

Antibiotic Resistance in Poultry Environment

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CONTENTS

S. No.	Topic	Page No.
1.	Pollution Monitoring Laboratory of CSE	3
2.	Introduction	3
3.	Review of Literature	4
4.	Objective of the Study	8
5.	Materials and Methods	8
6.	Results and Discussion	12
7.	Conclusions	24
8.	Recommendations	24
9.	References	29
	Annexure I - Details of samples collected from poultry farms	35
	Annexure II - Zone size interpretative chart for Antimicrobial Susceptibility Testing as per CLSI standards	37

1. POLLUTION MONITORING LABORATORY OF CSE

The Centre for Science and Environment (CSE), a non-governmental organization based in New Delhi, has set up the Pollution Monitoring Laboratory (PML) to monitor environmental pollution. PML is an ISO 9001:2008 accredited laboratory certified by SWISSCERT Pvt. Ltd. for conducting Pollution Monitoring and Scientific Studies on Environmental Samples. The Laboratory has highly qualified and experienced staff that exercise Analytical Quality Control (AQC) and meticulously follow Good Laboratory Practices (GLP). PML has all the equipments required for microbiological analysis of samples such as autoclave, laminar air flow, shaker incubator, BOD incubator and microscope etc. Further, it is equipped with most sophisticated state-of-the-art equipments for monitoring and analysis of air, water and food contamination, including Gas Chromatograph with Mass Detector, Gas Chromatograph (GC) with ECD, NPD, FID and other detectors, High Performance Liquid Chromatograph (HPLC), Atomic Absorption Spectrometer (AAS), UV-VIS Spectrophotometer etc. Its main aim is to undertake scientific studies to generate public awareness about food, water and air contamination. It provides scientific services at nominal cost to communities that cannot obtain scientific evidence against polluters in their area. This is an effort to use science to achieve ecological security.

2. INTRODUCTION

Antibiotics are generally used to treat microbial diseases in humans as well as in animals. However, the misuse and overuse of antibiotics results in resistance in pathogenic bacteria as well as in the endogenous flora of exposed individuals, be it humans or animals (Baldwin *et al.*, 1976; Howe *et al.*, 1976; Hinton *et al.*, 1982; Piddock, 1996 and Van den Bogaard, 1997). The antibiotic resistant bacteria have the ability to resist towards the actions of naturally occurring or synthetically produced compounds inimical to their survival (WHO ISDA, 2007).

The misuse and overuse of antibiotics is considered to be the most significant reason for emergence, selection and spreading of antibiotic resistant bacteria in both animals and

humans (Neu 1992; Witte 1998; Okeke *et al.*, 1999 and Moreno *et al.*, 2000). The resistant microbes may act as a potential source in the spread of antimicrobial resistance to human pathogens. It is also well established that antibiotics can lead to the emergence and dissemination of different resistant bacteria which can be passed on to people *via* food or direct contact with infected animals (Van den Bogaard and Stobberingh, 2000; Schroeder *et al.*, 2002).

Antibiotics are used extensively in poultry industry for different purposes. The enormous exploitation of antibiotics in the field of veterinary medicine has resulted in an increased number of resistant bacterial strains in recent years. In addition to antibiotic resistance increasing from natural selection, bacteria can receive genetic material through the process of Horizontal Gene Transfer (HGT). HGT conferring resistance to many classes of antimicrobials has resulted in a worldwide epidemic of nosocomial and community infections caused by multidrug-resistant microorganisms, leading to suggestions that mankind in effect is returning to the pre-antibiotic era (Warnes *et al.*, 2012). Transmission of plasmid mediated resistance widely takes place between different bacterial species and genera (Davies, 1994). There are a number of multidrug resistant strains, found in humans and animals (Amara *et al.*, 1995). However, the multiple drug resistant, non-pathogenic *Escherichia coli* found in the intestine is probably an important reservoir of resistance genes (Osterblad *et al.*, 2000). Further, the drug resistant *Escherichia coli* of animal origin may colonize the human intestine (Marshall *et al.*, 1990). This acquired multidrug resistance to antimicrobial agents creates extensive difficulties in management of intra and extra intestinal infections caused by the bacteria, resulting in illness, increased healthcare costs and death (Gupta *et al.*, 2001).

3. REVIEW OF LITERATURE

Poultry litter is a mixture of feces (which contains faecal microbial flora), wasted feed, bedding materials, and feathers (Wilkinson *et al.*, 2011; Kim *et al.*, 2012). It contains a large and diverse population of microorganisms. Microbial concentrations in poultry litter can reach up to 10^{10} cfu/g, and Gram-positive bacteria, such as Actinomycetes, Clostridia and Bacilli/Lactobacilli, account for nearly 90% of the microbial diversity (Bolan *et al.*,

2010). A variety of pathogens can be found in poultry litter or chicken litter-based organic fertilizers, such as *Actinobacillus*, *Bordetella*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Escherichia coli*, *Globicatella*, *Listeria*, *Mycobacterium*, *Salmonella*, *Staphylococcus*, and *Streptococcus* (Alexander *et al.*, 1968; Lovett *et al.*, 1971; Lu *et al.*, 2003; Stern and Robach, 2003; Ngodigha and Owen, 2009; Bolan *et al.*, 2010). Some of these bacteria such as *Salmonella*, *Campylobacter jejuni* and *Listeria monocytogenes* can potentially contaminate fresh produce or the environment and are frequently associated with food borne outbreaks (Chinivasagam *et al.*, 2010; Wilkinson *et al.*, 2011).

The use of various antibiotics as feed supplements is a common practice in livestock production (Roe and Pillai, 2003). Antibiotics may be administered to whole flocks rather than individual animals in intensively reared food animals. In addition, antibacterial agents may be continuously fed to food grade animals such as broilers and turkeys, as growth promoters. Therefore, the antibiotic selection pressure for resistance amongst bacteria in poultry is high and consequently their faecal flora contains a relatively high proportion of resistant bacteria (Van den Bogaard and Stobberingh, 1999).

The prevalence of some antibiotic-resistant bacteria in chicken litter or chicken litter based-organic fertilizers can reach more than 60% for selected microorganisms, while it should be noted that some bacteria, such as *Escherichia coli*, *Enterococcus*, and *Providencia*, are found to be multi-resistant to various antibiotics (Chen and Jiang, 2014). Moreover, as was observed by Khan *et al.* (2002), erythromycin-resistant Staphylococci, Enterococci, and Streptococci were only isolated from litter samples collected from poultry houses that had used the antibiotics.

