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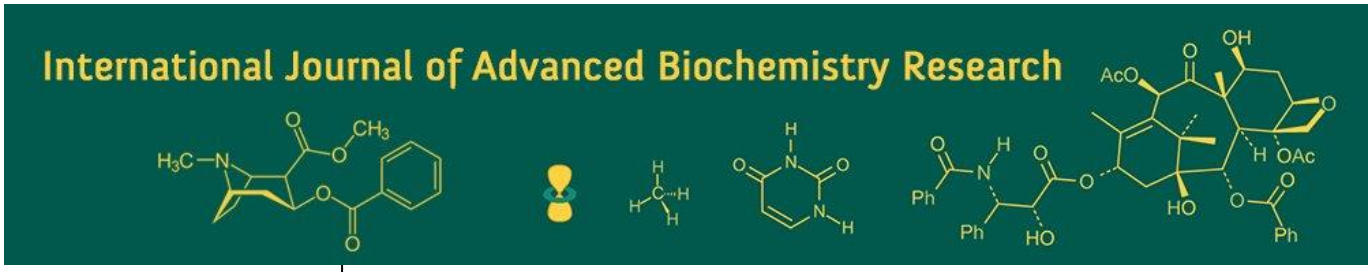

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Phenotypic characterization of *E. coli* isolates obtained from fecal samples of Egyptian vultures (*Neophron percnopterus*)

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Abstract
Egyptian vultures spending the winter in north-western India often stay for extended periods, and some of them gather at an unconventional and synanthropic location in Jorbeer, Rajasthan; a communal dumping ground for livestock carcasses. Local workers in Bikaner city are responsible for collecting, disposing, and skinning livestock carcasses from various clinical settings and dairies. These carcasses create opportunities for interactions among diverse predator host species, potentially facilitating the transmission of pathogens. All bacteria found in vultures, whether internally or externally, have the potential to cause diseases in both wildlife and humans, including *E. coli*. Among the six well-defined pathotypes of intestinal pathogens, Enteropathogenic *Escherichia coli* (EPEC) was the first described, causing bloody and watery diarrhea by developing attaching and effacing lesions on the intestinal epithelium. *Escherichia coli* containing both the LEE and Shiga toxins are referred to as enterohemorrhagic *Escherichia coli* (EHEC). This study aims to phenotypically characterize *E. coli* isolates obtained from fecal samples of Egyptian vultures.

Keywords: Egyptian vultures, fecal samples, *E. coli*, Shiga toxin, phenotypic characterization

Introduction
Vultures, often recognized as nature's sanitation team, play a vital role in upholding the ecological equilibrium within their habitats. Nevertheless, these magnificent birds can inadvertently carry and transmit diverse pathogens, shedding light on the intricate connection between vultures and the diseases they may harbour (Fadel *et al*., 2017) [3]. The bacteria found in vultures, whether internally or externally, have the potential to instigate diseases in both wildlife and humans, including *Escherichia coli* (Hubalek, 2004) [4]. *E. coli*, a ubiquitous inhabitant of the gastrointestinal tract, belongs to the family *Enterobacteriaceae*. It is a short gram-negative, non-spore-forming, peritrichous, and fimbriate bacillus that may possess a capsule or microcapsule. Extensive studies on *E. coli* have been conducted across various taxonomic classes of mammals and bird species. Infections caused by *E. coli* in the blood, respiratory tract, and soft tissues lead to diseases such as colibacillosis, air sacculitis, and cellulitis, often associated with high morbidity and mortality. Enteropathogenic *Escherichia coli* (EPEC) was the first-described *Escherichia coli* pathogen (Kaper *et al*., 2004) [5], inducing bloody and watery diarrhea through the development of attaching and effacing lesions. Additionally, Enterotoxigenic *Escherichia coli* (ETEC) stands out as the most prevalent cause of diarrhea worldwide (Turner *et al*., 2006) [11].
Shiga-like toxin-producing *E. coli* (STEC) is marked by essential virulence factors, including the chromosomal-encoded cytotoxins Shiga toxin 1 and 2 (encoded by stx1 and stx2 genes, respectively), the intimin protein (encoded by the chromosomal gene eae) responsible for bacterial attachment to intestinal epithelial cells, and the plasmid-encoded enterohaemolysin, also known as enterohemorrhagic *Escherichia coli* hemolysin (EHEC-hlyA), encoded by the hlyA gene (Law, 2001; Solomakos *et al*., 2009) [7, 10]. Adapted *E. coli* clones with various virulence factors exhibit enhanced adaptability to new environments, leading to a wide range of diseases. Infections with pathogenic *E. coli* strains result in three general clinical

syndromes: enteric or diarrheal disease, urinary tract infection, and sepsis or meningitis (Neher *et al*., 2016) [8]. Consequently, the current study was conducted to identify the phenotypic characteristics of *E. coli* isolates obtained from fecal samples of Egyptian vultures.

