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Research Article

Zoonotic Risk to Human Health

An Investigation of Zoonotic Bacterial Pathogens Associated with Rodents in Rural Areas of Nellore, India

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Abstract: Rodent species are known to harbour and host various zoonotic pathogens, including bacterial, viral, fungal, and protozoal species. Several investigations proved that commensal rats (*Rattus* spp.) are potential to transmit drug-resistant and hyper-virulent bacterial pathogens to humans. India's rapid urbanization and developmental activities facilitated rats to live near the human population. However, few information was known about bacterial species associated with rodents and their role in zoonotic risk to humans in India. The present study aimed to (i) investigate the presence of bacterial pathogens associated with rodents and (ii) infer the prevalence and diversity of potential bacterial pathogens in Nellore district, India. Bacterial prevalence was determined by isolation and identification techniques. The isolated bacterial cultures were submitted for phenotypic observation, biochemical identification using the VITEK 2 compact automated system, and molecular detection by DNA extraction and amplification of the 16S rRNA gene. A diversified bacterial community belonging to 14 species was detected from all collected animals. Bacterial species' prevalence was comparatively higher in black rats (n=66) than brown rats (n=27). 46 rats out of 93 were found to be positive (49.4%) for bacterial presence. A significant variation was found in the prevalence of bacterial species between both rodent species. The highest bacterial prevalence was recorded for *Bacillus* spp. (36%) followed by *E. coli* (29%). The prevalence of *Klebsiella pneumoniae* was found as 17%, of which 18% in black rats and 14% in brown rats. *Listeria* spp.'s prevalence was 23.6%, but a higher prevalence was observed in black rats (25.7%). Surprisingly, an uncommon pathogen, *Sphingomonas paucimobilis*, was detected in both rodent species. These results suggest that *Rattus* rats in Nellore were suspected to be potential carriers of transmitting zoonotic bacterial species to humans.

Keywords: Rodent-borne zoonosis, Bacterial pathogens, Zoonotic diseases, *Rattus* rats, Bacterial prevalence

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1. INTRODUCTION

Zoonotic diseases have impacted public health globally, with an increasing proportion of emerging infectious diseases over many years. The World Health Organisation (WHO) reported that 60% of zoonotic origin of all human infections and 75% of all emerging diseases were transmitted from animal origin[1,2]. A wide range of animal species are involved and responsible for transmitting diseases to humans. The frequency of contact between animal reservoirs and humans is the key factor of zoonotic transmission. The transmission may also be occurred by other factors, including arthropod vectors and animal excretes[3]. Rodents are widespread and represent the second-largest mammalian group on Earth. Rodents are well-known species to host and spread zoonotic pathogens, including bacteria, viruses, protozoa, fungi, and rickettsia. The major rodent bacterial pathogens were reported, including *Ehrlichia, Coxiella, Anaplasma, Leptospira, Bartonella, Borrelia, Francisella,* and *Rickettsia*[3]. Rodents were also known to carry *Salmonella, Campylobacter*, and *Listeria* spp[4]. The events of rapid urbanization constitute rodent adaptation to urban environments; specifically, the Norwegian and black rats (*Rattus* spp.) live near humans[2]. Major zoonotic diseases reported in India were rabies, cysticercosis, leptospirosis, brucellosis, scrub typhus, toxoplasmosis, trypanosomiasis, and Crimean-Congo haemorrhagic fever[5]. However, little was known about rodent-associated pathogens and their role in zoonosis in India. The plague outbreak in Himachal Pradesh in 2002 was due to the unhygienic lifestyle and hunting practices on rodents and treatment practices through faith healers[6]. The delay in initiating effective treatment of plague cases was a major factor that led to the spread of the disease[7]. This report explained the unawareness of people residing in remote areas and the lack of surveys on rodent-borne pathogens. Balakrishnan *et al.* (2008) suggested that zoonotic agents, especially *Bartonella* spp. were the prevalent causative organisms of blood culture–negative endocarditis in India and recommended serologic screening for antibodies to zoonotic microorganisms as diagnostic tools for this disease in India[7]. A high prevalence of leptospirosis in rodents in Mumbai and the southern part of India (Kerala and Tamil Nadu) was observed, proving these animals' possible role in transmitting leptospirosis to humans. Hence, it is imperative to design necessary control measures to prevent human leptospirosis[8]. The zoonotic risks associated with *R. norvegicus* in wetland agroecosystems such as Kerala cannot be ignored in the wake of emerging zoonotic and fungal potent carriers of dermatophytes and other opportunistic fungi[9]. Therefore, the present study aimed to investigate bacterial pathogens' presence, prevalence, and diversity in commensal rats and the zoonotic risk of humans living in the surrounding rural areas of Nellore City in India.

