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Prevalence, Molecular Diagnosis and Management of *Klebsiella* species in Captive Sloth Bears (*Melursus ursinus*)

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ABSTRACT

Genus *Klebsiella* from faeces of sloth bears was screened by using culture morphology, Gram's staining, biochemical tests and polymerase chain reaction. Our results showed that out of 60 samples collected, 22 samples (36.67%) were cultured on *Klebsiella* Selective Agar Base with *Klebsiella* Selective Supplement and Gram's stain revealed rod-shaped Gram-negative organism with purple-magenta colony - like colonies. The biochemical tests of cultured samples revealed negative to indole production and methyl red test, positive to Voges-Proskauer test, positive to Simmon citrate utilization test, negative to H_2S production and that produced acid over acid reaction in TSI agar and positive to urea production in cultured samples. All *Klebsiella* species isolates were sensitive to azithromycin followed by enrofloxacin and resistant to clindamycin and methicillin. The *gyr*A gene was amplified by PCR for the genus *Klebsiella* and found to be positive of 36.67%. This study may provide information for developing strategies in the future in the control of *Klebsiella* species infections in sloth bears.

Keywords: Antibiogram, *Klebsiella* spp., *gyr*A gene, Sloth Bear, Ursidae

Genus *Klebsiella* is Gram-negative, non-motile, usually encapsulated rod-shaped bacteria, belonging to the family Enterobacteriaceae and the family members are generally facultative anaerobic. The Genus *Klebsiella* includes 6 species with 3 subspecies and it consists of 77 capsular antigens (K antigens), leading to different serogroups (Janda *et al*., 2006). Genus *Klebsiella* are increasingly important opportunistic pathogens associated with severe hospital-acquired infections (nosocomial bacterial infections) such as septicaemia, pneumonia and urinary tract infections (Brisse and Verhoef, 2001). Enterobacteria are considered part of the intestinal tract of mammals and some bird species, and they are capable of spreading in the environment and becoming ubiquitous under the appropriate conditions (Gerlach, 1994). Information on *Klebsiella* bacteria of sloth bear (*Melursus ursinus*) is generally lacking and no information could be accessed on *Klebsiella* specifically from the Indian subcontinent. The aim of this study was to investigate *Klebsiella* spp. infection in sloth bears.

MATERIALS AND METHODS

During this study in 2017, the faecal samples (n=60) of captive sloth bears (*Melursus ursinus*) from 15 juveniles, 15 sub-adults, 15 adults and 15 geriatric animal groups and these animals are housed in different enclosures (n=5) at Bannerghatta Bear Rescue Centre (BBRC), Wildlife SOS, Bangalore, Karnataka were collected in nutrient broth using sterile swabs and kept at 4 ºC until further processing. The swabs were incubated at 37ºC for 24 hours under aerobic conditions. A loop full of cultured broth was streaked aseptically onto Hichrome™ *Klebsiella* Selective Agar Base with *Klebsiella* Selective Supplement under laminar floor hood and incubated at 37°C for 24hrs under aerobic conditions. The isolated colony was stained with Gram's stain and biochemical tests (HiIMViC™ Biochemical Test Kit, TSI agar and urea agar) and was confirmed by Polymerase Chain Reaction (PCR).

Antimicrobial resistance pattern of *Klebsiella* species isolates were studied by Modified Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006) for the following antibiotics: amoxicillin/clavulanic acid (30mcg), azithromycin (15mcg), cefotaxime (30 mcg), clindamycin (2 mcg), gentamicin (10 mcg), enrofloxacin (10 mcg), methicillin (5 mcg), streptomycin (300 mcg) and tetracyline (30 mcg).

