Original Research Article
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Identification of Transposable Elements Responsible For AMR in Food Borne Pathogen

Priyanka K Thakur [(1)*], Ranjeet Parihar [(1)], Seema Rani Pegu [(2)], Rajib Deb [(2)], Jagana Niharika [(1)], Pranab Jyoti Das [(2)], Gyanendra Singh Sengar [(2)], Joyshik Sonowal [(2)], Vivek Kumar Gupta [(2)]

[(1)] All India Institute of Hygiene and Public Health, Kolkata, West Bengal, India; Present address: Rajbari – 02, Madhesh Pradesh, Saptari, Nepal, [(2)] ICAR-National Research Centre on Pig, Guwahati, Assam, India

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Abstract

Despite the fact that the presence of silent genes, Transposable elements (TEs) and antibiotic resistance are related, only a small number of studies have examined the presence and distribution of transposable elements in animal food production chains, including pig and poultry farms. It is well known that there is a correlation between the spread of TEs carrying harmful bacteria from farms to food animals and from food animals into the cycle of production of animals for food. The present study identified the presence of five TEs namely Tn6763 (Accession number: OQ565300), Tn6764, (Accession number: OQ565299), Tn6765 (Accession number: OQ409902), Tn2003 (Accession number: OQ503494) and Tn6020 (Accession number: OQ503493) in (N= 235) samples collected from piggery farm and chicken meat shops from Kolkata and some regions of Assam. The identified food borne pathogens mainly belongs to *Enterobacteriaceae* family. Four highly zoonotic food borne pathogens with AMR was identified, 42% (77) *Salmonella enterica*, 26% (49) *E. coli*, 14% (26) *Proteus mirabilis*, 12% (22) *Klebsiella pneumoniae* and 6% (11) *K. pneumonia* were positive and carrying Tn6765, Tn6764, Tn6763, Tn6020 and Tn2003 respectively. Given the presence of TEs responsible for AMR in food borne pathogen and their relative contamination from pig farms and poultry farm to meat shops was described to be a major public health threat. Thus, preventative measures are vital for avoiding the spread of mobile genetic resistance determinants in the Livestock and Animal food production sector and to monitor their emergence.

Key words: Transposable elements, AMR, Food borne pathogens

Introduction:

The mobilization of TEs can pose a risk to the integrity of the genome as they have the potential to cause silent mutations, non-allelic homologous recombination (NAHR), alternative splicing, and various epigenetic changes. This can lead to genomic instability which in turn can accelerate the development of various diseases such as genetic disorders, psychiatric disorders, and cancer (Mukherjee et al., 2004). Antimicrobial-resistant diseases have a huge global health impact. The World Health Organization projects that if no action is taken, there will be 10 million fatalities per year due to drug-resistant bacterial infection by 2050, stressing the crucial need of understanding multidrug-resistant (MDR) microorganisms (World Health Organization, 2019).

Around the world, antibiotic-resistant bacteria are a major contributor to infections connected to healthcare, and resistance has also been observed in infections in the general population. Morbidity, mortality, and medical expenses are all significantly raised by infections brought on by multi resistant organisms. Molecular analyses have revealed that widespread multi resistance has commonly been achieved by the acquisition of pre-ex determinants followed by amplification in response to selection. Mobile genetic elements (MGE), also known as elements that encourage intracellular DNA mobility(for example, from the chromosome to a plasmid or between plasmids), as well as those that enable intercellular DNA mobility, play a significant role in the capture, accumulation, and dissemination of resistance genes (Partridge et al., 2018).

In the present study we collected the different samples from the Pig slaughter houses, pork meat shops, poultry farms and chicken meat shops. Then enriched the samples and further cultured and characterization of bacteria was done. The current study only targeted the gram-negative bacteria, some members of the family Enterobacteriaceae carrying TEs.

Materials and Methods:

Study design, Study area and sampling: The present study was carried from November 2022 to May 2023. Study area was Kolkata metropolitan area and random type of sampling was done (Figure 1).

