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# Preliminary study on inhibitory activity of *Enterobacter sp.* strain KD111 isolated from the Cow feces

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## Abstract

**The objective of the present study was to isolate *Enterobacter sp.* from the faecal matter of cow followed by its screening for antimicrobial activity against Gram-positive and Gram-negative bacteria having clinical importance. On the basis of morphological and biochemical characterisation of seven isolates obtained from cow dung sample, isolate KD111 was probably identified as *Enterobacter sp.* and screened for its inhibitory activity against 14 test organisms comprising both Gram-positive and Gram-negative types using cross-streak method, in which both i.e. test organisms and isolates were streaked perpendicularly on nutrient agar plates followed by measurement of inhibition zone between the streaks after incubation. The preliminary screening revealed significant antimicrobial activity of *Enterobacter sp.* against *Salmonella typhi* (MTCC 3216), *Escherichia coli*, *Staphylococcus aureus* (MTCC 7443) and *Bacillus cereus* (MTCC 6728) with highest inhibition against *Salmonella typhi* (MTCC 3216) and *Bacillus cereus* (MTCC 6728). Our results indicate that *Enterobacter sp.* may act as a producer of bioactive antimicrobial metabolites and therefore should be analysed further for its possible application as therapeutic agent.**

***Key words: Enterobacter sp., Cross-streak method, Antagonistic activity, Cow dung***

## Introduction

Microbial secondary metabolites are chemical compounds produced naturally by the microorganisms during idiophase which ensure their survival in a competing environment by regulating growth processes, replications and inhibiting life cycle of other microbes. Presence of these metabolites in the microbial extracts have played a major role in the development of new antimicrobial drugs (Kleinkauf and Dohren, 1990; Higgs *et al.*, 2001; Esikova *et al.*, 2002; Berdy, 2005; Ilic *et al.*, 2007; Gupta and Rana, 2016). Secondary metabolites from many microorganisms have been successfully developed and produced up to commercial level in the form of antibiotics such as Gramicidin and Rifampin being produced by *Bacillus* and *Streptomyces spp.* respectively (Waites *et al.*, 2008; Awais *et al.*, 2010; Mahajan and Balachandran, 2012; Gupta and Rana, 2017). Pathogens are continually becoming resistant towards antibiotics, however, this problem is further increased by continuous and independent use of antibiotics that pose serious health implications on patient care in terms of increasing substantial morbidity and mortality (Maataoui *et al.*, 2014; Balachandran *et al.*, 2015; Gay *et al.*, 2017; Ekwanzala *et al.*, 2018). Resistance towards third-generation cephalosporins and monobactams (aztreonam) is evident by production of ESBL which have the ability to hydrolyze them. Thus emergence of drug resistant pathogens is a major concern in both hospitals and at community level resulting in an alarming scarcity of new antibiotics (Singh *et al.*, 2014). At present, the researchers are trying to screen microorganisms from unexplored habitats in order to isolate microbes with new antimicrobial properties (Watve *et al.*, 2001; Hozzein *et al.*, 2011; Khanna *et al.*, 2011; Wadetwar and Patil, 2013; Maataoui *et al.*, 2014).

As per literature, many bacteria isolated from soil for example *Bacillus lentus*, *Micrococcus roseus*, *Enterobacter aerogenes*, *Bacillus pumillus*, *Bacillus alvei* and *Bacillus amylolquefaciens* have shown bioactivity against *Shigella spp.*, *Staphylococcus aureus*, *Pseudomonas spp.*, *Proteus spp.*, *Corynebacterium diphtheriae*, *Streptococcus pneumonia*, *Salmonella* group D, and *Vibrio*

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*parahaemolyticus* (Abdulkadir and Waliyu, 2012; Boottanun *et al.*, 2017). Therefore, screening microorganisms from new or less exploited sources with reference to antimicrobial metabolite production may provide new leads for drug discovery.

