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Dr. Harish Kalla
Ph.D, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Dr. Mythri Paila
BVSc and AH, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Corresponding Author:
Dr. Harish Kalla
Ph.D, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

A study of *Klebsiella pneumoniae* isolated from water samples in and around Tirupati

Dr. Harish Kalla and Dr. Mythri Paila

Abstract
The present study was undertaken to isolate the *Klebsiella pneumoniae* from water samples collected from different farms and nearby water bodies of Tirupati. Out of 112 water samples collected from different sources, 16 (14.3%) isolates from water samples were positive for *Klebsiella pneumoniae* by cultural isolation and conducting biochemical tests. The study found that all the 16 isolates were found positive for *K. pneumoniae* by PCR targeting for *rpoB* gene, which encodes for RNA polymerase B. Biofilm producing ability of *K. pneumoniae* was detected by Congo red agar method. In this study, out of 16 water isolates, five (31.2%) isolates were found as biofilm producers. Among 16 *K. pneumoniae* isolates from water samples, maximum resistance was observed for azithromycin (100%), ampicillin (87.5%), followed by cefazolin (56.3%), amoxyclav (37.5%), cefotaxime (12.5%) and tetracycline (6.3%). This study shows that water sources were contaminated with *K. pneumoniae* and anti-microbial resistance is a serious public health concern. In this study almost all samples were resistant to azithromycin and ampicillin which may implies to treatment failures in community acquired pneumoniae caused by *K. pneumoniae*.

Keywords: *Klebsiella pneumoniae*, trypticase soya broth, MacConkey-inositol-carbenicillin agar, IMVC tests, RNA polymerase B, *rpoB* gene, Congored agar, muller hinton agar

Introduction
K. pneumoniae is a gamma proteo bacterium, belonging to the family *Enterobacteriaceae*. *K. pneumoniae* is a Gram-negative, facultative anaerobic, encapsulated and non-motile rod-shaped bacteria. *K. pneumoniae* can colonize in wide range of animal hosts, but can also be found in association with plants, soil, water and drains. *K. pneumoniae* colonizes a diversity of body sites including the respiratory tract, gut, nasopharynx, oropharynx, and skin (Bagley, 1985; Podschun and Ullmann, 1998) [5, 13]. It is considered as an opportunistic pathogen, with the majority of infections such as urinary tract infections (UTI), pneumonia and wound or soft-tissue infections in neonates, elderly, and immunocompromised individuals and is the most frequent cause of hospital-acquired infections (Podschun and Ullmann, 1998) [13].

2. Materials and methods
In the present study, a total of 112 water samples were collected in and around Tirupati, Andhra Pradesh. The water samples were inoculated into trypticase soya broth and incubated aerobically for 24h at 37 ℃ (Garedew *et al.*, 2012) [7]. A loopful of inoculum from tripticase soya broth was streaked on MacConkey-inositol-carbenicillin agar and incubated aerobically at 24-48h at 37 ℃ (Hameed, 2017) [8]. Pink, mucoid colonies produced in Mac Conkey-inositol-carbenicillin agar were considered as *Klebsiella* positive (Figure-1). Gram's staining was performed to differentiate Gram negative bacteria from Gram positive bacteria (Figure-2). Capsular staining was performed to differentiate *Klebsiella* from other Gram negative bacteria. In capsular staining, *Klebsiella* isolates showed a refractile zone surrounding the bacterial cell (Figure-3). A battery of biochemical tests such as catalase test (Figure-4), oxidase test (Figure-5), IMVC tests (Figure-7), Triple sugar iron test (Figure-8), Nitrate reductase test (Figure-9), and Urease test (Figure-10) were performed for the confirmation of *Klebsiella pneumoniae* in conventional method. For molecular level confirmation of *K. pneumoniae*, DNA isolation followed by PCR targeting for *rpoB* gene with amplicon size of 108 bp was performed (Figure-11; Table-1, 2 and 3). Biofilm producing ability of *K. pneumoniae* isolates was tested by congored agar method (Figure-12). Antibiotic sensitivity test was performed for all *K. pneumoniae* isolates by Kirby Baur disc diffusion method using Muller Hinton agar (Figure-13, 14 and 15; Table-4).