In a similar trend, Sridevi Dhanarani *et al.*, (2009) isolated one hundred twenty isolates of bacteria from poultry litter samples and investigated the antibiotic resistance and its mode of transmission. Susceptibility pattern of these isolates was determined against different antibiotics such as Ampicillin, Streptomycin, Erythromycin, Tetracycline, Chloramphenicol, Kanamycin, Tobramycin, and Rifampicin. The overall resistance pattern showed that all 120 isolates had different patterns of resistance to antibiotics. The resistance pattern was found as Streptomycin (75%), Erythromycin (56.6%), Tobramycin

(54.1%), Ampicillin, (50%), Rifampicin (45.8%), Kanamycin (40%), Tetracycline (25%) and Chloramphenicol (3.33%). *Staphylococcus*, *Streptococcus*, and *Micrococcus* were randomly selected and examined for plasmids and plasmid-curing and plasmid-induced transformation studies. *Streptococcus* and *Micrococcus* harbored a plasmid of 4.2 and 5.1 kb, respectively, whereas *Staphylococcus* did not harbor any plasmids. This study showed that the mechanisms of horizontal gene transfer between bacteria in poultry litter, are either conjugation or transformation.

Moreover, Hemen *et al.* (2012) isolated *Shigella*, *Salmonella* and *Escherichia coli* from poultry litter and tested their antibiotic sensitivity patterns against Septrin, Chloramphenicol, Sparfloxacin, Ciprofloxacin, Amoxycillin, Augmentin, Gentamicin, Pefloxacin, Tarivid and Streptomycin. *Escherichia coli* were found to be resistant against 8 out of 10 drugs against which their antibiotic sensitivity pattern was tested followed by *Shigella* (6 out of 10) and *Salmonella* (3 out of 10). *Shigella* and *Salmonella* were completely resistant to Chloramphenicol, Augmentin, Pefloxacin, Amoxycillin. *Shigella* was also resistant to all the antibiotics except Septrin and Ciprofloxacin. Percentage antibiotics susceptibility pattern of Gram negative bacteria showed that all bacterial isolates (100%) were resistant to Chloramphenicol while most of the isolates were susceptible to Amoxycillin.

A low level of antibiotic(s) may cause bacteria to select for resistance in the gastrointestinal tract of the food animal and also under *in vitro* conditions when antibiotic-laden manure is applied to the agricultural land (Levy, 1992).

Therefore the concern about the presence of antibiotic-resistant bacteria in animal manures from both on-farm exposure and off-farm contamination is increasing. Widespread dispersal of chicken litter or chicken litter-based organic fertilizers harboring antibiotic-resistant food borne pathogens can be a serious environmental hazard. Furthermore, horizontal transfer of mobile antibiotic resistance genes from one bacterium to another can possibly occur (Rensing *et al.*, 2002).

In a similar trend, von Wintersdorff *et al.* (2016) mentioned in his review that the emergence and spread of antibiotic resistance among pathogenic bacteria has been a rising

problem for public health in recent decades. It is becoming increasingly recognized that not only antibiotic resistance genes (ARGs) encountered in clinical pathogens are of relevance, but rather, all pathogenic, commensal as well as environmental bacteria—and also mobile genetic elements and bacteriophages—form a reservoir of ARGs (the resistome) from which pathogenic bacteria can acquire resistance via horizontal gene transfer (HGT). HGT has caused antibiotic resistance to spread from commensal and environmental species to pathogenic ones, as has been shown for some clinically important ARGs. Of the three canonical mechanisms of HGT, conjugation is thought to have the greatest influence on the dissemination of ARGs. While transformation and transduction are deemed less important, recent discoveries suggest their role may be larger than previously thought. Understanding the extent of the resistome and how its mobilization to pathogenic bacteria takes place is essential for efforts to control the dissemination of these genes.

In the year 2009, Okamoto *et al.* analyzed 100 samples of *Salmonella enteritidis* (SE) isolated from avian material aiming at detecting the class 1 integron gene, the integron involved in antibacterial resistance, by means of polymerase chain reaction (PCR), and comparing it with plate inhibition test. Subsequently, SE samples were evaluated for their capacity to horizontally transfer this gene and it was observed that there was no direct relationship between the presence of the class 1 integron gene and SE resistance to the 14 antimicrobials tested, as 80% of the studied samples were resistant to up to three antimicrobials, and did not present the aforementioned gene. However, horizontal transfer of this gene was accomplished *in vitro* (from *Escherichia coli* to *Salmonella enteritidis*), demonstrating that class 1 integron gene can be disseminated among enterobacteria.

Furtula *et al.* (2013) collected 12 surface water and 28 ground water samples in the Abbotsford area of British Columbia, Canada, near poultry farms and berry farms that used poultry litter as fertilizer, as well as a reference site in a residential area in Port Moody, British Columbia. They also collected litter samples from two different poultry farms, one broiler farm and one layer farm. *Enterococci* were isolated from these samples and tested for resistance to 16 clinical antibiotics. Overall, 86% of litter isolates, 58% of surface water isolates and 100% of ground water isolates were resistant to more than one antibiotic.

Resistance to Lincomycin, Tetracycline, Penicillin and Ciprofloxacin in poultry litter isolates was high as 80.3%, 65.3%, 61.1% and 49.6%, respectively. Resistance in the surface water to the same antibiotics was 87.1%, 24.1%, 7.6% and 12.9%, respectively.

From the above available literature, it becomes very evident that poultry litter is a very prominent source of microorganisms bearing high level of antibiotic resistance; which if untreated and used as agricultural manure may pose a serious threat of horizontal spread of resistance factors.

4. OBJECTIVE OF THE STUDY

The main objective of the present study was to first understand the extent of ABR in the poultry environment and then establish if the resistant bacteria is moving out of poultry farms into the environment through waste disposal.

5. MATERIALS AND METHODS

5.1 Sampling

Sample collection was done from 12 poultry farms during 2016-17. These farms were distributed within 4 different states (comprising of nine districts) namely, Haryana (Jind, Panipat & Gurugram), Uttar Pradesh (Meerut, Bulandshahr & Ghaziabad), Rajasthan (Alwar & Jaipur) and Punjab (Ludhiana). All farms were located in different clusters, *i.e.* villages with at least three to four broiler farms. The number of birds in farms ranged from 3,000–21,000. Antibiotics were used in all the farms but the exact package of practice was not disclosed.

Samples collected from the farms were uniform except for the samples from Jaipur farm. From each farm one litter (inside shed), one poultry farm soil (outside shed) and one agricultural soil sample where reportedly litter is being used as manure, were collected. However, in case of the Jaipur farm, the agricultural soil sample could not be collected as there were no agricultural lands near the poultry farm.

Additionally, for control, 12 more samples were collected from soil (from a nearby road) about 10–20 km from the respective farms. There were no apparent poultry farms nearby

and reportedly litter was not thrown. Details of samples collected are presented in *Annexure-I*.