Ruminant animals have stable microbial ecological balance system and are rich in microbial diversity (Li *et al.*, 2018). Cow dung is a bovine excreta generated after the digestion of consumed food materials containing crude fibre, crude protein, cellulose and various types of macro (N, K, S), and micro nutrients such as traces of P, Fe, Co, Mg, P, Cl and Mn (Nene, 1999). Cow dung micro-flora is usually diverse containing abundant number of *Bacilli, Lactobacilli* and *Cocci* and some identified and unidentified fungi and yeasts (Muhammad and Amusa, 2003). Various microorganisms such as *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, Bacillus subtilis, Enterococcus diacetylactis* have been reported earlier from lower part of the gut of the cows (Ware *et al.*, 1998). Other than these, *Bacillus, Bifidobacterium* and yeasts are also present in the rumen of the cow for better rumen fermentation (Kung, 2004; Yang *et al.*, 2017). Antifungal substances isolated from cow dung inhibit the growth of coprophilous fungi (Dhama *et al.*, 2005; Joseph and Sankarganesh, 2011; Dhama *et al.*, 2013). *Eupenicillium bovifimosum*, that produces patulodine-like compounds viz. CK2108A and CK2801B was also isolated from cow dung (Dorothy and Frisvad, 2002). Laukova *et al.*, (1998) isolated *Enterococcus faecalis* V24 from cow dung that produced a heat stable, largely hydrophobic antimicrobial substance possessing antimicrobial activity against pathogenic Gram-negative bacteria. Similarly Teo and Teoh (2011) also isolated a strain from cow dung i.e. K4 showing antibacterial activity against *Escherichia coli*. Therefore, in the present work, considering all these facts, an attempt has been made to determine antagonistic activity of *Enterobacter sp*. from the gut of cow by collecting cow dung samples.

## Material and Methods

**Sample collection:** Dung sample of desi cow (breed Gaolao) was collected from Saharanpur. Sample was collected aseptically in sterile container and analysed immediately after transporting to the laboratory (Yang *et al.*, 2017).

**Isolation of Bacterial Species:** For isolation purpose, serial dilution technique was employed in which 1g of cow dung was mixed in 9ml of sterile saline (0.85%) and dilutions were made from $10^{-2}$ to $10^{-8}$. Aliquot (0.1 ml) from each dilution was spread on Nutrient Agar Medium (NAM) followed by incubation at $37^{O}$C for 24-48 h. Different bacterial colony were selected and purified by repeated streak plate method. Until further use, the slants were kept at $4^{O}$C (Sawant *et al.*, 2007; Das, 2010).

**Morphological and Biochemical characterization:** Morphological and Biochemical characterization of all the seven isolates was done in order to determine *Enterobacter spp.* Morphological characterization (Gram-staining) was undertaken as described by Beveridge (2001). Biochemical characterization was performed according to the criteria given in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

**Detection of inhibitory activity of *Enterobacter sp.*:** Antmicrobial activity of the isolated *Enterobacter sp.* designated as KD111 was evaluated using cross-streak method (Gupta and Rana, 2017) against 14 test organisms i.e., *Vibrio cholerae* (MTCC 3904), *Salmonella typhi* (MTCC 3216), *Salmonella typhimurium* (MTCC 3231), *Escherichia coli* (clinical isolate), *Escherichia coli* (MTCC 118), *Staphylococcus aureus* (MTCC 7443), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 6728), *Proteus vulgaris* (MTCC 426), *Enterococcus faecalis* (MTCC 439), *Pseudomonas aeruginosa* (MTCC 424), *Shigella flexneri* (MTCC 1457), and *Streptococcus pyogenes* (MTCC 442). *Enterobacter sp.* KD111 was streaked onto NAM plates as a single streak in the centre and incubated at 37°C for 24h. The plates were then reinocluated with each test organism as a single streak perpenducluar to the *Enterobacter sp.* KD111. The microbial inhibitions were observed after 24h by measuring the distance of the inhibition zone between KD111 and test organisms.

## Results and discussion

**Isolation and morphological characterization of gut bacteria:** In the present study, seven isolates of

gut from cow were isolated from the cow dung sample by serial dilution method. Morhphological characterisation of seven isolates revealed only KD111 to be Gram-negative rod while others were found to be Gram-positive cocci (KD112, KD113 and KD114), Gram-positive rod (KD115 KD116) and Gram-negative cocci (KD117). Therefore, KD111 was selected for biochemical charcterisation regarding identification of *Enterobacter sp.* Detailed results of morphological characterization are summarised in the Table 1.

**Table 1. Morphological analysis of the bacterial isolates.**

| Isolates /Investigation | Gram Reaction | Shape |
|---|---|---|
| KD111 | Gram-negative | Rod |
| KD112 | Gram-positive | Cocci |
| KD113 | Gram-positive | Cocci |
| KD114 | Gram-positive | Cocci |
| KD115 | Gram-positive | Rod |
| KD116 | Gram-positive | Rod |
| KD117 | Gram-negative | Cocci |

**Biochemical characterization of isolate KD111:**

Apart from being motile, and positive for amylase as well as catalse production, our isolate KD111 could also able to utilise glucose, lactose, sucrose and citrate as carbon source. Therefore, isolate KD111 was identified as *Enterobacter sp.* (Holt *et al*., 1994). Results of biochemical characters are comprised in the Table 2.