Table 1: Details of species specific oligonucleotide primers used in this study

Target gene	Primer sequence 5'-3'	Amplicon size (bp)	Reference
rpoB	ATCAACCGAGATTCCCCCAGT	108	Suresh *et al.* (2020)[17]
	TCACTATCGGTCAGTCAGGAG		

Table 2: Optimized reaction mixture for PCR targeting species specific gene (*rpoB)* of *K. pneumonaie*

Components	Volume (µl)/ reaction
PCR Master mix (2X)	12.5
Forward primer (10 pmol/µl)	1.0
Reverse primer (10 pmol/µl)	1.0
Template DNA	2.0
Nuclease free water	8.5
Total	25

Table 3: Standardized thermal cycling conditions for specific specific gene (*rpoB*) of *K. pneumoniae*

Steps	Standardized conditions	No. of cycles
Initial denaturation	94 °C for 3 min	1
Denaturation	94 °C for 30 sec	
Primer annealing	60 °C for 30 sec	30
Chain extension	72 °C for 30 sec	
Final extension	72 °C for 7 min	1
Hold/ Stand by	4 °C	

3. Results and Discussion

The study reported that out of 112 water samples collected from different sources, 16 (14.3%) isolates from water samples were positive for catalase, Voges-Proskauer test, citrate utilization test, triple sugar iron test, nitrate reduction test, urease test and negative for oxidase test, motility test, indole test and methyl red test. This kind of biochemical test reactions is the confirmatory for *K. pneumoniae.* In this study, a 14.3% prevalence of *K. pneumoniae* was identified in water samples collected from different farms and slaughterhouses. An almost similar finding was reported by Chaudhry *et al.* (2020) [6] (10.7%) from slaughterhouse wastewater.

Biofilm producing ability of *K. pneumoniae* was detected by Congo red agar method. In this study, out of 16 water isolates, five (31.2%) isolates were found as biofilm producers. Compared to the present study, higher per cent biofilm-producing isolates were reported by Niveditha *et al.* (2012) [11] (62.5%), Ammar *et al.* (2020) [3] (81.8%) and Preethi *et al.* (2021) [14] (67.3%) while lower per cent of biofilm producers were reported by Ruchi *et al.* (2015) [15] who have recorded 16.7%.

Among 16 *K. pneumoniae* isolates detected from water samples, maximum resistance was observed for azithromycin (100%) and ampicillin (87.5%). Compared to the present study, Ahmed *et al.* (2020) [1] recorded 65% of the resistance. The patients who are not following entire treatment regime leads to development of azithromycin resistance in gut bacteria. The other factor in concern is transfer of azithromycin resistant genes from one bacterial colonies with other bacterial colonies through R-plasmids during conjugation and environmental uptake of azithromycin resistant genes by the transformation process (Huddleston, 2014) [9]. Compared to the present study, higher ampicillin resistance among the isolates of *K. pneumoniae was* observed with the finding of Al-Shara *et al.* (2011) [2], Imtiaz *et al.* (2021) [10], Patilaya *et al.* (2019) [12], who have recorded 91.3%, 94%, and 95% resistance, respectively. Compared to the present study, lower resistance was reported by Soroush and Ghane (2017) [16] and Ayatollahi *et al.* (2020) [4] with 69.2%, and 75% resistance, respectively. Ampicillin is the broad-spectrum antibiotic and is widely used as the treatment of choice for various diseases like mastitis, urinary tract infection in cattle caused by *K. pneumoniae* and also used for *E.coli* and *Proteus* infections. Ampicillin-resistant genes of *E. coli* and *Proteus* may be transferred to the *K. pneumoniae* by transformation (Huddleston, 2014) [9].

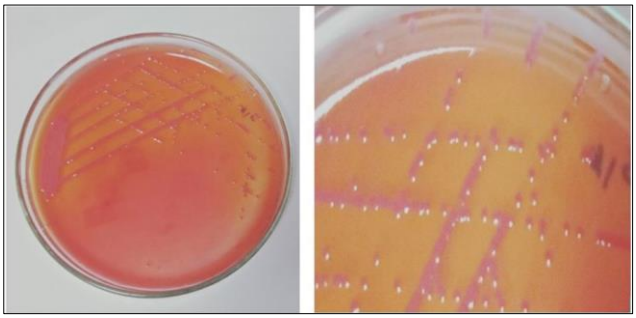

Fig 1: Plate showing growth of pink, mucoid colonies of *Klebsiella* on MacConkey-inositol-carbenicillin agar

