EVALUATION OF THE ANTIMICROBIAL RESISTANCE OF *Salmonella enterica* SEROVAR *enteritidis* ISOLATED FROM POULTRY

BS FYP THESIS

By:

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Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

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2020

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A thesis submitted to the National University of Sciences and Technology, Islamabad, in partial fulfillment of the requirements for the degree of

Bachelor of Sciences in Applied Biosciences

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

2020

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Dedication

Dedicated to our dear parents who have continuously supported us, and to the future bio scientists to help encourage them in their pursuit of science.

<u>Acknowledgements</u>

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LIST OF ABBREVIATIONS

- AMX: Amoxicillin

- AMK: Amikacin

- AP: Ampicillin

- CD: Clindamycin

- CDC: Centre for Disease Control

- CEF: Cefepime

- CFM: Cefixime

- CHL: Chloramphenicol

- CIP: Ciprofloxacin

- CLSI: The Clinical and Laboratory Standards Institute

- CN: Gentamycin

- EFSA: European Food Safety Authority

- ECDC: European Centre for Disease Prevention and Control

- ENR: Enrofloxacin

- ER: Erythromycin

- *E. coli: Escherichia coli*

- GDP: Gross Domestic Product

- HGT: Horizontal Gene Transfer

- IMP: Imipenem

- K: Kanamycin

- LZD: Linezolid

- MEM: Meropenem

- MH: Mueller Hinton

- MIN: Minocycline

- ND: Nalidixic Acid

- OX: Oxacillin

- RD: Rifampicin

- SS agar: Salmonella-Shigella agar

- ST: Streptomycin

- SXT: Sulphamethoxazole

- *S. Typhi: Salmonella typhi*

- SIM: Sulphite Indole Motility

- TET: Tetracycline

- V: Vancomycin

- WHO: World Health Organization

1. Abstract

Salmonella enteritidis is one of the major food-borne pathogens in the poultry industry which is now exhibiting increased antimicrobial resistance. This antimicrobial resistance can easily be transferred to other bacteria and humans through contaminated meat, eggs, and feces. The objective of this study was to evaluate the antimicrobial resistance of seven isolates of *Salmonella enteritidis* obtained from different sources against 23 different antimicrobials. For this purpose, first of all, few confirmatory tests were performed on *Salmonella enteritidis* isolates that were already identified and provided by *Abubakar et al.,* This was followed by antibiotic resistance testing by disc diffusion method in which antimicrobial discs were placed on Mueller-Hinton (MH) agar after bacterial spreading. The results showed that *Salmonella enteritidis* isolates were 100% resistant to 15 antibiotics including amikacin, tetracycline, gentamycin, nalidixic acid, sulfamethoxazole, vancomycin, streptomycin, kanamycin, erythromycin, teicoplanin, rifampicin, enrofloxacin, oxacillin, clindamycin, and minocycline. There was 100% susceptibility towards meropenem. For cephalosporin, ciprofloxacin, and imipenem, one of the seven isolates showed resistance towards them. Most of the *Salmonella enteritidis* isolates were also resistant to the rest of the antibiotics. Concluding, *Salmonella enteritidis* has high resistance even towards extended spectrum antibiotics and can be classified as multi-drug resistant. This is of importance as it can lead to antibiotic resistance in other microbes and humans as well which can be a great public hazard.

2. Introduction

Poultry industry plays a major role in the national economy with a 1.3% contribution to the national GDP and is one of the most vibrant sectors of the Pakistan agricultural industry. It is one of the most well-established sectors of agriculture with a 26.8% contribution to the annual meat production, and 1.3% to the GDP, with an annual growth rate of 10-12% as of 2017. This has increased many folds up till now.

Meat obtained from animals especially poultry plays a significant role in our daily diet. It provides us with many macronutrients and micronutrients. The most common and cheapest source of animal meat in Pakistan is also poultry which includes chicken meat and eggs. Shops selling poultry meat can be seen in every market at every corner. Poultry industry plays a very important role in Pakistan's economy (IK).

However, the poultry industry is facing devastating hazards due to the poor hygienic conditions of the poultry farms and meat selling shops. The feces of the birds can be seen lying around everywhere which is a major source of spread of pathogenic bacteria. Lack of disease control programs is one of the greatest hazards (IK). Mishandled eggs, meat, and other poultry products along with waste products can result in really harmful outcomes due to the presence of many pathogens at each step for raising chicks to their transport. The food-borne bacterial zoonotic diseases are especially harmful if consumed by humans (Sohail).

One such example of the harmful pathogen infecting the poultry is *Salmonella*. This gram negative, non-spore forming bacilli that belong to the family of Enterobacteriaceae has 2500 distinct serotypes and is the major cause of food-borne illnesses around the world. It has a wide host range from birds and mammals to humans. Broadly classifying, the genus contains two species; *Salmonella* enterica and *Salmonella* bongori. *S. enterica* has six subspecies and these cause most of the infections in humans. *Salmonella enterica* has sub-species *Salmonella enterica* which has *serotype enteritidis*. Non-typhoidal *Salmonella* is one of the major causes of food-borne illnesses around the world. The degree of the pathogenicity (infection) depends upon the adaption of a particular bacterial species towards a particular host. For example, *S. typhi* is more

adapted to cause diseases in humans rather than animals, while opposite is the case for *S. gallinarum* and so on.

Salmonella usually cause intestinal distress and food poisoning. Global data also suggests that *Salmonella* related infections have increased massively among the last decade (Administrator). Massive *Salmonella enteritidis* outbreaks in human populations have been observed because of their ability to be transmitted easily through eggs, consumed by humans, without even any discernible illness in poultry. Most of these occur due to the consumption of contaminated eggs and meat (Velge, Cloeckaert, & Barrow, 2005).

Besides humans, poultry is also contaminated with *Salmonella* leading to Salmonellosis. The contamination may arise during production due to vertical (contamination of egg yolk, egg shells, membranes etc.) or horizontal transmission (transmission through gut or contaminated feces), cutting of the meat using the same knives, transport or fecal contamination. *Salmonella* inhabit the intestinal tract of chickens. In young birds, symptoms that are seen include drowsiness, depression, low feed consumption rate, and diahorrea. Adult birds also show lesions in certain infected organs like liver and intestine etc (Sohail).

One of the reasons why it is difficult to control *Salmonella* is because of the numerous sources of infection and product contamination. It is really difficult to control and prevent it throughout from poultry farm to the market and finally to the table.

Antibiotics are given to the birds to treat them against *Salmonella enteritidis* and other serovars as well (tubitak). Another major reason of injecting birds with antibiotics is to promote their growth. Almost 90% usage of all antimicrobial agents is in animal food. But, now it starting to develop resistance against these antibiotics. Multi-drug resistance is also a much known term related to *Salmonella* around the globe (Afshari, Baratpour, Khanzade, & Jamshidi, 2018). This resistance is very dangerous not just for the birds, but for the humans as well who consume chicken as a food source. It is important to investigate which antibiotics are showing higher resistance towards *Salmonella* so their usage can be controlled meanwhile working for alternate ways to control, prevent and treat *Salmonella*.