5.2 Equipments

- ❖ Autoclave
- ❖ Laminar Air Flow
- ❖ Shaker Incubator
- ❖ Bacteriological Incubator
- ❖ Compound Microscope

5.3 Chemicals

All the chemicals and media used for the study were of analytical grade and purchased from HiMedia, India. The biochemical identification kits were also purchased from HiMedia. Ultrapure water used was obtained from Elga USF Maxima Ultra Pure DI Water System.

5.4 Glassware

Glassware used *viz.* measuring cylinders, beakers, conical flasks, funnels, pipettes, watch glasses and glass rods were obtained from Borosil. The measuring cylinders and pipettes used were calibrated. All the glasswares were cleaned with detergent followed by rinsing thoroughly with double distilled water (ddw) before use.

5.5 Standards

Antibiotic discs were purchased from HiMedia, India.

5.6 Sample Preparation

One gram of each of litter and soil samples were aseptically added separately into different sterile vials containing 9 mL of sterile normal saline. Further, they were subjected to 10 fold serial dilution.

5.7 Isolation of bacteria from collected samples their characterization and Identification

Samples collected from poultry farms were subjected to their microbial analysis for the isolation of *Escherichia coli*, *Klebsiella* sp., and *Staphylococcus* sp. These samples were

also subjected to microbial analysis for Total Viable Count of Bacteria. Standard methodologies were used for the isolation of different bacteria which are listed below:

<i>Escherichia coli</i>	:	IS 5887 (Part I) – 1976 (Reaffirmed 2005)
<i>Staphylococcus</i> sp.	:	IS 5887 (Part 8/Sec 1): 2002
<i>Klebsiella</i> sp.	:	On Klebsiella Selective Agar Media (HiMedia)

Isolated cultures from all the samples were characterized and identified using a combination of colony characteristics, morphology, and different biochemical tests using biochemical identification kits of HiMedia.

Identity of over 10% of the isolated bacteria (selected on the basis of geographical and frequency distribution) was confirmed by 16S rDNA sequence analysis. The 16S rDNA sequence analysis of the shortlisted cultures was done by a third party *i.e.* Chromous Biotech Pvt. Ltd., Bangalore, Karnataka. During the analysis, the PCR product (~1500bp) was sequenced using ABI PRISM Big Dye Terminators v 3.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instruction employing 16S rDNA universal primers. The comparison of the nucleotide sequences of the fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast>).

5.8 Antibiotic susceptibility test

The antibiotic susceptibility pattern of all the isolated bacteria from each farm as well as from control samples was determined using the disk diffusion method according to the Bauer - Kirby technique (Bauer *et al.*, 1966).

Pure cultures were grown in nutrient broth separately. Further, to grow a homogeneous mat of the bacterium on Muller Hinton Agar plate, pure cultures were swabbed onto the plates using sterile swabs. Discs of different antibiotics were placed aseptically on swabbed plates (3 discs on 1 plate) and incubated at 37⁰C for 24 hours. All the three targeted bacteria were subjected to antibiotic susceptibility tests against different antibiotics in triplicate (Table 1).

Table 1: Antibiotics used against the three targeted bacteria

S. No.	Antibiotic and its concentration	Antibiotic Class	<i>E. coli</i>	<i>Klebsiella sp.</i>	<i>Staphylococcus sp.</i>
1.	Doxycycline Hydrochloride (DO – 30 µg)	Tetracyclines	√	√	√
2.	Amoxyclav (AMC – 30 µg)	Penicillins	√	√	√
3.	Nitrofurantoin (NIT – 100 µg)	Nitrofurans	√	√	√
4.	Levofloxacin (LE – 5 µg)	Quinolones	√	√	√
5.	Ciprofloxacin (CIP – 5 µg)		√	√	√
6.	Chloramphenicol (C – 30 µg)	Amphenicols	√	√	√
7.	Cefuroxime (CXM – 30 µg)	Cephalosporins - 1st and 2nd generation	√	√	√
8.	Cefotaxime (CTX – 30 µg)	Cephalosporins - 3rd, 4th and 5th generation	√	√	√
9.	Ceftriaxone (CTR – 30 µg)		√	√	√
10.	Amikacin (AK – 30 µg)	Aminoglycosides	√	√	√
11.	Gentamicin (GEN – 10 µg)		√	√	√
12.	Co-trimoxazole (COT – 25 µg)	Sulfonamides, dihydrofolatereductase inhibitors and combinations	√	√	√
13.	Meropenem (MRP – 10 µg)	Carbapenems	√	√	√
14.	Clindamycin (CD – 2 µg)*	Lincosamides	-	-	√
15.	Linezolid (LZ – 30 µg)*	Oxazolidinones	-	-	√
16.	Azithromycin (AZM – 15 µg)*	Macrolides and ketolides	-	-	√

Note*: Not tested against *E. coli* and *Klebsiella sp.* due to the unavailability of the standards.

Table 1 shows that all the isolates of *Staphylococcus* sp. were analysed for antibiotic susceptibility test against a total of 16 antibiotics. However, the isolates of *E. coli* and *Klebsiella* sp. were tested for their antibiotic susceptibility test against 13 antibiotics *i.e.* all mentioned above except for CD, LZ and AZM (due to the unavailability of standards).

The zones of inhibition obtained (in mm) for each bacterium was compared with the standards of Clinical and Laboratory Standards Institute (CLSI) and where CLSI standard was not available, European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards were used.

6. RESULTS AND DISCUSSION

6.1. Isolation, characterization and Identification of bacteria

All the samples collected from poultry farms were subjected to the isolation of three different genera [*Escherichia coli* (E), *Klebsiella* sp. (K) and *Staphylococcus* sp. (S)] using their specific media. The total bacterial population of all poultry samples was also noted. The results obtained are presented in Table 2a & b.

Table 2a: Bacteria isolated from samples collected from poultry farms and nearby agricultural soil

Place of Sampling	S. No.	Sample ID	Total Viable Count (cfu/g)	<i>E. coli</i>	<i>Klebsiella</i> sp.	<i>Staphylococcus</i> sp.
Jind, Haryana	1.	F 1a	279 X 10 ⁵	5	5	1
		F 1b	44 X 10 ⁵	-	-	-
		F 1c	43 X 10 ⁴	-	-	-
Panipat, Haryana	2.	F2a	142 X 10 ⁶	5	6	4
		F2b	72 X 10 ⁵	-	-	-
		F2c	38 X 10 ⁵	2	-	2
	3.	F3a	107 X 10 ⁶	1	1	2
		F3b	80 X 10 ⁵	1	2	1
		F3c	53 X 10 ⁵	1	1	1
Gurugram, Haryana	4.	F4a	192 X 10 ⁶	2	2	1
		F4b	225 X 10 ⁴	1	-	1
		F4c	88 X 10 ⁶	1	2	1
Meerut,	5.	F5a	281 X 10 ⁵	5	16	6

