Genomic analysis of multidrug resistant non-typhoidal *Salmonella enterica* strains isolated from broiler chicken in Pakistan

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By

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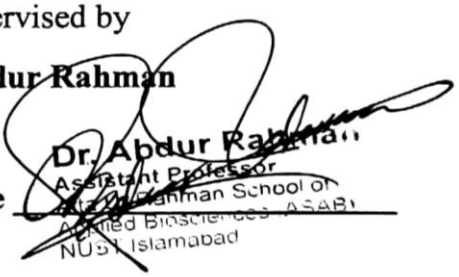
Supervisor's Signature 

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MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by **Roomana Ali & Reg No. 00000275117** **Titled:** "Genomic analysis of multidrug resistant nontyphoidal *Salmonella enterica* isolated from broiler chicken in Pakistan" be accepted in partial fulfillment of the requirements for the award of **MS degree in Industrial Biotechnology** and awarded grade (A grade).

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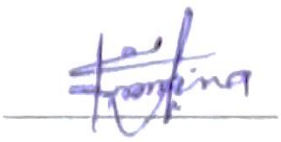
DECLARATION

I, **Roomana Ali,** declare that this research work titled "**Genomic analysis of multidrug resistant non-typhoidal *Salmonella enterica* strains isolated from broiler chicken in Pakistan***"* is my own work. The work has not been presented elsewhere for assessment. The work here in was carried out while I was a post-graduate student at Atta-Ur-Rahman School of Applied Biosciences, NUST under the supervision of Dr. Abdur Rahman. The material that has been used from other sources has been properly acknowledged / referred.



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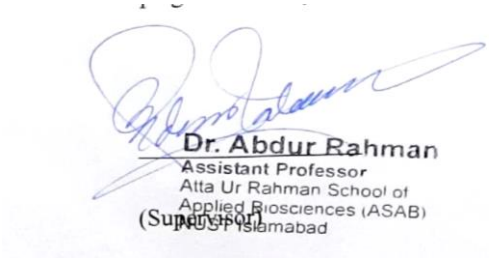

Dr. Abdur Rahman

For my mother

Rafia Khatoon

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Praise to **Allah** and blessings be upon the **final Prophet Muhammad (P.B.U.H.)**

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Roomana Ali

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AMR	Antimicrobial Resistance
ARGs	Antimicrobial resistance genes
CDC	Centers for Disease Control and Prevention
CDs	Coding sequences
DRS	Direct Repeated Sequences
EFSA	European Food Safety Authority
ESBL	Extended spectrum beta lactamase
FDA	Food and Drug Administration
Mb	Megabyte
MGE	Mobile Genetic Elements
TTSS	Type three secretion system
T3SS-1	Type three secretion system encoded by SPI1
SPI	*Salmonella* pathogenicity island
WGS	Whole genome sequencing
WHO	World Health Organization

ABSTRACT

Multidrug resistant non-typhoidal *Salmonella enterica* is zoonotic pathogenic bacteria that poses threat to public and animal health. This study represents first whole genome sequencing based analysis of multidrug resistant *S. enterica* strains isolated from broiler chicken in Pakistan for identification of anti-microbial resistance determinants and genomic components like virulence factors, plasmids, phages, pathogenicity islands and mobile genetic elements. Based on genome assembly quality report, 2 out of 5, *S. enterica* strains (*S.* Enteritidis RW50 and *S.* Enteritidis KHR57) were used for these assessments. Both genomes were of approximately 4.8 Mb having 52% GC content. A variety of resistance determinants like *QnrS1, golS, Tet(A), AAC (6')-Iy, KpnF, cmlA1, Arr-3* were identified in them that confer resistance to fluoroquinolone, beta-lactam, tetracycline, aminoglycoside, macrolide, chloramphenicol and rifampin antibiotics. Resistance determinants were most common to fluoroquinolones, followed by tetracycline and cephalosporins. Mismatches between phenotypic and genotypic resistance profiles were observed in *S.* Enteritidis KHR57, which was phenotypically susceptible against beta-lactams and chloramphenicols despite harboring *golS* and *cmlA1* genes. Similarly, *S.* Enteritidis RW50 showed phenotypic susceptibility for carbapenems despite having *golS and mdsA*. A large number of mobile elements, insertion sequences, pathogenicity islands and prophages were observed that can enhance genomic mobility and antimicrobial resistance in these *S.* Enteritidis isolates. Virulence factors including type three secretion systems and effector proteins (*Sips, Sops*) were identified in both isolates that are known to increase the entero-pathogenicity and zoonotic potential of *Salmonella* strains.

CHAPTER 1

INTRODUCTION

Salmonella enterica is zoonotic pathogen of major public health concern worldwide. This rod-shaped gram-negative bacterial species is most common cause of foodborne illness worldwide. *Salmonella* has higher rate of causing diseases in a wide range of animals including poultry and humans (Knodler & Elfenbein, 2019). *S. enterica* is further subdivided into six subspecies of which subspecies *enterica* stands out because of disease causing ability. This subspecies is composed of more than 1500 serovars but *S.* Enteritidis and *S.* Typhimurium have been given prime importance by researchers because of their pathogenicity towards humans and other animals (Lamas *et al*., 2017). *S.* Enteritidis was ranked first among infection causing serovars of *Salmonella* in 2015 by CDC (Hu *et al*.,2017).

S. enterica has ability to invade and colonize epithelial cells of host thus causing severe infections in GI tracts of humans as well as poultry (Ikejiri *et al*., 2020). Poultry is an important source of *S.* Enteritidis dissemination in humans. According to EFSA in 2018, all cases of food borne illness by consumption of egg or egg products were traced back to *S.* Enteritidis (EFSA, 2020).The number of *Salmonella* infections by consumption of poultry meat or direct contact with poultry have been increasing rapidly. *Salmonella* can reside in almost every part of GI tract, but it has been frequently isolated from crop and caeca. Caecum provides most favorable environmental conditions for *Salmonella* colonization as have been supported by many recent research's conducted on *Salmonella* interaction with poultry (Micciche *et al*., 2018). It can be colonized within 6 hours in esophagus, duodenum and caeca and will be cleared in approximately 20 hours however caeca requires up to 48 hours for clearance (EFSA, 2019).

Non-typhoidal *Salmonella* is usually confined to gastrointestinal infection in humans but invasive infection also occurs which may prove if patients are old aged or children less than four years and immunocompromised (Ashton *et al*., 2017). *Salmonella enterica* usually causes self-limiting infections but sometimes the infection becomes uncontrolled, and antibiotics must be administered to control the infection. Use of antibiotics becomes the choice of physicians for high-risk groups. Usually Fluoroquinolone, third generation cephalosporin and penicillin are used for infections caused by *Salmonella* along with macrolides and trimethoprim-sulfamethoxazole (Mukherjeer *et al*.,2019).

The use of antibiotics for treatment of *Salmonella* induced infections in poultry has decreased the chances of zoonotic transmission of disease to humans. Moreover, it also has resulted in lesser mortality rate sever gastrointestinal infections and invasive infections caused by *Salmonella*. But eventually we have to face the consequences of all these therapeutic measures in the form of increased antimicrobial resistance. Today, antimicrobial resistance is among the top 10 global public health threats humanity is facing (WHO, 2020).

In recent years antibiotic resistance *Salmonella* strains have been found in food chain which is a great indication of failure of complete treatment of salmonellosis. (Liljebjelke *et al*., 2017; CDC, 2017). Antibiotics have been used in poultry industry for controlling infections and promoting growth (Adhikari *et al*., 2019). There is a great diversity in antimicrobial resistance profile of drug resistant bacteria isolated from broiler poultry farms. Recent studies performed inside Asian and in non-Asian countries revealed that broiler chicken is disseminating pathogenic multi drug resistant *Salmonella* in humans (EFSA,2019; García-Soto,2020).

Pakistan produces 1.2-million-ton poultry meat annually and 25.8% of its total meat production is associated with poultry making poultry the second largest industry of Pakistan. Poultry sector contributes to 4.81% of agriculture GDP & 12% of livestock GDP of Pakistan. In last few decades *Salmonella* induced infections in poultry are repeatedly observed. This may cause a great loss to the agriculture-based economies of Pakistan because *Salmonella* infection in young birds is often fatal and results in white diarrhea, low feed consumption rate along with depression and retarded growth. Young birds may also experience intestinal or liver infections. Mortality rate due *Salmonella* is much lower in adult birds comparatively, but it affects their egg production rate. (Murakami *et al*., 2017).

In last few decades, a continuous increase in detection of *Salmonella* serovars has been detected in poultry of Pakistan (Hussain *et al*., 2020). . In almost all developed countries WGS of pathogenic bacteria is a routine practice. But due to limitation of resources despite of detection of high number of *Salmonella* serovars from different geographical regions of Pakistan, WGS based studies on non-typhoidal *Salmonella* serovars from Pakistan is negligible and there's no national consensus data on this problem. So, to address this problem, in this study WGS based analysis of multidrug resistant non-typhoidal *Salmonella* serovars isolated from Pakistan is conducted. WGS is a cost effective and rapid technique to screen whole bacterial genome. This allows researchers to identify novel regions in bacterial genome which might be related to antimicrobial resistance or pathogenicity.

WGS could be helpful to achieve long desired global one health goal by earlier detection of pathogens. Since human health, environmental health and animal health are very much interlinked and more than half of human emerging diseases have animal origin ,so this further increases the importance of WGS in current time. WGS can

identify intra and inter specie similarity of food borne pathogens. With the aid of WGS researchers can compare genomes of closely related species and serovars of same species can also be observed much easily. WGS can eventually be used to get more in-depth knowledge of genomic basis of antimicrobial resistance by identifying plasmids, prophages, virulence factors and genomic islands.

To achieve objectives of our study, WGS date of five multi-drug resistant *Salmonella enterica* strains and their phenotypic antimicrobial resistance data was provided by Food microbiology laboratory NUST Islamabad.

RESEARCH OBJECTIVES

The research objectives of current study are listed below.

1. Genomic characterization of multidrug resistant non-typhoidal *Salmonella enterica* strains isolated from poultry in Pakistan and their comparison with reference strain
2. WGS-based predictions of antimicrobial resistance determinants and its comparison with phenotypic antimicrobial resistance

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of *Salmonella enterica*

2.1.1 Microbial Characteristics

Salmonella is a bacterial genus that comprises gram negative bacteria which can grow both aerobically and anaerobically. *Salmonella* can survive for many years in dry shady conditions. Its multiplication temperature varies from 7 degree Celsius to 45 degrees Celsius (Xi *et al.*, 2019).

2.1.2 Classification of *Salmonella*

Salmonella bongori and *S*. enterica are the only two species of genus *Salmonella*. The specie *enterica* is then subdivided into six subspecies which are named as *enterica, salamae , arizonae , diarizonae , houtenae* and *indica* and have been given Roman numerals I,II,IIIa,IIIb, IVand VI respectively. *S*. enterica subspecies enterica is most studied *Salmonella* subspecies because it is responsible for more than 99% of human salmonellosis cases. *S*. Infantis, *S*. Enteritidis and *S*. Typhimurium are more commonly detected serovars of enterica subspecies (Sabag-daigle *et al*., 2016; Koutsoumanis *et al*., 2019). The subspecies enterica is mainly present in warm blooded animals such a poultry and livestock whereas non-enterica subspecies are more common in cold blooded animals like snake and reptiles (Lamas *et al*., 2017).

2.1.3 Diagnosis

Salmonella infections require appropriate lab testing because the salmonellosis symptoms are often like those caused by other food borne enteric pathogens and these symptoms usually comprise of diarrhea, fever, pain in abdomen, nausea and in a few cases vomiting. For confirmation of salmonellosis patient's fecal sample is tested.

Presence of *Salmonella* in must to confirm salmonellosis. In humans usually these symptoms may appear in 6 hours to 72 hours. Once *Salmonella* has been ingested the illness remains for for 2 to 7 days (Stanaway *et al*., 2019).

2.1.4 *Salmonella* enterica as pathogen

Every year salmonellosis causes 1 million food borne diseases only in United States (Han *et al*., 2020). Enteric diseases are responsible for 2.2 million life losses each year (Laing *et al.,* 2017). Foodborne *Salmonella enterica* is estimated to cause 155,000 deaths globally. (Nguyen *et al*., 2019). Over 1500 serovars of *S. enterica* causing a wide range of disease manifestations ranging from enterocolitis to typhoid fever are a serious concern for human health. The enterica subspecies is responsible for 99% of salmonellosis infections in both humans and other animals. The enterica subspecies members have virulence factors which are either absent or modified in their counter non-enterica subspecies members (EFSA-ECDC, 2016). *S. enterica* is widely known for its pathogenicity. It can invade and colonize epithelial cells of host thus causing severe infections in GI tracts of humans as well as poultry (Ikejiri *et al.,* 2020). Repeated detection of antibiotic resistance *Salmonella enterica* in poultry has been observed. (Forkus *et al*., 2017; Nair *et al*., 2018).

2.1.5. Global prevalence of *Salmonella* enterica

Table 1 Relevant research studies showing global prevalence of non-typhoidal *Salmonella* enterica.

Location	Serotypes	Source	Description	Reference
Nigeria	23	Broiler farms	47.9% of samples were *Salmonella* positive	Jibril *et al*.,2020
Bangladesh	*S.* Typhimurium	Broiler farms	35 out of 100 samples were positive	Alam *et al*.,2020
China	*S.* Newport	Human feces	4 out of 287 *Salmonella* strains were colistin resistant	Elbediwi *et al*.,2019
China	18	Broiler farms	280 out of 923 were positive	Yu *et al*.,2020
Maxico	N. A	Chicken meat	Prevalence increased from 13.7% to 27.1% 3 over years	Pineda *et al*.,2020
Egypt	7	Broiler farms	120 out of 420 positive samples with 76.7% strains were MDR	Elkenany *et al*, 2019
Pakistan	3	Broiler	239 out of 340 in 1 year	Wajid *et al*., 2019
Brazil	11	Slaughter-house	230 *Salmonella* strains isolated in 5 years from chicken	Rodregues *et al*.,2020
Iran	11	Human ,chicken and cattle	242 strains isolated in 6 years	Ghoddousi *et al*.,2019
Eucador	3	Layer farm	31 *Salmonella* strains identified	Salazar *et al*.,2020
Israel	13	Poultry(16 438), Human (27489)	New antibiotic resistance genomic islands identified	Cohen *et al*.,2020

2.1.6. Prevalence of *Salmonella* in Pakistan

In Pakistan, several serovars of poultry had been detected. According to Nazir 22% samples were *Salmonella* spp positive in Sawat (Nazir *et al*.,2018). While in another study conducted in Faisalabad, *Salmonella* Typhimurium (28.4%) and *Salmonella* Enteritidis, (9.2%) were prevalent serovars in local poultry farms (Wajid *et al*.,2018). In Kohat out of a total of 150 broiler chicken samples, the prevalence of *S.* enteritidis was 23.3% (Asif *et al*., 2017). In Rawalpindi *Salmonella* pullorum and *Salmonella* Gallinarium were 5.35% in total samples (Shoaib *et al*., 2019). Whereas out of 100 *Salmonella* positive samples, the most prevalent sero-groups identified were *S.* Enteritidis (44.4%) followed by *S.* Typhimurium (30.6%), *S.* Gallinarum (19.4%), *S.* pullorum (5.6%) and *S.* typhi (0%) (Samad *et al*., 2019).