3. Literature Review

3.1 Importance of Poultry:

Pakistan's poultry business has been known to grow continuously over the past few years; generating a turnover of 1,168 billion rupees (Pakistan Poultry Association, 6/29/2020). Based on the fact that Pakistan's economy is mostly agriculture based, this makes a good contribution to the national GDP, approximately around 1.3% (HUSSAIN, RABBANI, ASLAM, & AHMAD, 2015). A significant portion (around 40-45%) of meat that is consumed by people comes from poultry because it's an affordable source of protein (Pakistan Poultry Association, 6/29/2020). Additionally, the poultry sector is the source of livelihood for over one and a half million people nationwide. The overall contribution of the poultry industry to the total meat production, agricultural industry ad GDP is given in Table 1 (HUSSAIN et al., 2015)

Sector	Poultry Sector Contribution
Total Meat Production	26.8%
Agricultural Sector	5.76%
Overall GDP	1.4%

Table 1: Poultry sector's contribution

3.2 Food Borne Pathogens:

Infection from foodborne pathogens affect 10% of the global population with 33 million deaths annually ("World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010," 2020b).

One of the most important hurdles to optimum production is the threat from pathogens that affect poultry animals. The notorious infectious agents are many known species of *E. coli, Listeria*, *Cyclospora* and *Salmonella*. *Non-typhoidal Salmonella* serovars; most common cause of food poisoning in humans are of primary importance ("Salmonella, Non-Typhoidal Species (S.

Choleraesuis, S. Enteritidis, S. Hadar, S. Typhimurium) - Infectious Disease and Antimicrobial Agents," 6/29/2020). *Salmonella* is non-spore-forming, facultative anaerobic bacilli that is gram negative and a part of the family of *Enterobacteriaceae*. Because of these thousands of serotypes that *Salmonella* have, they have a variety of hosts that include humans and poultry birds. Some *Salmonella* serotypes, such as *typhi* and *paratyphi* are highly adapted to humans and have no other known natural hosts. Others, such as *typhimurium* and *enteritidis*, have a broad host range and can infect a wide variety of animal hosts. Some *Salmonella* species cause salmonellosis, which is a disease that causes gastroenteritis (stomach flu). *Salmonella enterica* has non-typhoidal serovars as well which are responsible for numerous food-borne illnesses that cause diarrhea in humans globally (Clayton et al., 2008) These infections are very common in poultry farm animals and are considered a major health problem besides the current control measures (Antunes, Mourão, Campos, & Peixe, 2016a). *Salmonella* serovars which have the ability to be transferred to humans are transmitted through infected poultry animals' meat and eggs (F.Akhtar, 2009)

3.3 Non-Typhoidal *Salmonella enterica*:

Salmonella species are one of the most significant food borne pathogen and since it is zoonotic, it is easily transmissible from poultry animals to humans through consumption of contaminated poultry meat but its mortality rate is considerably low (Crump, Sjölund-Karlsson, Gordon, & Parry, 2015). Out of all *Salmonella* species, *Salmonella enteriditis* was found to be the most common cause of non-typhoidal salmonellosis in humans that was identified in poultry animals as well (F.Akhtar, 2009).

The first or second most frequently prevalent *Salmonella* specie in many countries is *Salmonella enteritidis (Braden, 2006).* In the past few years, the most important infectious agent for causing gastroenteritis in humans has been known to be none other than *Salmonella enteritidis (Clayton et al., 2008).*Handling of eggs from poultry animals has a lot to do with contamination by *Salmonella* species as well. The manner in which these eggs are handled, cooked and eaten plays an important role in human infection. The consumption of eggs is sometimes slightly undercooked for some dishes and this can be associated with transmission of *Salmonella enteritidis* infection to human beings (Braden, 2006).

3.4 Global Prevalence:

On a global scale, the data presenting occurrence of *Salmonella* infections in humans with a wide range of food variety has helped in building an epidemiological link between poultry food products and salmonellosis. This includes various serovars of *Salmonella* that are spread amongst both humans and poultry animals (Antunes, Mourão, Campos, & Peixe, 2016b) Data collected from European Food Safety Authority (EFSA) reports: humans (2011–2013) and turkey/broiler meat (2013) was used to depict the distribution of some major serotypes of non-typhoidal *Salmonella* that were linked human salmonellosis cases and poultry meat in EU, 2011 to 2013 (Antunes et al., 2016a). On performing analysis of the occurrence of various *Salmonella* serovars in poultry animals as well as in humans, it was noticed that the prevalence of the infectious agents aligned with each other in some cases, as shown in the figure. (Figure 1).
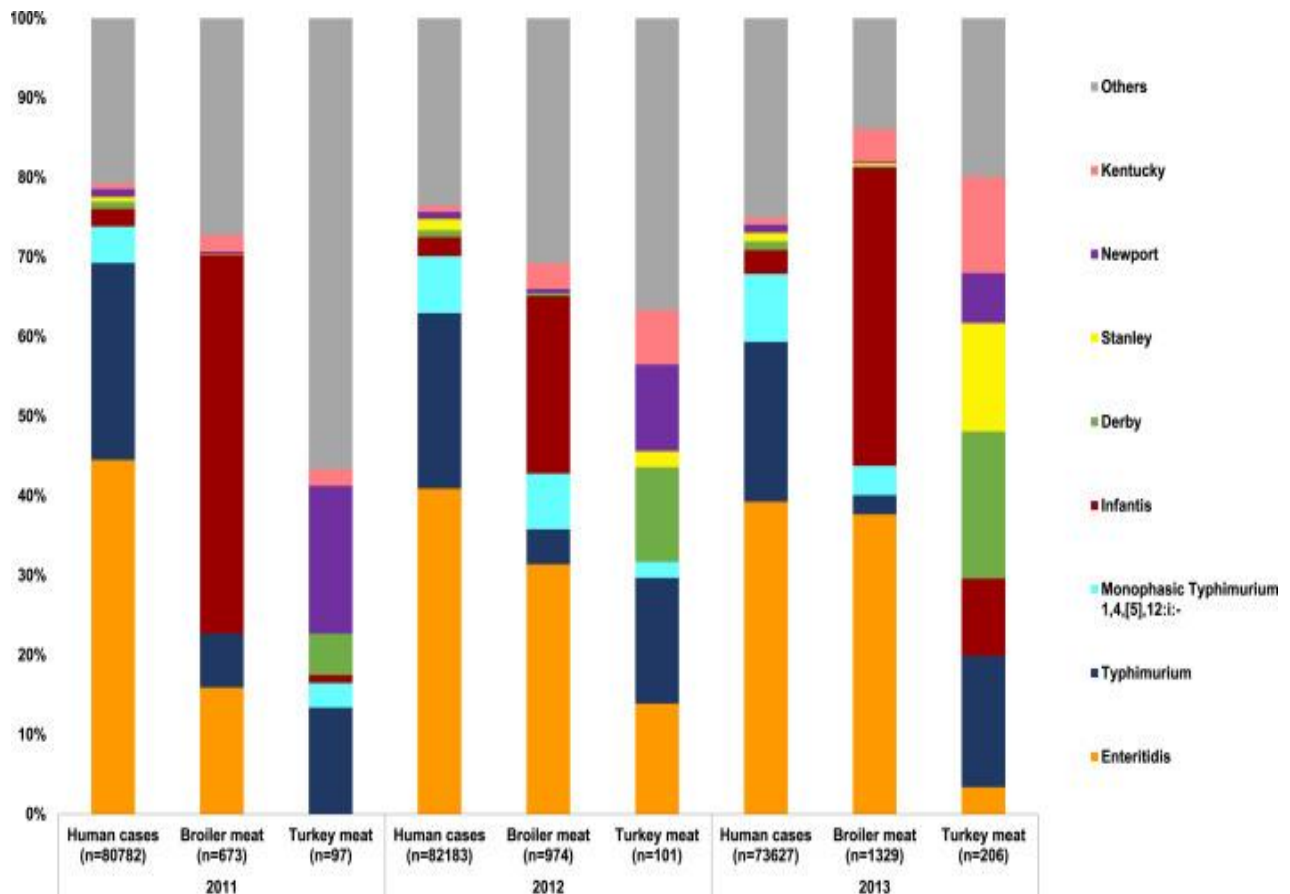

Figure 1: Association of *Salmonella* serovars with human salmonellosis cases (Antunes et al., 2016a)

According to European Food Safety Authority (EFSA), Salmonellosis was the second most commonly reported gastrointestinal infection in humans in the EU (91,857 cases reported) ("Salmonella the most common cause of foodborne outbreaks in the European Union," 2019).