For the PCR reaction, the DNA was extracted from the isolates by using boiling method (Medici *et al*., 2003). One ml of the pre-enriched culture was transferred to a 1.5ml micro-centrifuge tube. The cell suspension was centrifuged for 10 min at 10,000rpm. The supernatant was discarded carefully. The pellet was resuspended in 300 µl of DNase-RNase- free water by vortexing. The tube was centrifuged at 10,000 rpm for 5 min, and the supernatant was discarded carefully. The pellet was resuspended in 200 µl of DNase-RNase-free water by vortexing. The microcentrifuge tube was incubated for 15 min at 100°C and immediately chilled on ice. The tube was centrifuged for 5 min at 10,000 rpm at 4°C. The supernatant was carefully transferred to a new microcentrifuge tube and incubated again for 10 min at 100°C and chilled immediately on ice. Further, it was stored at -20°C. An aliquot of 5 µl of the supernatant was used as template in the PCR.

The molecular weight of the *gyr*A gene corresponding to the *Klebsiella* species was 441bp (Brisse and Verhoef, 2001). The following primers were used to amplify the *gyr*A gene (Forward Primer: *gyr*A-A 5'-CGCGTACTATACGCCATGAACGTA–3' and Reverse Primer: *gyr*A-C 5'-ACCGTTGATC ACTTCGGTCAGG–3'). The PCR was performed in a 25 µl reaction mixture consisting of 12.5 µl of 2X PCR mastermix, 1 µl of each primer, 2 µl of extracted DNA and finally volume was adjusted with nuclease free water. Amplification was carried out in thermocycler with initial denaturation 94°C for 4 min followed by 30 cycles each of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 50 seconds with a final extension period of 10 minutes at 72°C. 10 µl of each PCR products was electrophoresed on 1.5% agrose gel containing ethidium bromide in the presence of 100 bp ladder. The presence specific amplicon of 441 bp was viewed under UV transilluminator.

The PCR amplifies the *gyr*A gene of the one of the isolate was sequenced using commercial sequencing service.

RESULTS AND DISCUSSION

Out of 60 samples collected, 22 samples (36.67%) were cultured on Hichrome™ *Klebsiella* Selective Agar Base with *Klebsiella* Selective Supplement and purple-magenta colony - like colonies (Fig. 1) were picked for Gram's staining and revealed Gram-negative rod-shaped organism.

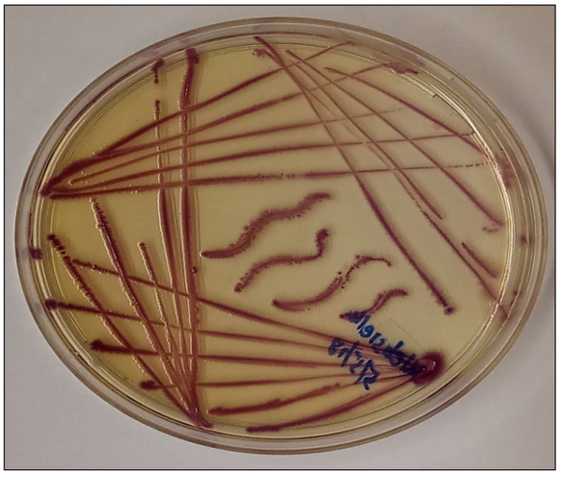

Fig. 1: *Klebsiella* species on Hichrome™ *Klebsiella* Selective Agar Base with *Klebsiella* Selective

Usage of biochemical tests in HiIMViC^{TM} Biochemical Test Kit, TSI agar and urea agar revealed that were negative to indole production and methyl red test, positive to Voges-Proskauer test, positive to Simmon citrate utilization test, negative to H_2S production and that produced acid over acid reaction in TSI agar and positive to urea production in cultured samples. All *Klebsiella* species isolates which were studied have been found sensitive to azithromycin followed by enrofloxacin. They were found resistant to clindamycin and methicillin.

Supplement

Further, based on the PCR assay (Fig. 2) among the enclosures, a higher prevalence of *Klebsiella* species infection was noticed in Chitrakuta Block (50.00%) followed by Jambhava Block (42.86%) and Dr. GKV Block (38.46%) (Table 1). Statistically, there was no significant difference among enclosures (P>0.05). Similarly, the highest infection (40.00%) was found in both sub-adult group and adult group followed by 33.33% of infection in juvenile group and geriatric group. There was no significant difference among animal groups (P>0.05) (Table 2). In male sloth bears, 40.74% of *Klebsiella* species infection was found during this study. The prevalence of *Klebsiella* species in male and female did not differ significantly (Table 3).

