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ISOLATION AND IDENTIFICATION OF ANTIBIOTIC RESISTANT BACTERIA WITH HIGH TOLERANCE FROM COMMERCIALLY REARED CHICKEN RECTAL SWAB SAMPLES

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Abstract: Antibiotic resistance has become a threat to modern medicine and excessive use of antibiotics in humans, animals and agriculture are the reasons for this evolution resulted in the acceleration for natural process of antibiotic resistance by the bacteria and made them to tolerate. Antibiotics are given in chicken's feed regularly at farms, as they promote the weight of the chicken and to prevent the bacterial infections. Hence, the chickens reared by modern farming methods have become a potential source of antibiotic resistant bacteria and this was proved by isolation of resistant strains from feces of such chicken. This study was aimed to isolate and identify the antibiotic resistant bacteria from the poultry chicken rectal swab sample. A total of 25 chicken rectal swabs samples were collected, inoculated and incubated in peptone water. The bacterial isolates were subjected to primarily screening of antibiotic resistance from the spread plate of the samples. After screening, the resistance of all the isolates was noted and those isolates, which showed resistance against many antibiotics, was selected for confirmation of tolerance to high concentration antibiotic solution by well diffusion method. Then the antibiotic resistant isolate with tolerance to highest concentration of antibiotics was identified by molecular identification 16S rRNA sequencing and was found to be *Klebsiella pneumonia* with tolerance to 5mg concentration of amoxicillin and erythromycin antibiotics. This study was successful in isolating three drug resistant bacteria. In this analysis we found that 60 percent of the organisms isolated from the rectum of poultry chicken reared by modern methods using antibiotic treatment and feeds, were antibiotic resistance bacteria.

KEYWORDS: Antibiotic resistant bacteria, Antibiotic tolerance, Bacterial isolation

1. INTRODUCTION

Antibiotic resistance has become a growing threat of the world in the current century (Matthew *et al*., 2021). Misusage, over usage, inappropriate selection of drug, insufficient dosage, inadequate adherence to guidelines, animal production with antibiotics in feed (Muhammad *et al*., 2009), etc are the factors responsible for the development of antibiotic resistance (Nguyen *et al*., 2017, Asghar *et al*, 2018). Antibiotics used in food producing animals for growth promotion and mass prophylaxis, and antibiotics use in agriculture to avoid. Due to this public health microbiology laboratories used a common terminology for grading various antimicrobial resistance profiles such as MDR, XDR and PDR. Multi Drug Resistant (MDR), Extensively Drug Resistant (XDR) and Pan Drug Resistant (PDR) bacteria which had been found in healthcare associated antimicrobial resistant bacteria has been used in medical literature to characterize their different patterns of resistance. MDR (Multi Drug Resistant) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. XDR (Extensively Drug Resistant) was defined as non-susceptibility to at least one

agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two categories) and PDR (Pan Drug Resistant) was defined as non-susceptibility to all agents in all antimicrobial categories (Magiorakos *et al*., 2012).

2. MATERIALS AND METHODOLOGY

The rectal swab samples of poultry chicken were collected. A total of 25 samples from five different farms were collected and dipped into peptone broth tubes (Lukas *et al*, 2015). After incubation for 24 hours at 37℃, 1 ml of the sample from each tube was subjected to serial dilution and bacterial organisms were isolated using spread plate technique. Based on the colony morphology, pure cultures of the isolates were maintained in nutrient agar slants for further analysis.

2.1. Screening of Antibiotic Resistant Bacteria and its Tolerance to different concentrations of different types of Antibiotics:

The bacterial isolates were tested for their resistance to antibiotics by streaking them onto the nutrient agar impregnated with antibiotics. Three antibiotics – one belonging to the aminopenicillin class - amoxicillin, one from macrolides class - erythromycin and the third one from fluroquinolones - ciprofloxacin were utilized for this study. All the antibiotics were taken in different concentrations - 10microgram, 15microgram, 20microgram, 50microgram, 200microgram, 1milligram, 2milligram, 3milligram and 5milligram per ml were used (Sarannia *et al*, 2020). A total of three sets of nutrient agar plates for the three antibiotics were prepared and all the bacterial isolates were inoculated by single streak line on to the agar surface and observed for growth after incubation at 37℃ for 18-24 hours. After noting the resistance of all the isolates, the isolate with resistance and maximum tolerance to highest concentration was selected for further steps.