Uttar Pradesh		F5b	90 X 10 ⁴	-	1	1
		F5c	39 X 10 ⁴	-	1	-
Bulandshahr, Uttar Pradesh	6.	F6 (a)	41 X 10 ⁶	2	-	3
		F6 (b)	44 X 10 ⁴	-	-	2
		F6 (c)	79 X 10 ⁴	1	3	3
	7.	F7 (a)	47 X 10 ⁶	3	3	4
		F7 (b)	112 X 10 ⁴	-	-	3
		F7 (c)	146 X 10 ⁵	1	-	2
Ghaziabad, Uttar Pradesh	8.	F8a	56 X 10 ⁷	2	2	2
		F8b	103 X 10 ⁵	1	2	
		F8c	45 X 10 ⁶	2	-	1
Alwar, Rajasthan	9.	F9a	185 X 10 ⁶	10	4	-
		F9b	51 X 10 ⁴	-	-	-
		F9c	74 X 10 ⁵	-	4	-
Jaipur, Rajasthan	10.	F10a	291 X 10 ⁵	3	2	2
		F10b	121 X 10 ⁵	-	-	3
Ludhiana, Punjab	11.	F11a	165 X 10 ⁷	4	4	3
		F11b	38 X 10 ⁷	-	-	2
		F11c	65 X 10 ⁷	3	-	2
	12.	F12a	78 X 10 ⁷	4	4	2
		F12b	43 X 10 ⁵	-	-	3
		F12c	47 X 10 ⁵	2	-	2
Total bacterial isolates				62	65	60

Note: - = Absent; a= poultry litter; b= poultry farm soil; c= agricultural soil

Table 2b: Isolation of bacteria from control samples

Place of Sampling	Sample ID	Total Viable Count (cfu/g)	<i>E. coli</i>	<i>Klebsiella</i> sp.	<i>Staphylococcus</i> sp.
Jind, Haryana	Control (C1)	52 X 10 ⁵	-	-	2
Panipat, Haryana	Control (C2)	55 X 10 ⁵	-	1	1
Gurugram, Haryana	Control (C3)	129 X 10 ⁵	-	-	1
Meerut, Uttar Pradesh	Control (C4)	56 X 10 ⁴	-	-	6
	Control (C5)	32 X 10 ⁴	-	-	-

Bulandshahr, Uttar Pradesh	Control (C6)	107 X 10 ⁶	-	-	2
	Control (C7)	97 X 10 ⁶	-	-	3
Ghaziabad, Uttar Pradesh	Control (C8)	64 X 10 ⁶	-	-	-
Alwar, Rajasthan	Control (C9)	128 X 10 ⁶	-	4	-
	Control (C10)	111 X 10 ⁷	-	4	1
Jaipur, Rajasthan	Control (C11)	48 X 10 ³	-	-	3
Ludhiana, Punjab	Control (C12)	120 X 10 ⁴	-	-	2
Total bacterial isolates			0	9	21

Note: - = Absent

It is clear from the observations presented in Table 2a that, there was a variation in the isolates of bacteria detained and that the frequency of occurrence of *Klebsiella* sp. (65 isolates) was higher followed by that of *E. coli* (62 isolates) and *Staphylococcus* sp. (60 isolates). Further, it was also observed that the total viable count of bacteria was higher in Litter followed by Agricultural soil and poultry farm soil, respectively. In case of litter samples, all the 3 target bacteria were present in 10 out of 12 samples; whereas in case of agricultural soil samples the 3 bacteria were present in 3 out of 11 samples. Further, in case of poultry farm soil all the 3 bacteria were found in 1 out of 12 samples.

On the other hand, observations presented in Table 2b show that, the pattern of isolates of the target bacteria obtained from the control samples were quite different to that observed in the poultry environmental samples. No isolates of *E. coli* were obtained in the control samples. In addition to this, a total of 9 isolates of *Klebsiella* sp. were obtained (only in the control samples from Panipat and Alwar, not in the other samples). On the contrary, isolates of *Staphylococcus* sp. were widely distributed (total 21 in number) in the control samples.

Based on the biochemical identification results and on comparing with standard biochemical test results of the three genera of concern; the isolates which demonstrated maximum frequency with standard biochemical tests; such isolates were further subjected to molecular identification / confirmation through 16S rDNA sequence analysis.

The report of the 16S rDNA sequence analysis confirms the identity of the isolated cultures. However, the summarised identification report is presented in Table 3 a, b & c.

Table 3a: Identification report for *E. coli* isolates

S. No.	Isolates no.	Source sample identity	Identified as similar to
1.	E11	Poultry Litter (Jind, Haryana)	<i>Escherichia coli</i>
2.	E57	Agricultural soil (Ludhiana, Punjab)	<i>Escherichia coli</i>
3.	E98	Poultry Farm Soil (Gurugram, Haryana)	<i>Escherichia coli</i>
4.	E69	Agricultural soil (Panipat, Haryana)	<i>Escherichia coli</i>
5.	E85	Agricultural soil (Bulandshahr, Uttar Pradesh)	<i>Escherichia coli</i>
6.	E90	Poultry Litter (Jaipur, Rajasthan)	<i>Escherichia coli</i>
7.	E32	Poultry Litter (Meerut, Uttar Pradesh)	<i>Escherichia coli</i>

Table 3b: Identification report for *Klebsiella* sp. isolates

S. No.	Isolate No.	Source Sample identity	Identified as similar to
1.	K117	Agricultural soil (Bulandshahr, Uttar Pradesh)	<i>Klebsiella pneumoniae</i>
2.	K159	Poultry Litter (Panipat, Haryana)	<i>Klebsiella</i> sp.
3.	K172	Poultry Farm Soil (Ghaziabad, Uttar Pradesh)	<i>Klebsiella pneumoniae</i>
4.	K5	Poultry Litter (Jind, Haryana)	<i>Klebsiella</i> sp.
5.	K121	Poultry Litter (Bulandshahr, Uttar Pradesh)	<i>Klebsiella pneumoniae</i>
6.	K143	Poultry Litter (Ludhiana, Punjab)	<i>Klebsiella pneumoniae</i>
7.	K68	Poultry Litter (Alwar, Rajasthan)	<i>Klebsiella pneumoniae</i>
8.	K28	Agricultural soil (Alwar, Rajasthan)	<i>Klebsiella pneumoniae</i>
9.	K113	Poultry Litter (Jaipur, Rajasthan)	<i>Klebsiella</i> sp.
10.	K98	Control soil (Alwar, Rajasthan)	<i>Klebsiella pneumoniae</i>