Sample collection: A total two hundred thirty five (N=235) samples were collected from different locations in Kolkata metropolitan area. One hundred eighty five (185) samples from pig farms and slaughter houses containing feces, soil samples, slaughter effluents,

drainage water, pork carcass/meat washing water, drinking water of animals and pork meat samples. Fifty samples from Chicken meat shops and poultry farms containing Drainage water, feces, soil samples and chicken carcass/meat washing water and chicken meat. All the samples were collected aseptically, samples containing fluids (such as washing water, drainage water etc.) were collected in closed sterile plastic vials and solid (such as feces, soil and meat samples) were collected in sterile polyethylene bags. The samples were collected using sterile cotton swab sticks (HiMedia, India) in sterile vials containing transport medium (HiMedia, India) in accordance with the normal procedure (OIE 2002).The samples were brought to the laboratory in ice pack maintaining proper cold chain. They were held at 4°C for not more than 48 hr before processing.

Enrichment of the samples and culture: The collected samples were enriched in the MacConkey media (broth), (HiMedia, India) and incubated at 37°C for 18-24 hours. Next day growth has been observed in the broth. The enriched samples in MacConkey were sub cultured in the different types of medias such as MacConkey agar, Brilliant Green Agar (BGA), Eosin Methylene Blue Agar (EMB) and *Klebsiella* Blue Agar (KBA) to obtain the pure culture to grow and count microbial cells and to cultivate and select microorganism for further identification.

Gram's staining was used for the initial identification of all the pure isolate cultures in nutrient broth. Gram staining was carried out according to protocol using a smeared slide. Gram positivity or negativity, shape and size of the organisms were taken into consideration. The current study is only targeted towards gram negative bacteria containing Tes responsible for AMR in food borne pathogens

Biochemical assay of the bacterial culture: Various types of biochemical tests such as Indole test, Methyl-Red test, Voges-Proskauer test, Citrate utilization test, Catalase test, Oxidase test and urease tests were done to further characterization of the bacterial isolates.

Primer designing: Primers were designed for cataloguing of different transposable elements existing in the isolated bacterial DNA. Insertion sequences of *Salmonella*, *E. coli*, *Proteus*, *Klebsiella*, *Proteus* of *Enterobacteriaceae* family was retrieved from Isfinder database (Isfinder, https://www-is.biotoul.fr/). Table 1 contains information on the oligonucleotide primers and PCR conditions that were utilized for the identification of transposable elements from *Enterobacteriaceae* family obtained from various sources of piggery waste. The details of the PCR conditions and primers used are provided in the table.

DNA extraction and PCR: The genomic DNA was isolated from an overnight growth bacterial culture using Chen and Kuo's (1993) method with minor modifications. At the end of the exponential phase of growth, bacterial cells were extracted by centrifugation for 5 minutes at 12,000 rpm. The cell pellets were resuspended in lysis buffer (40 mMTris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, and 1% SDS), vigorously shaken, and centrifuged for 10 minutes at 4°C at 12,000 rpm. The supernatant was transferred to a new vial, which was then filled with an equal volume of chloroform and inverted many times until the solution appeared milky. The mixture was then centrifuged at 12,000 rpm for 3 minutes, and the supernatant was transferred to another tube for DNA precipitation with 100% ethanol, washed twice with 70% ethanol, air-dried, and resuspended in 1 x TE buffer. Spectrophotometric readings were used to assess the quality and amount of the isolated genomic DNA. Extracted genomic DNA was treated to polymerase chain reaction (PCR) with primers specific for distinct transposable elements. In a 25 µl reaction volume, 10 x PCR buffer (50 mMKCl, 10 mM Tris-HCl, 0.1% Triton X), 5 µlTaq DNA Polymerases, 1 mM $MgCl_2$, 1.5 µM each primer, and 200 µM each dNTP was used. PCR was done for 40 cycles of amplification, with denaturation at 95 °C for 5 minutes, annealing at 55-57 °C for 15 seconds, 72 °C for 15 seconds, and extension at 72 °C for 10 minutes. The PCR results were validated using agarose gel electrophoresis.