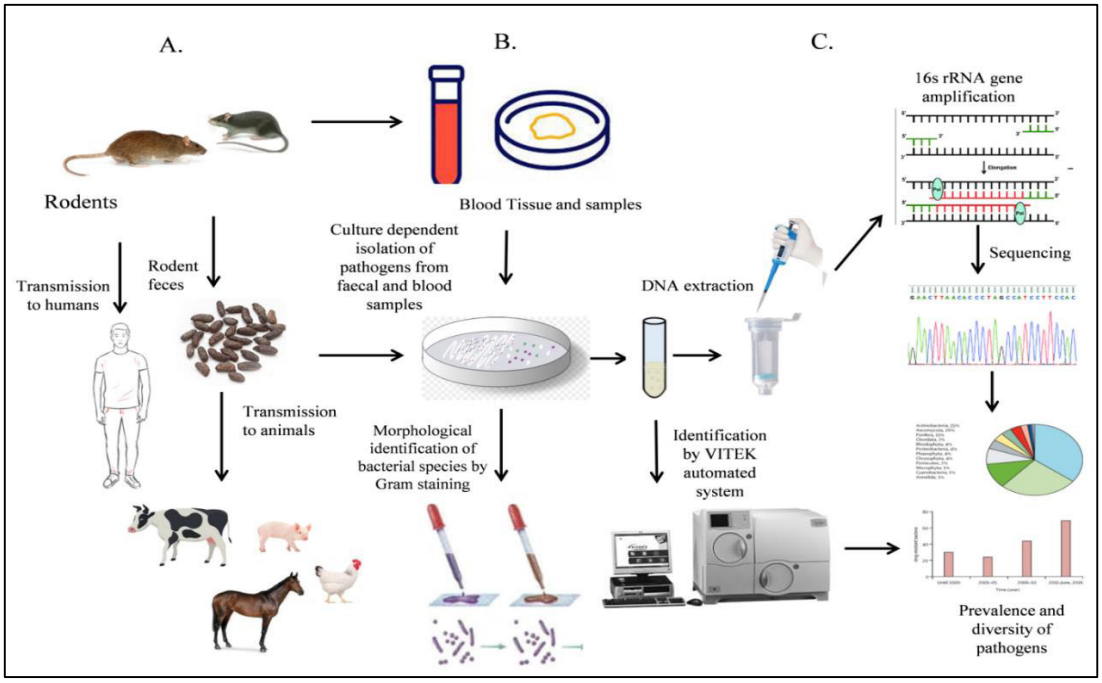

Fig 1: Representation of the overall study design in the flowchart. (A): Rodents as potential zoonotic carriers and transmit pathogens to humans and animals by direct or indirect contact. (B): Culture-dependent isolation of possible bacterial pathogens from rodent blood, tissue, and faecal samples. C): Biochemical identification of bacterial pathogens by VITEK 2 compact system, inferring their prevalence and diversity.

2. MATERIALS AND METHODS

2.1. *Animal Ethical Committee Approval*

All experiment was carried out by the guidelines of the Committee for Control and Supervision on Experiments on Animals, Government of India (CPCSEA 2003) and approved by the Institutional Animal Ethical Committee (1837/PO/RcBiBt/S/15CPCSEA).

2.2. *Rodent Trapping and Sample Collection*

The present study was conducted in rural areas of the Nellore district associated well with agricultural activities, including different varieties of crop fields. Rats were captured in 4 villages (Figure 2), including Allur, Muttukur, Kodavalur, and Golagamudi, around Nellore town in southern India between 2018 and 2022. The areas of animal trapping include households, crop fields, and village dumps. Animals were trapped alive using locally available mesh-made traps

(LxWxH; 24x12x10 cm) and brought to a laboratory at the Department of Biotechnology, Vikrama Simhapuri University, Nellore, India. Animals were identified morphologically to gender and species level[3] and anesthetized with 0.1 mL of Ketamine+Xylazine (9:1) per 100 g of body weight[10]. Blood samples were collected aseptically by cardiac puncture in sterile EDTA-containing tubes. Then the rats were euthanized by cervical dislocation, and tissue samples, including heart and liver, were collected. A loopful of the faecal sample was also collected from the rectum of each animal. The overall work of the study is represented by a schematic workflow (Figure 1), and the specimens used are mentioned in Table 1.