According to an annual report that was submitted by the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), *Salmonella* was responsible for causing almost one in three foodborne outbreaks in the year 2018 in the European Union ("Salmonella the most common cause of foodborne outbreaks in the European Union," 2019).

In different parts of the world such as Europe and the USA, the occurrence of human infection by *Salmonella* that caused food poisoning has increased with passing time, with poultry products such as eggs and meat being the major source of infection (Arora et al., 2015).

If the reporting of the foodborne infections is taken into consideration, then globally it is under-reported. But this situation is even worse in developing countries where the data collected and recorded for analysis is reported so inefficiently that understanding the degree of disease and infection is made considerably more difficult. This has to do with the insufficient diagnostic techniques and resources and absence of efficient personnel that can manage the whole investigation (Barbour et al., 2015).

3.5 Prevalence in Pakistan:

Pakistan is one of the developing countries that are not safe from the infections of *Salmonella*. A research based in Quetta to check prevalence of *Salmonella spp*. reported contamination in both fresh and frozen poultry meat samples. The percentage of occurrence of different species is given in Table 2 (Dr. Abdul Samad, 2018)

The detection of *Salmonella typhimurium* and *Salmonella enteriditis* in a study that was conducted on poultry in Kashmir indicated that poultry could be a potential source of infection of *Salmonella* for humans. It was suggested in the paper that more frequent surveillance of *Salmonella* species should be performed in poultry to make sure that it is safe for human consumption (Mir et al., 2010a).

Salmonella species	Frozen Poultry (30%)	Fresh Poultry (36%)
S. typhi	3.3%	0%
S. enteritidis	43.3%	44.4%
S. typhimurium	30.0%	30.6%
S. gallinarum	13.3%	19.4%
S. pullorum	10.0%	5.6%

Table 2: Percentage occurrence of different *Salmonella* species

3.6 Conventional Therapies:

For the control of *Salmonella* in poultry, antibiotics are commonly used.

3.6.1 Antimicrobial Therapy:

Antimicrobials are agents that act against pathogens to either inhibit their growth or to kill them. They are classified into different groups based on which microorganism they act against. Antibiotics act against bacteria, antifungals act against fungi, anti-protozoan against protozoa and so on. Currently, antimicrobial therapy is one of the most important control measures for reducing the rate of morbidity and mortality from pathogens, including *Salmonella* species in poultry and humans. There is a need to use antimicrobials smartly to maintain effective treatment and to prevent the growing development of drug resistance among the clinical bacterial isolates (Mir et al., 2010a). Antibiotics work in different ways to overcome a bacterial infection. They either work to kill the bacteria or inhibit their growth or reproduction. Different target sites for different classes of antibiotics are shown in Figure 2 (Ebimieowei Etebu, 2016).
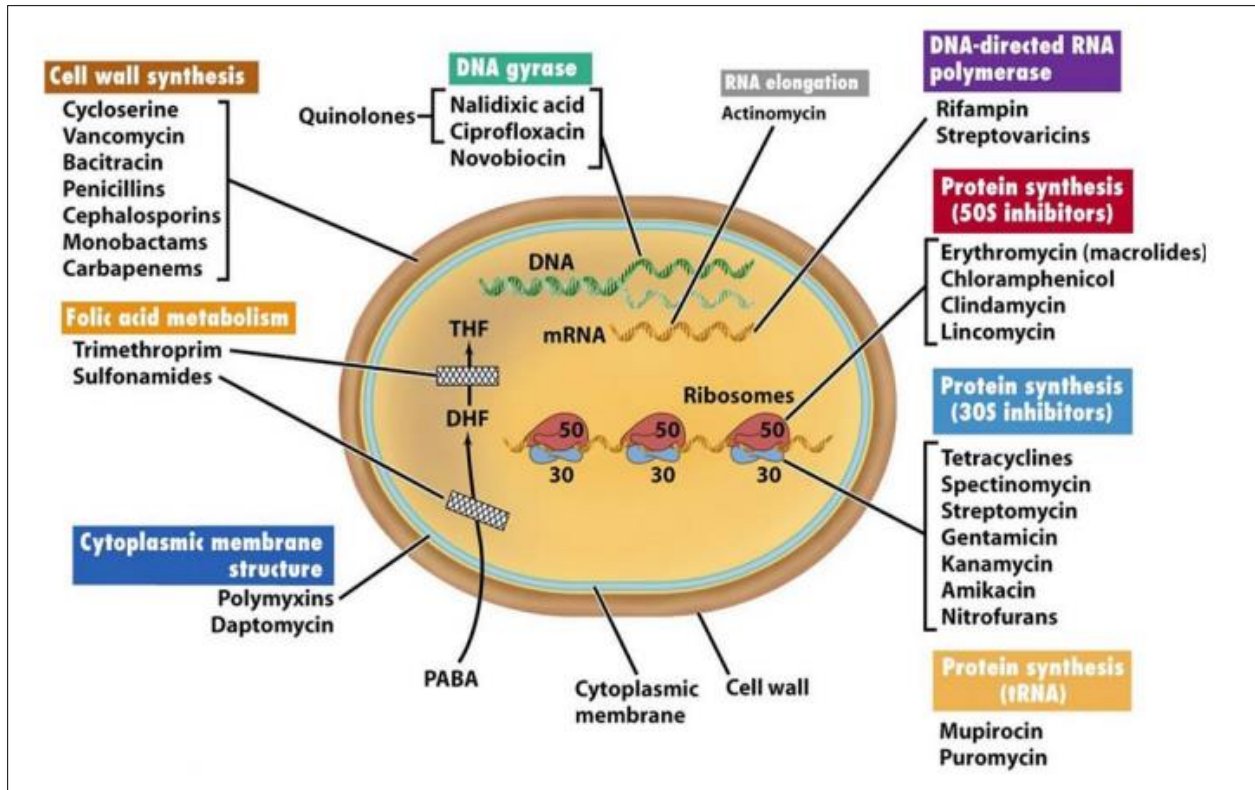
Figure 2: Target Sites for different antibiotics

Antibiotics can be classified in different ways. However, they are mostly classified based on:

a) molecular structures

b) mode of action

c) spectrum of activity

3.7 Growing Antimicrobial Resistance in Foodborne Pathogens

The advent of antimicrobial drugs proved miraculous in preventing and treating stubborn and sometimes deadly infectious diseases especially because of their specific targeting of pathogens through well-known mechanisms. The specificity, predictability, and consistency of the effects of antimicrobials on disease made them a popular therapeutic option across the world in a very short span. This also opened doors for the use of antimicrobials in animals and antimicrobials found their way to animal agriculture. Today, it's impossible to produce the current volume of animal meat and other products without considering the role of antimicrobials. However, as the usage increased, the pathogens concurrently developed mechanisms to cope and the indiscriminate and unregulated

usage of clinically important antimicrobials in animals has lead the infectious pathogens to become resistant to their effects (Sharma, 2011).