Sanger's sequencing and gene bank submission of positive samples: The amplified products of different transposable elements were subjected for Sanger's sequencing for tracing the microbial identity.

Antibiotic susceptibility test: Bacterialisolates encoding different transposable elements were tested for sensitivity/resistance to various common antibiotics such as Chloramphenicol, Amikacin, Gentamycin, Vancomycin, Ofloxacin, Tetracycline, Trimethoprim and sulfamethoxazole., Ampicillin, Nalidixic acid, Cefpodoxime and Colistin (CLSI, 2014).

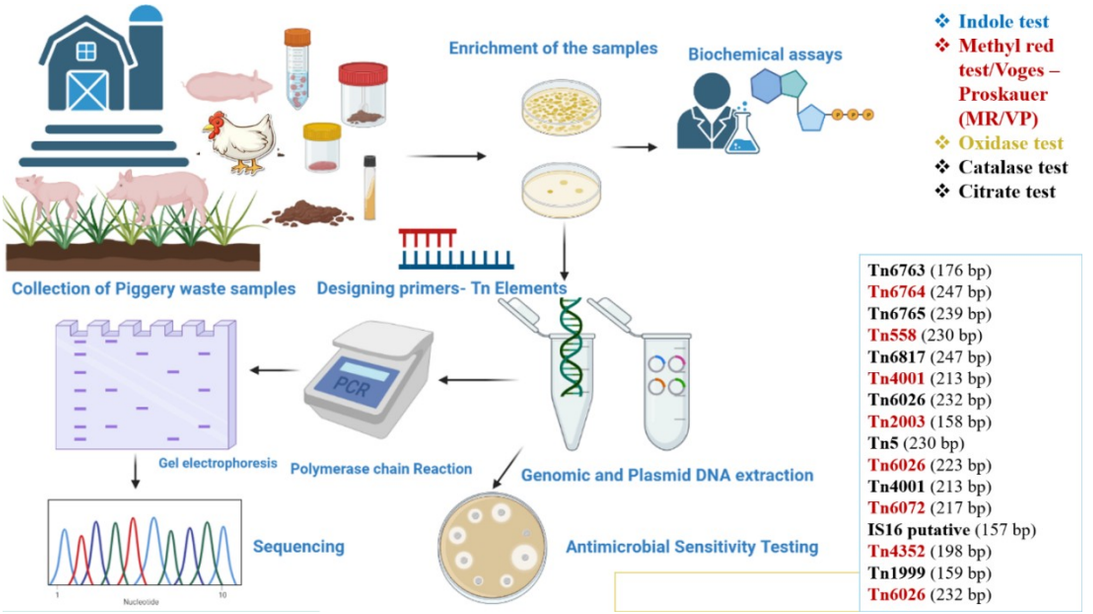


Figure 1: Diagrammatic representation of the methodology used in the study

Table 1: List of the primers used for the current study

TE Name	Forward primer	Reverse primer
1. *IS16* gene	ACACTGAGTGGCTGGCTTCT	GCCATGCCTTTCTTTCTGAG
2. *Tn 558*	CTACCCGACATTCCACGACT	ACGGAGTGTGGAGGTTGTTC
3. *Tn 4001*	TAGCGCGTGAGGCTAAAAAT	GATTGGGGAAGATGCGAATA
4. *Tn6026*	CACCGGCAGGTGAAGTATCT	CAATGCCAAAAGCTCTCTCC
5. *Tn 6020*	CAACGTGAAGAAGTGGCAGA	ACCTTTGATGGTGGCGTAAG
6. *Tn2003*	GAGCTGCACATGAACCCATT	CAGCGGTAAATCGTGGAGTG
7. *Tn5*	CGGATCGAGGAGTTCCATAA	TTCTACGTGTTCCGCTTCCT
8. *Tn2006*	GAGTCACTGGCGTGTTCTCA	CGTCCGGAAAAGTTCATTGT
9. *Tn6072*	AACGCGGTTTACAAGGTACG	GCCTCACGCGCTAAGTTAAT
10. *Tn5384*	AGATCTGAAGAGACCTGCGG	AGATCAGGCACCTTCAACGA
11. *Tn1999*	CAACAGTCCAGAGCGATTCG	CCCAAGCGAATCGTTGAGAG
12. *IS Sav10*	GCTTCCTCTGCCATTCGATG	CCGATGTTCTGCGACGAATT
13. *Tn 6763*	GTTGGTGCACAAAACATTCG	TGCTTTTCAGAAGGCAAGGT
14.*Tn 6764*	TTCAGGCACGTATGTGGGTA	GCTTGAGGCGTTTATTCAGC
15. *Tn 6765*	ACCGACAGCGATACGGTAAG	ACGGTGTGTATGTCGAGCAA
16. *Tn 6718*	GCTTGAGGCGTTTATTCAGC	TTCAGGCACGTATGTGGGTA