2.2 Colonization of *Salmonella* enterica in Poultry gut

The intestines of poultry are colonized by *Salmonella* spp. because of a horizontal or vertical transmission of bacteria at the stage of primary production. The horizontal route of infection includes contaminated feed and water while vertical route includes direct infection of offspring by its flock. *Salmonella* spp. may be present in as much as 65% of individuals in a flock (Raehtz *et al.*, 2018). *S.* Enteritidis can colonize in young chicks more easily as compared to adults. Chicken gut must perform two important functions at a time: absorbing the nutrients from food and protecting against the harmful Pathogens. This homeostasis is achieved with the help of microflora of gut. Immune organs in poultry start to develop during embryogenesis and become functional by the age of 2 to 3 weeks (Han *et al*., 2020).

Microbiota of young chicks is very closely associated with development of immune health. It is now a well-established fact that *S.* Enteritidis may alter the gut microbiome

of young chicks because *S.* Enteritidis promotes the growth of bacteria belonging to Enterobacteriaceae family. To reduce the invasion of *S.* Enteritidis in gut bacterial communities such as Bifidobacterium, Lactobacillus, bacillus and Blautia play important role. As the chick ages it becomes more immune against *S.* Enteritidis infection and that's why the mortality rate is higher in young chicks (Liu *et al*.,2018).

2.2.1 Anatomy and Physiology of Poultry Gut

Crop is very important segment in upper region of GI tract as it has a pH of 4.5 which acts as acid barrier. This segment is also used for fermentation and hydrolysis of starch to sugar. Food can be temporarily stored in crop (Borda-molina *et al*., 2018). Crop region harbors Firmicutes along with Actinobacteria and Bacteroidetes here, Lactobacillus and Bifidobacterium bacteria break starch and initiate lactate fermentation. In crop feed is retained for only 8 minutes. Gizzard is more acidic than crop with pH of 2.6 to enable grinding of food. Here feed remains for just 50 minutes. Firmicutes is dominant phyla in gizzard region (Micciche *et al*., 2018).

More than 900 species of bacteria comprise Gut microbiome of chicken (Xi *et al*., 2019). Some gut microbes release hydrolytic enzymes which can easily degrade complex polysaccharides which would be otherwise very difficult to digest while other bacteria that reside in GI tract release SCFA which ferment these degraded polysaccharides, hence making the food available to their host (Borda-molina *et al*., 2018) Intestinal microflora also protects against harmful pathogens ,thus ensuring the well-being of their host (Xi *et al*., 2019).

GI tract of poultry harbors complex microbial communities, but bacterial species dominates all these. Pathogenic bacteria also reside in GI tract of poultry and are a major source of infections (Shang *et al.*, 2018).It's a known fact that bacterial

communities in poultry vary depending upon age of chick, its diet and sex. Additionally immune system and genetics also substantially alter the microbiome of broiler chicken(Rehman *et al.*, 2018). At the age of 20 to 30 days GI health related issues are observed such as less and poor weight gain and wet Recent studies showed that external factors such as litter, housing condition, hygiene condition of farm, access to water and climate litter (Ranjitka *et al*., 2016).

Caecum is most complex and diverse part of chicken GI tract as it harbors a great variety of bacterial species, including Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria and methanogenic Archaea and gram-positive bacteria. As the broiler ages, the diversity of bacterial communities in caecum also increases, this was supported by a study which showed that bacterial genera increased from 50 to 200 genera when compared on day 1 and day 42 (Xi *et al*., 2019;Li *et al*., 2019). In caecum polysaccharides like cellulose and starch are broken down, digested, and then absorbed. Feed retains for 12 to 20h in caecum. This is the longest time for retaining feed as compared to any other region. (Borda-molina *et al*., 2018).

Studies have shown that small intestine is dominated by Flexibacter, Firmicutes, Proteobacteria and cyanobacteria. Lactobacillus and Escherichia are major genera in large intestine from Firmicutes and Proteobacteria phylum respectively (Shang *et al*., 2018).

2.3 Antimicrobial-resistant NTS and poultry linkage

When a microbe is declared as AMR it means that the microbe is indifferent to the available antimicrobials against the infection caused by that microbe. Anti-microbial resistance infections are spreading rapidly these days and according to an estimate it is responsible for death of more than 700,000 people annually and if the recent trend

didn't change then by 2050 the figures could be as high as 10 million people. In other words, more people would die from drug resistance infections then from cancer. (Ragheb *et al*., 2019). According to recent reports of EFSA and CDC, AMR causes 25000 deaths annually in in EU and 23000 deaths in USA (CDC, 2019).

Salmonellosis had long been treated with antibiotics such as ampicillin and chloramphenicol but now these antibiotics had been replaced with fluoroquinolones and extended spectrum Cephalosporins. *S.* Typhi and *S.* Paratyphi cause severe infections which require Cefixime, Chloramphenicol and Aztreonam, Cefotaxime antibiotics(Gut *et al*., 2019).

According to Medella, *S.* Enteritidis accounted for more than fifty percent of ciprofloxacin resistant infections in data obtained from CDC from 2004 to 2012.In the same study, *S.* Newport, *S.* Typhimurium and *S.* Heidelberg were found to be responsible for 75% of resistance infections against ceftriaxone and ampicillin antibiotics (Medella *et al*.,2016).

Antibiotics have been used in poultry industry for controlling infections and promoting growth (Adhikari *et al*., 2019).In recent years antibiotic resistance *Salmonella* strains have been found in food chain which is a great indication of failure of complete treatment of salmonellosis. There is a great diversity in antimicrobial resistance phenotypes in poultry farms (Liljebjelke *et al.*,2017; CDC,2017). Recent studies performed inside Asia and in non-Asian countries revealed that broiler chicken is disseminating pathogenic multi drug resistance *Salmonella* in humans (EFSA, 2019; García-Soto, 2020). It is now a known fact that cattle provide a safe environment where antimicrobial resistance bacteria grow, multiply and then disseminate in surroundings. This is very concerning because humans have a long history of raising cattle's for milk

and beef (Sabino *et al*., 2019).Recent research's reveals that ground beef is great reservoir of pathogenic bacteria and specifically *S.* enterica(Adhikari *et al*., 2019).

Though the salmonellosis cases reported from fresh produce are very less as compared to animal sources, yet this factor cannot be ignored (Liu *et al*.,2017). Another important vehicle for transmission of *Salmonella* strains is sea food. Since, pathogens are not confined to geographical borders so imported sea food is causing inter-continental transmission of *Salmonella* (Hassan *et al*.,2018).

2.4 Routes of antibiotic resistance

There are two basic routes of antibiotic resistance in bacteria i.e., either by some mutation in chromosomal genes or by acquiring plasmid (Katiyar *et al*., 2020). The resistant genes that are present on the mobile genetic elements cause a further increase in resistance profile of several microbes (Frost *et al*.,2019)

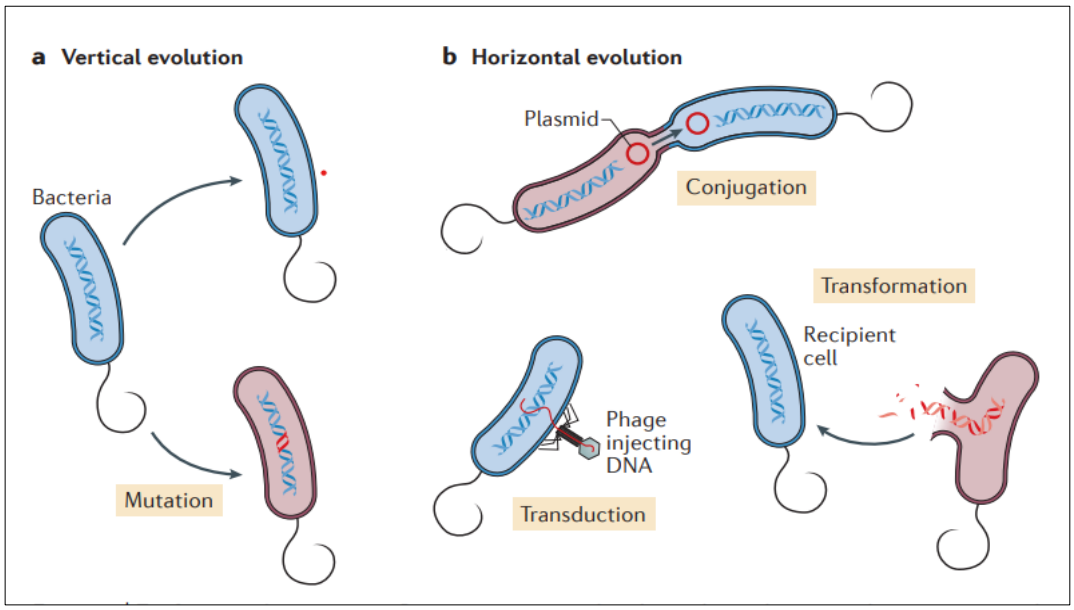


Figure 1 Routes of evolution of resistance. Image from Sommer *et al*., 2017.

2.4.1 Antibiotics and classification of Antibiotics

2.4.1.1. Cell wall targeting antibiotics

Cell wall synthesis inhibitors target the cell wall of bacteria. They are grouped as intracellular wall inhibitor, β-lactams, and glycopeptide.

2.4.1.2. Beta-lactam antibiotics

β-lactam antibiotics possess a very reactive four membraned beta lactam ring β-lactam are important due to their ability to bind PBP enzyme and thus ultimately inhibiting bacterial cell wall Synthesi*s*. PBP enzymes are present in both gram negative and gram-positive bacteria and are present on external side of cytoplasm. They have transpeptidase and transglycosylase activity. PBP are also involved in peptidic carboxypeptidation and endopeptidation (Lima *et al*., 2020). Commonly β-lactam antibiotics are grouped as penicillin, Cephalosporin, Carbapenem and Monobactam. Four major type of resistance mechanisms have been observed for β-lactams.

1. Reduction in permeability of membrane
2. Modification if PBP
3. Bypassing an important step in the pathway by LDT enzymes
4. Degrading the antibiotic with Beta lactamases (Nikolaidis *et al*.,2014; Sarkar *et al*., 2017).

2.4.1.3. Glycopeptide

vancomycin and teicoplanin are first generation glycoproteins having very similar structure*S.*Vancomycine has special importance for its ability to fight MDR gram positive bacterial infection *S.* Telavancin and Dalbavancin are second generation glycopeptides (Lima *et al*., 2020).

2.4.1.4. Antibiotics targeting protein translation

Protein synthesis blocking antibiotics target the ribosome and thus blocking the synthesis of protein. Bacterial ribosome is made up of smaller 30S subunit and larger 50S subunit. Tetracyclines and Aminoglycosides target the smaller 30S subunit whereas Lincosamides, Macrolides and Chloramphenicols target the 50S ribosomal subunit(Lima *et al*., 2020).

Tetracyclines block bacterial translation. TetM and TetO genes are key players in tetracycline resistance. Bacteria may become resistant to tetracyclines by two important possible mechanisms: active efflux of antibiotics and targeted protection (Wilson *et al*.,2020).

2.4.1.5. Antibiotics targeting DNA replication

Quinolone antibiotics are effective against both gram-positive and gram-negative bacteria. Quinolones target bacterial topoisomerases gyrase enzyme and topoisomerase IV enzyme (Gutierrez *et al*.,2018). Bacterial topoisomerases gyrase and topoisomerase IV enzymes modulate the supercoliling of DNA and decatenate the daughter chromosomes after they have undergone replication. Topoisomerases are involved in causing double strand break in DNA. Quinolones perform their action by stabilizing DNA–enzyme cleavage complexes and thereby halting process of DNA ligation. This event stalls replication fork. Gram negative bacteria have developed resistant to quinolones by mutating the genes encoding gyrase enzymes and topoisomerase IV enzymes (Dhiman *et al*., 2019). Latest emerging technologies in biotechnology and bioinformatics have provided us insights into development against quinolone antibiotics and it has been observed that qnr, the qep, or the oqx genes which are acquired and cause resistance to Quinolones (Martínez & Igrejas ,2019).

2.4.1.6 Folic acid metabolism inhibitors

In 1935 an experiment was done by Domagk in which he came to know that hydrolysis of prontosil released sulfonamides in tissues which ultimately interfered in bacterial folate synthesis and cell growth of bacteria. In 1956 trimethoprim was made important in folate production, an inhibitor of dihydrofolate reductase. (Sköld *et al*., 2017)

2.4.1.7. Antibiotics targeting mRNA synthesis

Rifampicin was introduced back in 1967, in which it was concluded that rifampicin plays a vital role in the therapy treatments of various widespread diseases such as leprosy, tuberculosis and many others because it has specific antibacterial activity because of specific bacterial RNA polymerase inhibition (Kohli *et al*., 2021).

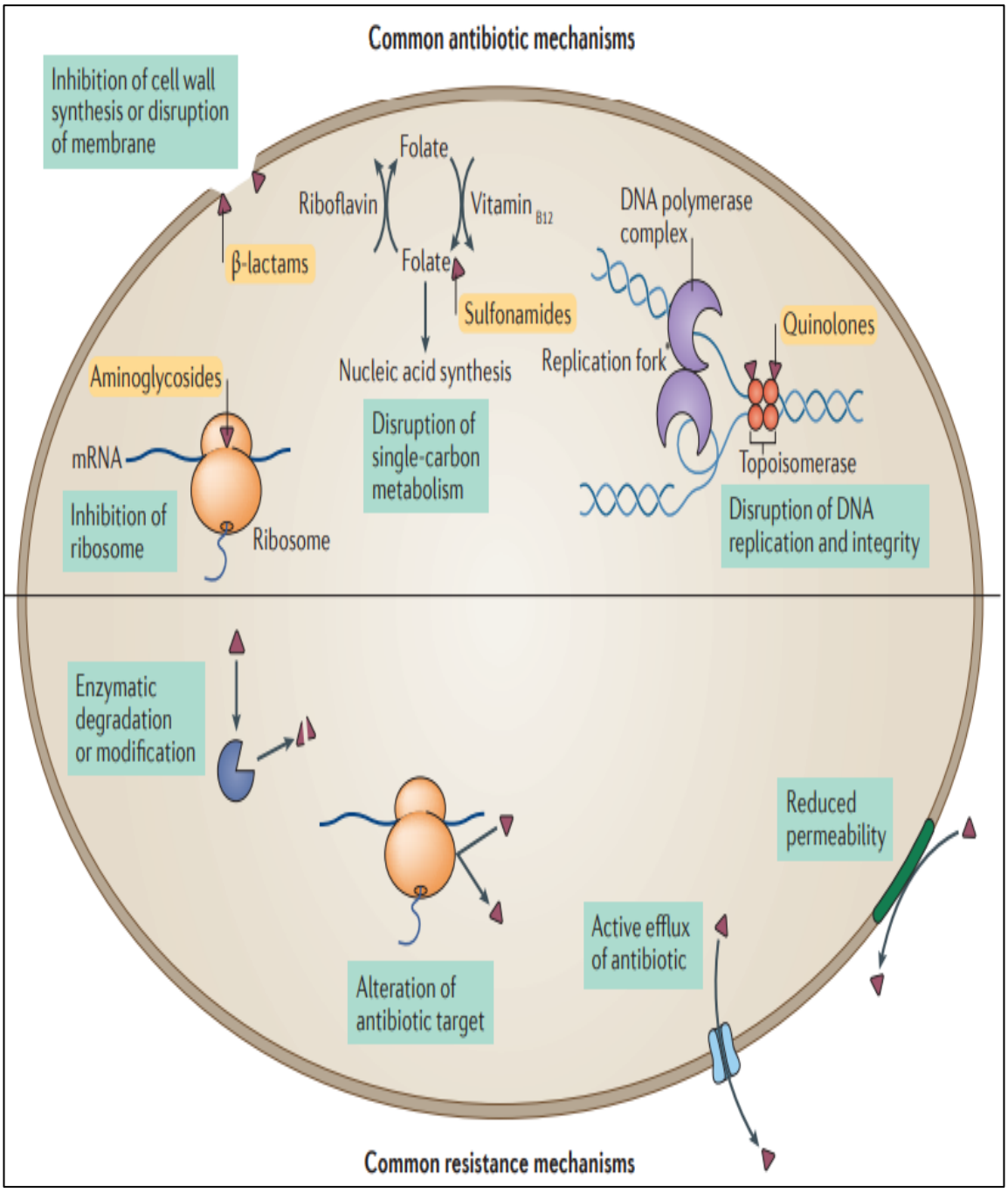


Figure 2 Common mechanisms of antibiotic action and antibiotic resistance. Image from (Crofts *et al*., 2017)

2.7. Antibiotic resistance determinants of *Salmonella* Enteritidis

2.7.1 Plasmids

Plasmids are given prime importance in HGT studies as host range of bacterial plasmid shows the evolutionary process. In antibiotic resistance studies route of plasmid transmission is much emphasized (Redondo-Salvo *et al*.,2020). Naturally occurring plasmids can easily be exchanged between bacterial cells, thereby providing a basis for transfer of virulence and pathogenicity genes. Their analysis is of key consideration for bacterial characterization (Galata *et al*.,2019). Environment specific metal resistance genes are also known to be present in plasmids. Plasmids are found in almost all types of samples ranging from human and bacteria to environmental samples (Tatiana *et al*., 2019).