Drug-resistant bacteria can spread rapidly and quite unpredictably in both animal and human populations, which is worsened by trade, travel, and globalization as a whole ("9789241509763_eng").

Antimicrobial resistance is one of the leading public health concerns not only because of the increasing prevalence of resistant pathogens but also because many clinically important antimicrobials have been rendered ineffective in humans, causing devastating health and economic setbacks.

3.8 Types of Resistance

<u>3.8.1 Natural resistance:</u> Natural resistance in bacteria is an innate feature of a population and can either be intrinsic (always present) or induced (stimulated by the action of an antimicrobial) (Reygaert, 2018a). This type of resistance is not a global health concern.

<u>3.8.2 Acquired resistance:</u> Bacteria that are originally susceptible to certain antimicrobials may develop resistance mechanisms against them, either by undergoing mutations within its chromosomal DNA, or acquiring resistance-carrying genetic material from the neighboring environment through transformation, conjugation and transposition, also known as Horizontal Gene Transfer (HGT) (Reygaert, 2018b). Acquired resistance is the focus of the global strategy to combat Antimicrobial resistance because it is unpredictable, spreads rapidly through the environment, and can be extremely hard to control.

3.9 Emergence and Mechanisms of Resistance:

Bacteria have the following mechanisms of combatting the action of and developing resistance against antimicrobials they were previously susceptible to:

1) Restricting access to the drug by undergoing structural changes, sometimes modifying the entryways or limiting the number of entryways for the drugs.
2) Getting rid of antibiotics by producing efflux pumps in their cell walls to remove drug components that enter the cell.
3) Destroying the antibiotics through enzymatic action.

4) Bypassing the effects of the antimicrobial by developing new cell processes that avoid using the antibiotic's target (Cdc, 2020).

3.10 Dissemination of Antimicrobial resistance in Non-typhoidal *Salmonella*

Several factors contribute to the emergence of antimicrobial resistance in Non-typhoidal *Salmonella* and its dissemination to other strains and species, animals, humans, and the environment.

1) <u>Horizontal Gene Transfer:</u> The horizontal transmission of resistant genes, especially via plasmids, is the most direct cause of the dissemination of resistant genes, single or multiple, to other strains and species. Resistance can be transferred via plasmids, transposons (mobile genetic elements that carry resistance genes and can induce recombination of resistance genes with plasmids and chromosomes of other bacteria) and integrons (genetic elements that contain integrase enzyme, a recombination site, and a promoter). Additionally, conjugation activity helps the integration and spread of resistance to other strains and species via plasmids (V T Nair, Venkitanarayanan, & Kollanoor Johny, 2018b).

2) Overuse and misuse of antimicrobials in poultry: The indiscriminate and unchecked use of antimicrobials contributes greatly to the spread of antimicrobial resistance from poultry to other animals and humans.

3) The unregulated sale and use of antimicrobials.

4) Substandard hygiene practices.

5) Improper waste disposal: Wastewater and other waste material in not decontaminated and are disposed openly into the environment from where it travels to the surrounding areas and comes into contact with humans and other animals. Sometimes, this contaminated wastewater seeps into agricultural soil, affecting plants, from where it spreads to humans upon consumption (Choudhury, Panda, & Singh, 2012)

3.11 Resistance of Non-typhoidal *Salmonella* to Clinically Important Drugs

Non-typhoidal *Salmonella* species have gained resistance against most common antimicrobials over the last few decades. However, in 2018, the Centre for Disease Control revealed data showing that it is now developing resistance to Ciprofloxacin and Cephalosporin (3rd generation) at an alarming rate (U.S. Centers for Disease Control and Prevention) This a major and immediate threat to public health because ciprofloxacin and extended

Figure 3: Drug resistance of non-typhoidal Salmonella, CDC, 2018

spectrum cephalosporin are administered as a last resort treatment option for invasive salmonellosis in adults and children, respectively. (Figure 3) (V T Nair, Venkitanarayanan, & Kollanoor Johny, 2018c).

So, quite understandably, if non-typhoidal *Salmonella* species were to become completely resistant to clinically important drugs, the spread of Salmonellosis and other infectious diseases will become increasingly hard to control, resulting in not only loss of health and lives, but will also give rise to devastating economic losses. It will also be a major setback to the process of antimicrobial developments (Cuypers et al., 2018).

Based on all the information we gathered, in our study, we aimed to evaluate the antimicrobial resistance in non-typhoidal *Salmonella* obtained locally.

4. Objective:

Phenotypic analysis of antimicrobial resistance of *Salmonella enteritidis* in poultry in Pakistan.

5. Materials and Methods

5.1 Sample Collection:

Fecal samples were obtained from the food microbiology and biotechnology lab of Atta Ur Rahman School of applied biosciences. These samples were provided by *AbuBakr et al.,* and collected from different poultry farms in Islamabad, Rawalpindi, Khairpur, Ghotki, Jhelum, and Rahim Yar Khan.

5.2 Isolation and Selection of Bacteria

1ml of fecal samples were added in 9ml of distilled water and diluted up to10^{-7}. 1ml of the diluted samples were spread on the *Salmonella* specific SS Agar and incubated at 37°C for 24 hours. Samples were purified by streaking isolated colonies on SS agar and incubating at 37°C for 24 hours. These purified colony plates were stored at 4°C for Identification tests.

5.3 Phenotypic Identification

5.3.1 Colony Morphology:

Pure cultures were spread on SS agar plates and left overnight in an incubator at 37°C. Colony color and morphology was observed under the magnifying glass.

5.3.2 Gram Staining:

The standard protocol was performed to stain the bacteria. A single colony was picked from pure culture by using a sterile loop and dispensed in a drop of distilled water on a glass slide. Smear was formed and it was heat fixed. Smear was treated with crystal violet for 1 minute and washed with distilled water. After washing iodine was applied for 45 seconds and washed again. Deodorizing agent ethanol was applied for 5 seconds and washed again with distilled water. Safranin was applied for 40 seconds and washed with distilled water. After drying glass slide was observed under a microscope at 100x magnification power. Color and shape of the bacteria were observed, pink colored bacteria were gram negative, and purple colored were gram positive bacteria.

5.4 Biochemical Identification

5.4.1 Catalase Test:

The standard protocol was performed for the catalase test. A single colony was picked form the SS agar plate and transferred on a clean glass slide with a sterile loop. 3% H_2O_2 was applied to the colony and mixed well. The colony was observed for the bubble formation. Bubbles formation confirms a positive catalase test and no bubbles formation shows a negative catalase test.

5.4.2 Urease Test:

Urease test differentiates *Salmonella enterica* serovars from *Proteus vulgaris* based upon the ability to split urea by urease enzyme. 5ml urea broth was prepared and added to the test tube. The bacterial colony was suspended in urea broth and incubated for 24 hours at 37°C. After incubation urea broth was observed for color change. If the urea broth turns pink then its urease positive test.