Results and Discussion:

Isolation of Enterobacterials: To isolate *Enterobacteriaceae* bacteria, we used various agar media MacConkey, EMB, BGA, and *Klebsiella* selective agar. Out of the total samples collected, 185 (78.72%) were found to have grown on the fore mentioned media and were selected for primary identification through Gram staining (Figure 2).

DNA extraction and PCR based detection of TEs: Out of the total number of samples (N=235), 185 were found to be positive for Enterobacterial family. To identify any transposable elements, present in these samples, we screened each sample using 16 primers that were designed specifically for this purpose. As a result of this screening, we obtained positive with five transposable elements (Figure 3).

Transposable elements identified in samples and their percentage positivity: Out of 185 samples, 42% (77) *Salmonella enterica*, 26% (49) *E. coli*, 14% (26) *Proteus mirabilis*, 12% (22) *Klebsiella pneumoniae* and 6% (11) *K. pneumonia* were positive and carrying Tn6765, Tn6764, Tn6763, Tn6020 and Tn2003, respectively (Figure 4).

Sanger sequence analysis and gene bank submission: The sequencing results were analyzed Figure 5 and Figure 6, and the sequence was submitted to www.ncbi.nlm.nih.gov to obtain an accession number. Results of sequencing and accession no. obtained. The present study identified the presence of five TEs namely Tn6763 (Accession number: OQ409902), Tn6764, (Accession number: OQ565299), Tn6765 (Accession number: OQ409902), Tn2003 (Accession number: OQ503493) and Tn6020 (Accession number: OQ503493)

Antibiotic resistance or susceptibility pattern and determination of minimum inhibitory concentration of bacteria: All the bacteria which were identified by the sequencing & were carrying transposable elements positive were screened with antibiotics. *Klebsiella* spp. Carrying Tn6020 and Tn2003 isolates were resistant (100%) to Nalidixic acid, vancomycin, ceftriaxone, ceftazidime, cefotaxime, cefpodoxime, ceftizoxime, cefixime, amoxicillin-clavulanate combination, tetracycline, gentamicin, ciprofloxacin, levofloxacin, amikacin, doxycycline and cefepime/tazobactam. Again, *Proteus mirabilis* is Carrying Tn6763 (Figure 7) were resistant to piperacillin/tazobactam, and cefepime, chloramphenicol, streptomycin and trimethoprim/sulfamethoxazole, For Tn6764 &Tn 6765 which was carrying bacteria *E. coli* and *Salmonella enterica* were resistance Ofloxacin (Figure 7) frequently noted to other antibiotics like norfloxacin (85%), tobramycin (65%), cotrimoxazole (60%) and cefoxitin (60%). It is noteworthy that almost all the bacteria isolates were intermediate resistant to imipenem. Most of the isolates were sensitive to Chloramphenicol (60%), Amikacin (100%), meropenem (100%) and colistin (100%).

To spread bacterial AMR, Tn7-like transposons are crucial mobile elements. The information currently available on the thorough analysis of Tn7-like transposons in Enterobacterales isolates is still insufficient when compared to studies about the transposition mechanism of the Tn7-like transposons. In this study, we evaluated the percentage positivity of Tn7-like transposons in isolates of the genus Enterobacterales isolated from various farms of pigs raised for meat. Among the 235 sample 180 were positive for Enterobacterales isolates. The positive rate of Tn7-like transposons in *Proteus* spp. (50.6%) was significantly higher than other bacteria. This finding was consistent with previous reports that genomes of *Proteus* spp. exhibited strong plasticity facilitating high-frequency insertion of mobile genetic elements like Tn7-like transposons. (Dong et al., 2019, Gu et al., 2020).