2.7.2 Genomics islands

Genomic Island is that region in a bacterial chromosome that has been horizontally acquired and is not native to the bacterial chromosome.GI can be differentiated from rest of genome because of their different GC content and dinucleotide frequency (Bertelli *et al*.,2019). GI have been classified based on their gene content in many subtypes. Genomic Islands that have resistant determinants are called to be resistance islands while GIs that have virulence factors are called Pathogenicity islands. Genomic Islands have also been classified as Catabolic and Symbiosis islands. Metabolic islands contain genes that encode for metabolic proteins (Partridge *et al*.,2018).

Pathogenicity islands are most studied genomic island among all the studied genomic islands as they have ability to change the phenotype of bacteria (Juhas *et al*., 2019). The excision process of pathogenicity Islands is shown in figure 1. Fig1(A) shows that Pathogenicity Islands have DRS or att sites at their ends. Integrases and excisionases

recognize these sites and catalyze the excision of these sites. Fig1(B) is depicting that after excision pathogenicity islands (episomal element) contains one of the att site while the other att site remains attached to chromosome. Fig1(C) shows that change in excision rate alters the expression of island genes and also the passage of episomal element to other bacteria is increased. Based on their role genomic islands are grouped into Replacement genomic islands those that are acquired by homologous recombination or Additive genomic islands that re acquired by non-homologous recombination in specific sites such as rRNA or tm RNA (Filho *et al*.,2018). Usually, genomic islets are 10kb to 100kb in size and those Genomic islands that fall below this lower limit are referred to as Genomic Islets (Desvaux *et al*., 2020).

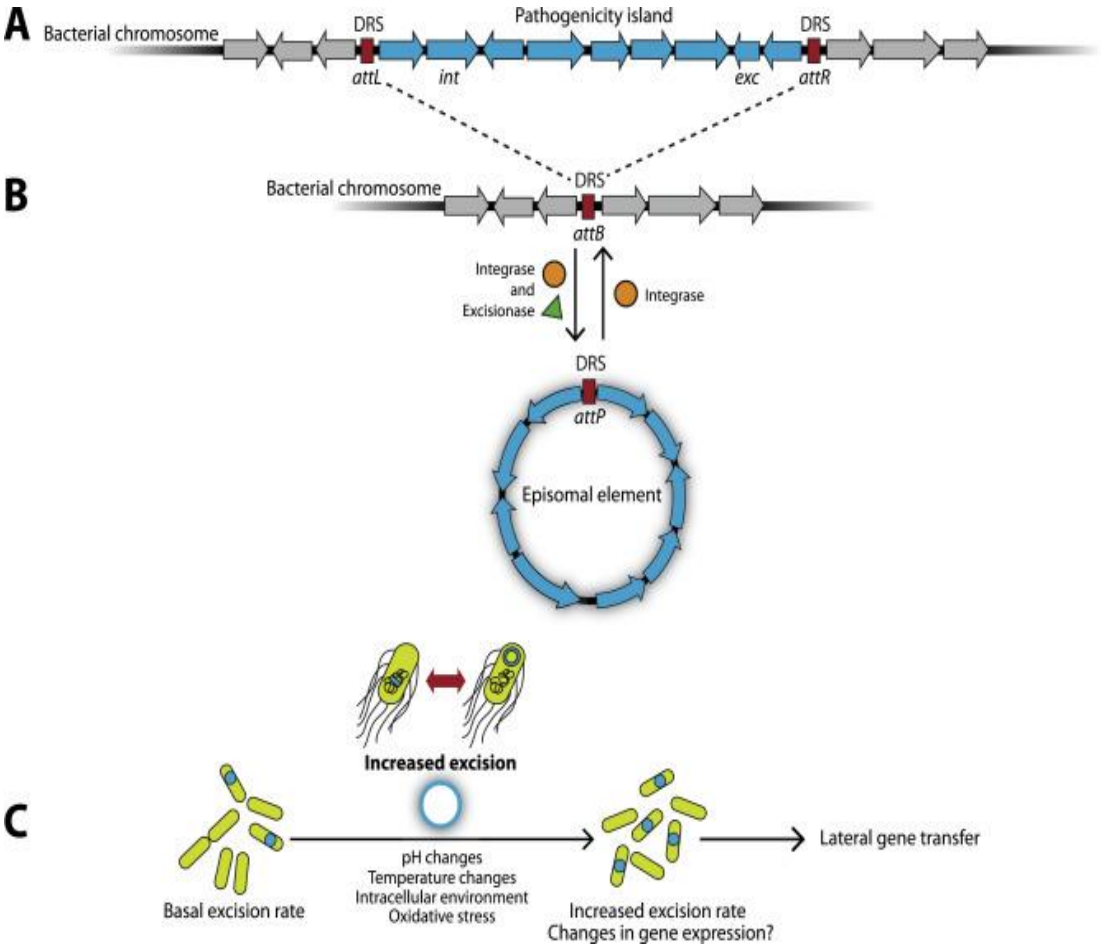


Figure 3 The excision process of pathogenicity islands (Pamela *et al*., 2019).

2.7.3 *Salmonella* pathogenicity islands

Salmonella pathogenicity islands are usually large genomic islands ranging from 10kb to 200 kb in size. They help Salmonella species in infecting macrophages and dendritic cells. Their presence is often serovar specific. Five type of secretion systems are present in bacteria that help in delivering virulence factors in the host cells. Different SPIs have different kind of secretion systems (Lou *et al.*,2019).

2.7.3.1 SPI-1

All *S.* enterica bacteria have pathogenicity islands 1(SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2). SPI-1 has a special significance due to its role in interaction with the host as it facilitates the host epithelial cell invasion which is a special virulence characteristic of *S.* enterica (Kaur *et al*.,2016). SPI-1 encodes for T3SS which is involved in enteritis production. SPI-1encoded T3SS has great significance in Gastrointestinal diseases. SPI-1encoded T3SS cannot disseminate infection. T3SS is also essential for suppressing early proinflammatory cytokines expression in macrophages. SPI-1 genes are required to colonize intestinal tract and persist the infection in liver and spleen of chicken (Dieye *et al*., 2009).

2.7.3.2 SPI-2

SPI-2 encodes for one of the two T3SS encoded by *Salmonella* enterica which is most important of all the *Salmonella* virulence factor*S.*SPI-2 encoded T3SS is involved in intestinal infections as well as dissemination of functions (Jennings *et al*.,2017).SPI-2 genes are found to important for *S.* Enteritidis colonization in liver and spleen of chicken just like SPI-1 (Dieye *et al*., 2009).SPI-2 T3SS have been found to interfere with innate immune response of host (Cerny & Holden, 2019).Those *Salmonella* strains that do not have SPI-2 have shown less virulence (Nieto *et al*.,2016).

2.7.3.3 SPI-3

SPI-3 encodes for type 5 secretion system also called autotransporter. SPI-3 encodes for functionally non-related proteins (Kirchweger *et al*.,2019).

2.7.3.4 SPI-4

SPI-4 encodes for type 1 secreston system(T1SS). TTSI encoded by SPI-4 together with T3SS encoded by SPI-1 infects polarized epithelial cells (Kirchweger *et al*.,2019). SiiE is the substrate protein of TISS and this adhesin protein is largest protein of *Salmonella* proteome because its size is 595 k Da Virulence factors associated with SPI-4 are needed to survive in macrophages (Barlag & Hensel, 2015).

2.7.3.5 SPI-5

SPI-5 is also an important pathogenicity island as it required for different stages of Salmonellosis. SPI-5 has genes that encode for SopB effector proteins of T3SS of SPI-1. SopB is expressed for promoting membrane invasion. SPI-5 also has pipB which is translocated by T3SS encoded by SPI-2. It has been observed that pipB comes in active form when the bacteria is inside host cells.(Ilyas *et al*.,2017;Schmidt & Hensel, 2004).

2.7.4. Insertion sequences

Insertion sequences are simplest mobile genetic elements found in bacteria as well as archaea that can move within a genome or between genomes. Horizontal transfer of Insertion sequences becomes possible when they become part of phages or plasmids (Vandecraen *et al*., 2017).

Insertion sequences only have genes that they need for transposing themselves thus, enabling them to have such a small size (Carlie *et al*., 2020). When Insertion sequences randomly move to new positions, they often carry resistance genes along with

transposase genes thereby facilitating the horizontally acquired bacterial resistance. It has been recently confirmed that Insertion sequences influence the resistance genes by adding a promoter region upstream of them (Harmer & Hall, 2019).Insertion sequences are grouped on basis of their mechanism of transposition as well as motif present at active site of transposase gene. In cut and paste mechanism the excised cut region from donor is simply pasted into the recipient while the copy and paste mechanism involves the replication of IS to join donor with recipient in a cointegrate which is later resolved to give IS to both donor and recipient. Another mechanism of transposition of IS is copy-out -paste in in which the IS is replicated to form double stranded intermediate. This circular intermediate is later integrated into recipient (Partridge *et al*.,2018).

CHAPTER 3

METHODOLOGY

3.1 Genomic characterization

3.1.1 Sample collection

Whole genome sequence data of five isolates of *S. enterica* were provided by Food microbiology laboratory, ASAB. Source of *S.* Enteritidis ISB 8 isolated from broiler farm in Islamabad was poultry liver. The source of *S.* Enteritidis KHR 57, *S.* Enteritidis VHR 2 and *S.* Enteritidis SKR 54 isolated from Khairpur, Vihari and Sukkhar respectively was poultry feces whereas the source of *S.* Enteritidis RW 50 isolated from poultry farms in Rawalpindi was eggshell. At the time of original isolation all isolates were characterized using standard methods of biochemical and molecular characterization and then antibiotic susceptibility test was performed on them. Genomic DNA from these isolates was extracted by Kit method using Thermo Scientific kit K0721 as per instructions given by manufacturer. NanoDrop in ASAB laboratory NUST was used to analyze quality of genomic DNA. Genomic DNA was subjected to Illumina Hi seq 2500 platform. With collaboration of Dr. Erica Ganda libraries were prepared at department of animal sciences in Pennsylvania state university USA located at Park Road USA.

3.1.2 Assembly and Annotation

Quality of sequenced genomes was assessed using **FASTQC** with default parameters (Andrews & Simon, 2010). **Trimmomatic** 0.3 was used to trim the raw reads using standard settings (Bolger *et al.*, 2014).

Further filtering and preprocessing of sequenced data were performed by using **FASTP** tool on default settings except for quality phred score, 20 and minimal read length, 50 base (Chen *et al.*,2018). Based on genome quality report only *S.* Enteritidis

RW50 and *S*. Enteritidis KHR57 were used for downstream analysis. Genomes were *De novo* assembled by Shovill pipeline (v.1.1.0) using **Spades** as the assembly method (Bankevich *et al*., 2012; Seeman T. 2019). "Trim read" option was turned on and minimum length of contig was set to 200 bp.

Quality of assembly files were checked using **QUAST** (v. 5.0.2) Annotation of assembled genome was performed to identify and label important genomic feature. Annotation of assembled genome was performed using **Prokka** at Galaxy Australia pipeline. (Seemann, 2014; Brettin *et al.,* 2015). Prokka can perform fast functional annotation of bacterial genomes. *In silico* serotyping was performed with **SeqSero** v1.2 available at (https://cge.cbs.dtu.dk/services/SeqSero/) (Zhang *et al*., 2015).Both strains were subjected to *in silico* **Multi-Locus Sequence Typing** (MLST) v2.0 (https://cge.cbs.dtu.dk/services/MLST/) by using fastq files (Larsen *et al*., 2012).

3.1.3 Reference based assembly

Salmonella enterica subsp. enterica serovar Enteritidis str. P125109 complete genome sequence having NCBI reference number; NC_011294.1 was downloaded from GENBANK database of NCBI (http://www.ncbi.nlm.nih.gov/genome)(Carolina *et al*., 2019). MAUVE was used for reference-based assembly for which input files were in gbk format with default parameters. Move contig tool was used for aligning sequences in MAUVE which generated output files in fasta (.fas, .fna, .faa) format to conduct downstream analysis. Multi-fasta sequence of *S*. Enteritidis RW50 and *S*. Enteritidis KHR57 was saved (Darling *et al*., 2004). MAUVE tool was preferred for alignment due to its sensitivity and ease of use. MAUVE aligns the genome sequences with respect to the reference genome.

3.2 Phylogenetic analysis

Phylogenetic analysis was performed based on 16 S ribosomal RNA sequence and whole genome alignment. 16S r RNA was predicted using **Type strain genome server** (TYGS) which is a fully automated online platform for phylogenetic analysis server available at https://tygs.dsmz.de/ with default parameters (Meier-Kolthoff *et al*., 2019). It is a high-throughput web server which can be used for genome based taxonomic analysis to infer genome scale phylogenies in a very user friendly mode.

For genome based phylogeny and 16 S rRNA analysis, genomes of complete few *Salmonella* Enteritidis strains of poultry origin were manually uploaded at the time of genome submissions in FASTA format in TYGS and rest of genomes were automatically selected based on closest type of genomes. Phylogenetic tree was then calculated at 100 bootstrap value.

3.3 Identification and comparison of prophage sequences

Prophages are important contributors of genome plasticity and to identify prophage sequences **PHASTER** (Phage search tool enhanced release) tool that is available at (http://phast.wishartlab.com/) was used with default parameters (Arndt *et al*., 2016). PHASTER can predict and annotate prophages in microbial genomes. Prophages predicted by PHASTER were then checked for presence of virulence factors and antimicrobial resistance genes as well. PHASTER is an upgraded version of PHAST web server.