5.4.3 SIM Motility Test:

Sulfide indole motility (SIM) test was performed to differentiate *Salmonella enterica* serovars from other typhoidal *Salmonella* serovars based upon motility because of flagella. 5ml SIM media was prepared in a test tube and the colony was inoculated in the center of the test tube with a sterile straight wire. Test tubes were incubated at 37°C for 24 hours. After incubation, these tubes were observed for color change and the spread of bacteria. If black color appears in the test tube it means Sulphide is present and if bacteria do not spread in the tube it means non-motile bacteria are present.

After getting Phenotypic and biochemical identification tests these isolated colonies were used for overnight culture in LB broth at 37°C. The overnight culture was used for 40% glycerol stocks preparation. Stocks were stored at -20°C and -80°C for further genotypic Identification and antimicrobial sensitivity testing assays.

5.4.4 Antibiotic Resistance Profiling:

Antibiotic resistance profiling of the 7 *Salmonella enteritidis* isolates was assessed. Antibiotics were available in disc form. The resistance pattern was checked against most commonly antibiotics of first, second, third, and fourth generation used in the poultry industry. Bacterial cultures were inoculated in the LB Broth and incubated for 24 hours at 37°C. Bacterial suspension with a density of 0.5 compared to McFarland standard was swabbed on Muller Hinton (MH) agar using a sterile

cotton swab. Antibiotic discs were placed at a sufficient distance so that the zone doesn't interfere and placed in an incubator for 24 hours at 37° C. zones of inhibition were measured after 24 hours, using zone diameter interpretive criteria (CLSI guidelines 2018) isolates were deliberated as resistance, intermediate susceptible or sensitive to the antibiotics. Given below is the list of antibiotics used in this study in table form (Table 3).

Antibiotic	Detail (generation, class)	Antibiotic	Detail (generation, class)
1. Oxacillin (OXA) 5µg	(2nd generation) Penicillin	13. Ciprofloxacin (CIP) 10 µg	(2nd generation) Quinolone
2. Amoxicillin/clavulanic acid (AMC) 10 µg	(2nd generation) Penicillin	14. Enrofloxacin (ENR) 30 µg	(2nd generation) Quinolone
3. Ampicillin (AMP) 10 µg	(3rd generation) Penicillin	15. Minocycline (MIN) 30 µg	(2nd generation) Tetracycline
4. Cefixime (CFM) 5 µg	(3rd generation) Cephalosporin	16. Tetracycline (TE) 30 µg	Tetracycline
5. Cefepime (CEF) 30 µg	(4th generation) Cephalosporin	17. Erythromycin (ER) 30 µg	Macrolide
6. Meropenem (MEM) 10 µg	Carbapenem	18. Clindamycin (CD) 10 µg	Macrolide
7. Imipenem (IMP) 10 µg	Carbapenem	19. Chloramphenicol (CHL) 30 µg	Phenicol

8. Vancomycin (VA) 30 µg	Glycopeptide	**20. Rifampicin (RD) 30** µg	Rifampicin
9. Streptomycin (ST) 25 µg	Aminoglycoside	**21. sulfamethoxazole (SXT) 25** µg	Sulfonamide
10. Gentamycin (CN) 30 µg	Aminoglycoside	**22. Linezolid LZD 30** µg	Glycopeptide
11. Kanamycin (K) 30 µg	Aminoglycoside	**23. Nalidixic acid (ND) 30** µg	(1st generation) Quinolone
12. Amikacin (AMK) 30 µg	Aminoglycoside		

Table 3: List of antimicrobials used for antimicrobial resistance profiling

6. Results

6.1 Sample Selection and Isolation:

Samples collected from different poultry farms were homogenized and spread on SS agar plates. Different colonies of isolates appeared on plates. *Salmonella* colonies were isolated by further streaking as they were black in their appearance. These isolates were further processed for identification. (Figure 4)
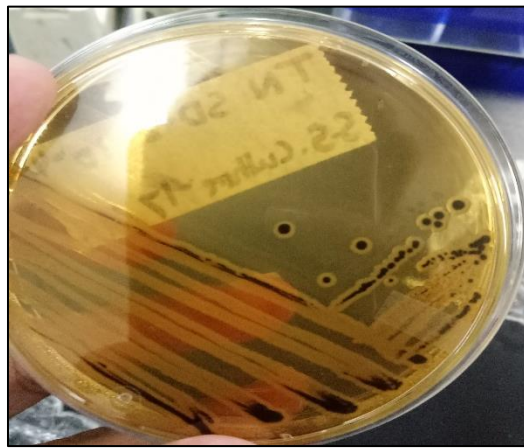

Figure 4: Isolated bacterial colonies on SS agar plate

6.2 Phenotypic Identification

6.2.1 Colony Morphology:

Salmonella isolates colonies showed a definite black round appearance which confirms that these isolates belong to *Salmonella* specie. (Figure 5)
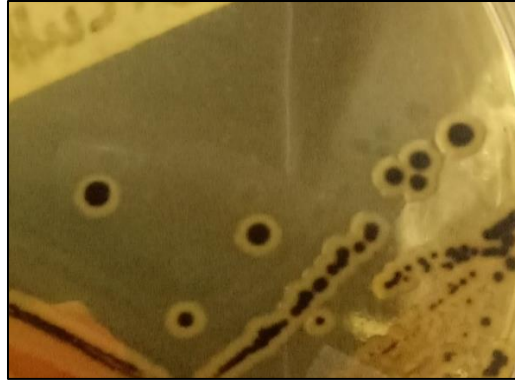
Figure 5: Colony morphology of isolated bacteria

6.2.2 Gram Staining:

Gram staining test performed for all the isolates showed gram positive and Negative rods and cocci under the 100x power of the microscope. Negative rod isolates were further processed for genotypic identification. (Figure 6)
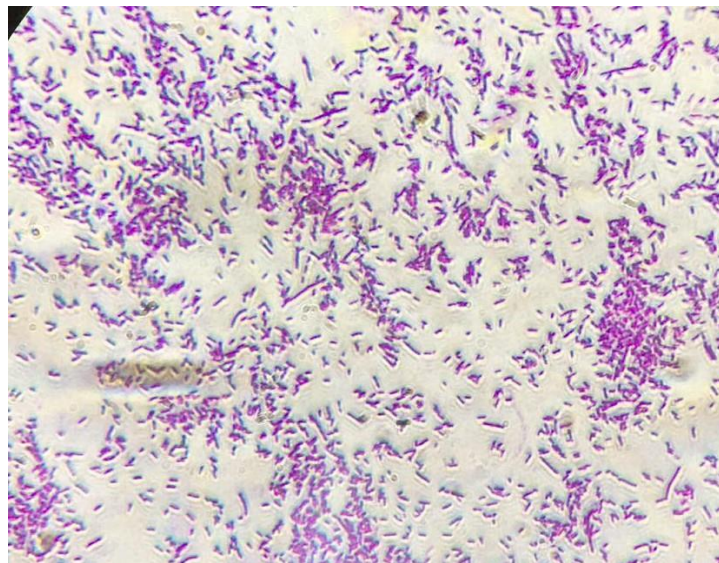

Figure 6: Gram negative rods observed under a microscope at 100X resolution.