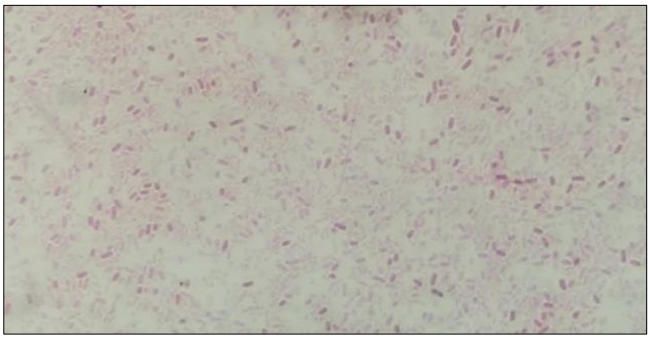

Fig 2: Gram's staining showing characteristic Gram-negative, straight, short rod-shaped bacteria indication of *Klebsiella* spp.

Fig 3: Figure showing capsule as refractile zone surrounding a cell indicative of *Klebsiella* species

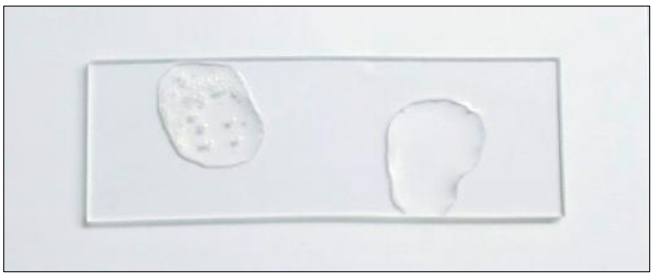

Fig 4: Figure showing positive catalase reaction for *Klebsiella* species

Left side: Catalase Positive Right side: Negative Control

Fig 5: Figure showing negative oxidase reaction for *Klebsiella* species

Left side: Oxidase Negative
Right side: Positive Control (*Pseudomonas* culture)

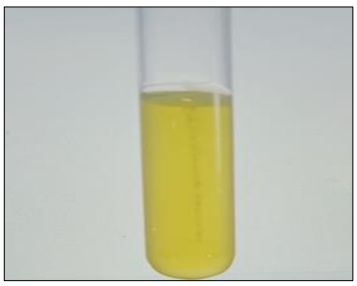

Fig 6: Figure showing *Klebsiella* species growth at the site of inoculation in nutrient agar indicative of non-motile nature of te bacteria.

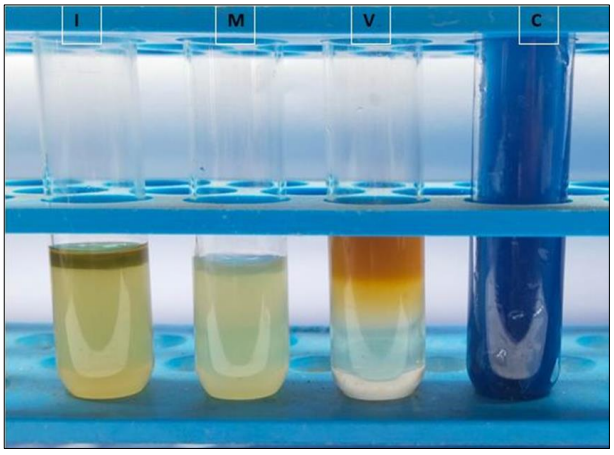

Fig 7: IMViC test showing "-, -, +, +" reaction for *Klebsiella pneumoniae*
I: Indole M: Methyl red;
V: Voges Prosquor; C: Citrate utilization test

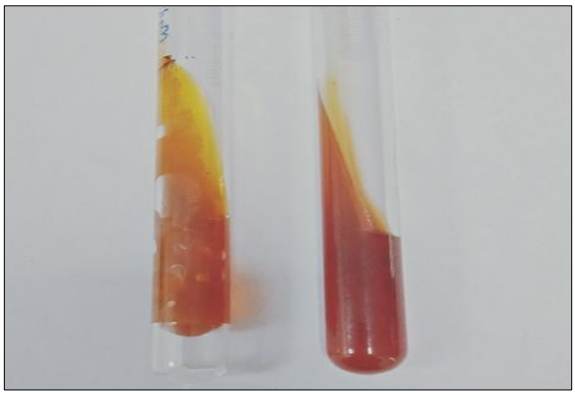

Fig 8: Figure showing positive TSI reaction of *Klebsiella species*

Tube 1: TSI positive (Yellow slant, Yellow butt with production of gas)
Tube 2: Negative control