3.4 Identification and comparison of virulence factors

Virulence factors add to the virulence of pathogenic bacteria. They determine the disease causing capability of a pathogen. Pathogenic bacteria use virulence factors to integrate and survive in their host and this ultimately destroys body of their host.

Mostly they are integrated in microbial genomes and are also acquired by horizontally. To identify virulent factors in genome of *Salmonella* Enteritidis RW50 and *Salmonella* Enteritidis KHR57, **VFDB** (virulent factors database) available at (http://www.mgc.ac.cn/VFs/) was used (Chen *et al*., 2005).VFDB gives very inclusive information of virulence factors of input sequence file. Many reference genomes of different pathogenic bacteria are already annotated on VFDB for presence of virulence factors and description of these virulence factors is also available on database.

The input files were in GENBANK format and BLASTn option with E value 0.0001 was chosen. Matrix BLOSUM 62 option was selected in next step. To obtain more precise and accurate results, only results with threshold value of 1e-20 or lower were considered. Likewise Bit score value of 100 and percent identity of more than 35 was selected.

3.5 Identification and comparison of Genomic islands

Genomic Islands are regions that are actually horizontally acquired from other species. These genomic islands can acquire several genes which can be later cause increase in antibiotic resistance and virulence of food borne pathogens like *Salmonella enterica*. Genomic islands were identified using **IslandViewe**r 4 at https://www.pathogenomics.sfu.ca/islandviewer/ address with default input settings (Bertelli *et al*., 2017). Genomic islands were predicted by using GENBANK file as input sequence file. At the time of file submission *S*. Enteritidis P125109 was chosen as reference strain. IslandViewer 4 predicted genomic island regions in circular image form. Island regions were also identified in tabular file format which was downloaded for later use.

To identify Pathogenicty islands in our isolates **SPIFinder 2.0** available at Center of Genomic Epidemiology CGE (https://cge.cbs.dtu.dk/services/SPIFinder/) was used. Selection criteria was minimum 95% identity and 60% sequence length. SPIFinder detects pathogenicity islands which are often species and subspecies specific and they impact the pathogenicity of *Salmonella* genomes .

3.6 Identification and comparison of ICE

Integrative and conjugative elements are genomic regions that promote intercellular mobility of DNA. For prediction of ICE sequences in our genomes web-based **ICEberg** 2.0 was used. Default parameters were chosen to run the tool. ICEberg provides detailed information of integerative and conjugative elements (Bi *et al*., 2012).Furthermore, ICE region was also checked for antimicrobial resistance genes and virulence factore.

3.7 Identification and comparison of plasmid

Plasmids were found by using online **Plasmid finder** tool v.2 available at CGE (https://cge.cbS.dtu.dk/services/PlasmidFinder/) (Carattoli *et al*., 20018).Threshold for minimum percent identity was 95% and minimum 60% sequence coverage selection criteria were used .

3.8 Identification and comparison of Insertion sequences

Insertion sequences are discrete DNA regions that contribute to genome plasticity. Insertion sequences are simplest mobile elements of prokaryotic genomes.

They have ability to transpose themselves to new positions. Insertion sequences in genomes of *S.* Enteritidis RW50 and *S.* Enteritidis KHR57 were studied by using **ISfinder** with standard settings (Partridge *et al*., 2018; Siguier, 2006).

3.9 Whole-Genome sequence comparison using BRIG

Whole-genome sequence comparison of local methicillin-resistant *S.* Enteritidis RW50 strain and *S.* Enteritidis KHR57 was performed with *S.* Enteritidis P125109 genome using, BRIG (Blast Ring Image Generator) (http://brig.sourceforge.net/). BRIG is a free cross-platform software used to generate and display circular map of bacterial gnomes.

Circular map is generated by performing BLAST against uploaded genome sequences and similarity between a reference sequence and other sequences is shown in the form of circular map. Alignment was performed by using NCBI BLAST+ with Upper and Lower threshold values at 90% and 70%, respectively (Alikhan *et al*., 2011).

3.10 Identification and comparison of resistance genes

To predict the resistance genes in the assembled *Salmonella* genomes, resistance gene identifier (RGI) from the Comprehensive Antibiotic Resistance Database (CARD, available at https://card.mcmaster.ca/analyze/rgi) was employed. RGI-CARD has curated collection of AMR genes and mutations along with computational biology models to detect them in genomic data. In RGI CARD "perfect and strict hit criteria" was selected to predict the antimicrobial resistance genes.To identify acquired resistance genes ResFinder (https://cge.cbS.dtu.dk/services/ResFinder/) webserver 3.0 was utilized. ResFinder can detect resistance genes in both raw reads and draft gnome assemblies. Cut-off criteria of ≥ 60% sequence length and ≥90% sequence identity was selected .

CHAPTER 4

RESULTS

4.1 General genomic Characterization

4.1.1Whole Genome Sequencing, Assembly and Annotation

Illumina whole genome sequencing generated paired end reads which were checked for their quality. Genomes were identified as *S.* Enteritidis with MLST type 11. Based on genome quality report 2 whole genomes of *S.* Enteritidis were used for downstream analysis out of 5. The general genomic features of *S.* Enteritidis strains used in this study have been summarized in table 2.

Table2 General genomic features of *Salmonella* genomes used in study

Feature	KHR57	RW50	P125109
Size	4840646	4859596	4685848
GC content	51.96	51.9	52.2
N50	231420	126095	4685848
Contigs	55	80	1
CDs	4538	4564	4405
RNAs	92	95	104

A graphical representation of virulence genes and antimicrobial resistance genes of *S.* Enteritidis genomes is shown (Figure 4; Figure5).

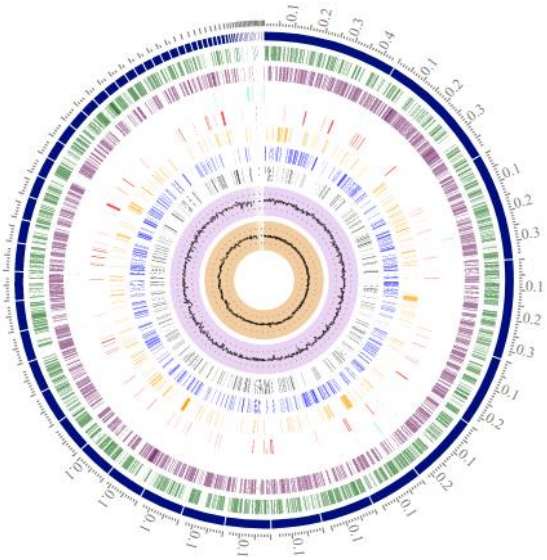


Figure 4 Circular view of *Salmonella* Enteritidis KHR57.

Moving from inwards to outwards 1.GC skew (brown). 2.GC content.(mauve) 3.Drug target(black). 4.Transporter. 5.Virlence factors genes(mustard). 6.AMR genes(red). 7.Non-CDS features(turquoise) .8.CDS reverse(purple). 9. CDS forward(green)

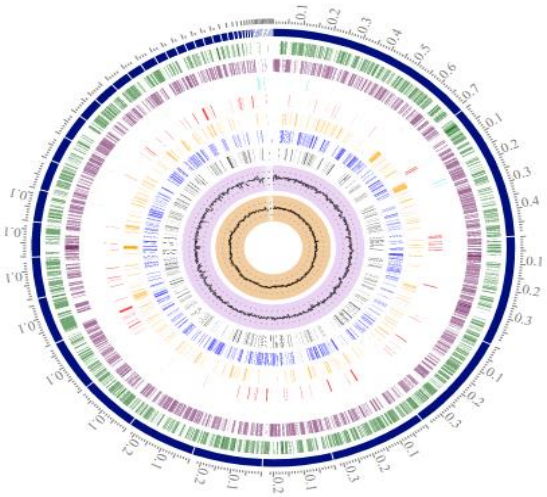


Figure 5 Circular view of *Salmonella* Enteritidis RW50

Moving from inwards to outwards 1.GC skew (brown). 2.GC content.(mauve) 3.Drug target(black). 4.Transporter. 5.Virlence factors genes(mustard). 6.AMR genes(red). 7.Non-CDS features(turquoise) .8.CDS reverse(purple). 9. CDS forward(green).

4.1.2 Reference based alignment

The sequences were then aligned with *Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109 complete genome sequence having NCBI reference number; NC_011294.1 via MAUVE. Alignment of *S.* Enteritidis RW50 is shown in (Figure 6) and alignment of *S.* Enteritidis KHR57 with reference genome *S.* Enteritidis P125109 is shown in (Figure 7). MAUVE tool generated a multi-fasta file which was converted to single assembled fasta file using ARTEMIS.

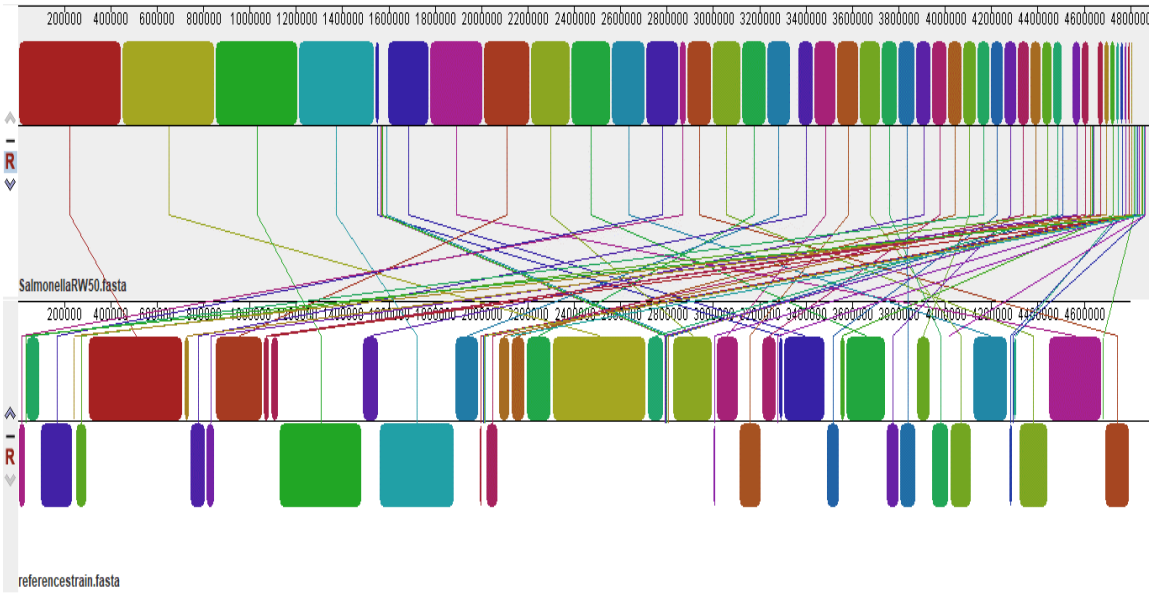


Figure 6 Reference based alignment of *S.* Enteritidis RW50 with reference strain *S.* Enteritidis P125109 using MAUVE tool

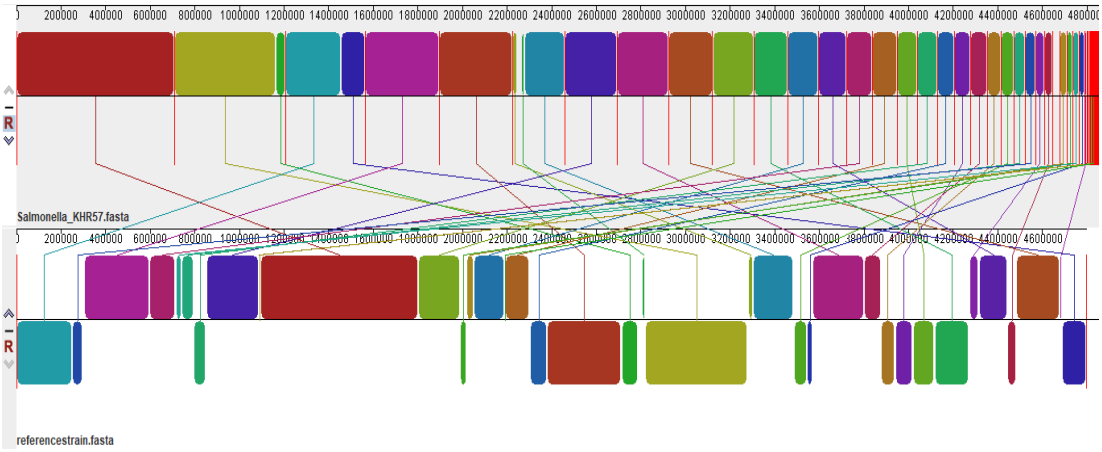


Figure 7 Reference based alignment of *S.* Enteritidis KHR57 with reference strain *S.* Enteritidis P125109 using MAUVE tool

4.2 Phylogenetic analysis

4.2.1 Phylogenetic analysis based on 16S rRNA

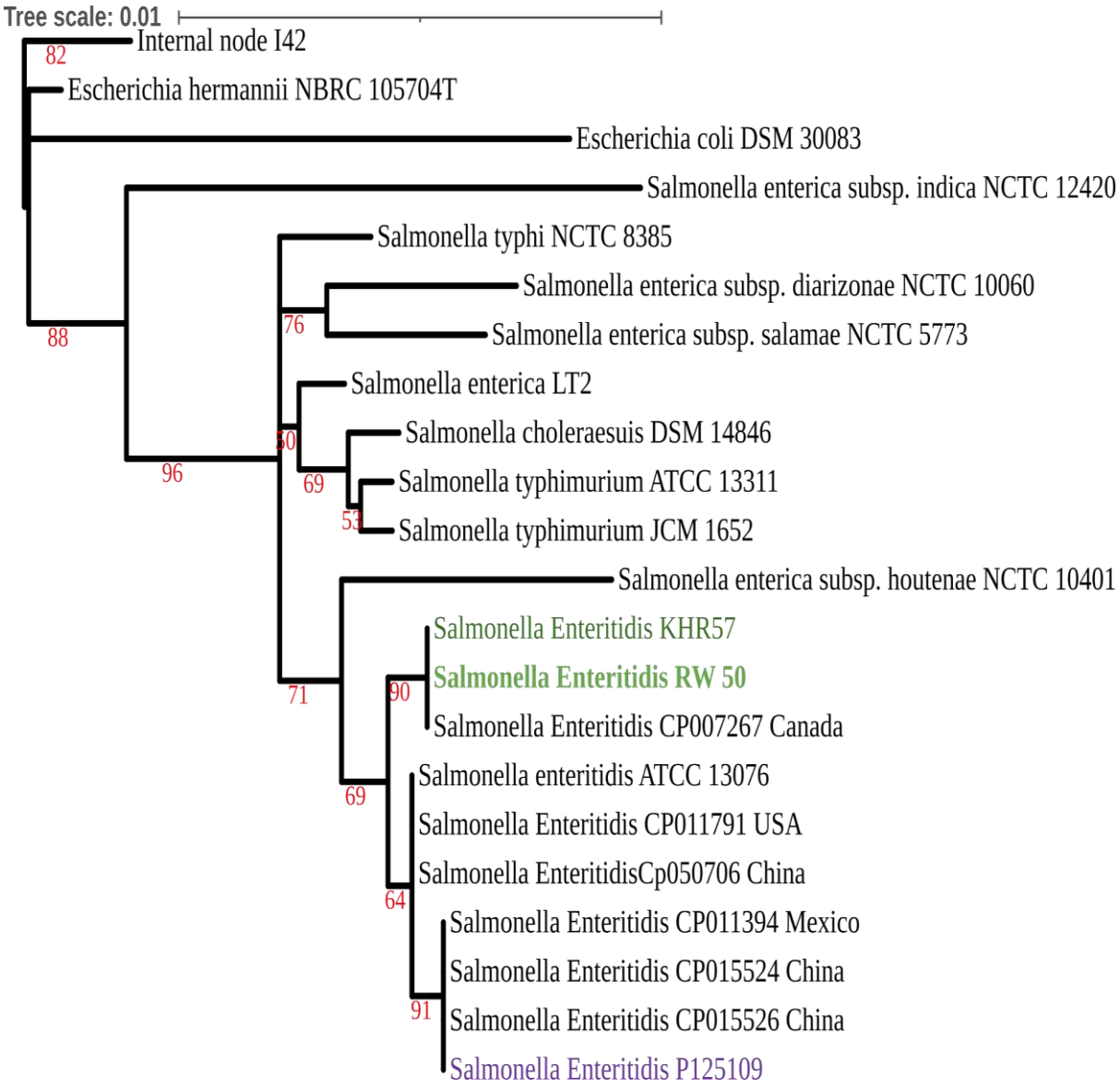


Figure 6 Phylogenetic analysis of *Salmonella* Enteritidis strains based on 16S rRNA

Phylogenetic analysis of *S.* Enteritidis shown here is inferred with FastME 2.1.456. Bootstrap support is shown here for main lineages. Bootstrap values shhown here are representing the percentage of compatible bootstraps from 1,00 iterations. *S.* Enteritidis KHR57 and *S.* Enteritidis RW50 are shown with green colour whereas reference strain *S.* Enteritidis P125109 is shown with mauve color.