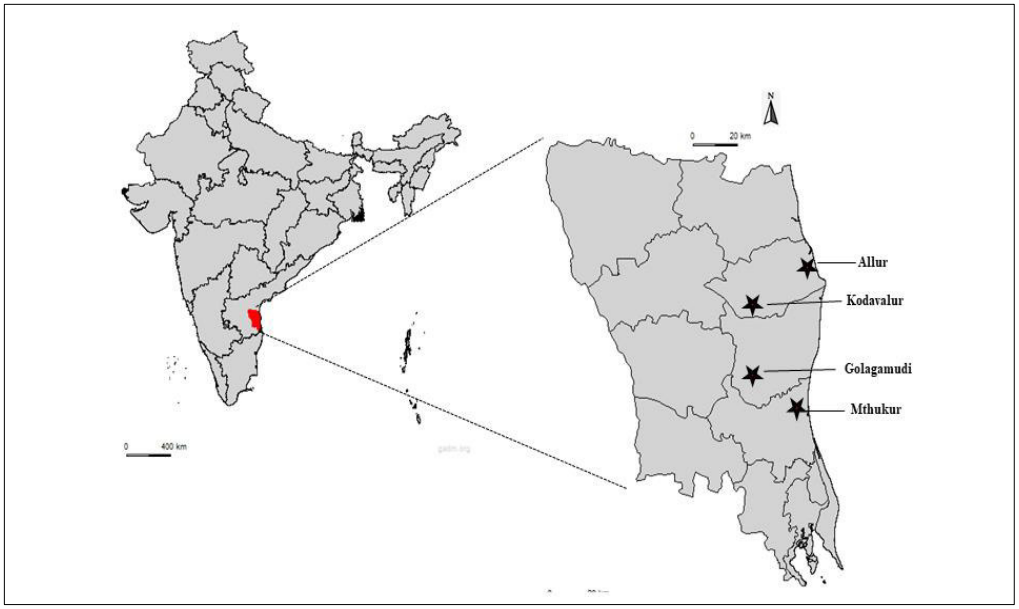

Fig 2. Map of Nellore district indicating the location of the villages where the animals were captured.

2.3. *Isolation of Bacteria*

The collected tissue samples were homogenized (25 to 30 mg of each tissue) using a sterile mortar and pestle in 200 µl of PBS buffer[11]. One-hundred microliters of homogenized solution and 100 µl of whole blood samples were inoculated onto blood agar plates (HiMedia, India) from each sample and incubated at 37°C for 24 to 48 hours. The collected faecal samples were inoculated into the nutrient broth (NB) and incubated aerobically at 37°C for 24 to 48 h. Then, a loopful of resultant growth in NB was streaked onto MacConkey agar and nutrient agar plates and incubated as mentioned above. After incubation, single colonies were picked up from each plate, inoculated onto new MacConkey agar, blood agar, and nutrient agar plates, and incubated under the same conditions[4].

2.4. *Biochemical Characterization of Bacteria*

The isolated bacterial cultures were subjected to Gram-staining according to the standard procedure. All bacterial isolates were identified by biochemical analysis with VITEK 2 compact automated system (BioMérieux, India) using GN and GP cards following the manufacturer's instructions[12,13].

2.5. *Molecular Detection*

2.5.1. *Nucleic Acid Extraction*

Genomic DNA was extracted from all bacterial isolates using QIAamp UCP Pathogen Mini Kit (Qiagen, India) according to the manufacturer's instructions[14]. Briefly, 1 ml (2 x 10^9/ml) of overnight grown cultures were added to a 2 ml centrifuge tube and centrifuged at 14,000 x g for 5 min. Then, the supernatant was discarded, and the pellet was suspended with 400 µl of ATL buffer. The suspended pellet was treated with 40 µl Proteinase K, and 200 µl of APL2 buffer was added to the tube and mixed by pulse vortex for 30 s and incubated at 70°C for 10 min. After incubation, 300 µl of ethanol was added to the content. The mixture was vortexed and transferred to UCP mini spin column placed in a 2 ml collection tube followed by centrifugation at 6000 x g (8000 rpm) for 1 min. The column was then washed with 600 µl APW 1 and with APW 2, followed by centrifugation each step at 8000 for 1 min and 14000 rpm for 3 min, respectively. DNA was eluted to a new collection tube by adding100 µl of AVE buffer to the centre of the column, followed by centrifugation for 1 min at 20,000 x g (14000 rpm). The concentration and quality of the purified DNA were checked with NanoDrop-2048 (Microvolume UV-Vis Spectrophotometer, Thermo Scientific, India).