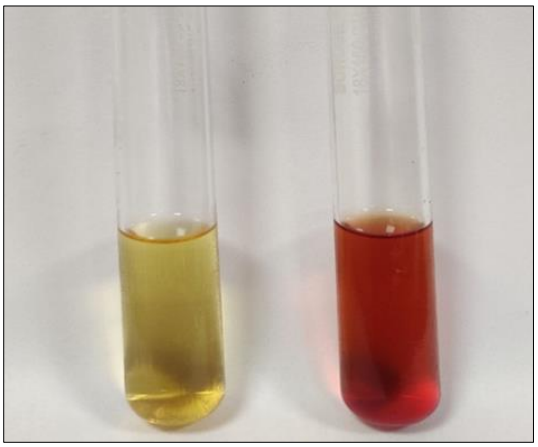

Fig 9: Figure showing positive nitrate reduction test reaction for *Klebsiella* species

Tube 1: Nitrate Positive, Tube 2: Negative Control

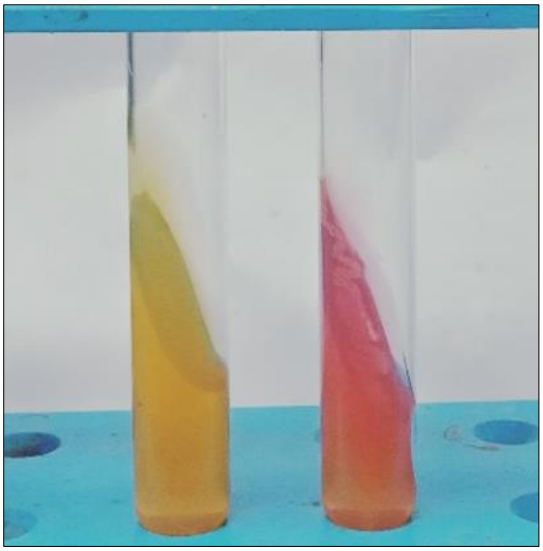

Fig 10: Figure showing positive urease test reaction for *Klebsiella* species.

Tube 1: Urease Positive, Tube 2: Negative Control

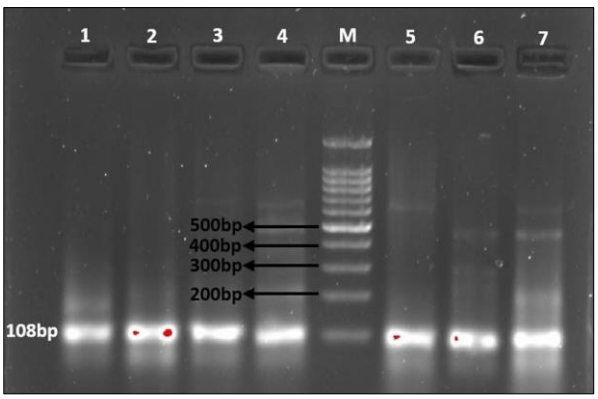

Fig 11: Figure showing amplified *rpoB* gene of *K. pneumoniae* by PCR

Lane M : Molecular weight marker (100 bp)

Lane 4: Positive control for *rpoB* gene (108 bp) (*K. pneumoniae* ATCC 700603)
Lane 1, 2, 3, 5, 6, and 7: water samples showing positive result for *rpoB* gene

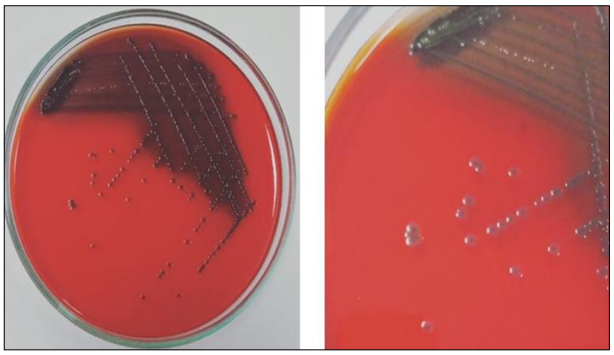

Fig 12: Figure showing biofilm production by *Klebsiella pneumoniae* on Congo red agar