6.3 Biochemical Identification

6.3.1 Catalase Test:

All *Salmonella* isolates were treated with 3% H_2O_2 showed bubble formation which confirms that all isolates were catalase positive compared with a negative control which showed no bubble formation. (Figure 7)

Figure 7: Catalase activity of *Salmonella* isolates on a glass slide

6.3.2 Urease Test:

All *Salmonella* isolates showed no color change in the test tubes which confirms that these isolates were urease negative compared with urease positive control which showed pink color formation. (Figure 8)
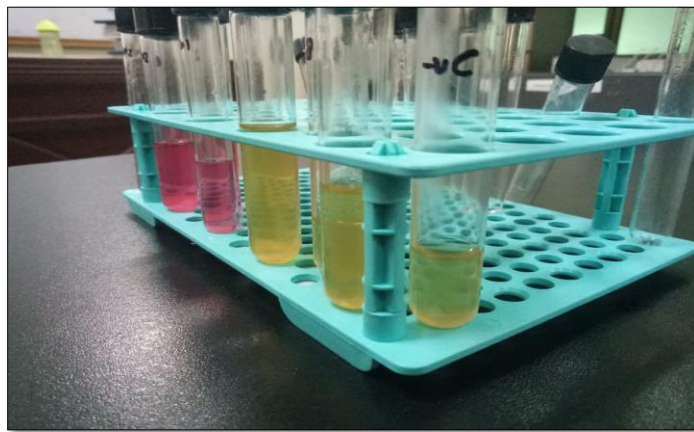

Figure 8: Yellow color confirms the urease negative test compared with pink colored positive control

6.3.3 SIM Motility Test:

All *Salmonella* isolates were SIM positive as the black color appeared in test tubes compared with the negative control. Black color spread confirms that these isolates were motile due to flagella presence. (Figure 9)
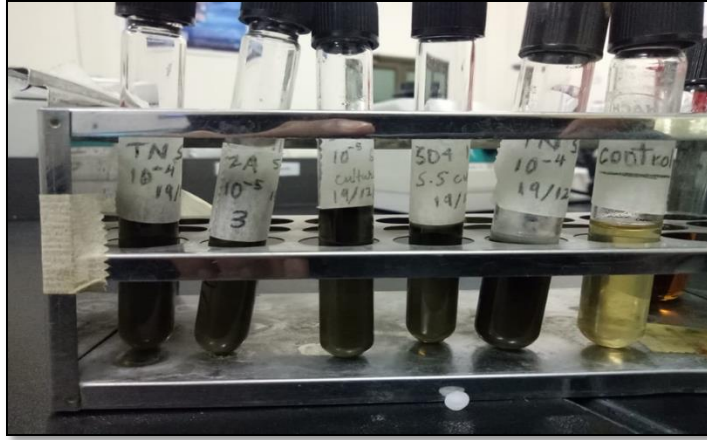
Figure 9: Black color appearance confirms SIM positive test for motility compared to the negative control

6.3.4 Antimicrobial Resistance Profiling:

Inhibition zones were measured using digital Vernier caliper and compared with standards (CLSI guidelines 2018) and using zone diameter interpretive criteria isolates were deliberated as resistance, intermediate susceptible, or sensitive to the antibiotics. Given below is the antibiogram of the *Salmonella enteritidis* isolates. (Table 4)

isolates code	Antibiotics							
	Penicillin		Carbapenem		fluoroquinolones	Cephalosporins		chloramphenicol
	AMX	AMP	MEM	IMP	CIP	CFM (3gen)	CEF (4gen)	CHL
F1	Sensitive	resistant	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	resistant
F2	resistant	resistant	Sensitive	Sensitive	Sensitive	Sensitive	resistant	resistant
F3	resistant	resistant	Sensitive	resistant	resistant	resistant	Sensitive	resistant
F4	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
F5	resistant	resistant	Sensitive	Sensitive	Sensitive	resistant	resistant	resistant
F6	resistant	resistant	Sensitive	Sensitive	Sensitive	resistant	Sensitive	resistant
F7	Sensitive	resistant	Sensitive	Sensitive	Sensitive	resistant	Sensitive	resistant
	TE	ENR	OX	CN	K	VA	RD	CD
F1	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
F2	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
F3	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
F4	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
F5	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
F6	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
F7	resistant	resistant	resistant	resistant	resistant	resistant	resistant	resistant
	ST	AMK	SXT	ND	ER	MIN	LZD	
F1	resistant	resistant	resistant	resistant	resistant	resistant	resistant	
F2	resistant	resistant	resistant	resistant	resistant	resistant	resistant	
F3	resistant	resistant	resistant	resistant	resistant	resistant	resistant	
F4	resistant	resistant	resistant	resistant	resistant	resistant	resistant	
F5	resistant	resistant	resistant	resistant	resistant	resistant	resistant	
F6	resistant	resistant	resistant	resistant	resistant	resistant	resistant	
F7	resistant	resistant	resistant	resistant	resistant	resistant	resistant	

Table 4: Antibiogram of *Salmonella enteritidis* isolates

KEY: ▇ = Sensitive   ▇ = resistant

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The bar graph below shows a summary of the findings of this research. *Salmonella enteritidis* was 100% susceptible to meropenem, highlighted in green. Against ciprofloxacin, imipenem and cephalosporin highlighted in yellow, only 15% was observed. Amoxicillin and cefepime showed around 57% resistance. While for ampicillin and chloramphenicol, 85% resistance can be observed. (Figure 10)
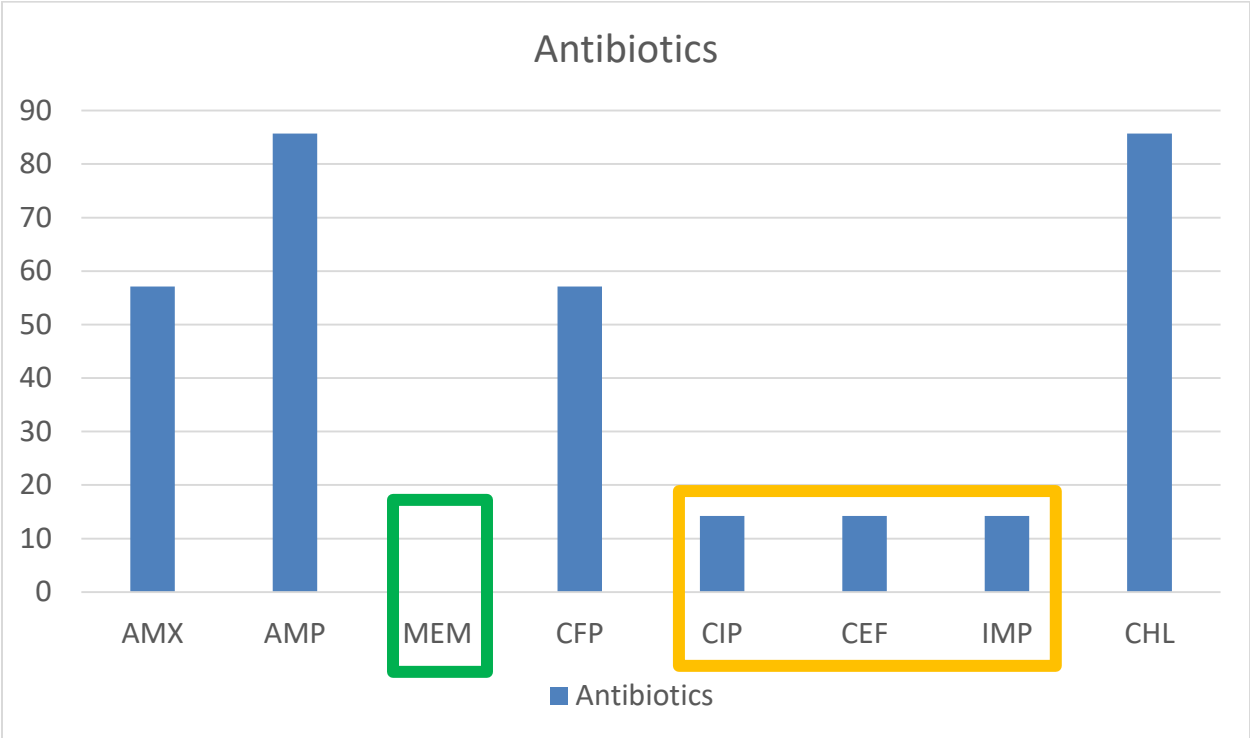