2.5.2. *PCR Amplification of 16S rDNA and Sequencing*

The extracted genomic DNA was diluted to 50 to 70 ng/µl with nuclease-free water for PCR amplification. The 16S rRNA gene was amplified using bacterial universal primers, including P8 and Pc1544[15]. The PCR conditions were 93°C for 5 min (initial denaturation) and 30 cycles of each consisting of 93°C for 40 s (denaturation), 58°C for 1 min (annealing), extension at 72°C for 1 min, and final extension at 72°C for 7 min. Then the PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, India) following the manufacturer's instructions. Amplified DNA was sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Whitefield-Bangalore, India). The sequences obtained were searched against the nucleotide database in BLAST (https://blast.ncbi.nlm.nih.gov) for taxonomic detection[14].

3. RESULTS

3.1. Rodent Species and Bacterial Isolates

A total of 93 rats were collected from all the selected areas, of which 57 were male, 36 were female rats, 66 were black rats (*Rattus rattus*), and 27 were brown rats (*Rattus norvegicus*). The number of rodents trapped in different villages is given in Table 2.

Table 1. Bacterial species isolated from blood, tissue, and faecal samples of rats

S.No.	Bacteria species	No. of positive samples / No. of samples tested				Total number of rats positive (%) (n=93)
		Blood	Heart	Liver	Faecal	
1	*Escherichia coli*	15/93	7/93	2/93	24/93	27 (29)
2	*Yersinia enterocolitica*	7/93	0/93	0/93	11/93	12 (13)
3	*Klebsiella pneumoniae*	2/93	5/93	0/93	13/93	16 (17)
4	*Pseudomonas oryzihabitans*	9/93	4/93	0/93	0/93	12 (13)
5	*Pseudomonas aeruginosa*	14/93	7/93	0/93	4/93	17 (13)
6	*Sphingomonas paucimobilis*	12/93	2/93	8/93	0/93	16 (17)
7	*Serratia plymuthica*	9/93	3/93	0/93	6/93	12 (13)
8	*Proteus mirabilis*	3/93	0/93	0/93	11/93	12 (13)
9	*Bacillus* spp.	22/93	9/93	14/93	28/93	34 (36)
10	*Clostridium* spp.	10/93	0/93	2/93	6/93	11 (12)
11	*Staphylococcus arlettae*	2/93	0/93	0/93	6/93	6 (6.5)
12	*Staphylococcus aureus*	14/93	0/93	2/93	12/93	19 (21)
13	*Alcaligenes faecalis*	3/93	0/93	0/93	16/93	16 (17)
14	*Listeria* spp.	15/93	2/93	7/93	13/93	22 (23.6)

Table 2. Prevalence and distribution of pathogenic bacterial agents identified from rats and their captured sites.

Bacterial species	*Rattus rattus* (Black rats)				*Rattus norvegicus* (Brown rats)				Total Prevalence (%)
	Village 1 (n=19)	Village 2 (n=13)	Village 3 (n=22)	Village 4 (n=12)	Village 1 (n=9)	Village 2 (n=4)	Village 3 (n=8)	Village 4 (n=6)	
Escherichia coli	6	4	8	3	0	2	3	1	27 (29.03)
Yersinia enterocolitica	6	0	3	0	0	3	0	0	12 (12.9)
Klebsiella pneumoniae	3	2	3	4	0	1	0	3	16 (17.2)
Pseudomonas oryzihabitans	2	0	7	1	0	0	1	1	12 (12.9)
Pseudomonas aeruginosa	3	2	8	2	0	1	0	1	17 (18.3)
Sphingomonas paucimobilis	3	0	1	0	0	0	2	0	6 (6.5)
Serratia plymuthica	2	1	5	0	0	3	0	1	12 (12.9)
Proteus mirabilis	2	2	6	0	1	0	0	1	12 (12.9)
Bacillus spp.	7	4	12	1	3	4	1	2	34 (36)
Clostridium spp.	1	3	5	0	1	1	0	0	11 (12)
Staphylococcus arlettae	1	2	0	2	0	1	0	0	6 (6.5)
Staphylococcus aureus	4	4	6	1	2	1	0	1	19 (21)
Alcaligenes faecalis	3	2	8	0	2	0	1	0	16 (17)
Listeria spp.	4	4	6	3	1	1	2	1	22 (23.6)