**Table 2. Biochemical characterisation of the bacterial isolate KD111.**

| Test/Isolates | KD111 |
|---|---|
| Motility | Motile |
| Glucose Fermentation | + |
| Lactose Fermentation | + |
| Sucrose Fermentation | + |
| Gelatine Liquification | - |
| Starch Hydrolysis | + |
| Indole | + |
| Methyl-Red | - |
| Vogeus-Proskeur | - |
| Citrate Utilisation | + |
| Catalase Production | + |
| Oxidase | - |
| Urease | - |

**Inhibitory activity of *Enterobacter sp.* by cross-streak method:** Out of seven isolated bacteria, KD111 was identified as *Enterobacter sp.* and subjected to antagonistic activity by cross-streak method against a panel of test bacteria having medical importance. Among test organisms include *Salmonella typhi* that causes Typhoid and paratyphoid fever (Hsiao *et al.,* 2016) and *Escherichia coli,* which is responsible for urinary tract infection, food poisoning and diarrhoea (Alteri *et al.,* 2009; Teo and Teoh, 2011). Isolate KD111 demonstrated significant antimicrobial activity as evident by the inhibition of seven out of fourteen test organisms. Maximum inhibition was recorded against *Salmonella typhi* (8.0mm), *Salmonella typhimurium* (8.0 mm) and *Bacillus cereus* (8.0 mm), while minimum (3.0 mm) was against both strains of *Staphylococcus aureus* (Table-3).

Isolate KD111 inhibited both Gram-positive and Gram-negative test organisms, thereby indicating the secretion of broad spectrum antimicrobial agents. The difference in the sensitivity of Gram-positive and Gram-negative bacteria against *Enterobacter* sp. strain KD111 may be due to the morphological differences in their outer membrane (Gebreyohannes *et al.,* 2013). Qiu *et al.,* (2017) stated that bacteria belonging to *Enterobacteriaceae* may revert the disrupted structure of the gut microbiota to stabilize diversity. *Enterobacter aerogenes* isolated from orchard soil have shown significant in vitro antagonistic activity against several plant pathogens as reported by Utkhe and Sholberg (1986). One important observation of KD111 was made against *Escherichia coli*, when 5.0 mm inhibition was recorded. However, still antimicrobial activity of *Enterobacter spp.* is not well reported in the literature but other researchers have shown similar findings by using different producer organisms. Abdulkadir and Waliyu (2012) isolated *Bacillus lentus* and *Bacillus alvei* isolated from soil showing antibacterial activity against *Staphylococcus aureus*. Bacitracin, a commercially available antibiotic produced by *Bacillus sp.* inhibits *Escherichia coli* and *Staphylococcus aureus* (Prescott *et al.,* 2008). *Enterococcus faecium* isolated from traditional rigouta cheese was also found effective against *Staphylococcus aureus* (Ghrairi *et al.,* 2008). *Bacillus* species isolated from cow dung showed significant antimicrobial effect

**Table 3. Inhibitory activity of *Enterobacter sp.* KD111 against test bacteria.**

| Isolates/ Test organisms | *V. cholerae* MTCC 3904 | *S. typhi* MTCC 3216 | *E. coli (clinical isolate)* | *S. aureus* MTCC 7443 | *B. subtilis* MTCC 441 | *B. cereus* MTCC 6728 | *P. vulgaris* MTCC 426 | *E. fecalis* MTCC 439 | *P. aeruginosa* MTCC 424 | *E. coli* MTCC 118 | *S. fexneri* MTCC 1457 | *S. typhimurium* MTCC 3231 | *S. pyogenes* MTCC 442 | *S. aureus* MTCC 3160 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KD111** | - | 8mm | 5mm | 3mm | - | 8mm | - | - | - | 5mm | - | 8mm | - | 3mm |

against *Vibrio Cholerae* (MTCC 3904), *Salmonella typhi* (MTCC 3216), *Escherichia coli*, and *Bacillus cereus* (MTCC 6728) (Gupta and Rana, 2017). Gupta and Rana (2018), observed inhibitory activity of *Alcaligenes fecalis* and *Alcaligenes latus* isolated from cow dung against *Salmonella typhi*, *Escherichia coli* and *Bacillus subtilis* (MTCC 441). Antibiotic resistance among clinical pathogens for example, *Salmonella typhi*, *Vibrio choleraee*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus* has been reported recently against not only 1st and 2nd generation antibiotics but also against 4th generation antibiotics (Mare and Coetzee, 1964; Alteri *et al.*, 2009; Puah *et al.*, 2016; Uppal *et al.*, 2017).

## Conclusion

Isolated strain *Enterobacter* sp. KD111 possess potential for producing antimicrobial substances which might be effective against these disease causing bacteria. Although the genus *Enterobacter* is generally associated with hospital-acquired infections, however, their bioactive metabolites should be analysed further to confirm their possible application as therapeutic agent against bacterial species.

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