2.2. Confirmation of Tolerance to High concentration Antibiotic solution by Well Diffusion Method: The tolerance capacity of the isolate to high concentrations of antibiotics was confirmed by agar well diffusion method. After lawn culturing the 24 hours broth of the selected organisms, the Muller Hinton agar plate surface is punched aseptically with a sterile cork borer with a diameter of 6 to 8mm for well formation and then a volume of about 20 to 100 microliters of antibiotic diluted solution at desired concentration was loaded into that well. Then the agar plates were incubated at 37℃ for 18-24 hours and observed for the growth around the wells (Mounyr *et al*, 2015)

2.3. Identification of Extreme Antibiotic Resistant Isolates by Morphological and Biochemical Characterization: The best antibiotic resistant isolates with tolerance to highest concentration of antibiotics were first identified by conventional characterization methods - grams staining techniques, biochemical characterization by catalase test, oxidase test, indole test, methyl red test, Voges Proskauer test, citrate test, urease test, triple sugar test, nitrate reduction test and carbohydrate fermentation tests (Aprilia *et al*, 2021).

2.4 Molecular Identification of Selected Extreme Antibiotic Resistant Bacteria by 16SrRNA Sequencing: The isolate which showed excellent growth in the higher concentrations for three antibiotics was sequenced to determine the species of it, which had been preliminarily identified by biochemical test. DNA was isolated from the culture of the organism which was found to have extreme antibiotic resistance.

Forward and reverse DNA sequencing reaction of polymerase chain reaction amplicon was carried out with 16SrRNA-Forward and 16SrRNAReversed primers using BDT v3.1 cycle sequencing kit on ABl 3730xl genetic analyzer. Consensus sequence of 16SrRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST (Basic Local Alignment Search Tool) with the ‘nr’ database of NCBI Gene Bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. distance matrix and phylogenetic tree was constructed using MEGA 10.

3. RESULTS

The peptone water tubes showed good bacterial growth after incubation period. A total of ten isolated bacterial colonies with different colony morphologies were selected from the spread plates. These different bacterial cultures habituating the rectum region of the bacterial cultures habituating the rectum region of the poultry birds

reared by the commercial methods were stored and used to check their ability to resist antibiotics with tolerance to high concentrations of antibiotics as well.

3.1 Screening of Antibiotic Resistant Bacteria and its Tolerance to different Concentrations of different types of Antibiotics: Among the selected ten bacterial isolates, three isolates (5^{th}, 8^{th} and 10^{th} isolates) were observed to be resistant to all three antibiotics. Three out of the remaining seven isolates showed resistance to one antibiotic and four isolates were found to be sensitive for all three antibiotics with no growth in the test medium. It has been observed that fifth isolate showed more resistance for all the concentrations of three antibiotics. Whereas eighth isolate and tenth isolate showed resistance for six concentrations only, up to 1mg. Hence fifth isolate which showed extreme resistance for three antibiotics has been sub cultured by quadrant streaking method and maintained for further analysis.

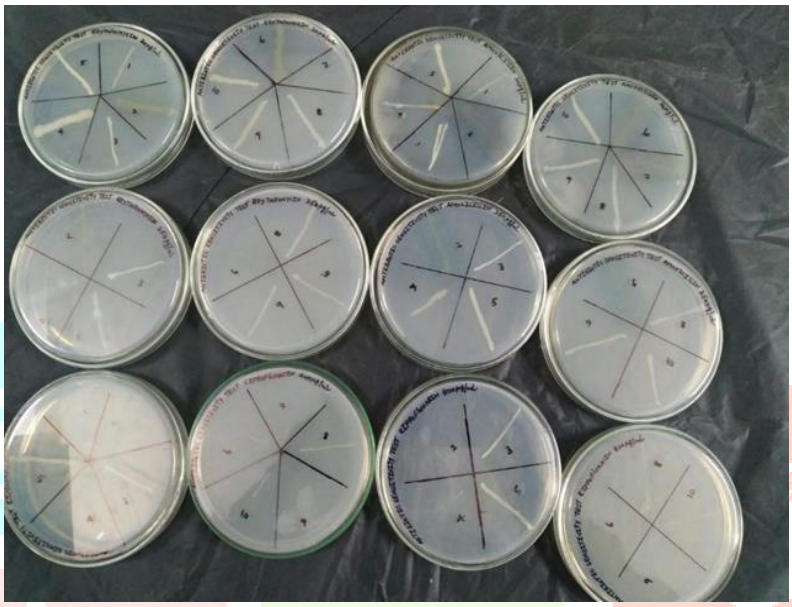

Fig: A. Screening of antibiotic resistant bacteria and its tolerance to different concentrations of different types of antibiotics