Materials and Methods

1. Fecal Sample Collection: In the Jorbeer conservation area, Bikaner (Rajasthan), 38 freshly voided fecal samples were gathered from Egyptian vultures. The collection process maintained aseptic conditions, utilizing a sterile spatula to transfer each sample into a sterile test tube.

2. Isolation and Identification of *E. coli*: The procedures for isolating and identifying the organisms followed the methodologies outlined by Cowan and Steel (1975) [2], Buchanan and Gibbons (1974) [1], and Quinn *et al*. (1994) [9]. Gram's staining served as the primary identification test, and colonies appearing pink were streaked on Eosin Methylene Blue agar (EMB) plates in primary, secondary, and tertiary stages to achieve isolated bacterial colonies. The plates were then incubated for 24 hours at 37 °C to facilitate aerobic cultivation. The subsequent day, the growth was assessed for colonial morphology and pigmentation. Various colony types were sub-cultured onto separate plates to ensure the purity of cultures. Smears from each sub-cultured colony were fixed through gentle heat, Gram-stained, and examined under oil immersion to verify the cultures' purity.

3. Tests for Generic Identification of Bacteria

a. **Aerobic Growth:** To confirm the ability of bacterial isolates to thrive under aerobic conditions, their growth in air was assessed.
b. **Gram's Reaction:** Smears from young bacterial culture isolates were stained using a modified Gram's staining method.
c. **Morphology:** After staining with Gram's method, the morphology of bacterial isolates was recorded.
d. **Motility:** Bacterial isolates, from a six-hour-old broth culture incubated at 37 °C, were examined in a hanging drop preparation under 40X magnification to observe bacterial motility.
e. **Catalase Activity:** Catalase activity was determined by mixing a loopful of a young bacterial culture with a drop of 3% hydrogen peroxide on a clean glass slide. The presence of gas bubbles or effervescence within a few seconds indicated catalase positivity.
f. **Oxidase Test:** The oxidase test involved placing one drop of oxidase reagent on a filter paper, rubbing a loopful of the test bacterial culture against the wet surface, and noting the appearance of dark purple colour within a few seconds as oxidase positive. The absence of colour or delayed appearance was considered oxidase negative.

4. MAR Index: The MAR indexing method was applied to evaluate all isolates. This index aids in risk assessment by identifying contamination from high-risk environments. A MAR index greater than 0.2 suggests that strains originated from an environment with antibiotic use, indicating a high-risk potential source of multi-drug resistance (MDR) spread.

5. Metabolic and Biochemical Reactions (Secondary Tests): To confirm *E. coli* isolates, a set of 12 biochemical tests from the Hi *E. coli*™ Identification Kit (HiMedia, Mumbai) was employed.

6. Genotypic Identification of *Escherichia coli*: Following primary and secondary biochemical tests, isolates underwent polymerase chain reaction for 16S rRNA ribotyping, using species-specific primers to confirm their identity as *Escherichia coli*.

Results and Discussion

1. Prevalence and Occurrence of *Escherichia coli* in Egyptian Vultures (Sample Collection, Isolation & Confirmation): In this study, 30 out of the 38 fecal samples collected from Egyptian vultures at Jorbeer conservation area in Bikaner (78.94%) were presumptively identified as *Escherichia coli* using conventional methods. Subsequently, genotypic confirmation was conducted through 16S rRNA typing.

2. Cultural and Biochemical Properties

All 30 *E. coli* isolates obtained from the fecal samples of Egyptian vultures underwent aerobic cultivation on MacConkey agar and Eosin Methylene Blue (EMB) agar for 24 hours at 37 °C. Pink colonies indicative of lactose fermentation observed on MacConkey agar (Fig. 3). Lactose-fermenting colonies were selected, identified by gram stain and motility, and then streaked on EMB agar. Metallic green sheen, characteristic of *E. coli*, was observed for all isolates on EMB agar (Fig. 4).

Isolates displaying the metallic sheen on EMB agar were subjected to standard biochemical tests, including Voges-Proskauer (VP), Methyl Red (MR), and indole production tests, for confirmation of *E. coli* using the HiE.coli™ Identification Kit (HiMedia, Mumbai). All 30 *E. coli* isolates exhibited the typical IMViC pattern, with positive results for Indole and MR tests, and negative results for VP and Citrate tests (++--). (See Table 1 for details).