Figure 10: Bar graph representation of the resistance pattern

For the rest of 15 antibiotics, *Salmonella enteritidis* showed 100% resistance,
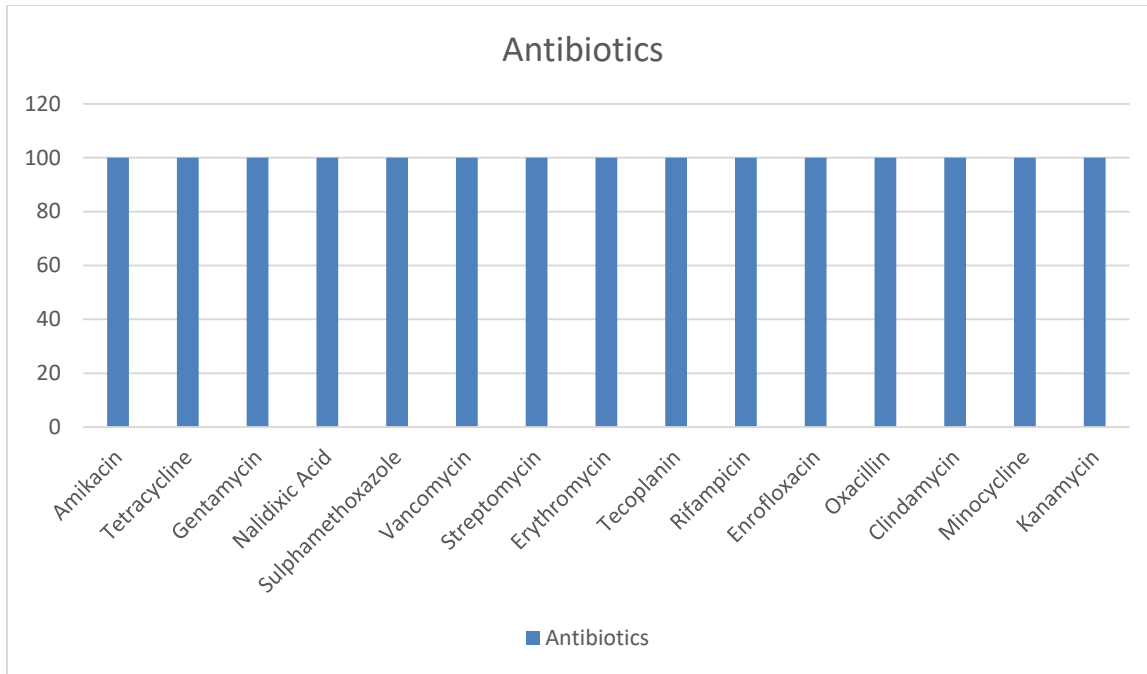
Figure 11: Bar Graph representation of antibiotic resistance.

7. Discussion

Salmonella is the second largest pathogenic bacteria posing threat to poultry and a hazard to public health as well. Therefore, preventing and controlling *Salmonella* in the poultry industry is of the utmost importance. However, consistent use of antimicrobials as a prophylactic and growth promoter has resulted in antimicrobial resistance. For that purpose, first of all, it is important to find out which antibiotics are showing resistance. Then we will be able to find out about the resistance and transmission along with measures to control this.

In this study, seven *Salmonella enteritidis* isolates were tested. Spreading of bacterial colonies was performed on SS agar for identification purposes. Black colonies of *Salmonella enteritidis* were observed which confirmed it to be *Salmonella.* These colonies are comparable to the ones that were obtained during *Salmonella* identification performed by *Thanes Gunasegaram et al,*. (2011) after this step, confirmatory tests were performed (Gunasegaran et al., 2011).

For this purpose, Gram staining was done to identify pink gram negative rod-shaped *Salmonella*. Next, the catalase test was performed which resulted in bubble formation. This shows the presence of oxygen formed as a result of the catalase enzyme which is a characteristic of *Salmonella*. It was followed by a urease test in which negative results were obtained as *Salmonella* lacks a urease enzyme, so no ammonia was produced. The solution remained the same in color. However in the control, Proteus vulgaris, the solution turned pink due to the presence of urease enzyme. The third test that was performed, was the sulfur indole motility test to differentiate the motile *Salmonella* from its non-motile specie. The results came out to be black growth in the media which confirmed that it was indeed motile *Salmonella* i.e. *Salmonella enterica or Salmonella typhi.* Catalase and SIM tests performed by us provided with the results similar to that of confirmation tests performed by *Jane Francis et al,*. (Akoachere, Tanih, Ndip, & Ndip, 2009)

After performing these confirmatory tests, antimicrobial resistance of *Salmonella enteritidis* which was the main focus of the project was carried out by using 23 different antimicrobials belonging to different classes and generations including penicillin, cephalosporin, carbapenem,

quinolones, and others explained above. At present, antimicrobial therapy is the primary way to reduce morbidity and mortality in poultry by pathogenic bacteria. However for the treatment to be effective and to reduce the risk of antimicrobial resistance, these antimicrobials must be used wisely.

For this purpose disc diffusion method was performed, as utilized by *Jane Francis et al,.* (Akoachere et al., 2009). Bacterial colonies gave different inhibition zones after overnight incubation. This helped in identifying susceptible and resistant antibiotics.

The results here, represent that all seven isolates of *Salmonella enteritidis* were 100% susceptible to only meropenem. Against Ciprofloxacin, cephalosporin, and imipenem, around 15% resistance can be seen. A similar study was conducted in Thailand by Nata Pratama and Cucunawangsih which exhibited that *Salmonella entrerica* serovars obtained from a hospital showed a little resistance towards imipenem and meropenem. Our isolates were, however, completely resistant to meropenem and isolated solely from poultry. The reason for that could be that meropenem might not be utilized very commonly here in our region for poultry. It may also be due to the chance that none of our isolates showed resistance towards meropenem (Hardjo Lugito & Cucunawangsih, 2017)

Carbapenemase producing *Salmonella* are a huge threat to these antibiotics. And the resistance mutation is horizontally transferrable together with co and cross-selection mechanisms. Carbapenem resistance also transfers this resistance to extended spectrum cephalosporin (Fernández, Guerra, & Rodicio, 2018).