Village 1-Allur, Village 2-Kodavalur, Village 3-Golagamudi, Village 4-Muthukur.

3.2. VITEK 2 Detection and Molecular Identification of Bacterial Cultures

About 46 rats out of 93 were found to be positive (49.4%) for bacterial presence. A wide range of bacterial cultures with different colony morphologies were isolated from the trapped animals. After Gram-staining of pure cultures, phenotypic observation yielded Gram-positive and Gram-negative bacterial isolates with different shapes, including cocci and rods. VITEK 2 Compact automated system detected 93% of total isolates to species level with 87% to 98% probability (Table 3). Further, molecular identification by sequencing of 16S rDNA gene from all isolated bacterial cultures identified to species level except the genera *Bacillus*, *Clostridium*, and *Listeria* (Table 1). The similarities of BLAST hits from the nucleotide database against the isolates ranged from 97.4% to 100 % (Table 3). The positive rate and percentage of prevalence of bacterial species varied from 6.5 to 29% between black rats and brown rats and between the villages. The number of animals positive for various bacterial species across the villages is given in Table 2.

3.3. Bacterial Diversity and Prevalence

Based on biochemical and molecular identification, all the bacterial communities belonging to 14 species were identified from all positive rodents. The Gram-negative bacterial species, including *Escherichia coli, Yersinia enterocolitica, Klebsiella pneumoniae, Pseudomonas oryzihabitans, Pseudomonas aeruginosa*, *Sphingomonas paucimobilis*, *Serratia plymuthica*, *Porteous mirabilis,* and *Alcaligenes faecalis* were identified in the collected animals. Similarly, Gram-positive bacteria such as *Staphylococcus aureus, Bacillus* spp., *Clostridium* spp., *Staphylococcus* Arlette, and *Listeria* spp. were detected in the rats. Bacterial species' prevalence was comparatively higher in black rats (n=66) than brown rats (n=27). The highest prevalence rate was recorded for *Bacillus* spp., with 34% positivity, followed by *E. coli*, with 29% positivity in the studied animals. The prevalence of *Bacillus* spp. and *E. coli* was 36% and 31% in black rats and 37% and 27% in brown rats, respectively (Table 1).