3.2. Confirmation of Tolerance to High Concentration Antibiotic Solution by Well Diffusion Method: The fifth isolate was lawned on Muller Hinton agar plate through sterile swab for well diffusion method. Different concentrations such as 10microgram, 15microgram, 20microgram, 50microgram, 200microgram, 1milligram and 2milligram, of antibiotics were loaded into the wells and incubated.

Fig: B. Plate showing tolerance to high concentrations of ciprofloxacin by fifth isolate

After incubation, no zone of inhibition was observed till 1 milligram concentration. Hence, the fifth isolate was confirmed to possess resistance to highest concentration of amoxicillin till 1mg. This isolate was selected for identification and characterization.

3.3. Identification of antibiotic resistant isolates by morphological and biochemical characterization: The fifth, eighth and tenth isolates were selected for identification as these organisms were resistant to all three antibiotics of three different classes. The results were given below in the table.

Table 1: Biochemical test results

S.NO	BIOCHEMICALS TEST	FIFTH ISOLATE	EIGHTH ISOLATE	TENTH ISOLATE
1.	Oxidase	Positive	Positive	Positive
2.	Catalase	Positive	Positive	Positive
3.	Indole	Negative	Negative	Negative
4.	Methyl red	Negative	Negative	Negative
5.	Voges Proskauer	Negative	Positive	Negative
6.	Citrate	Positive	Positive	Negative
7.	Urease	Positive	Negative	Negative
8.	Triple sugar iron	A / A	Negative	Negative
9.	Nitrate reduction	Positive	Positive	Positive
10.	Sugar Fermentation Maltose	Positive	Positive	Positive
	Mannitol	Positive	Positive	Positive
	Sucrose	Positive	Positive	Positive
	Glucose	Positive	Positive	Positive

Based on the biochemical test results the isolates has been identified as *Klebsiella species* (fifth isolate), *Bacillus species* (eighth isolate) and *Bacillus species* (tenth isolate).

3.4. Molecular Identification of Selected Extreme Antibiotic Resistant Bacteria by 16S rRNA Sequencing: The fifth isolate, *Klebsiella species*, was subjected to Molecular identification by 16S rRNA sequencing to confirm the genus and to identify its species. Based upon the phylogenetic tree and sequence producing significant alignments, 98% the organism matched with the *Klebsiella pneumoniae* species. So, the fifth isolate

which was identified as *Klebsiella* by biochemical test, was identified to be *Klebsiella pneumonia* by molecular identification of bacteria using 16S rRNA sequencing.

Table: 2. Table of Sequence producing significant alignments:

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Klebsiella pneumoniae strain DSM 30104	2741	2741	100%	0.0	99.87%	NR_117683.1
Klebsiella pneumoniae strain DSM 30104	2719	2719	100%	0.0	99.60%	NR_036794.1
Klebsiella pneumoniae strain DSM 30104	2719	2719	100%	0.0	99.60%	NR_117686.1
Klebsiella pneumoniae strain DSM 30104	2713	2713	100%	0.0	99.53%	NR_117684.1
Klebsiella pneumoniae strain DSM 30104	2708	2708	100%	0.0	99.46%	NR_117685.1
Klebsiella pneumoniae strain DSM 30104	2708	2708	100%	0.0	99.46%	NR_117682.1
Klebsiella pneumoniae strain DSM 30104	2680	2680	98%	0.0	99.52%	NR_114715.1
Klebsiella pneumoniae strain NBRC 14940	2680	2680	98%	0.0	99.59%	NR_113702.1
Klebsiella pneumoniae strain JCM 1662	2675	2675	98%	0.0	99.59%	NR_113240.1
Klebsiella quasipneumoniae strain 01A030	2658	2658	98%	0.0	99.38%	NR_134062.1