The resistance of *Salmonella enteritidis* isolates for amoxicillin, ampicillin, cefepime, and chloramphenicol can be seen to be very high. For the rest of 15 antibiotics including amikacin, tetracycline, gentamycin, nalidixic acid, sulfamethoxazole, vancomycin, streptomycin, erythromycin, teicoplanin, rifampicin, enrofloxacin, oxacillin, clindamycin, minocycline, kanamycin, there was 100% resistance. Varying resistance percentage was also shown towards kanamycin (28%), penicillin (28%), streptomycin (7%), erythromycin (100%), ampicillin (21%) and novobiocin (100%) at 100 µg/disc concentration of antibiotics. Gentamycin did not exhibit resistance at this concentration. These results were presented in the experiment performed by F. Akhtar et al., Faisalabad, Pakistan (Administrator) For some of these antibiotics, resistance was

only observed at high *Salmonella enteritidis* concentrations. Moreover, the number of isolates that showed resistance was also varying among these antibiotics. These results are slightly different from what we obtained as your isolates showed complete resistance towards these antibiotics. Again this could be because the study was conducted in 2009 and since then, the use of antimicrobials has increased many folds. Similarly in Chile, during the year 2016, high resistance by *Salmonella enteritidis* was proved against sulphamethoxazole. The study also proved that it was mainly resistant to tetracycline (López-Martín Juana Isabel).

However, a study conducted in Kashmir by *I.A Mir et al,.* (2010) shows that majority *Salmonella* isolates including serovar *typhymurium* and *enteritidis* were susceptible to chloramphenicol, tetracycline, and cefixime, that is contrary to our study. The reason for this could be that the use of these antibiotics increased afterward and they were utilized more in the regions from where we collected our samples (Mir et al., 2010b).

The study by Ikram et al,. (1993) presents Kanamycin as a viable option for *Salmonella* control in poultry, but now things have changed drastically. Kanamycin can't be the drug of choice as it is now also completely resistant. Our claim is also supported by a study conducted by F. Akhtar et al., (2009) in Faisalabad (Administrator).

These results indicate that *Salmonella enteritidis* is now exhibiting resistance to most of the antibiotics. Even for the 3rd and 4th generation antibiotics, the susceptibility is starting to decrease now. As in our case, out of all the 23 antibiotics, *Salmonella enteritidis* was 100% to almost two-third of the antibiotic while 100% susceptible to just one antibiotic. Therefore, *Salmonella enteritidis* shows high resistance as indicated by the results of all the isolates.

Since *Salmonella enteritidis* in current research showed resistance to one and more antibiotics belonging to more than two different categories, so, therefore, it is safe to say that it is now a Multi-drug resistant serovar. Multidrug resistance had also been shown by *Muhammad Asif et al,.* (2017) in 54.8% *Salmonella enteritidis* isolates obtained from Kohat, Pakistan (Asif et al., 2017).

These results of antibiotic resistance of *Salmonella enteritidis* resistance are comparable to results found in other studies. A study by Atta Hussain Shah and Nazar Ali conducted in Karachi

represents that *Salmonella enteritidis* is showing resistance and susceptibility to somewhat similar categories of antibiotics as shown by this study (Atta Hussain Shah).

Another study by *Mohammad Asif et al,.* (2017) shows similar kind of results. These and many other such studies conducted prove that antimicrobial resistance of *Salmonella enteritidis* is expanding and is becoming a huge problem worldwide (Asif et al., 2017).

It is important to know how the resistance is transmitted to other strains and species. First of all, it may be a bacterial genetic mutation that may lead to resistance. The mechanism for transfer of this resistance within *Salmonella enterica* species is also necessary to understand. Horizontal transmission is of utmost importance here. The resistant gene within the plasmid or chromosome can be horizontally transmitted. Resistance transmission through plasmid is the most efficient method that can also result in the transmission of multiple resistance genes at a time. These genes are then capable of being transferred to other strains and species as well (V T Nair, Venkitanarayanan, & Kollanoor Johny, 2018a).

High antimicrobial resistance of *Salmonella enteritidis*, as indicated by the results is a growing hazard. This bacteria is a part of gut microflora and with it, are many other microbes of the same or different family e.g. *E.coli, Lactobacillus, Eubacteria, Campylobacter* and many other. A similar study by *Roderick et al,. (2017)* carried out in the United Kingdom indicates the transmission of resistance from *Salmonella* to *E. coli*. This resistance can be transferred to other species of the gut microbiota as well (Card et al., 2017). Another hazard is the transmission of this resistance to microbes other than the gut when they are exposed to the environment. Eventually, all of this will be transmitted to human which poses a much greater public health concern. Zoonotic and environmental transmission into humans is a real threat and they do happen. This was proved by a study performed in Uganda (2016) (Afema et al., 2016). WHO has also provided detailed guidelines regarding *Salmonella* transmission and outbreaks which shows much of a real and big problem this is ("Salmonella (non-typhoidal)," 2020a). This resistance can lead to many outbreaks and pandemics. Quinolone, chloramphenicol, and ciprofloxacin-resistant *Salmonella* outbreaks in humans have occurred in the United States and Taiwan which is worrisome as it poses a much serious threat to humans in the future. Since quinolone is the choice of the drug against invasive salmonellosis in adults, it is of great concern (Su, Chiu, Chu, & Ou, 2004). We have seen cases of extended drug resistance typhoid already in Pakistan in the

last few years. Antimicrobial resistance has now crippled the treatment with conventional antibiotics like ampicillin, chloramphenicol, and sulphamethoxazole. This can resistance arising through the poultry industry along with overuse in humans as well. All this situation is of great concern and needs to be addressed as soon as possible to prevent future generations from a huge disaster.

8. Future Prospects

As already discussed that *Salmonella enteritidis* is a huge problem and poses a great threat to poultry as well as humans, strict protocols and alternate control measures need to be taken to stop its spread. Furthermore, special attention is to be paid to the hygiene conditions of poultry farms and butcher shops which are the hub of disease transmission. Monitoring of the birds for the presence of the bacteria can also help predict the health status and therefore, predict the measures that need to be taken.

Carrying out genomic identification of resistant bacterial genes can help us in identifying and pinpointing those genes that are responsible for this and see for ourselves whether this resistance is acquired or induced.

Another option is to do carry out whole genome sequencing of *Salmonella enteritidis* to know more about their diversity that can help in identifying the emergence of resistance, different serovars that are present and how are they showing resistance.

Bacteriophage therapy can be a useful technique as well. Bacteriophage selectively engulfs only the harmful pathogenic bacteria for which they are specifically designed.

The fourth alternative is the use of probiotics as a preventive control measure for *Salmonella enteritidis.* Probiotics are living bacteria inside the gut of organisms. They live there inside the gut and confer health benefits to the host for example prevention of disease, enhanced growth, efficient nutrient uptake, and healthy gut health, etc. They can be used to control and reduce the growth of *Salmonella enteritidis* in the gut as they reduce the bacteria by a different mechanism like competitive adhesion and release of certain chemicals. A lot of work is now being conducted in this area as well by many researchers. One such study (*Divek et al,.* 2018) also shed some light on the use of probiotics as an alternative to antimicrobials (V T Nair et al., 2018b).

In the end, it has to be mentioned again that the limited and controlled use of those antibiotics that have not yet shown resistance to *Salmonella enteritis* is very important. As we know that the poultry industry depends so much on these antibiotics, therefore, eliminating them might not be a very feasible and practical option and may lead to heavy financial losses. So, strict controls and standard protocols to limit the use only to the treatment of disease in severe conditions, along with check and balance by the authorities need to be done. Initially, the limited use of antibiotics

in combination with probiotics seems like a feasible solution. And slowly with the passage of time and more research on utilization of probiotics for this purpose, we can eventually limit the use of antibiotics to just emergency cases and worst situations for example if the animal is too sick or if the hygienic conditions of the farm are the worst.

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