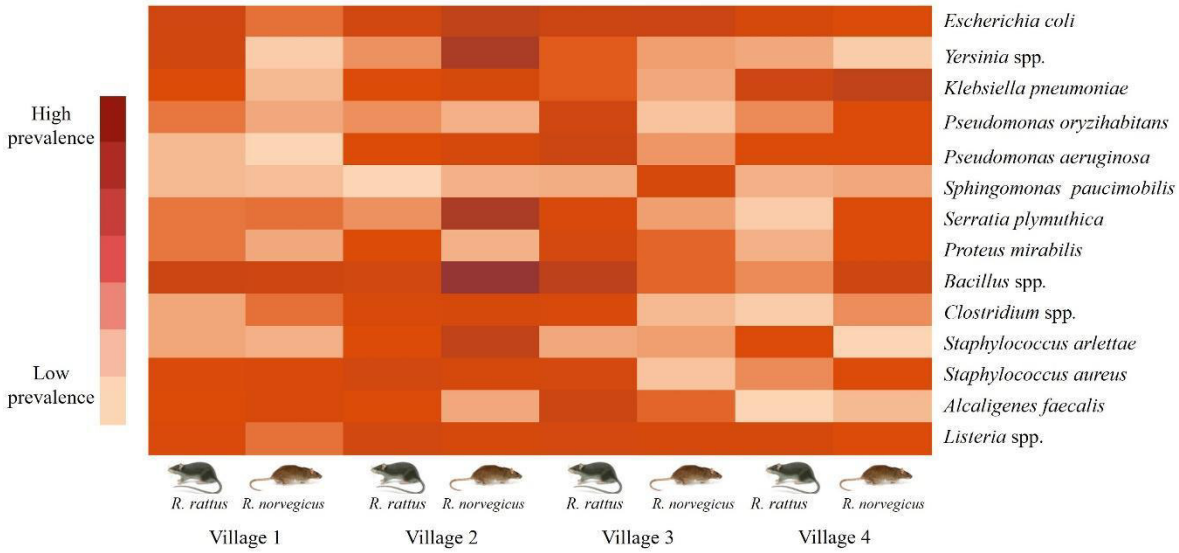

Fig 3. Heatmap of the prevalence of bacterial species identified by VITEK 2 automated system and 16S rDNA sequencing in rodent species collected from rural villages. Low relative prevalence is represented in light colour, and those with high prevalence are represented in dark colour.

The other bacterial pathogens, including *Staphylococcus aureus* (21%), *Klebsiella pneumoniae* (17%), and *Yersinia enterocolitica* (13%), were also detected with significant prevalence rates (Figure 4). The overall prevalence of bacterial species was comparatively higher in black rats (39.3%) than in Norway rats (37%), except for *Bacillus* spp. Surprisingly, we also detected a rare and uncommon pathogen, *Sphingomonas paucimobilis*, and a Gram-positive coccus and coagulase-negative *S. arlettae*, which were not reported in rodents anywhere else previously. A high prevalence of *Listeria* spp. (23.6%) was also noticed from both rodent species from all the villages. The prevalence of all bacterial species was also represented in the heat map based on the positive percentages of animals (Figure 3). Co-infections with multiple bacterial species were also observed in rodents with at least two pathogens in each animal. Overall, we observed the presence of pathogens in rats collected from all villages with at least one organism in each rat.

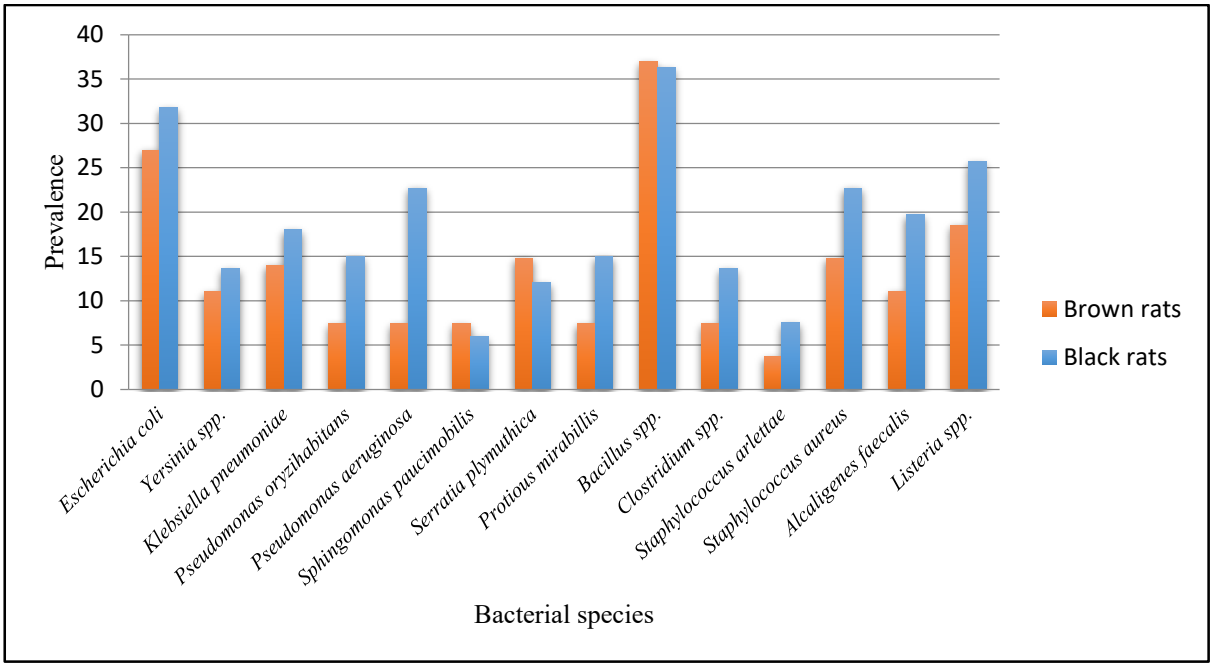

Fig 4. Prevalence of bacterial pathogens in the trapped animals.