Table 1: Biochemical tests performed by Hi *E. coli*™ Identification Kit

Well No.	Test	Original colour of the medium	*E. coli* positive isolates
1.	Methyl red	Colourless	Red
2.	Voges Proskauer's	Colourless/ Light yellow	Colourless/ slight copper
3.	Citrate Utilization	Green	Green
4.	Indole Colourless	Reddish	pink
5.	Glucuronidase	Colourless	Bluish green
6.	Nitrate Reduction	Colourless	Pinkish red
7.	ONPG	Colourless	Yellow
8.	Lysine utilization	Olive green	Purple/Light Purple
9.	Lactose Pinkish	Red/ Red	Yellow
10.	Glucose	Pinkish	Red/ Red Yellow
11.	Sucrose	Pinkish	Red/Red Yellow
12.	Sorbitol	Pinkish	Red/ Red Yellow

Genotypic Confirmation

Escherichia coli organisms can be cultivated on bacteriological media and identified using biochemical tests, but these procedures are known for their time-consuming nature. To address this, confirmation can be achieved through a genotypic method, known for its increased accuracy and efficiency. In the current study, out of the 38 collected samples, 30 isolates were verified using PCR through 16S rRNA ribotyping, as described by Khaled *et al*. (2010) [6]. The resulting amplicon had a size of 662 bp, as illustrated in Figure 5. The primer pair sequence utilized in this process was as follows: Forward Primer - 5' GCT TGA CAC TGA ACA TTG AG 3' Reverse Primer - 5' GCA CTT ATC TCT TCC GCA TT 3'

Fig 1: Egyptian vultures at Jorbeer conservation area, Rajasthan

Fig 2: Sample collection

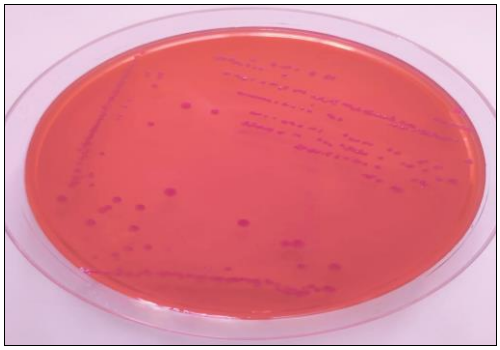

Fig 3: Pink colonies on MacConkey agar

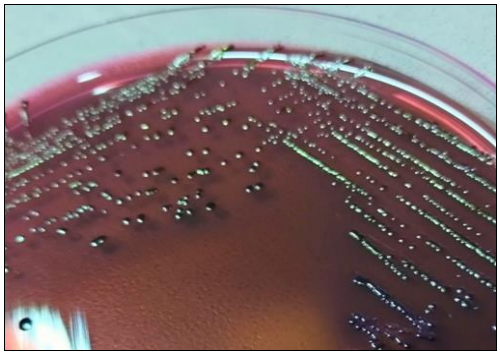

Fig 4: Metallic green sheen on EMB agar

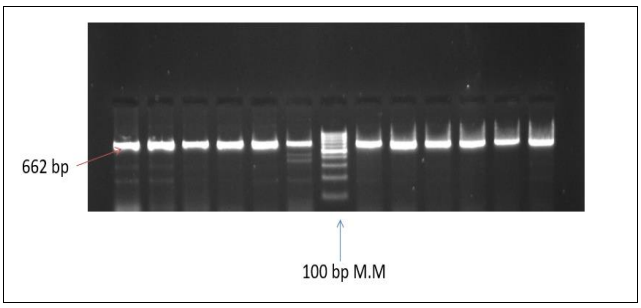

Fig 5: Amplicon of 662 bp confirming them to be *E. coli*

Summary and Conclusion

In the current investigation, 30 out of the 38 freshly voided fecal samples collected from Egyptian vultures in the Jorbeer conservation area, Bikaner (Rajasthan), yielded *E. coli* isolates, representing a prevalence of 78.94%. The identification of these isolates was initially conducted through conventional methods, encompassing primary tests, as well as cultural, morphological, and biochemical assessments, including growth on MacConkey and Eosin Methylene Blue (EMB) agar media. Notably, all 30 *E. coli* isolates exhibited pink colonies on MacConkey agar and displayed a metallic sheen on EMB agar. Subsequently, the isolates underwent polymerase chain reaction (PCR) using species-specific primers for 16S rRNA-based ribotyping. The resulting amplicon of 662 bp confirmed the genotypic identity of these isolates as *E. coli*.

Further scrutiny of the genotypically identified isolates focused on cultural and biochemical properties. Those exhibiting a metallic sheen on EMB agar were subjected to standard biochemical tests, including Voges-Proskauer (VP), Methyl Red (MR), and indole production tests, using the HiE.coli™ Identification Kit (HiMedia, Mumbai). These isolates adhered to the typical IMVic pattern, with positive results for indole and Methyl Red tests, and negative results for VP and citrate utilization tests.

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