4.2.2 Phylogenetic analysis based on whole genome

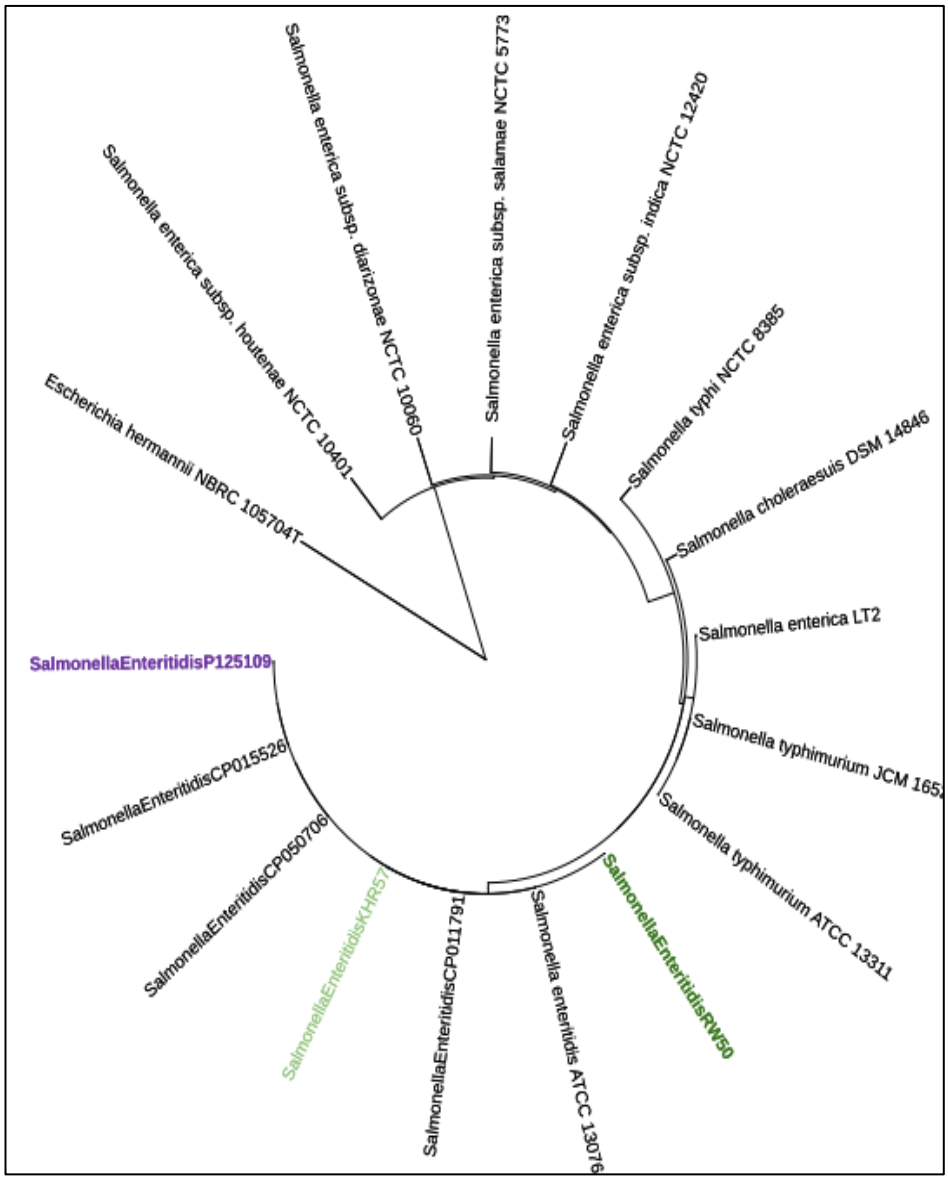


Figure 7 Genome based phylogenetic analysis of *Salmonella* Enteritidis isolates

TYGS results for genome based phylogeny are shown here. *S.* Enteritidis RW 50 is shown with dark green color and *S.* Enteritidis KHR57 is shown with light green colour whereas reference strain is shown is purple colour.

4.3 Prophage identification and comparison

Three intact phages were detected in *S.* Enteritidis RW50.The most common phage types were *Salmonella* phage_g341c, Escherichia phage 186 and *Escherichia* phage pro483 (Figure 10) Two intact prophages were detected in *S.* Enteritidis KHR57 and most common phages were *Salmonella* phage SW9 and *Salmonella* phage c341. In reference strain only one intact prophage and most common phage type was *Salmonella* Typhimurium phage ST64B (Figure 11). Comparison of prophages predicted by PHASTER is shown (Table 3). A list of all the predicted phages by PHASTER in both strains in given in supplemental material (Appendix A:Table B1, B2)

Table 3 Comparative analysis of phages predicted in *Salmonella* genomes

Strain	Region Length	Protein	Discription	GC (%)
***S.* Enteritidis P125109**	62.6Kb	58	transposase, tail,head,plate,capsid,portal, terminase,integrase	47.8
***S.* Enteritidis KHR 57**	38.2Kb	47	protease, tail,plate,lysis,head,terminase, capsid,	52.1
	38.6 Kb	53	portaltail, terminase,portal,coat	47.8
***S.* Enteritidis RW 50**	38.6Kb	52	tail, coat,portal,terminase	47.8
	38.2 Kb	49	protease, tail,plate,capsid,terminase	52.1
	30.5Kb	41	capsid,terminase,head,tail,lysin, plate,integrase	52.70

4.4 Identification and comparison of Virulence factors

In all the three strains virulence factors were present. Table 4 summarizes the virulence factors in all three strains.

Table 4 Comparative analysis of virulence factors

Virulence factors	***S.* Enteritidis P125109**	***S.* Enteritidis KHR57**	***S.* Enteritidis RW50**
Fimbrial adherence determinants	+	+	+
Capsular proteins	_	_	_
Serum resistance	_	_	_
Regulation	+	_	_
Toxin	_	_	_
Macrophage inducible genes	+	+	+
Magnesium uptake	+	+	+
Non-fimbrial adherence determinants	+	+	+
TTSS (SPI-2 encode)	+	+	+
TTSS (SPI-1 encode)	+	+	+

4.5 Genomic islands prediction analysis:

Genomic Islands were predicted by using Island viewer 4 and results were exported in both circular and tabular forms. *Salmonella* Enteritidis RW50 had 32 genomic islands whereas *S.* Enteritidis KHR57 had 31genomic island regions. Reference strain *S.* Enteritidis P125109 had 27 genomic islands. Genomic islands predicted in all strains are shown in figures below.

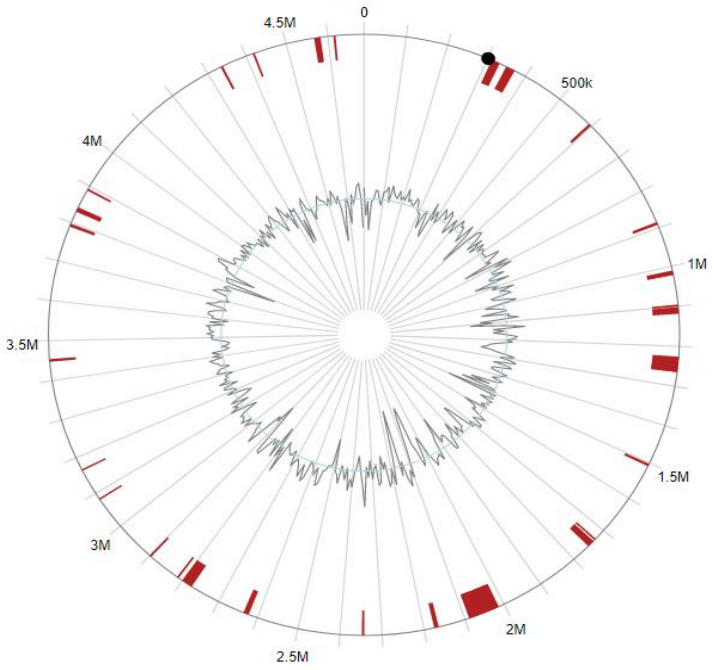


Figure8 Circular visualization of 27 genomic islands predicted in *Salmonella* Enteritidis P125109 by IslandViewer4 are shown with integrated prediction method.

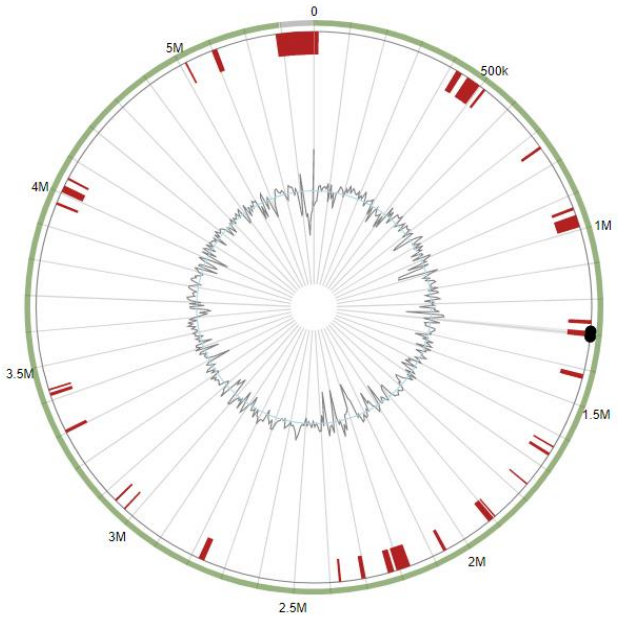


Figure9 Genomic islands predicted in studied *Salmonella* Enteritidis KHR57 isolate by IslandViewer 4 server by integrated prediction method .The outer green ring is showing alignment with reference genome. Maroon color blocks are showing 31 Genomic islands of *S.* Enteritidis KHR57 isolate

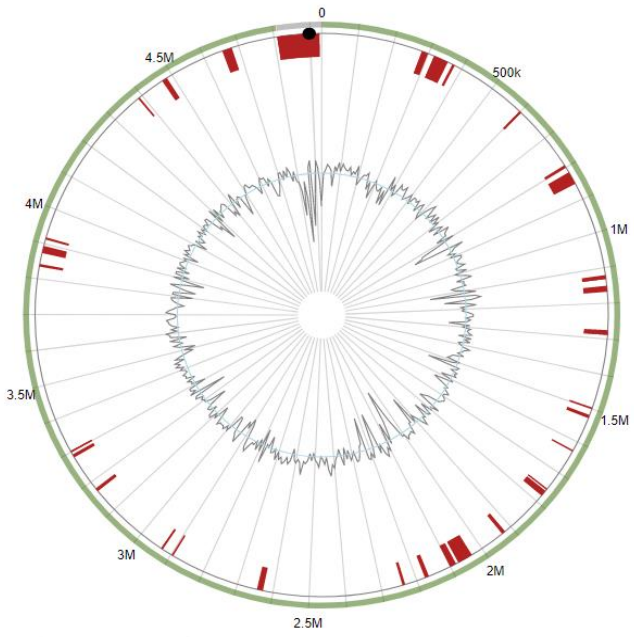


Figure10 Circular visualization of Genomic islands predicted in the studied *Salmonella* Enteritidis RW50 by IslandViewer 4 server. Outer green ring is showing alignment with reference genome. 32 genomic islands are shown in maroon color.

4.6 Identification of integrative and conjunctive mobile elements

In all the three strains integrative and conjunction mobile elements were present. *S.* Enteritidis RW50 had putative Integrative mobile element region without any identified repeats. *S.* Enteritidis KHR57 and reference strain *S.* Enteritidis P125109 had direct integrative mobile elements with direct repeat as depicted in table below (table4). Position of putative mobile regions is shown in supplemental material (Appendix A: figure A1 and A2)

Table 5 Identification of Integrative and conjugative elements in *Salmonella* Enteritidis strains and comparison with reference strain

Strain	***S*. Enteritidis P125109**	***S*. Enteritidis RW50**		***S*. Enteritidis KHR57**
Region	1	1	2	1
Length	26496	30083	95578	29918
Identity	Putative IME element with direct repeat attL and attR	Putative IME with identified direct repeat attL and attR	Putative ICE with T4SS	Putative integrative mobile element with direct repeat attL and attR
Insertion site	tRNA	tRNA	Predicted ORF	tRNA
proteins	27	28	114	24
GC	37.6	39.97	50.77	39.98

4.6.1 Identification of genes encoding for putative virulence factors

The identified ICE and IME regions were also viewed for virulence factors which are naturally present in food borne microbial pathogens. When checked for virulence factors in VFDB no hits were found against putative IME regions identified in *S.* Enteritidis KHR 57 and that of reference strain. Contrary to that diverse virulence factors were found in putative ICE region predicted in *S.* Enteritidis RW50. These virlence factors including fimbrial usher protein(bcfC), fimbrial adhesin protein(bcfD), fimbrial chaperon(bcfG)(bcfB) , fimbrial subunit(bcfA)(bcfE) (bcfF). (cheD), methyl-accepting chemotaxis protein (fimD) and usher protein (FimD).

4.7 Plasmids identification

No plasmid was detected in both genomes. Reference strain *S.* Enteritidis P125109 also has no plasmid.

4.8 Identification and comparison of Insertion sequences

Comparative analysis of Insertion sequences revealed that IS 1 family having origin from *Yersinia pseudotuberculosis* in reference strain was absent in both *S.* Enteritidis RW50 and *S.* Enteritidis KHR57.