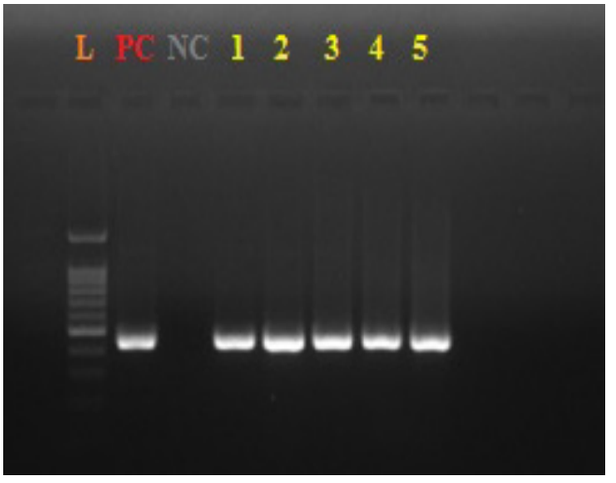

Fig. 2: Amplification of *gyr*A gene of *Klebsiella* species by PCR in 1.5% agarsoe; Lane L: DNA ladder (500bp), Lane PC: Positive control, Lane NC: Negative control, Lane 1-5: *gyr*A gene amplification (441bp)

Table 1: Prevalence of *Klebsiella* species among enclosures

Enclosure	n	*Klebsiella* species
Panchavati Block	12	2 (16.67%)
Chitrakuta Block	10	5 (50.00%)
Kishkinda Block	11	4 (36.36%)
Dr. GKV Block	13	5 (38.46%)
Jambhava Block	14	6 (42.86%)
Overall	60	22 (36.67%)
χ^2		3.08^{NS}

n – Number of samples collected; NS - Not Significant (P>0.05).

Table 2: Prevalence of *Klebsiella* species among animal groups

Animal Groups	n	*Klebsiella* species
Juvenile	15	5 (33.33%)
Sub-adult	15	6 (40.00%)
Adult	15	6 (40.00%)
Geriatric	15	5 (33.33%)
Overall	60	22 (36.67%)
χ^2		0.29^{NS}

n – Number of samples collected; NS - Not Significant (P>0.05).

Table 3: Prevalence of *Klebsiella* species among animal genders

Animal genders	N	*Klebsiella* species
Male	27	11 (40.74%)
Female	33	11 (33.33%)
Overall	60	22 (36.67%)
χ^2		0.35^{NS}

n – Number of samples collected; NS - Not Significant (P>0.05).

The *gyr*A gene was chosen for amplification since presence of this gene indicates the *Klebsiella* species. All the isolates from the sloth bears were positive for the presence of *gyr*A gene. The sequence results was compared with that of *Klebsiella* species isolate sequences using nBLAST (Nucleotide local alignment service tool) available at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch. The sequence data is provided as supplement.

The *Klebsiella* organisms revealed during the culture related studies carried out with the fecal samples obtained from captive sloth bears reared at BBRC had purple-magenta colonies and were positive for catalase test and citrate test, in addition to Voges-Proskauer test. These biochemical identification made during the study was in accordance to the reports furnished by Davies *et al*. (2016) who opined that *Klebsiella pneumonia* was consider as one of the most important gram negative opportunistic pathogens and isolates were revealed to have the ability to catabolise citrate promote hydrolysis of urea and decarboxylation of lysine and were also associated with negative indole production. Encountering the rod shape organisms of *Klebsiella* sp. in this study was also in accordance with the reports presented by Abdel-Aziz *et al*. (2017) who opined about this rod shaped bacterium and were the gram-negative and facultative anaerobic organisms. The colonies identified as *Klebsiella* sp. using standard biochemical procedures were also subjected to molecular confirmation by polymerase chain reaction, in this study.