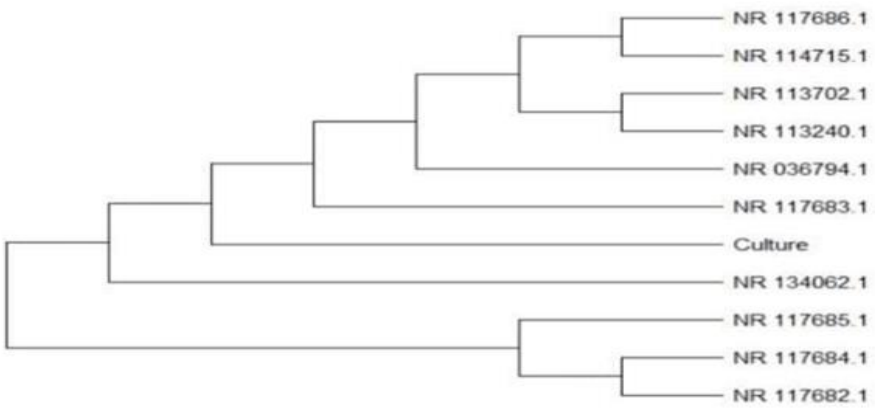

Fig:C. Phylogenetic tree

4. DISCUSSION

The bacteria those have lost the susceptibility to the growth inhibitory properties or killing ability of the antibiotic agent are termed to be antibiotic resistant bacterial strains. Hence the infection caused by these bacterial strains has become untreatable and life threatening. A total of 25 chicken rectal swabs were collected from different poultry chicken following the procedures of Lukas *et al*, 2015. Then the samples were dipped in peptone broth and incubated for bacterial growth. Prominent growth was obtained in the broths, from which serial dilutions were prepared and chosen for spread plate method. Ten selected isolates were primarily screened for antibiotic resistance and tolerance to the highest concentration of antibiotics son nutrient agar plates containing antibiotics at different concentrations. After incubation, four (2, 4, 6 and 9) bacterial isolates failed to grow even in the low concentration antibiotics impregnated nutrient agar plates and three (1, 3 and 7) were able to grow in the less concentrations – up to 20microgram, of amoxicillin and erythromycin antibiotics impregnated nutrient agar plates. Final three isolates (5, 8 and 10) were able to grow in all three antibiotics up to 1mg concentration and one isolate (5th isolate among the total ten isolates) among these three was able to grow up to 5mg of amoxicillin and erythromycin with high tolerance. Lukas *et al*, 2015 were also successful in isolating drug resistant *E. coli* from the chicken. A similar work was been carried out by Sarannia *et al*., 2020 using raw chicken meat, with a little in screening protocol. Based on colony morphology different strains were collected by them in the pure form and antibiotic resistance was screened by Kirby Bauer method with different concentration of antibiotics. In the present study nutrient agar impregnated with antibiotics were used by single streak line method to check antibiotic tolerance capacity of the resistant bacteria as many isolates can be accommodated easily in few media plates. The tolerance of the extreme resistant isolate to the antibiotics was reconfirmed by agar well diffusion for the antibiotics at high concentrations. The isolate showed higher resistance and no zone of inhibition was formed. The morphological and biochemical identification revealed the genus to be bacillus for isolate 8 and 10, and Klebsiella for isolate 5. As fifth isolate was been the most tolerant with extreme antibiotic resistance, molecular identification was done for it and its species level identification was confirmed, as done by Aprilia *et al*, 2021. The procedure followed by Sarannia *et al*, 2020 and Aprilia *et al*, 2021 was adapted and 16SrRNA sequencing was done to the strain for species level identification. Through phylogenetic tree – by maximum likelihood method the species was confirmed to be *Klebsiella pnuemoniae*.

5. CONCLUSION

This study was successful in isolating three extreme drug resistant bacteria. In this analysis we found that 60 percent of the organisms isolated from poultry chicken reared by modern methods using antibiotic treatment and feeds, were antibiotic resistance bacteria. Among these, 50 percentage of the isolates were found resist to three different classes of antibiotics and are of extreme drug resistant bacterial category. Whereas remaining belonged to multidrug resistant category. As well as, 20 percentage of the extreme drug resistant isolates were with tolerance to high concentrations of antibiotics revealed their hazardous property. Hence we conclude that the poultry chickens are serving as a source for the development and spreading of deadly antibiotic resistant bacteria in the environment.

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