Table 3. Overview of the identification methods and the similarity percentages with the database

S.No	Bacterial species	Detection method & % similarity	
		VITEK 2 compact system (Probability)	PCR and 16S rRNA gene sequencing
	Gram-positive bacteria		
1.	*Bacillus* spp.	91 – 97	97.8 – 98.5
2.	*Clostridium* spp.	93 – 97	97. 6 – 99.1
3.	*Staphylococcus arlettae*	91 – 95	98.4 – 99.7
4.	*Staphylococcus aureus*	94 – 97	99.1 – 99.8
5.	*Alcaligenes faecalis*	UI	98.4 – 99.5
6.	*Listeria* spp.	UI	97.4 – 99.2
	Gram-negative bacteria		
7.	*Escherichia coli*	93 – 98	98 – 100
8.	*Yersinia enterocolitica*	91- 97	98 – 99.6
9.	*Klebsiella pneumoniae*	93-97	98.8 – 99.8
10.	*Pseudomonas oryzihabitans*	87- 91	97.6 – 99.8
11.	*Sphingomonas paucimobilis*	92- 98	97.5 – 99.4
12.	*Serratia plymuthica*	91- 94	97.4 – 98.5
13.	*Pseudomonas aeruginosa*	92 – 96	97.4 – 99.4
14.	*Proteus mirabilis*	87 – 94	98.2 – 99.9

UI-Unidentified

4. DISCUSSION

Rodents are distributed and exist in both urban and rural environments in large populations. Commensal rats (*Rattus* spp.) are commonly adopted to live near humans and spread infectious pathogens from contaminated environments and spoiled foods[16]. Both roof black rats (*Rattus rattus*) and brown Norway rats (*Rattus norvegicus*) were important in spreading zoonotic diseases[17]. In this study, we investigated the presence of rodent-associated bacterial pathogens in both black and brown rats. The study results revealed rodents harboured various potential human pathogenic bacterial species. We described the presence of 14 different bacterial species, such as *E. coli, Y. enterocolitica, Klebsiella pneumonia, Listeria* spp., *S. aureus, P. aeruginosa*, *P. oryzihabitans*, and *P. mirabilis* including an uncommon pathogen *S. paucimobilis* from rats in rural villages of Nellore district. *E. coli* is generally treated as a commonly present gut microflora in animals and humans and is sometimes responsible for gastrointestinal disruption. However, *E. coli* (serotype O157:H7) has emerged as a foodborne and zoonotic pathogen and has been reported to cause haemolytic uremic syndrome and haemorrhagic colitis[18]. This strain was isolated from wild rodents[19] and reported to have emerged as a multidrug-resistant organism with an extended spectrum of β-lactamases (ESBLs), representing a major threat to public health[20]. *K. pneumoniae* was considered an important nosocomial pathogen that causes various human infections, including pneumonia, sepsis, urinary tract, and abdominal infections[21]. This bacterium causes human diseases and is reported to cause infection in animals, including cattle, dogs, cats, and horses[22]. In humans, *K. pneumoniae* was reportedly present in the nasopharynx and intestine as a saprophyte and rarely found on the skin. Several reports were also made about *K. pneumonia* emergence as a multidrug-resistant pathogen against carbapenemases or