Table 3c: Identification report for *Staphylococcus* sp. isolates

S. No.	Isolate No.	Source Sample identity	Identified as similar to
1.	S80	Poultry Farm Soil (Meerut, Uttar Pradesh)	<i>Staphylococcus lentus</i>
2.	S210	Poultry Litter (Bulandshahr, Uttar Pradesh)	<i>Staphylococcus sciuri</i>
3.	S218	Poultry Litter (Bulandshahr, Uttar Pradesh)	<i>Staphylococcus lentus</i>
4.	S223	Poultry Farm Soil (Jaipur, Rajasthan)	<i>Staphylococcus lentus</i>
5.	S257	Poultry Farm Soil (Ludhiana, Punjab)	<i>Staphylococcus lentus</i>
6.	S270	Poultry Farm Soil (Ludhiana, Punjab)	<i>Staphylococcus lentus</i>
7.	S280	Poultry Litter (Gurugram, Haryana)	<i>Staphylococcus lentus</i>
8.	S38	Control Soil (Jind, Haryana)	<i>Staphylococcus lentus</i>

It is clear from the details of Table 3a & b that the isolated bacteria (intended to be *E. coli* and *Klebsiella* sp.) have been identified as *Escherichia coli* and *Klebsiella pneumoniae*, respectively. Similar reports have been published by Kim *et al.* (2005), Ogunleye *et al.* (2008), Akond *et al.* (2009), Duru *et al.* (2013), Adelowo *et al.* (2014) and Ibrahim and Hameed (2015) wherein, *Klebsiella pneumoniae* or *Klebsiella* spp. and *E. coli* have been isolated from poultry environment/origin.

Table 3c represents that the isolated bacteria (intended to be *Staphylococcus* sp.) that has been majorly identified as *Staphylococcus lentus*. These results are supported by the fact that *S. lentus* is a commensal bacterium colonizing the skin of several animal species. It has commonly been isolated from food-producing animals, including poultry and dairy animals (Zhang *et al.*, 2009 and Huber *et al.*, 2011), and from their food products (Perreten *et al.*, 1998 and Mauriello *et al.* 2004). In addition to this, the identification of one isolate (S210) as *Staphylococcus sciuri*, is also supported by the report of Stepanovic *et al.*, 2003 and 2005 wherein it is mentioned that *S. lentus* and *S. sciuri* are from the same group (*S. sciuri* species group). In addition to this the characteristics of *S. lentus* and *S. sciuri* are quite similar (Adegoke, 1986). *S. lentus* is generally reported to be an opportunistic pathogen to immune-compromised patients (<http://www.tgw1916.net/Staphylococcus/lentus.html> as viewed on 20.06.17). However,

there are reports of human infections caused by *S. lentus* (Koksal *et al.*, 2009; Mazal and Sieger 2010; Rivera *et al.*, 2014). In addition to this, reports are also there to show the transfer of resistance from *S. lentus* to the most common human pathogen *S. aureus* (Schwendener and Perreten, 2012).

Thus, on the basis of 16S rDNA sequence analysis report, now onwards the isolated bacteria *i.e.* *E. coli*, *Klebsiella* sp. and *Staphylococcus* sp. should be referred to as *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Staphylococcus lentus* (*S. lentus*), respectively.

6.2 Antibiotic susceptibility test of bacteria

The detailed observations were recorded as zone of inhibition (mm) formed by different bacterial isolates against the antibiotics and their interpretations were compared with CLSI standards to determine the resistance pattern (EUCAST standards were used where CLSI standard was not available). Further, the compiled results of total percentage resistance of all the three bacteria against different antibiotics are presented in **Table 4**.

Table 4: Total percentage resistance of different bacteria (from poultry environmental samples) against different antibiotics

S. No.	Antibiotics	Total percentage of isolates showing resistance (%)		
		<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. lentus</i>
1.	Doxycycline hydrochloride (DO)	88.7	65.3	51.7
2.	Amoxyclav (AMC)	90.3	88.9	35.0
3.	Nitrofurantoin (NIT)*	33.9	44.4	58.3
4.	Levofloxacin (LE)	87.1	70.8	45.0
5.	Ciprofloxacin (CIP)	91.9	76.4	48.3
6.	Chloramphenicol (C)	46.8	36.1	23.3
7.	Cefuroxime (CXM)	62.9	54.2	21.7
8.	Cefotaxime (CTX)	77.4	73.6	5.0
9.	Ceftriaxone (CTR)	66.1	58.3	13.3
10.	Amikacin (AK)	27.4	26.4	20.0
11.	Gentamicin (GEN)	19.3	26.4	3.3

12.	Co-Trimoxazole (COT)	93.5	75.0	46.7
13.	Clindamycin (CD)	-	-	71.7
14.	Linezolid (LZ)	-	-	25.0
15.	Azithromycin (AZM)	-	-	65.0
16.	Meropenem (MRP)	100	77.8	13.3

Note: *EUCAST standards were followed. - : Standards not available

Observations presented in Table 4 clearly indicate that in general all the three bacteria show very high level of resistance (expressed as percentage resistance) towards almost all the antibiotics tested. Precisely, the percentage resistance in *E. coli* was higher than that of *K. pneumoniae*. In case of *E. coli* 100% resistance was observed against Meropenem. Also, more than 85% resistance was observed against Doxycycline hydrochloride, Amoxycyclav, Levofloxacin, Ciprofloxacin and Co-Trimoxazole. Further, in case of *K. pneumoniae*, 75% or more resistance was observed against Amoxycyclav, Ciprofloxacin, Co-Trimoxazole and Meropenem. The percentage resistance of *S. lentus* against all the antibiotics was comparatively lesser. Further, percentage resistance of the three targeted bacteria against all the antibiotics in terms of sample types were analysed, which is presented in **Table 5a, 5b, 5c & 5d**.

Table 5a: Total percentage resistance of different bacteria from poultry litter

S. No.	Antibiotics	Total percentage of isolates showing resistance (%)		
		<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. lentus</i>
1.	Doxycycline hydrochloride (DO)	86.9	73.5	56.7
2.	Amoxycyclav (AMC)	91.3	91.8	33.3
3.	Nitrofurantoin (NIT)*	26.1	36.7	53.3
4.	Levofloxacin (LE)	84.8	83.7	46.6
5.	Ciprofloxacin (CIP)	91.3	87.8	53.3
6.	Chloramphenicol (C)	36.9	36.7	16.6
7.	Cefuroxime (CXM)	52.2	55.1	16.6
8.	Cefotaxime (CTX)	76.1	75.5	3.3
9.	Ceftriaxone (CTR)	58.7	53.1	13.3
10.	Amikacin (AK)	26.1	24.5	20.0

11.	Gentamicin (GEN)	13.0	30.6	0.0
12.	Co-Trimoxazole (COT)	91.3	79.6	63.3
13.	Clindamycin (CD)	-	-	86.6
14.	Linezolid (LZ)	-	-	26.6
15.	Azithromycin (AZM)	-	-	70.0
16.	Meropenem (MRP)	100.0	89.8	6.6