The present study revealed increased sensitivity of the *Klebsiella* sp. organisms to azithromycin followed by enrofloxacin. The findings of comparatively a high sensitivity towards enrofloxacin by *Klebsiella* sp. organisms in this study was in agreement with the reports furnished by Du *et al*. (2014) who additionally detailed that in case of Chinese hares, the antibiotic agents like ampicillin, penicillin, polymyxin B, imipenem, meropenem and vancomycin were resistant. However, the zoonotic bacteria including the *K. pneumonia* where quoted to be resistant to ceftiofur in a most prevalent manner, followed by ampicillin and ceftriaxone. Similar to the finding in this study related to enrofloxacin, sensitivity of the *Klebsiella* sp. organisms were reported by Seliskar *et al*. (2007) towards enrofloxacin, in addition to ciprofloxacin but at the same time, it was revealed that these organisms were resistant to azithromycin in dogs; however, in the current study with bears, these were found to be more sensitive to azithromycin. The variations in sensitivity might be due to different species, environment and virulence as well as nature of microbes present in the species.

The overall positivity of *Klebsiella* sp. organisms was 36.67 per cent among the captive sloth bears in the study encountering of genus *Klebsiella* during the study with captive sloth bears was an agreement with the findings reported by Du *et al*. (2014) who however revealed drug-resistance *Klebsiella pneumonia* in Chinese hares (*Lepus sinensis*) the findings of genus *Klebsiella* among the captive sloth bears in the study might be due to the opportunistic nature of these pathogens.

It becomes significant to mention the report of Brissee and Verhoef (2001) who specifically quoted that bacteria of the genus *Klebsiella* are increasingly important opportunistic pathogens associated with severe hospital-acquired infections such as septicaemia, pneumonia and urinary tract infections. Bacteria belonging to the genus *Klebsiella* had been reported in multiple species of wild fauna like red deer (Dias *et al*., 2018), hares (Du *et al*., 2014), wild birds (Matias *et al*., 2016) and other wild animals (Eze *et al*., 2018).

Similar to the case with detection of other bacterial organisms in this study, clinically affected captive sloth bears with bacteria of genus *Klebsiella* were however not encountered during this study at Bannerghatta Bear Rescue Centre, Bangalore. In this regard, the incidences of *Klebsiella pneumonia* were documented by different authors in different species. Species specific study with regard to genus *Klebsiella* might be more useful in revealing heterogeneity as well as the complex epidemiology of the *Klebsiella* sp. and however, studies on the phylogenetic relationships between *Klebsiella* sp. and subspecies among the captive sloth bears are highly lacking, in this regard.

Interestingly, the presence of genus *Klebsiella* was encountered among the captive sloth bears, regardless of the type of enclosures, sex of the animal and age group of the animal. However, the variation with regard to the presence of *Klebsiella* sp. revealed in this study failed to reveal any significance among age group or sex or enclosures.

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SUPPLEMENT

Partial nucleotide sequence of *gyr*A gene from *Klebsiella* species isolated from sloth bear (*melursus ursinus*)

>Consensus A A A T C A G C C C G T G T C G T T G G T G A C G T A A T C G G T A A A T A C C A C C C K C A T G G T G A C
T C T G C G G T A T A C G A C A C C A T T G T C C G T A T G G C T C A G C C G T T C T C G C T G CGAT A Y A T G C T G
G T G G A T G G Y C A G G G K A A C T T C G G T T C C A T C G A T G G C G A Y T C C G C G G C G G C G A T G C G T T A
T A C G G A A A T C C G T C T G G C G A A R A T T G C C C A T G A G Y T G A T G G C C G A T C T G G A A A A A G A G A C
G G T T G A T T T Y G T C G A C A A C T A T G A C G G C A C G G A A A A A A T Y C CW G A C G T Y A T G C C R A C C A A
A A T Y C C T A A C C T G Y T G G T A G A A C G G T T C G T T C C G G T A T C G C M G T A G G K A T G G C

IUPAC nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T (or U)	Thymine (or Uracil)
R	A or G
Y	C or T
W	A or T
K	G or T
M	A or C