The characterization of individual TEs and the recombination events they predict has been the subject of numerous studies. Positive antibiotic selection can easily identify such AR-causing events. However, focused analyses of global omics are required to fully understand the effect of TE-dependent changes on bacterial transcriptomes. Understanding the molecular underpinnings of the operation of a much larger pool of TEs, representing various families of elements, is equally crucial. The discovery of new pathways and mechanisms that activate silent genes may be made possible by a better understanding of their biology and unique properties.

Significant increases in the emergence and spread of bacteria that are resistant to multiple drugs as well as rising resistance to more recent drugs like fluoroquinolones and specific cephalosporins have been observed over the past two decades (von Baum et al., 2005). The development of antimicrobial resistance in *E. coli* has been attributed to a variety of mechanisms, with horizontal gene transfer through conjugative plasmids, transducing phages, and transposable elements carrying resistance genes making the biggest contributions to this genomic fitness.

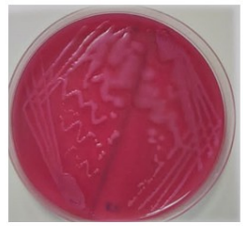
Our findings show that there may be a reservoir of Tn7-like transposons in the Enterobacterales strains, which is a risk that requires our attention. Antimicrobial susceptibility testing indicated that Enterobacterales strains carrying Tn7-like transposons exhibited high resistance to a variety of antibiotics, with a 54.9% multidrug resistance rate. Relatively high rates of MDR in *Proteus* spp. and *S. enterica* strains may be due to the prevalence of multiple mobile elements in both bacteria, which was confirmed in previous studies (Beutlich et al., 2011; Murgia et al., 2015; Schultz et al., 2015; Lei et al., 2018)

Additionally, it suggested that Tn7-like transposons were probably to appear concurrently with other mobile elements. The intI2-associated resistance gene cassette (aadA1, sat2, and dfrA1) carried by Tn7-like transposons is responsible for the high resistance to streptomycin (87.8%) and trimethoprim/sulfamethoxazole (74.0%,), (Tietze and Brevet et al.,1991; Kaushik et al., 2019).The gene cassettes of intI2 contained the aminoglycoside adenyltransferase (aadA1 and aadA2), dihydrofolatereductase (dfrA1), and streptothricinacetyltransferase (sat2) encoding genes, which are responsible for streptomycin-spectinomycin, trimethoprim, and streptothricin resistance, respectively (Zhang et al., 2019)

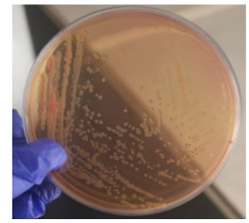
Compared with the resistance rates to streptomycin and trimethoprim/sulfamethoxazole, relatively low resistance rates to florfenicol (60.5%), gentamicin (24.4%), and ciprofloxacin (22%) were detected in the Tn7-like transposons positive strains. The majority of them

demonstrated susceptibility to the antibiotics imipenem, ceftazidime, cefoxitin, aztreonam, and amikacin. In China the abuse of antibiotics as feed additives in veterinary clinical practise and the transfer of movable components carrying drug-resistant genes between isolates, which has become a common problem in both human and veterinary clinical practise, are the main causes of the emergence of severe drug-resistant status (Rahmani et al., 2013).