Note: *EUCAST standards were followed. – : Standards not available

Table 5b: Total percentage resistance of different bacteria from poultry farm soil

S. No.	Antibiotics	Total percentage of isolates showing resistance (%)		
		<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. lentus</i>
1.	Doxycycline hydrochloride (DO)	66.7	60.0	43.8
2.	Amoxyclav (AMC)	33.3	60.0	43.8
3.	Nitrofurantoin (NIT)*	33.3	40.0	56.2
4.	Levofloxacin (LE)	100.0	60.0	37.5
5.	Ciprofloxacin (CIP)	66.7	80.0	43.8
6.	Chloramphenicol (C)	66.7	40.0	50.0
7.	Cefuroxime (CXM)	100.0	20.0	37.5
8.	Cefotaxime (CTX)	66.7	20.0	12.5
9.	Ceftriaxone (CTR)	66.7	20.0	12.5
10.	Amikacin (AK)	33.3	20.0	12.5
11.	Gentamicin (GEN)	0.0	20.0	12.5
12.	Co-Trimoxazole (COT)	100.0	80.0	37.5
13.	Clindamycin (CD)	-	-	62.5
14.	Linezolid (LZ)	-	-	25.0
15.	Azithromycin (AZM)	-	-	68.8
16.	Meropenem (MRP)	100.0	80.0	25.0

Note: *EUCAST standards were followed. – : Standards not available

Table 5c: Total percentage resistance of different bacteria from agricultural soil

S. No.	Antibiotics	Total percentage of isolates showing resistance (%)		
		<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. lentus</i>
1.	Doxycycline hydrochloride (DO)	100.0	72.7	50.0
2.	Amoxyclav (AMC)	100.0	90.9	28.6
3.	Nitrofurantoin (NIT)*	61.5	54.5	71.4
4.	Levofloxacin (LE)	92.3	54.5	50.0
5.	Ciprofloxacin (CIP)	100.0	63.6	42.9
6.	Chloramphenicol (C)	76.9	36.4	7.1
7.	Cefuroxime (CXM)	92.3	72.7	14.3
8.	Cefotaxime (CTX)	84.6	72.7	0.0
9.	Ceftriaxone (CTR)	92.3	72.7	14.3
10.	Amikacin (AK)	30.7	45.5	28.6
11.	Gentamicin (GEN)	46.1	27.3	0.0
12.	Co-Trimoxazole (COT)	100.0	63.6	21.4
13.	Clindamycin (CD)	-	-	50.0
14.	Linezolid (LZ)	-	-	21.4
15.	Azithromycin (AZM)	-	-	50.0
16.	Meropenem (MRP)	100.0	63.6	14.3

Note: *EUCAST standards were followed. – : Standards not available

Table 5d: Total percentage resistance of different bacteria from control soil

S. No.	Antibiotics	Total percentage of isolates showing resistance (%)		
		<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. lentus</i>
1.	Doxycycline hydrochloride (DO)	NA	77.7	38.1
2.	Amoxyclav (AMC)	NA	100	61.9
3.	Nitrofurantoin (NIT)*	NA	22.2	47.6
4.	Levofloxacin (LE)	NA	77.7	23.8
5.	Ciprofloxacin (CIP)	NA	77.7	9.5
6.	Chloramphenicol (C)	NA	66.7	33.3
7.	Cefuroxime (CXM)	NA	33.3	28.6
8.	Cefotaxime (CTX)	NA	77.7	19.0
9.	Ceftriaxone (CTR)	NA	66.7	28.6
10.	Amikacin (AK)	NA	44.4	23.8
11.	Gentamicin (GEN)	NA	33.3	4.7

12.	Co-Trimoxazole (COT)	NA	55.5	38.1
13.	Clindamycin (CD)	-	-	85.7
14.	Linezolid (LZ)	-	-	23.8
15.	Azithromycin (AZM)	-	-	71.4
16.	Meropenem (MRP)	NA	77.7	9.5

Note: *EUCAST standards were followed. - : Standards not available

It is clear from the observations presented in Table 5a, b, c & d that the antibiotic resistance percentage in the isolates from poultry litter samples is higher followed by the resistance in the isolates from agricultural soil which is further followed by the resistance in the isolates from poultry farm soil and control samples, respectively. Therefore, to understand the pattern of resistance in the isolates from different samples an analysis was done on the basis of sample type and drug resistance pattern. The resistance pattern of the three targeted bacteria against all the antibiotics tested in terms of sample type is presented in **Table 6**.

Table 6: Resistance pattern in bacteria from different sample types

<i>E. coli</i>			
Poultry Litter	Poultry Farm Soil	Agricultural Soil	Control Soil
Resistance was observed against all the 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in 19.6% of the isolates > 5-10 antibiotics in 73.9% of the isolates 3-5 antibiotics in 6.5% of the isolates < 3 antibiotics in none of the isolates	Resistance was observed against 12 out of 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in 33.3% of the isolates > 5-10 antibiotics in 66.7% of the isolates 3-5 antibiotics in none of the isolates < 3 antibiotics in none of the isolates	Resistance was observed against all the 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in 61.5% of the isolates > 5-10 antibiotics in 38.5% of the isolates 3-5 antibiotics in none of the isolates < 3 antibiotics in none of the isolates	--
<i>K. pneumoniae</i>			
Poultry Litter	Poultry Farm Soil	Agricultural Soil	Control Soil
Resistance was observed against all the 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in 18.4% of the isolates > 5-10 antibiotics in 67.3% of the isolates 3-5 antibiotics in 12.2% of the isolates < 3 antibiotics in 2% of the isolates	Resistance was observed against all the 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in none of the isolates > 5-10 antibiotics in 80% of the isolates 3-5 antibiotics in none of the isolates < 3 antibiotics in 20% of the isolates	Resistance was observed against all the 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in 54.5% of the isolates > 5-10 antibiotics in 9.1% of the isolates 3-5 antibiotics in 18.2% of the isolates < 3 antibiotics in 18.2% of the isolates	Resistance was observed against all the 13 antibiotics tested. Of which resistance was found against: >10 antibiotics in 22.2% of the isolates > 5-10 antibiotics in 66.7% of the isolates 3-5 antibiotics in 11.1% of the isolates < 3 antibiotics in none of the isolates
<i>S. lentus</i>			
Poultry Litter	Poultry Farm Soil	Agricultural Soil	Control Soil
Resistance was observed against 15 out of 16 antibiotics tested. Of which resistance was found against: >10 antibiotics in none of the isolates > 5-10 antibiotics in 53.3% of the isolates 3-5 antibiotics in 36.7% of the isolates < 3 antibiotics in 10% of the isolates	Resistance was observed against all the 16 antibiotics tested. Of which resistance was found against: >10 antibiotics in 12.5% of the isolates > 5-10 antibiotics in 43.8% of the isolates 3-5 antibiotics in 18.8% of the isolates < 3 antibiotics in 25% of the isolates	Resistance was observed against 14 out of 16 antibiotics tested. Of which resistance was found against: >10 antibiotics in none of the isolates > 5-10 antibiotics in 50% of the isolates 3-5 antibiotics in 14.3% of the isolates < 3 antibiotics in 35.7% of the isolates	Resistance was observed against all the 16 antibiotics tested. Of which resistance was found against: >10 antibiotics in 4.8% of the isolates > 5-10 antibiotics in 42.9% of the isolates 3-5 antibiotics in 42.9% of the isolates < 3 antibiotics in 9.5% of the isolates

