *fyuA* found among *E. coli* isolates confirms our previously reported findings in which *fyuA* was significantly more prevalent than *iroN* gene. Taken together, these data suggest that siderophores genes particularly *fyuA* is broad characteristic of bacteremic *E. coli* and more attention should be given to it to find its further details among captive wild animals. Both the siderophore genes are widely distributed among the *E. coli* isolates,

suggesting their important role in virulence and broad prevalence of both genes in highly virulent isolates could indicate that the protein products of these genes would be good vaccine targets for prevention of UTI but such data must be considered in combination with other virulence associated factors.

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