extended-spectrum of β-lactamases (ESBL)[23]. Zhong *et al.* (2020) reported that rodent species harbouring hypervirulent *K. pneumoniae* (hvKPs) strains with multidrug resistance against an extended spectrum of β-lactamases (ESBL). The prevalence of ESBL-carrying hvKPs was 7.94% in rodents, 12.79% in shrews, and 17% in humans. It was suggested that urban rodents could transmit drug-resistant *K. pneumoniae* to humans[24]. Our findings in this study suggested that 16% of rodents were carrying *K. pneumoniae. P. aeruginosa* was an important nosocomial pathogen responsible for serious acute and chronic infections with notable morbidity[25]. Infections of this pathogen were documented with high mortality in patients with cystic fibrosis, cancer, and severe burns[26]. Moreover, this pathogen is incriminated to cause septicaemia, urinary tract, and surgical wound infections[27,28]. This species was distributed widely in nature, colonizing different ecological niches in soil and water. *P. aeruginosa* was reported to survive in a broad range of hosts, including aquatic, terrestrial animals, plants, and humans[29]. Antibiotic-resistant strains of this pathogen were isolated from rodent species against many drugs with a 34.7% positive rate. The rate of antimicrobial resistance was found to be high in *Rattus* rats[30]. Our study elucidated a high prevalence of *P. aeruginosa* in black rats (22.7%) than in Norway rats (7.4%). A total of 13% of animals were positive for the presence of *P. aeruginosa* in our study (Table 1). *P. oryzihabitans* was another important nosocomial pathogen in the genus of *Pseudomonas* that we isolated in our study. This organism was considered to cause rare human infections, especially sepsis in children, bacteraemia in pathogen immunocompromised patients, and catheter-related infections[31]. However, *P. oryzihabitans* has become an important nosocomial pathogen with frequent case reports[32]. *Staphylococcus* spp. was ubiquitous and colonized in humans and animals[33]. This genus consists of pathogenic species, majorly *S. aureus*, and *S. epidermidis*, and cause diverse infections in humans ranging from minor food poisoning, skin and soft tissue infections to life-threatening bacteraemia and septicaemia[34], pneumonia, osteomyelitis, and endocarditis[35]. *S. aureus* was recognized as an asymptomatic colonizer of human nares and caused skin and soft tissue infections[36]. Though it was not found to cause invasive infections in most colonized individuals, it was reported to cause bloodstream infections in most infected individuals. It was also found to cause heart diseases, renal infections, and diabetes[37]. *S. aureus* was resistant to methicillin in wild and domestic animals and humans[38]. Besides the domestic animals and humans, methicillin-resistant *S. aureus* (MRSA) was isolated from rats [39,40]. The MRSA strains isolated from wild rats resembled strains associated with livestock, while the urban rats were found to carry the MRSA strains, which were prevalent in the human population[41]. In this study, we identified the presence of *S. arlettae* and *S. aureus* in rats collected in the populated rural areas of Nellore district. *S. arlettae* is a coagulase-negative organism isolated from the nares of the poultry goat. This species emerged as an important antibiotic-resistant pathogen to many β-lactam antibiotics[42]. This is the first report on isolating *S. arlettae* from rats and detecting bacterial pathogens from rodents. We also isolated an uncommon and rare pathogen, *Sphingomonas paucimobilis*, from the rats for the first time. It was found to be an opportunistic pathogen, which rarely infects hospitalised patients[43]. This organism now emerged as an important nosocomial pathogen and is represented to cause both nosocomial and community-acquired infections[44]. The infections by this organism, including bloodstream, urinary tract, cerebrospinal fluid, vagina, and cervix infections, have been reported[45]. The other bacterial pathogens, such as *Alcaligenes faecalis, Porteus mirabilis,* and *Serratia plymuthica,* were also isolated from rodent species and important rodent pathogen *Listeria* spp. in our study.

5. CONCLUSION

Rodents are well-known mammals to carry and transmit a wide variety of zoonotic pathogens. In India, no proper investigations were made on rodent pathogens except studies to investigate the presence of a single organism. This study constituted the first report of multiple bacterial pathogens associated with commensal rodents in India. Our investigation revealed the presence of diversified bacterial communities belonging to 14 species. Bacterial species, including *K. pneumoniae, S. aureus, S. arlettae, P. oryzihabitans, S. plymuthica, P. mirabilis,* and *Y. enterocolitica,* were detected for the first time in rodents in India. In the present study, an uncommon pathogen, *Sphingomonas paucimobilis*, was also detected for the first time in rodents. The results of this investigation indicate that rodents are potential sources of zoonotic and opportunistic bacterial pathogens in India and the Nellore district. Therefore, environmental biosecurity measures must be implemented in urban and rural areas to avoid pathogenic transmission. Further studies must be conducted to investigate the pathogens associated with rodents from different geographical locations in India and the potential risk to public health by the multiple infections.

6. FUNDING ACKNOWLEDGEMENTS

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7. AUTHORS CONTRIBUTION STATEMENT

Dr. Vijay A.K.B. Gundi conceptualized and designed the study. Mr. Manohar B. Vadela and Mr. Daveedu Thathapudi conducted all study experiments. Mr. Manohar B. Vadela, Dr. Satyanagalakshmi Karri, and Devi Bogireddy analysed the data and prepared the manuscript. All authors read and approved the final version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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