10 families of insertion sequences were present in *S.* Enteritidis RW50. IS 256, IS Azo13, IS 1380, IS 701 were found absent in *S.* Enteritidis RW50 and present in *S.* Enteritidis KHR57 and *S.* Enteritidis P125109. (Table 4)

S. Enteritidis KHR 57 has 14 families of insertion sequences .IS As1 was found to be unique in *S.* Enteritidis KHR57 as it was absent in *S.* Enteritidis RW50 and *S.* Enteritidis P125109 (Table 5)

Table 6 Insertion sequences predicted in *Salmonella* genomes

Isolate	*S*. Enteritidis P125109	*S*. Enteritidis RW 50	*S*. Enteritidis KHR 57
IS 3	+	+	+
IS 66	+	+	+
IS 630	+	+	+
IS 110	+	+	+
IS200/ 605	+	+	+
IS 91	_	+	+
IS 1	+	_	_
IS481	+	+	+
IS 256	+	+	+
Tn3	_	+	+
IS L3	_	_	+
IS 6	_	+	+
IS 4	+	+	+
IS Kra4	_	+	+
IS 5	+	+	+

4.9 Whole-Genome sequence comparison using BRIG

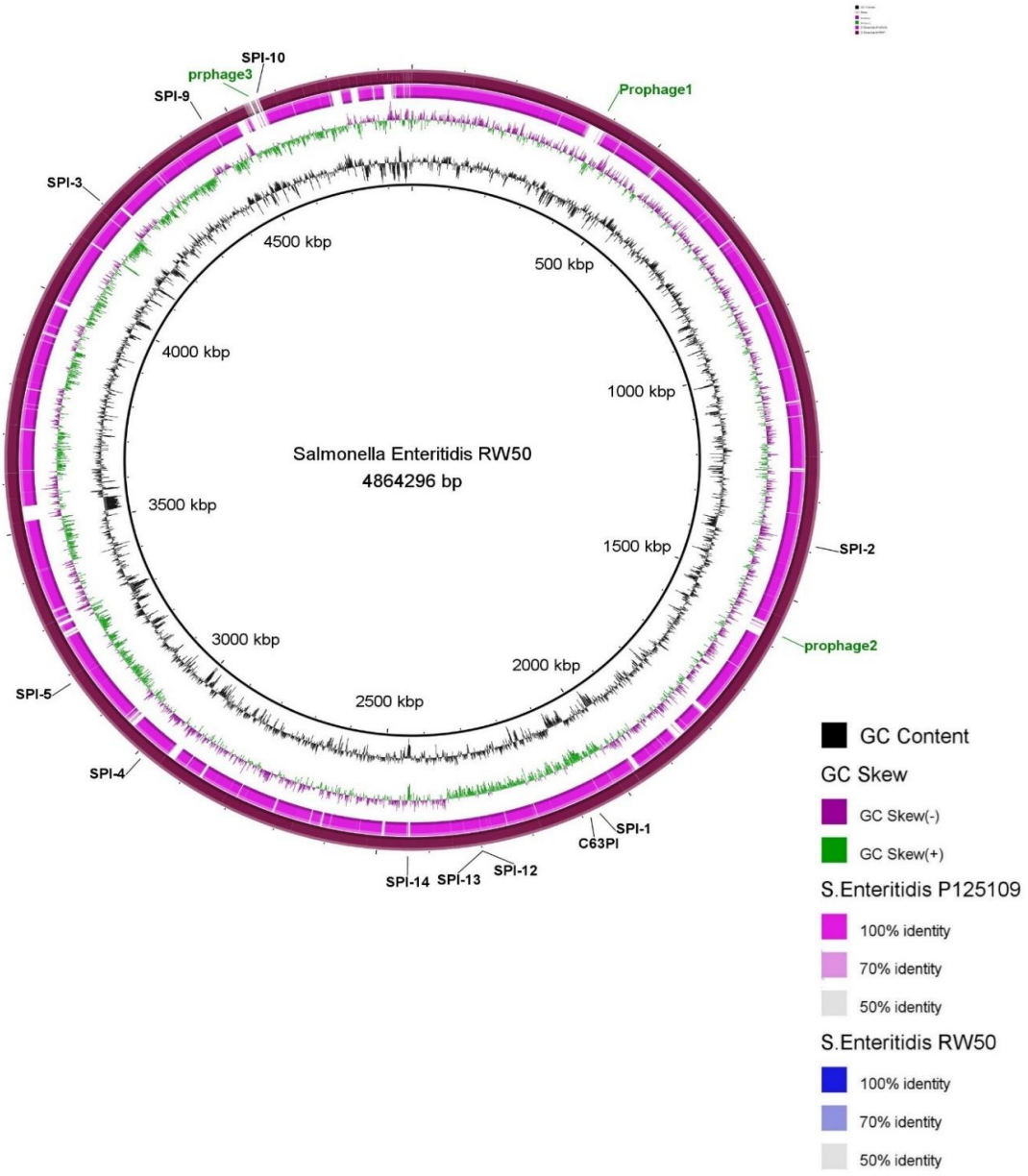


Figure 11 Circular comparison map of *Salmonella* Enteritidis RW50

Whole genome sequence comparison of S. Enteritidis RW50 is represented in figure. Each ring is corresponding to *Salmonella* Enteritidis genome shown in the legend. Salmonella pathogenicity islands and Prophage sequences of *S*. Enteritidis RW50 have been high lightened. The intra-species resemblance is illustrated by the strength of the color. Darker color displays greater similarity whereas dimmer colors represent lesser similarity of genomes.

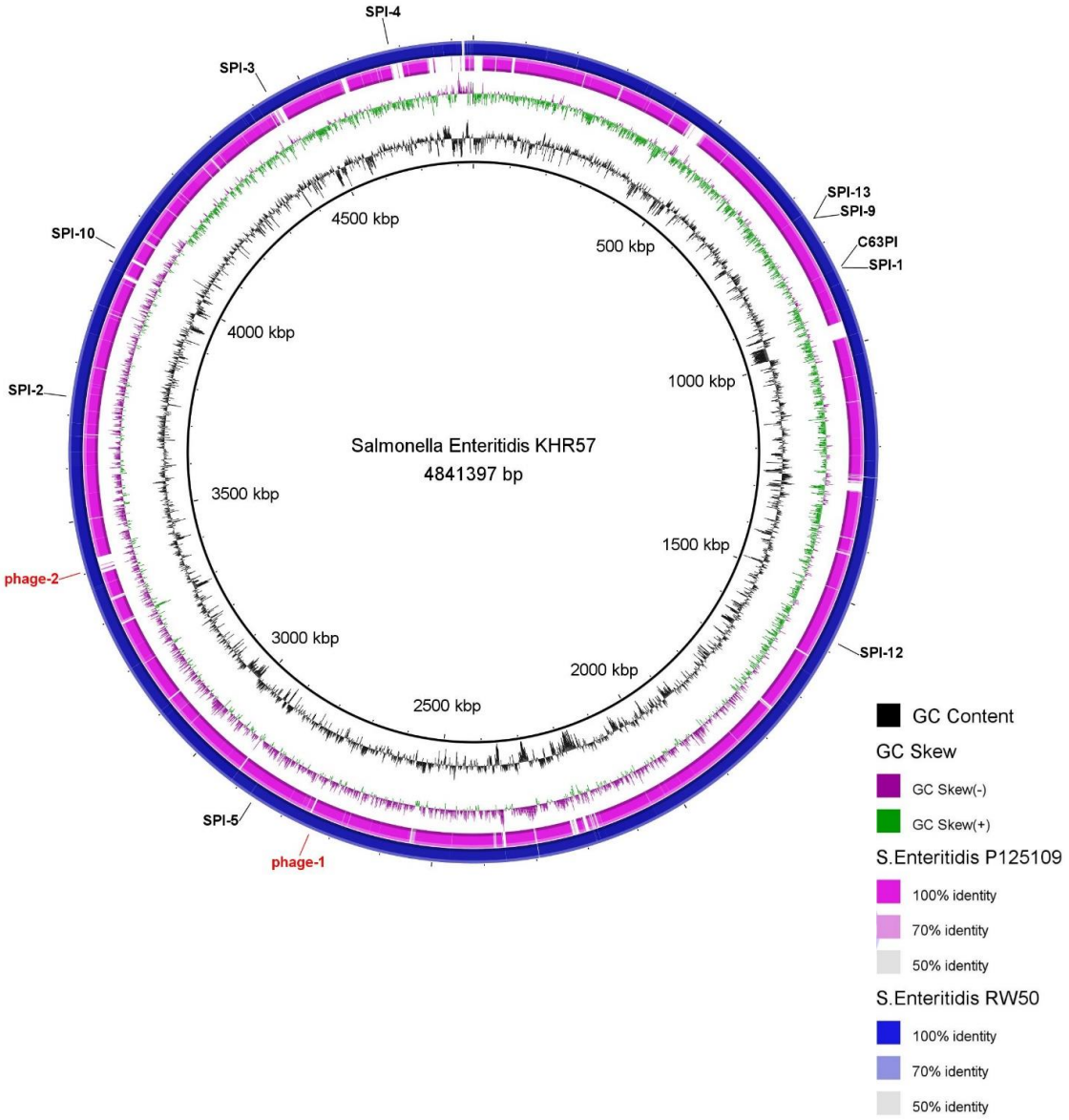


Figure 12 Circular comparison map of *Salmonella* Enteritidis KHR57

Whole genome sequence comparison of *S.* Enteritidis KHR57 is represented in figure. Each ring is corresponding to *Salmonella* Enteritidis genome shown in the legend. Salmonella pathogenicity islands and Prophage sequences of *S.* Enteritidis KHR57 have been high lightened. The intra-species resemblance is illustrated by the strength of the color. Darker color displays greater similarity whereas dimmer colors represent lesser similarity of genomes.

3.10 Identification and comparison of resistance genes

3.10.1 Resistance determinants of reference strain

Contrary to our strains reference strain had less resistance genes (n= 30). The pattern of highest frequency was same in all three strains howere the number of genes in reference strain for all the three classes was less. This indicates that our lab isolates might be more resistant. The highest frequency of resistance genes was observed for fluoroquinolones in reference strain with 14 genes, followed by cephalosporins (n=14) and third highest frequency of resistance genes was against tetracycline (n=12)

3.10.2 Resistance determinants of KHR57

Many antibiotic resistance genes were identified in genome. Resistance genes against flouroquinolone (n=17) antibiotic class were maximum in number (*QnrS1, sdiA, Salmonella enterica gyrA* conferring resistance to fluoroquinolones, *MdtK, CRP, rsmA, Escherichia coli soxR* with mutation conferring antibiotic resistance ,*emrB, Escherichia coli soxS* with mutation conferring antibiotic resistance, *marA, acrB, Escherichia coli marR* mutant conferring antibiotic resistance, *Escherichia coli acrA, emrA, rsmA, adeF and emrR*). This was followed by tetracycline resistance (n=16) and Cephalosporin resistance(n=16).

Tetracyline resisnace determinants were found to be *adeF, acrB, Escherichia coli acrA, Escherichia coli mdfA, sdiA, H-NS, marA, Klebsiella pneumoniae KpnF, Klebsiella pneumoniae KpnE,Tet(A),Escherichia coli marR* mutant conferring antibiotic resistance, *Escherichia coli soxS* with mutation conferring antibiotic resistance, *Escherichia coli soxR* with mutation conferring antibiotic resistance.

Cephalosporin resistance genes were *golS, OXA-10, mdsA, Escherichia coli ampH, acrB , Escherichia coli acrA, sdiA, H-NS, marA Klebsiella pneumoniae KpnF,*

Klebsiella pneumoniae KpnE, Escherichia coli ampC1 beta-lactamase, Haemophilus influenzae PBP3 conferring resistance to beta-lactam antibiotics, *Escherichia coli marR* mutant conferring antibiotic resistance, *Escherichia coli soxS* with mutation conferring antibiotic resistance, *Escherichia coli soxR* with mutation conferring antibiotic resistance.

Overall, 7 hits were found to cause antibiotic target inactivation ,11 hits were found against antibiotic target alteration,2 hits for reduced permeability to antibiotics ,26 hits were found against antibiotic efflux pump. For antibiotic target protection 1 hit was observed in RGI CARD. The resistance criteria along with percent identity is given in table 6

Table 7 Antibiotic Resistance mechanisms observed in *Salmonella* Enteritidis KHR57

Antibiotic Resistace mechanism	**criteria**	**percent identity**
Reduced permeability to antibiotic		
marA	Strict	95.24
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Antibiotic target alteration		
dfrA14	perfect	100
pmrF	Strict	87.74
bacA	Strict	97.07
Haemophilus influenzae PBP3 conferring resistance to beta-lactam antibiotics	Strict	51.85
Salmonella enterica gyrA conferring resistance to fluoroquinolones	Strict	99.89
Escherichia coli GlpT with mutation conferring resistance to fosfomycin	Strict	96.9
Escherichia coli UhpT with mutation conferring resistance to fosfomycin	Strict	95.68
Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin	Strict	97.79
Escherichia coli marR mutant conferring antibiotic resistance	Strict	92.36
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Escherichia coli soxR with mutation conferring antibiotic resistance	Strict	96.05

Antibiotic inactivation		
FosA7	Strict	97.14
Escherichia coli ampH	Strict	91.43
AAC(6')-Iy	Strict	99.31
Escherichia coli ampC1 beta-lactamase	Strict	72.69
ANT(3'')-IIa	Perfect	100
APH(3')-IIa	Perfect	100
OXA-10	Perfect	100
arr-2	Strict	72.69
Antibiotic target replacement		
dfrA14	Perfect	
Escherichia coli UhpT with mutation conferring resistance to fosfomycin	Strict	95.68
Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin	Strict	97.79
Escherichia coli marR mutant conferring antibiotic resistance	Strict	92.36
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Escherichia coli soxR with mutation conferring antibiotic resistance	Strict	96.05
pmrF	556.6	
bacA	Strict	97.07
Haemophilus influenzae PBP3 conferring resistance to beta-lactam antibiotics	Strict	51.85
Salmonella enterica gyrA conferring resistance to fluoroquinolones	Strict	99.89
Escherichia coli GlpT with mutation conferring resistance to fosfomycin		
Antibiotic efflux		
golS	Perfect	100
cmlA5	Perfect	100
emrA	Strict	90
emrB	Strict	95.7
rsmA	Strict	85.25
CRP	Strict	99.05
tet(A)	Strict	99.74
tet(A)	Strict	99.75
Escherichia coli marR mutant conferring antibiotic resistance	Strict	92.36
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Escherichia coli soxR with mutation conferring antibiotic resistance	Strict	96.05
adeF	Strict	60.57
mdsA	Strict	99.51

acrB	Strict	94.66
Escherichia coli acrA	Strict	91.69
kdpE	Strict	92.41
Escherichia coli mdfA	Strict	87.93
msbA	Strict	96.39
sdiA	Strict	99.58
H-NS	Strict	94.89
marA	Strict	95.24
Klebsiella pneumoniae KpnF	Strict	87.16
Klebsiella pneumoniae KpnE	Strict	77.5
mdtK	Strict	99.58
baeR	Strict	96.67
emrR	Strict	93.14
Antibiotic target protection		
QnrS1	Perfect	100

3.10.2 Resistance determinants of RW50

Overall, 36 resistance genes were found in RW50. Highest frequency of gene diversity was observed against flouroquinolones class, and overall 17 genes showed resistance against this class including *QnrS1, sdiA, Salmonella enterica gyrA* conferring resistance to fluoroquinolones, *MdtK, CRP, rsmA, Escherichia coli soxR* with mutation conferring antibiotic resistance ,*emrB, Escherichia coli soxS* with mutation conferring antibiotic resistance, *marA, acrB, Escherichia coli marR* mutant conferring antibiotic resistance, *Escherichia coli acrA, emrA, rsmA, adeF* and *emrR.* Second highest frequency of resistance genes was observed for Cephalosporins (n=15) and its resistance determinants were *golS, mdsA, Escherichia coli ampH, acrB* , *Escherichia coli acrA, sdiA, H-NS, marA Klebsiella pneumoniae KpnF, Klebsiella pneumoniae KpnE, Escherichia coli ampC1 beta-lactamase,Haemophilus influenzae PBP3* conferring resistance to beta-lactam antibiotics, *Escherichia coli marR* mutant conferring antibiotic resistance, *Escherichia coli soxS* with mutation conferring

antibiotic resistance, *Escherichia coli soxR* with mutation conferring antibiotic resistance

This was followed by Tetracycline drug class, which comprised 14 genes resistant against this class including *adeF, acrB*, Escherichia coli *acrA*, Escherichia coli *mdfA, sdiA, H-NS, marA, Klebsiella pneumoniae KpnF, Klebsiella pneumoniae KpnE, Tet(A),Escherichia coli marR* mutant conferring antibiotic resistance, *Escherichia coli soxS* with mutation conferring antibiotic resistance, *Escherichia coli soxR* with mutation conferring antibiotic resistance.