EMB agar showing dark, pink, mucoid colonies; with metallic sheen of *E. coli*

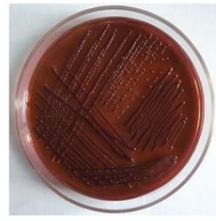


Rose pink mucoid colour colonies MacConkey agar



MacConkey agar with flat, smooth, colourless colonies

Magenta coloured colonies on *Klebsiella* selective agar



EMB agar showing dark, pink, mucoid colonies: - without metallic sheen- of *Klebsiella spp.*

Red colour colonies on Brilliant green agar

Figure 2: Isolation of the members of the family Enterobacteriaceae showing different bacterial strains such as *E. coli, Salmonella, Klebsiella* in differential and selective media

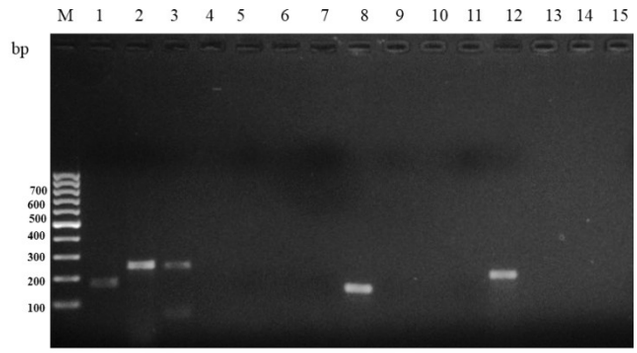


Figure 3: Amplification and detection of 5 Transposable elements; Lane 1: Tn6763(176bp); Lane 2: Tn6764 (247bp); Lane 3: Tn6765 (239bp); Lane 8: Tn 2003 (158bp) and Lane 12: Tn6020 (232bp) in enterobacterial isolate, (*Proteus mirabilis, Escherichia coli, Salmonella enterica, Klebsiella pneumoniae, K. pneumoniae*). Lane M: molecular mass marker (100 bp, ThermoFisher scientific) representing 176bp, 247bp, 239bp, 158bp and 232bp.

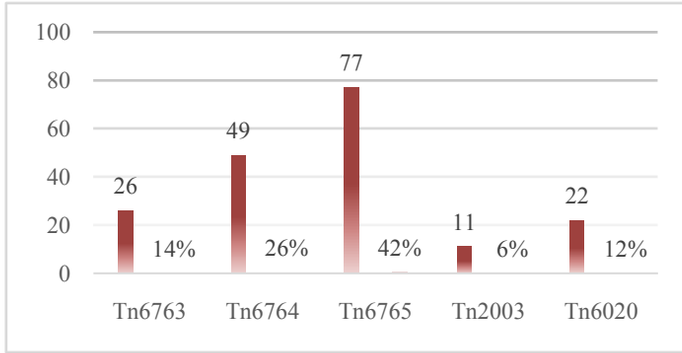


Figure 4: Percentage positivity with TEs; 42% (77) *Salmonella enterica*, 26% (49) *E. coli*, 14% (26) *Proteus mirabilis*, 12% (22) *Klebsiella pneumoniae* and 6% (11) *K. pneumoniae*were positive and carrying Tn6765, Tn6764, Tn6763, Tn6020 and Tn2003 respectively

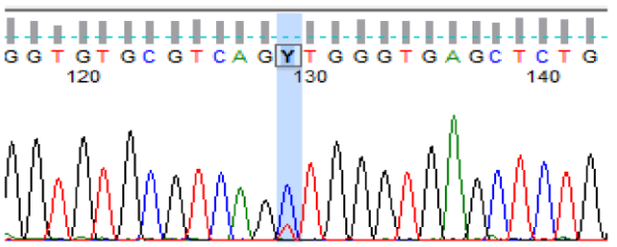


Figure 5: Direct sequencing of Tn6765 amplified Fragment Showing heterozygous locus at position 159.C > T

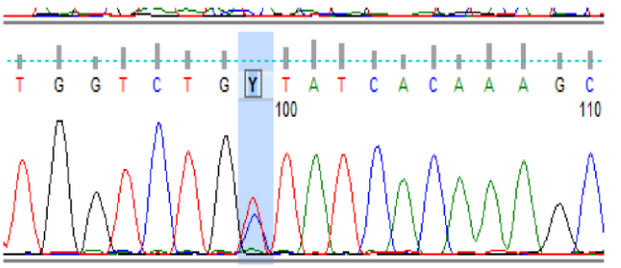


Figure 6: Direct sequencing of Tn6763 amplified Fragment Showing heterozygous locus at position 129.T > C