Note: — : Isolates were not found in those samples

Observations presented in Table 6, clearly show that there is very high level of multidrug resistance in most of the isolates of all the three target bacteria. In case of *E. coli*, 100% of the isolates were multidrug resistant as all of them were resistant against antibiotics of 3 or more classes. Wherein, most of the isolates (66.1%) of *E. coli* were resistant to 5-10 antibiotics. Further, 40% isolates were resistant to 10 or more antibiotics.

Of all the isolates of *K. pneumoniae*, 92.3% were multidrug resistant, of which 58.5% isolates were resistant towards 5-10 antibiotics followed by over 30% of the isolates showing resistant against 10 or more antibiotics. However, in case of *S. lentus*, 78.3% of the total isolates were multidrug resistant of which, 50% of the isolates were resistant against 5-10 antibiotics.

Moreover, on the basis of the resistance pattern of the isolates against different antibiotics, it was observed that there is a correlation in the resistance pattern of the isolates from poultry litter and in the isolates from agricultural soil (where litter is reportedly being used as manure). On the other hand, such a strong correlation was not observed in the resistance pattern of the isolates from poultry litter and poultry farm soil or even poultry farm soil and agricultural soil. In addition to that, the resistance in the isolates from control samples could not be compared as there was no proper distribution of isolates amongst the samples.

Table 7: Statistical correlation parameters for the target bacteria from litter vs agricultural soil

Name of Bacteria	p value	Pearson coefficient (r)
<i>E. coli</i>	0.08	0.88
<i>K. pneumoniae</i>	0.83	0.70
<i>S. lentus</i>	0.45	0.81

Furthermore, the statistical analysis of the results (Table 7) proved that in case of *E. coli* there is a strong statistical correlation (p value of 0.08; Pearson's correlation coefficient $r=0.88$) between the resistance pattern in the isolates from poultry litter and agricultural soil. This was also supported by the observation that there were very few isolates of *E. coli* from poultry farm soil. Both these facts in a way depict that untreated litter is being dumped in the agricultural land to be used as manure which eventually reflects the possible transfer of

antibiotic resistance from litter to agricultural land. On the other hand, such a correlation could not be drawn in case of *K. pneumoniae* and *S. lentus* from litter and agricultural land. Also, in case of *S. lentus* and *K. pneumoniae* from control soil and agricultural soil, as well as *S. lentus* from litter and poultry farm soil, the resistance pattern was different statistically.

7. CONCLUSIONS

- High multidrug resistance found in poultry environment (poultry litter, poultry farm soil and nearby agricultural soil). Overall, the highest resistance was found in *E. coli*, followed by *K. pneumoniae* and *S. lentus*.
- Multidrug resistance is moving from farms to agricultural fields in the case of *E. coli*. This is seen through presence of similar proportion of isolates, similar pattern of resistance and strong statistical correlation in *E. coli* resistance in both litter and agricultural soil.
- A statistical correlation could not be observed for resistance of *K. pneumoniae* and *S. lentus* in litter and agricultural soil. More studies are required to understand their behaviour in view of different sources of bacteria such as other animals and synthetic fertilizer and pesticides in the agricultural fields.

8. RECOMMENDATIONS

8.1 Recommendations to reduce antibiotic use in food animal production

PML's study of 2014 found antibiotic residues in chicken meat and based on which CSE had highlighted rampant use of antibiotics in chicken and had proposed a number of recommendations to regulate and limit the antibiotic misuse in poultry sector. Minimizing antibiotic use in food animal production is the most effective way to address resistance spread from farms. The Central and state animal husbandry departments, drug control departments and food safety departments must take a lead in this. Since no control on antibiotic misuse has been attained so far, a concrete action on the following recommendations must be considered:

- Non-therapeutic use of antibiotics for growth promotion and mass disease prevention should be prohibited. It should only be used to cure the sick, based on prescription of veterinarians.
- Antibiotics should not be allowed in feed and feed supplement. The government should set standards for animal feed, regulate the business.
- Antibiotics that are critical for humans should not be allowed for use in animals.
- The development, production and use of alternative antibiotic-free growth promoters, such as herbal supplements, should be encouraged.
- It should be ensured that licensed antibiotics reach registered users through registered distributors or stockists of veterinary medicines. All animal antibiotics should be traceable from the manufacturing site to user. Stringent control on import of antibiotics and feed supplements should be implemented.
- Good farm management practices should be followed to control infection and stress among the flock. Biosecurity guidelines of the Central Poultry Development Organisation should be improved and applied to all farms. Capacity of small farmers must be enhanced so that they can comply with the guidelines. The guidelines should be legally enforced on big companies.
- Alternatives to antibiotics should be explored and adopted. For example, vaccinations should be promoted against bacterial diseases.
- Veterinarians should be trained and educated on judicious use of antibiotics and infection prevention. The government should ensure that veterinarians do not get incentives for prescribing more antibiotics.
- There is a need to introduce a labelling system wherein poultry raised without use of antibiotics should be labelled through reliable certified schemes to facilitate consumer choice. Poultry produced with antibiotics must also be labelled accordingly. This would incentivize the farmer who can charge a premium and provide consumer with a healthy choice.

- Lack of data on the use of antibiotics and drug resistance is a major problem in India. It is necessary to create an integrated surveillance system to monitor antibiotics use and antibiotics resistance trends in humans, animals and food chain. A national-level database should be developed and kept in the public domain.

8.2 Recommendations to reduce the spread of ABR from farms

The Indian NAP-AMR aims to address the environmental aspect of antibiotic resistance through necessary laws and environmental surveillance. The implementation of it will be a bigger task and is yet to be seen. Management of waste from farms will therefore require adoption of a new ABR-centric approach and a greater leadership role of environment regulators such as the CPCB and SPCBs and the nodal ministry, i.e. MoEFCC. CSE being a stakeholder in implementation of NAP-AMR recommends that the following recommendations be considered.