2 hits were found for reduced permeability to antibiotic,10 for antibiotic targ*et al*teration,5 for antibiotic inactivation, 25 for antibiotic efflux and 1 for antibiotic target protection. Antibiotic resistance mechanisms observed in *S.* Enteritidis RW50 are given in table 7 listed below.

Table 8 Antibiotic Resistance mechanisms observed in *Salmonella* Enteritidis RW50

Resistace mechanism	**criteria**	**percent identity**
Reduced permeability to antibiotic		
marA	Strict	95.24
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Antibiotic targ*et al*teration		
pmrF	Strict	87.74
bacA	Strict	97.07
Haemophilus influenzae PBP3 conferring resistance to beta-lactam antibiotics	Strict	51.85
Salmonella enterica *gyrA* conferring resistance to fluoroquinolones	Strict	99.89
Escherichia coli GlpT with mutation conferring resistance to fosfomycin	Strict	96.9
Escherichia coli UhpT with mutation conferring resistance to fosfomycin	Strict	95.68
Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin	Strict	97.79

Escherichia coli marR mutant conferring antibiotic resistance	Strict	92.36
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Escherichia coli soxR with mutation conferring antibiotic resistance	Strict	96.05
Antibiotic inactivation		
FosA7	Strict	97.14
Escherichia coli ampH	Strict	91.43
AAC(6')-Iy	Strict	99.31
Escherichia coli ampC1 beta-lactamase	Strict	72.69
APH(3')-IIa	Perfect	100
Antibiotic efflux		
golS	Perfect	100
emrA	Strict	90
emrB	Strict	95.7
rsmA	Strict	85.25
CRP	Strict	99.05
tet(A)	Strict	99.75
Escherichia coli marR mutant conferring antibiotic resistance	Strict	92.36
Escherichia coli soxS with mutation conferring antibiotic resistance	Strict	95.33
Escherichia coli soxR with mutation conferring antibiotic resistance	Strict	96.05
adeF	Strict	60.57
mdsA	Strict	99.51
acrB	Strict	94.66
Escherichia coli acrA	Strict	91.69
kdpE	Strict	92.41
Escherichia coli mdfA	Strict	87.93
msbA	Strict	96.39
sdiA	Strict	99.58
H-NS	Strict	94.89
marA	Strict	95.24
Klebsiella pneumoniae KpnF	Strict	87.16
Klebsiella pneumoniae KpnE	Strict	77.5
mdtK	Strict	99.58
baeR	Strict	96.67
emrR	Strict	93.14
Antibiotic target protection		
QnrS1	Perfect	100

3.10.3 Comparative analysis of WGS based genotypic antibiotic resistance

A comparative analysis of genotypic antimicrobial resistance determinants predicted by Resfinder and RGI CARD in *S.* Enteritidis RW50 and *S.* Enteritidis KHR57 with refence strain was performed. The results of analysis are depicted in table 7.

Table 9 Comparative analysis of genotypic antimicrobial resistance in *Salmonella* isolates

Drug class			**Resistance determinants**	***S.* Enteritidis RW50**	***S.* Enteritidis KHR57**	***S.* Enteritidis P125109**
Beta Lactams		Penicillin	*mdsA, marA, blaOXA-10, golS*	R	R	R
		Cephalosporins	*golS, OXA-10,marA*	R	R	R
		Carbapenem	*mdsA, marA,golS*	R	R	R
No Lactam		Glycopeptides		S	S	S
Protein Synthesis	30S	Aminoglycosides	*APH (3') – lla, aadA1, AAC (6')-Iy*	R	R	R
		Tetracyclines	*Tet(A*)	R	R	R
	50S	Chloramphenicol	*CmlA1, floR, CmlA5*	R	R	R
		Macrolides	*H-NS, KpnF, KpnE, CRP*	R	R	R
		Lincosamides		S	S	S
		Oxazolidonones		S	S	S
		Fusidane		S	S	S
DNA topoisomerase		Fluoroquinolones	*QnrS1, gyrA, gyrB*	R	R	R
Folate pathway antagonist		Trimethoprim	*Dfra14*	S	R	S
RNA polymerase inhibitor		rifampin	*Arr3, Arr-2*	R	R	R

3.10.4 Phenotypic antibiotic resistance

Phenotypic antibiotic resistance date was provided by Microbiology laboratory NUST Islamabad is given in table below. R means resistance against antibiotic and S shows susceptibility for given antibiotic.

Table 10 Phenotypic antimicrobial resistance in *Salmonella* isolates

Drug class		**Antibiotics**	***S.* Enteritidis RW50**	***S.* Enteritidis KHR57**
Beta Lactams	Penicillin	Ampicillin	R	S
		Amoxicillin	R	S
	Cephalosporin	Cefepime	R	S
	Carbapenem	Meropenem	S	S
		Imipenem	S	S
No lactam	Glycopeptides	Vancomycin	R	R
30S inhibitor	Amino-glycosides	Gentamycin	R	R
		Streptomycin	R	R
	Tetracyclines	Tetracycline	R	R
		Minocycline	R	R
50 S inhibitor	Chloram-phenicol	Chloram-phenicol	R	S
	Macrolides	Erythromycin	R	R
	Lincosamide	Clindamycin	R	R
	Oxaz-olidonones	Linezolid	R	R
	Fusidane	Fusidic acid	R	R
DNA topoisomerase inhibitor	Flour-quinolones	Nalidixic Acid	R	R
Folate pathway antagonist	Sulfonamide	Sulfameth-oxazole	R	R
RNA polymerase inhibitor	Rifampim	Rifampicin	R	R

3.10.5 Comparison of phenotypic and genotypic resistance

Comparison of phenotypic and genotypic resistance was performed to evaluate the phenotypic resistance data. Results are given in table below and categorized as

- False negative (FN) results were obtained when WGS prediction did not detect an antibiotic resistance determinant but isolate was phenotypically resistant
- False positive (FP) results were obtained when WGS resistance testing predicted resistance genes but the strain was found to be phenotypically sensitive.
- True positive (TP) results were for antibiotics where genotypic resistance analysis predicted resistance gene and strain displayed resistant phenotype

Table 11 Comparative analysis of phenotypic and genotypic antibiotic resistance

Antibiotics	***S*. Enteritidis RW50**		***S*. Enteritidis KHR57**	
	Description	**Result**	**Description**	**Result**
Penicillin		TP	*mdsA*[1],*bla-Oxa10*[2]	FP
Cephalosporins		TP	*golS*[3], *OXA-10*,*marA*[4]	FP
Carbapenem	*golS*	FP	*marA, mdsA*	FP
Glycopeptides	No resistance determinants	FN	No resistance determinants	FN
Aminoglycosides		TP		TP
Tetracyclines		TP		TP
Chloramphenicol		TP	*floR*[5], *cmlA1*[6]	FP
Macrolides		TP		TP
Lincosamides	No resistance determinant	FN	No resistance determinant	FN
Oxazolidonones	No resistance determinant	FN	No resistance determinant	FN
Fusidane	No resistance determinant	FN	No resistance determinant	FN
Fluoroquinolones		TP		TP
Sulfonamides/ Trimethoprim	No resistance determinant	FN		TP
Rifampin		TP		TP

1.MdsA is the membrane fusion protein of the multidrug and metal efflux complex *MdsABC* 2. *Oxa beta lactamases*.3 *GolS* is a regulator activated by the presence of *golD*, and promotes the expression of the *MdsABC* efflux pump. 4 Multiple antibiotic resistance protein.5 Florfenicol Resistance Gene 6.chloramphenicol resistance causing gene.

CHAPTER 5

DISCUSSIONS

Salmonella is second most cause of foodborne gastrointestinal infections in humans following *Campylobacter*. It is one of the primary zoonotic pathogens causing foodborne disease associated with poultry products consumed by humans. New scientific research conducted on *Salmonella* reveled that both typhoidal and non-typhoidal *Salmonella* are becoming resistant to several classes of antibiotics. (Park *et al*., 2017; Cosby *et al*., 2015).

WGS has facilitated in rapid and comprehensive investigation of antimicrobial resistance in *Salmonella*. *Salmonella* enterica serovar Enteritidis has been found self-limiting in contrary to *S*. Typhimurium but in some cases the situation may worsen, and antibiotics must be administered specially when infection disseminates into blood (Deng *et al*., 2015) With the improvement in sequencing methods now whole genome sequencing of bacterial species can be easily performed which provides a very detailed information of genotype (Oakeson *et al*., 2017).

Results of this study showed that, in both isolates most common resistance determinants were detected against fluoroquinolone antibiotics followed by tetracyclines and cephalosporins. These results were in accordance with the resistance pattern of reference strain. Globally a very abrupt increase in incidence of resistance to fluoroquinolone antibiotics has been observed and this is one of the reasons that WHO has added Fluoroquinolone resistant *Salmonella* in the WHO Priority Pathogens List of antibiotic-resistant bacteria. This pathogen will be given high priority in developing new antibiotics (WHO, 2017).

Third generation antibiotics which had been showing promising effects against *S.* Enteritidis infection are now a days becoming less effective mainly due to misuse of antibiotics. In related studies several cases of salmonellosis have shown resistance to ampicillins along with fluoroquinolones. Surveillance reports from South Asian countries have shown that Ciprofloxacin resistance is common in the region. Fluoroquinolone enrofloxacin that had been allowed for poultry use,4 decades ago, resulted in higher number of reduced susceptibilities to ciprofloxacin in poultry recovered *S.* Typhimurium DT104 (Frost *et al*.,2019).

Cefepime (fourth generation cephalosporin) resistant *S.* Enteritidis has been reported in China. Though first case was observed in 2010 in diarrheal patient, but recently animal derived sources have also been confirmed which makes the situation even more threatening (Fu. *et al*, 2020). The study of resistance determinants for extended spectrum cephalosporins revealed diverse results. As per EFSA extended spectrum cephalosporin resistance is linked with presence of extended spectrum beta lactamase and plasmid mediated *ampC* genes or at times co-existence of both of them (EFSA, 2019). Extended spectrum cephalosporin resistant *Salmonella* has been reported from poultry globally (Wei *et al*., 2021).

bla_{OXA-10} were detected in *S.* Enteritidis KHR57 and absent in *S.* Enteritidis RW50 and *S.* Enteritidis P125109. Previous studies have reported variation among beta lactamase enzymes from different geographical regions. In China the bla_{OXA} genes were found to be less prevalent in poultry isolated *Salmonella* strains as compared to bla_{CTXM} and $bla_{\mathrm{TEM..}}$ Oxa type carbapanemases are known to cause resistance against penicillins and usually do not alter monobactam antibiotics and cephalosporins however they have been reported to have hydrolytic activity against carbapenems(Tang *et al*., 2014)

S. Enteritidis KHR57 strain had *ompA* virulence factors which was absent in other two strains. *ompA* has been associated with integrity of plasma membrane and thus resistance to chloramphenicol and nalidixic acid antibiotics although exact mechanism of resistance is yet unknown (Samani *et al*.,2014; Sabry *et al*.,2020). *ampC* and *ampH* beta lactamases were present in all three strains. *AmpC* cephalosporins are known to confer resistance against oxymino-cephalosporins and fourth generation cephalosporins.

Comparative analysis of phenotypic antimicrobial resistance with genotypic resistance profile found disagreements in both isolates. *S.* Enteritidis RW50 was predicted to be genotypically resistant against chloramphenicol antibiotics but found susceptible phenotypically. *S.* Enteritidis KHR57 showed phenotypic susceptibility against three antibiotic classes despite of prediction of AMR resistant determinants genotypically, indicating the possibility that AMR determinants showed either no expression or poor expression. Study also identified genotypic susceptibility but phenotypic resistance in both isolates. (Katiyar *et al*., 2020).

This is not the first time that disagreements in phenotypic and genotypic antimicrobial resistance is observed. Our results are in concordance with a a study conducted in Ibague, Colombia in 2017 and according to that study the difference between genotypic and phenotypic resistance analysis for a few antibiotics might be due to the fact that resistance genes found for few antibiotics are not confirmed by phenotypic testing due to limited resources (Vélez *et al*., 2017). Moreover, our present comparison of WGS based resistance with phenotypic antibiotic susceptibility is similar to a study conducted in England 76 isolates showed discrepant phenotypic and genotypic resistance data of NTS strains isolated from 2014 to 2015 (Neuert *et al*., 2018).

Mismatches of phenotypic antibiotic susceptibility and WGS prediction of antibiotic resistance were observed for 88 isolates in a related study (Zankari *et al*., 2013) .

In a nutshell genotypic method of determining antibiotic resistance provides deep insight not only into the mechanisms of resistance but also the possible routes of horizontal transmission between different strains (Katiyar *et al*., 2020). Resistance determinants detected by genotypic characterization are often very high, but their clinical relevance has to be verified. This is why phenotypic methods of antibiotic resistance need to be employed instead of relying on merely WGS (Lepuschitz *et al*., 2019) .Whereas phenotypic methods of antibiotic resistance have a few limitations because not all the drugs are routinely tested in laboratories due to their unavailability and often the mechanism of resistance of all drugs are not known (Katiyar *et al*., 2020).

We detected 51 Insertion sequences from 14 different families in *S.* Enteritidis RW5. IS 3, IS 66, IS 630, IS 110, IS200/605, IS 91, IS481, IS 256, Tn3, IS 6, IS 4, IS Kra4 and IS 5. 69 different insertion sequences from 15 families including IS 3, IS 66, IS 630, IS 110, IS200/605, IS 91, IS481, IS 256, Tn3, IS 6,IS l3, IS 4, IS Kra4 and IS 5 were detected. This number is higher than Insertion sequences reported by Jones-Dias *et al* in 2017; who reported 33 insertion sequences in *Salmonella* Enteritidis strain from 9 families (Jones-Dias *et al*., 2017).

Genome sizes of Phages detected in our isolates ranged from 30.5 Kb to 54.5Kb and their GC values ranged from 47.8% to 53.6%. These results concord with phages reported by Fong *et al* in *S. enterica* (Fong *et al*., 2019). To end discussions, all these elements (insertion sequences, genomic islands, prophages and virulence factors) contribute to pathogenicity and survival ability in diverse environmental conditions of *S.* Enteritidis RW50 and *S.* Enteritidis KHR57 and highlight the possibility of

horizontal acquisition of these traits and hence ultimately impacting on diversification of microbial genomes and their genetic evolution.

CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTS

The present study demonstrates genomic characterization carried out on non-typhoidal *S. enterica* strains previously isolated from poultry in Pakistan. This study showed that *S.* Enteritidis RW50 and *S.* Enteritidis KHR57, have variety of pathogenicity and virulence factors, insertion sequences, prophages and mobile genetic elements that influence the antibiotic resistance potential of these isolates. Both isolates were resistant against many classes of antibiotics and maximum number of resistance genes were found for fluoroquinolone antibiotics which is drug of choice for poultry and of great public health concern due to its genes transferring capability to enteric pathogens. The results of this study imply that poultry is a potential source of multi-drug resistant *S.* Enteritidis in Pakistan. In this study false positive results observed for beta lactams and carbapenems, which highlights the importance of WGS to combat antimicrobial resistance. For future resistance studies in Pakistan WGS based resistance studies along with phenotypic resistance assays are recommended. Both strains harbored *Sips* and *Sops* effector proteins that are involved in dissemination of salmonellosis in poultry birds and its zoonotic transmission to humans and other animals. For future studies and publications *S.* Enteritidis RW50 is renamed as FMBL26 and *S.* Enteritidis KHR57 is renamed as FMBL13. More comprehensive and detailed investigations are recommended to understand the dynamics of antimicrobial resistance in local *Salmonella* strains and device mitigation strategies to lower its impact on poultry and human healt

Appendix A: supplemental table and figures

Table A1 Insertion sequences predicted in *Salmonella* Enteritidis RW50

IS3	ISKra4	Tn3	IS605	IS200	IS91	IS4
S. enterica	*Klebsiella pneumoniae*	*Aeromonas salmonicida*	*Klebsiella pneumoniae*	*Yersinia enterocolitica*	*E. coli*	*Aeromonas media*
Aliivibrio salmonicida	*Xenorhabdus nematophila*	*Pseudomonas Aeruginosa*	*S. enterica*		*Shigella boydii*	*E. coli*
P. aeruginosa	*Escherichia sp.*	*Escherichia coli*	*E.coli*			
Aeromonas salmonicida		*Shewanella sp.*				
Citrobacter freundii						
E. coli		*S. enterica*				
Shigella dysenteriae		*Aeromonas salmonicida*				
Pectobacterium atrosepticum		*Shewanella frigidimarina*				
E. fergusonii		*Shewanella oneidensis*				
E. albertii		*Acinetobacter sp.*				

IS110	IS6	IS630	IS5	IS 256	IS 66	IS 481
K. pneumoniae	*Salmonella panama*	*Shigella sonnei*	*Enterobacter cloacae*	*K. pneumoniae*	*Citrobacter rodentium*	*Erwinia sp.*
S.enteritidis	*Proteus vulgaris*	*Shewanella putrefaciens*	*Primary endo-symbiont*	*Edwardsiella ictaluri*		
	Salmonella Typhimurium	*E. coli*		*Acinetobacter baumannii*		
	Acinetobacter calcoaceticus	*Citrobacter freundii*		*Glaesserella parasuis*		
		Yersinia pestis		*Paracoccus yeei*		
		Photorhabdus luminescens		*Aeromonas salmonicida*		

Table A2 Insertion sequences predicted in *Salmonella* Enteritidis KHR57

IS110	IS256	IS200/IS605	ISKra4	Tn3	IS6	IS3
S. Enteritidis	*Shewanella sp.*	*S. Enterica*	*Klebsiella pneumoniae*	*Aeromonas Salmonicida*	*Salmonella panama*	*Salmonella enterica*
Klebsiella pneumoniae	*Yersinia pestis*	*E. coli*	*Escherichia sp.*	*Erwinia amylovora*	*Salmonella typhimurium*	*Shewanella sp.*
	Klebsiella pneumoniae	*Klebsiella pneumoniae*	*Xenorhabdus nematophila*	*P. aeruginosa*	*Proteus vulgaris*	*Aliivibrio salmonicida*
	Acinetobacter baumannii	*Aeromonas salmonicida*	*unculture d bacterium*	*Shewa-nella sp.*	*Oligella urethralis*	*Enterobacter cloacae*
	Paracoccus yeei	*Yersinia enterocolitica*		*S. enterica*	*Acineto-bacter sp.*	*Dickeya dadantii*
	Primary endosymbiont	*Dickeya dadantii*		*Azospirillum sp.*	*Oligella urethralis*	*Klebsiella pneumoniae*
	Escherichia coli	*Shewanella frigidimarin a*		*Shewanella frigidimarina*	*Acidiphiliu m cryptum*	*Escherichia sp.*
	Glaesserell a parasuis			*Arthrobacter sp.*	*Proteus vulgaris*	*Pluralibacter gergoviae*
				Acinetobacter species	*Stappia aggregata*	*P. aeruginosa*
						Shigella flexneri
						Agrobacteriu m tumefaciens

IS481	IS4	ISL3	IS5	IS630	IS91	*Enterobactr cloacae*
Erwini a sp.	*Aeromonas media*	*E. coli*	*Primary endosymbiont*	*Shigella sonnei*	*E.coli*	*Pectobacterium atrosepticum*
	E. coli		*Sodalis glossinidius*	*E. coli*	*Vibrio Prosthecochloris*	*salmonicida aestuarii*
				Citrobacte r freundii		*Pseudomonas syringae*
				Yersinia pestis		*Yersinia pestis*
				Photorhabdus luminescens		*Aeromonas salmonicida*

Table B1 List of all phages predicted in *Salmonella* Enteritidis RW50

Phage	Length	Complete-ness	Score	Protein	Position	Most common phage	GC %
1	38.6Kb	intact	120	52	362485-401142	*Salmonella* phage g341c_NC_013059	47.8
2	11.6Kb	incomplete	40	11	1095214-1106895	*Salmonella* phage SEN1_NC_029003	50.6
3	38.2Kb	intact	150	49	1554471-1592739	*Escherichia* phage 186_NC_001317	52.1
4	30.3Kb	incomplete	60	17	1767798-1798117	*Enterobacteria* phage P4_NC_001609	50.9
5	20.4Kb	incomplete	50	29	3039631-3060052	*Burkholderia cenocepacia* phage_NC_005882	50.7
6	25.3Kb	incomplete	50	10	3880812-3906206	*Cronobacter* phage_vB_CsaM_GAP32_NC_019401	53.1
7	39.7Kb	Question-able	70	18	4424887-4464595	*Escherichia* phage_500465_1_NC_049342	53.6
8	30.5Kb	intact	150	41	4533409-4563966	*Escherichia* phage _pro483_NC_028943	52.7

Table B2 List of phages predicted in *Salmonella* Enteritidis KHR57

Phage	Kb	Complete-ness	Score	Protein	Region Position	Most common phage	GC (%)
1	38.2	intact	150	47	3385481-3423749	*Salmonella* phage SW9_ NC_049459	52.1
2	38.6	intact	120	53	453311-491968	*Salmonlla* phage c341_ NC_013059	47.8
3	54.5	questionable	70	18	2872408-29269288	*Escherichia* phage_ 500465_1 _NC_049342	53
4	38.4	questionable	80	29	4738287-4776784	*Escherichia* phage _P4 _NC_001609	52.1
5	22.1	incomplete	50	10	1103508-1125700	*Cronobacter* phage _vB_Cs aM_GAP32_N C_019401	53.6
6	20.4	incomplete	50	29	4425416-4445837	*Burkholderia cenocepacia* _ BcepMu_NC_ 005882	50.7
7	11.6	incomplete	40	11	1398855-1410536	*Salmonella* phage_SEN34 _NC_028699	50.6

Figure A1 Putative mobile elements in *Salmonella* Enteritidis RW50

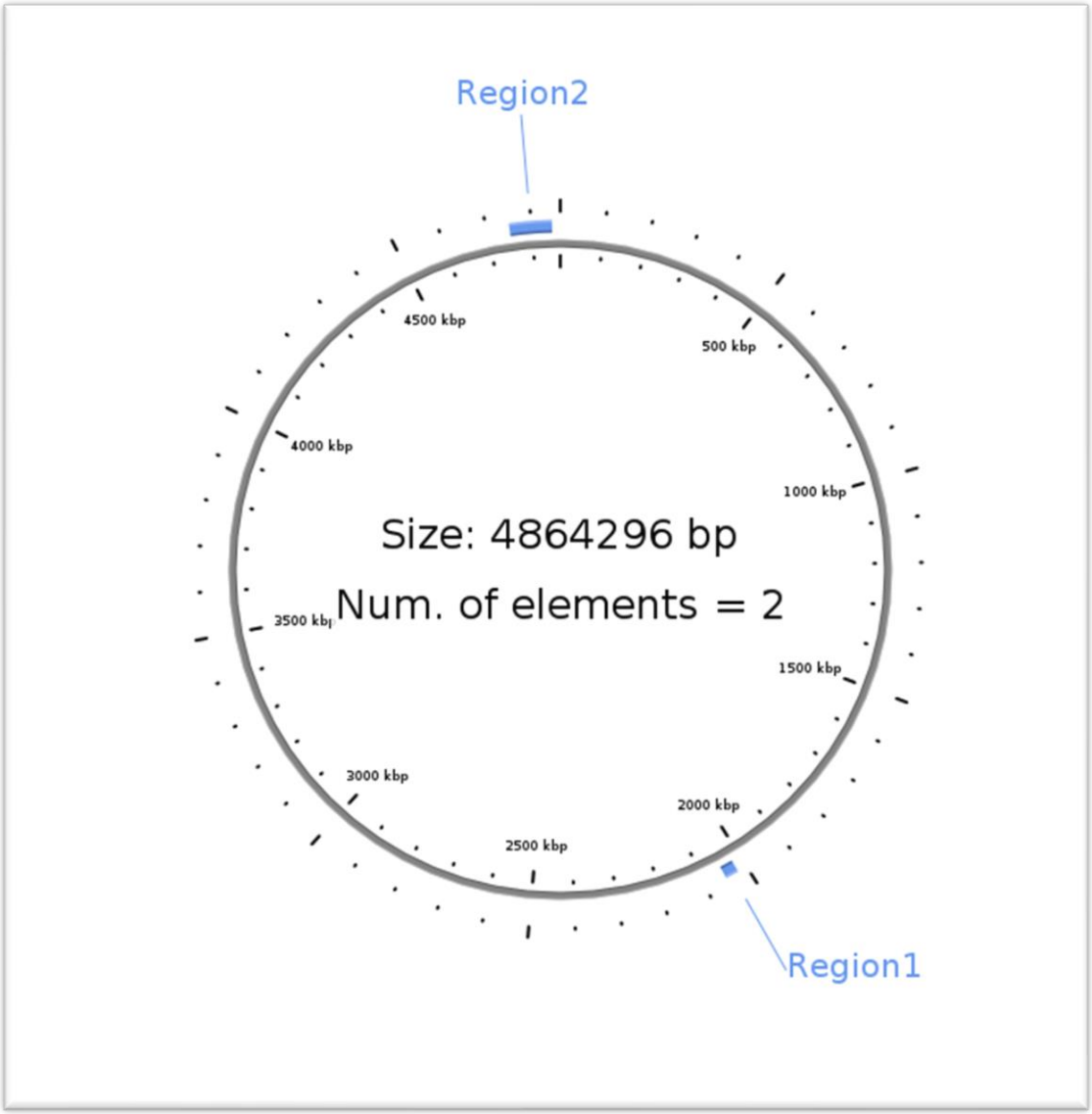
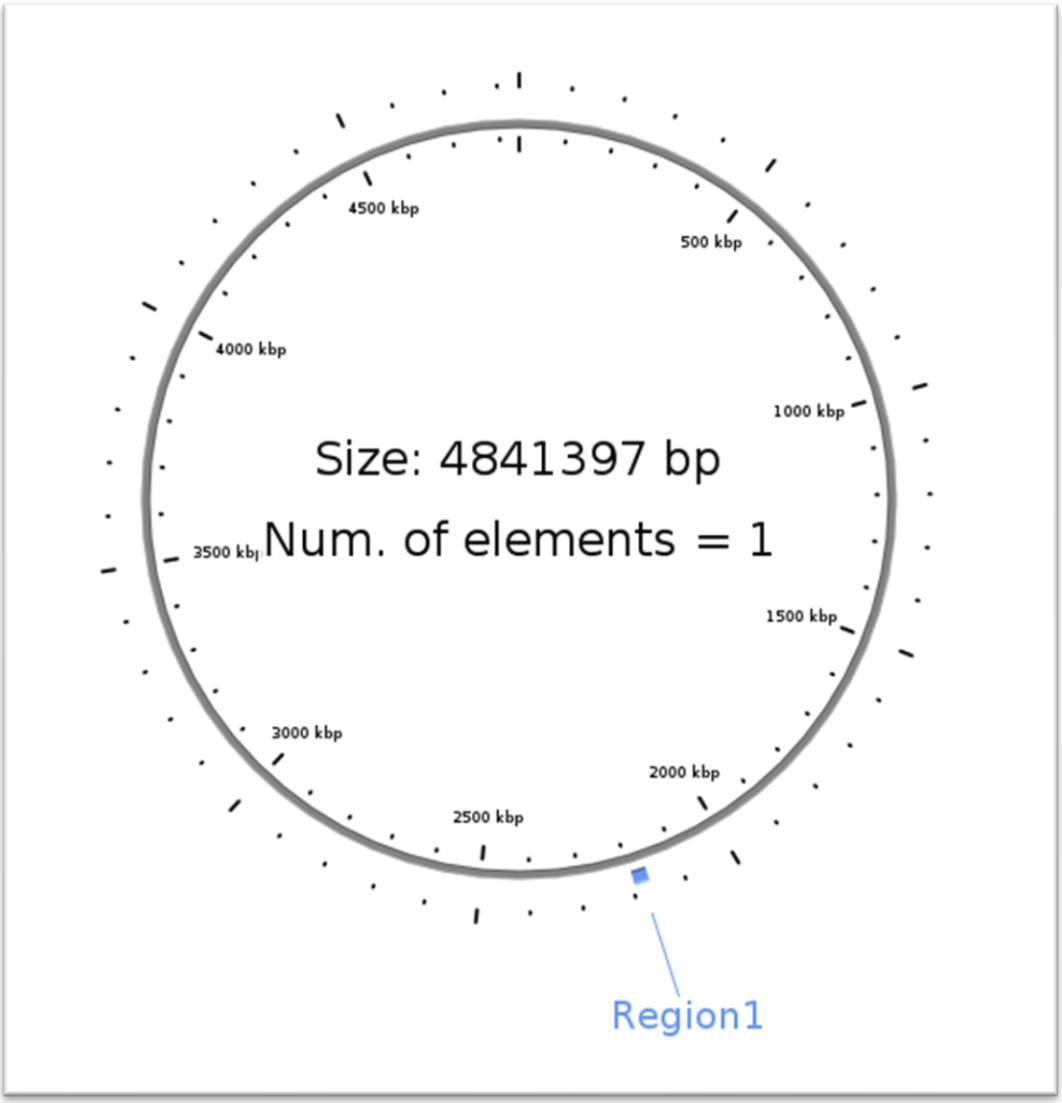


Figure A2 Putative mobile elements in *Salmonella* Enteritidis KHR57



CHAPTER 7

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Genomic analysis of multidrug resistant non-typhoidal *Salmonella enterica* strains isolated from broiler chicken in Pakistan

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