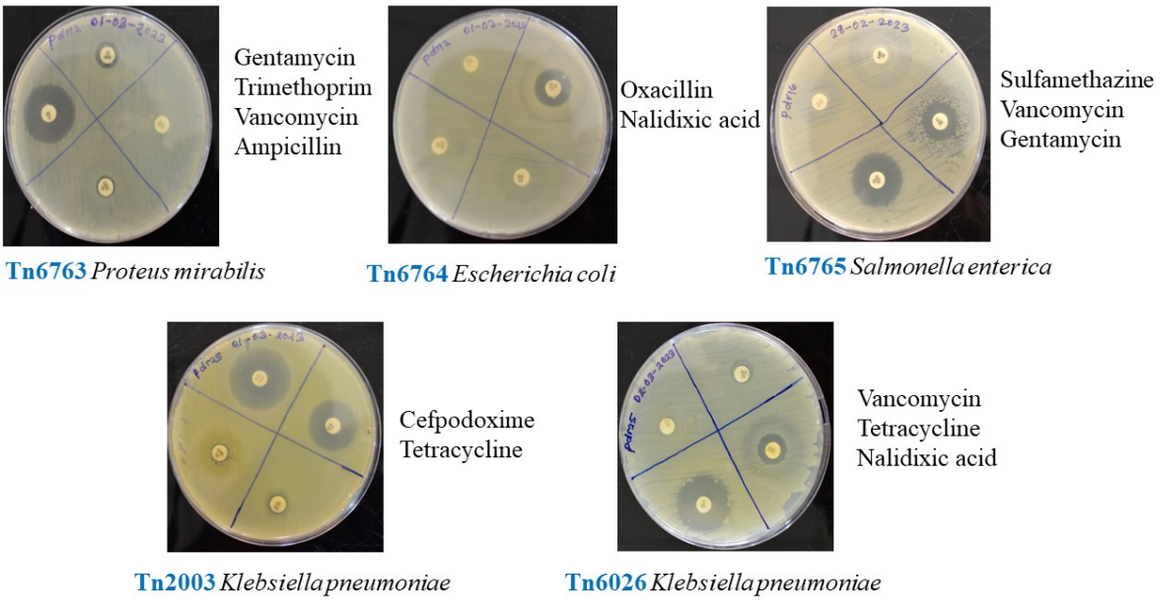


Figure 7: Antibiotic sensitivity tests of the positive samples (Tn6763 *Proteus mirabilis*resistant to Gentamycin, Trimethoprim and Ampicillin), (Tn6764 *E. coli* resistant to Oxacillin and Nalidixic acid), (Tn6765 *Salmonella enteric* resistant to Sulfamethazine, Vancomycin and Gentamycin), (Tn2003 *K. pneumonia* resistant to Cefpodoxime and Tetracycline) and (Tn6026 *K. pneumonia* resistant to Vancomycin, Tetracycline and Nalidixic acid)

Conclusion:

High rates of MDR in *Proteus* spp. and *S. enterica* strains was due to the prevalence of multiple mobile elements in the bacteria. Enterobacterales strains carrying Tn7-like transposons exhibited high resistance to the antibiotics. This study concluded that it is very crucial to standardize clinical drug application to increase the efficacy of new medications.

Conflict of interest:

Authors declare no conflict of interest for this investigation.

Data Availability:

All raw data are preserved at the ICAR-National Research Centre on Pig, Guwahati, Assam, India

Ethical Statements:

Authors maintained all ethical concern during sampling and collection of data throughout the experiments.

Author's Contribution:

PKT: Study Execution**, RP:** Sample collection**, SRP:** Sample analysis and supervision, **RD:** Molecular study**, JN:** isolation study**, PJD:** preparation of the manuscript**, GSS:** Isolation **, JS:** sequence analysis**, VKG:** revision of manuscript.

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***Corresponding author's email ID:** drpinkuthakur@gmail.com

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