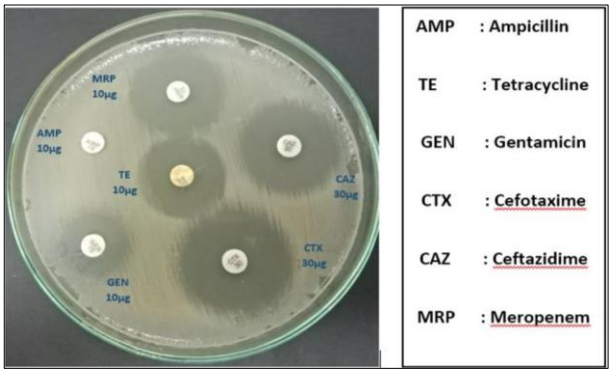

Fig 13: Antibiotic resistance patterns of the *K. pneumoniae* isolates

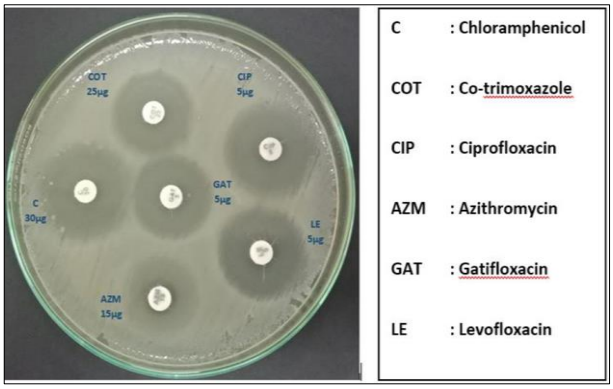

Fig 14: Antibiotic resistance patterns of the *K. pneumoniae* isolates

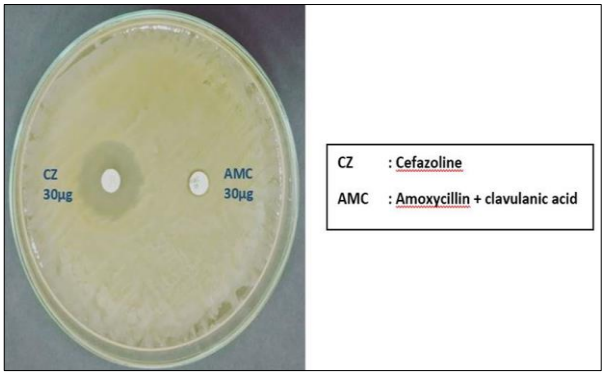

Fig 15: Antibiotic resistance patterns of the *K. pneumoniae* isolates

Table 4: Antibiogram pattern of *Klebsiella pneumoniae* isolates

S. No.	Antimicrobial agent	% of resistance	% of intermediate resistance	% of sensitivity
1	Ampicillin	87.5	0	12.5
2	Cefazolin	56	37.5	6.25
3	Cefotaxime	12.5	31.5	56
4	Ceftazidime	0	0	100
5	Meropenem	0	0	100
6	Amoxyclav	37.5	6.5	56
7	Azithromycin	100	-	0
8	Chloramphenicol	0	12.5	87.5
9	Ciprofloxacin	0	0	100
10	Cotrimoxazole	0	0	100
11	Gatifloxacin	0	0	100
12	Levofloxacin	0	0	100
13	Tetracycline	6	6.5	87.5
14	Gentamicin	0	0	100

4. Conclusion

In conclusion, *Klebsiella pneumoniae* detection in farm waste water and other drinking water sources may pose risk to farm workers and general public. Biofilm forming ability of *K. pneumoniae* may be the reason for community acquired pneumonia. This physical property may give a supreme power to gain more multi drug resistance, to evade host immune response, and to protect itself from environmental stress. In this present study, ampicillin and azithromycin resistance were recorded in almost all *K. pneumoniae* isolates. This study shows the antibiotic resistant *K. pneumoniae* in water sources can create a havoc in public health and causation for multidrug resistance in other Gram negative bacteria through transformation mechanism.

5. Acknowledgement

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