- The MoEFCC and CPCB should develop ABR-centric environmental regulations for farms and factories/industry. Additionally, for poultry sector, the existing CPCB guidelines, ‘Environmental Guidelines for Poultry Farm’ should be modified and strengthened in view of the below mentioned recommendations and notified. The SPCBs should make it mandatory in states and ensure its implementation.
 - Pollution causing potential from poultry farm sector should be recategorized and prioritized to provide the required mandate to develop laws and conduct ABR surveillance by CPCB and SPCBs.
 - Manure management approaches in poultry farms which pose lesser risk to the spread of ABR should be preferred than more risky approaches such as land application of manure. For example, biogas generation must

be the most preferred approach of managing litter/ manure from farms. Other options of waste to energy conversion can also be explored.

- Big/integrated poultry farms having large volumes of litter/manure must only be allowed to manage waste through in-house biogas generation plants. This should also become a part of criteria for licensing and renewal of farms going forward.
- Small poultry farmers, particularly those operating in a cluster should be encouraged to develop and manage a common biogas generation plant. This should be supported by a national-level programme which starts from key hubs and select poultry producing states.
- Land application of untreated litter must be prohibited through necessary laws, awareness and surveillance. Only application of treated litter/manure should be allowed if the option of biogas generation is not feasible.
- Proper composting for treatment of manure should be encouraged only under very high level of supervision. In this regard, laws in line with global best practices should be framed with reference to approval of composting sites, validation of treated manure and timing of application of litter/manure and type of land it could be applied to.
- In order to prevent resistance spread across food animal production settings, poultry litter must not be allowed to be used as feed for fishes in aquaculture. Central and state Fisheries departments must ensure this through necessary laws, awareness and surveillance.
- Finally, the ABR research agenda should include, understanding the impact of litter/manure treatment through composting/biogas generation on resistant bacteria and mechanism and movement of transfer of resistance from farms to environment through waste. This should be led by the scientific

community which includes those from the Indian Council of Agricultural Research, State colleges of veterinary sciences and environmental studies etc. with the support from regulatory surveillance.

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ANNEXURE-I**Details of Samples Collected from Poultry Farms**

S.No.	State	Sample Name	Sample Code	Location
1.	Haryana	Poultry Litter	F1 (a)	Safidon Town, Jind
		Poultry Farm Soil	F1 (b)	
		Agricultural Soil	F1 (c)	
2.		Poultry Litter	F2 (a)	Kawi Village, Panipat
		Poultry Farm Soil	F2 (b)	
		Agricultural Soil	F2 (c)	
3.		Poultry Litter	F3 (a)	Ahmadpur Majra Village, Panipat
		Poultry Farm Soil	F3 (b)	
		Agricultural Soil	F3 (c)	
4.		Poultry Litter	F4 (a)	Sanpka Village, Gurugram
		Poultry Farm Soil	F4 (b)	
		Agricultural Soil	F4 (c)	
5.		Control	C 1	10 Km far from Khedi Taloda (Jind)
6.		Control	C 2	10 Km far from Kawi
7.	Control	C 3	10 Km far from Sanpka	
8.	Poultry Litter	F5 (a)	Mamepur Village, Meerut	
	Poultry Farm Soil	F5 (b)		
	Agricultural Soil	F5 (c)		
9.	Poultry Litter	F6 (a)	Bhaipur Village, Bulandshahr	
	Poultry Farm Soil	F6 (b)		
	Agricultural Soil	F6 (c)		
10.	Poultry Litter	F7 (a)	Ranapur Village, Bulandshahr	
	Poultry Farm Soil	F7 (b)		
	Agricultural Soil	F7 (c)		
11.	Poultry Litter	F8 (a)	Kushalya Village, Ghaziabad	
	Poultry Farm Soil	F8 (b)		
	Agricultural Soil	F8 (c)		
12.	Control	C 4	10 Km far from Mamepur	
13.	Control	C 5	20-25 Km far from Meerut	
14.	Control	C 6	10-15 far away from	

				Bhaipur	
15.		Control	C 7	15-20 Km far from Ranapur	
16.		Control	C 8	10 Km far from Kushalya	
17.	Rajasthan	Poultry Litter	F9 (a)	Rangala Village, Alwar	
		Poultry Farm Soil	F9 (b)		
		Agricultural Soil	F9 (c)		
18.			Poultry Litter	F10 (a)	Moriya Village, Jaipur
			Poultry Farm Soil	F10 (b)	
19.			Control	C 9	10 Km far from Rangala
20.			Control	C 10	20 Km far from Rangala
21.			Control	C 11	10 Km far from Morija
22.	Punjab	Poultry Litter	F11 (a)	Kotla Shamshapur Village, Ludhiana	
		Poultry Farm Soil	F11 (b)		
		Agricultural Soil	F11 (c)		
23.			Poultry Litter	F12 (a)	Sangatpura Village, Ludhiana
			Poultry Farm Soil	F12 (b)	
			Agricultural Soil	F12 (c)	
24.			Control	C 12	10 Km far from Sangatpura

Note:

Total Litter samples : 12

Soil Outside shed : 12

Agricultural soil : 11

Control Soil : 12

ANNEXURE – II

Zone Size Interpretative Chart for Antimicrobial Susceptibility Testing as per CLSI Standards

S No	Name of Antibiotics	<i>Enterobacteriaceae</i> [#]			<i>Staphylococcus sp.</i>		
		S	I	R	S	I	R
1	Doxycycline hydrochloride (DO)	14	11-13	10	16	13-15	12
2	Amoxyclav (AMC)	18	14-17	13	20	NA	19
3	Nitrofurantoin (NIT)*	11	NA	11	13	NA	13
4	Levofloxacin (LE)	17	14-16	13	19	16-18	15
5	Ciprofloxacin (CIP)	21	16-20	15	21	16-20	15
6	Chloramphenicol (C)	18	13-17	12	18	13-17	12
7	Cefuroxime (CXM)	18	15-17	14	18	15-17	14
8	Cefotaxime (CTX)	26	23-25	22	23	15-22	14
9	Ceftriaxone (CTR)	23	20-22	19	21	14-20	13
10	Amikacin (AK)	17	15-16	14	17	15-16	14
11	Gentamicin (GEN)	15	13-14	12	15	13-14	12
12	Co-Trimoxazole (COT)	16	11-15	10	16	11-15	10
13	Clindamycin (CD)	NA	NA	NA	21	15-20	14
14	Linezolid (LZ)	NA	NA	NA	21	NA	20
15	Azithromycin (AZM)	NA	NA	NA	18	14-17	13
16	Meropenem (MRP)	23	20-22	19	16	14-15	13

Note:

S = Sensitive, zone of inhibition (mm or more); I = Intermediate, zone of inhibition (mm);

R = Resistant, zone of inhibition (mm or less)

* = EUCAST Standards.

Standards of Enterobacteriaceae family for all the drugs tested were used to interpret the zone of inhibitions for *Escherichia coli* and *Klebsiella sp.*