PHENOTYPIC AND GENOTYPIC ANALYSIS OF A NOVEL 4TH GENERATION CEPHALOSPORIN RESISTANT *SALMONELLA ENTERICA* SER. TYPHI CLINICAL ISOLATE

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Annex A to NUST Letter No.

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Declaration

I certify that this research work titled "Phenotypic and Genotypic Analysis of a Novel 4th Generation Cephalosporin Resistant *Salmonella enterica* ser. Typhi Clinical isolate" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

_____

Danish Gul

Dedicated to

Maa, Papa & Tabish

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To begin with, I would not be writing down these words at 7:20 PM, August 14, 2017 if it were not for God and His countless blessings. I might have stumbled over repeatedly but I know, He has been watching over me all this time. Thank You God for I know You are always by my side.

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List of Acronyms

Acronym	Generic Name
µg/mL	**Micrograms per Milliliter**
µg/L	**Micrograms per Liter**
w/v	**Weight per unit volume**
ESBL	**Extended Spectrum Beta Lactamase**
WGS	**Whole Genome Sequencing**
rRNA	**Ribosomal RNA**
tRNA	**Transfer RNA**
mRNA	**Messenger RNA**
Mb	**Mega bases**
Kb	**Kilo bases**
Bp	**Base pairs**
CDS	**Coding Sequences**
PCR	**Polymerase Chain Reaction**
bla	**Beta-lactamase**
S83F	**Serine substituted by Phenylalanine at position 83**
***gyr*A**	**Gyrase A**
qnrS	**Quinolone Resistance Protein S**
SDS	**Sodium Dodecyl Sulfate**
EtBr	**Ethidium Bromide**

ABSTRACT

A case of Typhoid fever was reported at a tertiary care hospital in Rawalpindi that was caused by an extended spectrum β-lactamase (ESBL) producing *Salmonella enterica* serovar Typhi (*S. typhi*) exhibiting resistance to ceftriaxone (3rd generation cephalosporin). Phenotypic analysis by antimicrobial susceptibility assay using KB Disk Diffusion and MIC assay by Broth Microdilution revealed the isolate to be exhibiting resistance to all commonly used antibiotics for treatment of Typhoid fever. The isolate showed susceptibility to Carbapenem class of antibiotics and was resistant to the rest of clinically prescribed antibiotics including Cefepime (4th generation Cephalosporin) and several fluroquinolone antibiotics suggesting the isolate to be classified as Extensively Drug Resistant (XDR). Genotypic analysis was done by identification of β-lactamases by PCR revealing the presence of $bla_{\text{CTX-M}}$. Alkaline Lysis and Plasmid Curing Assay confirmed the isolate to be lacking plasmids. Whole Genome Sequencing and downstream analysis was employed for further genotypic analyses. Whole Genome Annotation revealed the genome to be ~4.8Mb having 4,975 CDSs, 69 tRNA genes and 4 rRNA genes. Further downstream analysis revealed the presence of a putative novel resistance island harboring numerous resistance genes (incl. $bla_{\text{CTX-M}}$, bla_{TEM}, *qnrS*1), insertion elements and mobile element proteins without presence of an independent plasmid along with S83F *gyr*A mutation along with other resistance genes that provide resistance to aminoglycosides, sulfonamides, macrolides, isoniazid and mupirocin. These genetic elements explain for the increased antimicrobial resistance by the current bacterial isolate.

1 INTRODUCTION

Typhoid fever affected roughly 13.5 million individuals worldwide in year 2010 (Buckle, Walker, & Black, 2012). Typhoid is an "acute systemic infection" which spreads via fecal-oral route. The infection sustains in population, which do not have access to clean drinking water and good sanitation. It also spreads through ingestion of contaminated food. *S. typhi* is an invasive pathogen that survives through the gastric acid barrier followed by invasion of intestinal mucosal cells resulting in an inflammatory response. Infection is followed by fever ensued by abdominal symptoms like vomiting and diarrhea, headache and a high pulse. Distinct symptoms of typhoid fever include high fever with rose spots on the chest. If left untreated, in the later stages of infection, bacteria can also passage through the intestine to the blood causing a systemic disease (Anwar, *et al*., 2014; Dougan & Baker, 2014; Baron, 1996).

Salmonella belongs to the family of Enterobacteriaceae and comprises of the known human pathogens associated with causing enteric fever including typhoid and gastroenteritis (Ohl & Miller, 2001; Baron, 1996). This group contains facultative anaerobic, flagellated gram-negative bacilli divided into two species, *enterica* and *bongori*. *S. enterica* comprises of six subspecies, namely Enterica, Salamae, Arizonae, Diarizonae, Houtenae and Indica (Mastroeni & Maskell, 2006). Further characterized based on serotyping of O, H and Vi antigen divides these *enterica* subspecies into more than 2500 serovars e.g. *Salmonella enterica* serovar Typhi (*S. typhi),* the causative agent of Typhoid fever (Bangtrakulnonth, *et al*., 2004).

Apart from clinical symptoms, the conventional method for diagnosis and confirmation of *S. typhi* infection is through traditional microbiological procedures like blood/stool cultures followed by biochemical identification. Certain serological procedures are performed either too look for the presence of *S. typhi* antigens (O and H) or to look for

specific antibodies in the serum against the pathogen (Felix-Widal Test). Although, Widal test was widely used, it gave false positive results and therefore newer diagnostic tests were developed like IDL Tubex®, Thypidot® and Typhidot-M® (World Health Organization, 2003). These tests combined did increase efficiency of detection, still rapid and correct detection could prove vital for affected. With advancement in technology, better diagnostic methods using PCR and similar better technologies have been shown to be used for better detection of *S. typhi* ((Song *et al.*, 1993; Khan *et al.*, 2012).

An estimate of 217,000 mortalities occurred out of 22 million cases of typhoid fever (Crump *et al*., 2004). Two major endemic regions of typhoid fever are that of Asia and Africa. In Asia, although the disease burden varies across the region on the basis of age and location, most cases of typhoid fever are reported on the Indian Subcontinent with an annual reported cases of 412.9 per 100,000 persons in Pakistan (Ochiai, et al., 2008; Wain *et al.*, 2015).

If left untreated, typhoid could have a fatality rate of 10-30% but appropriate therapy with effective antibiotics can reduce the rate to 1-4% (Buckle, Walker, & Black, 2012). The first line of treatment emerged with "the introduction of chloramphenicol (in 1948), ampicillin (1961), co-trimoxazole" followed by introduction of third generation cephalosporins and fluoroquinolones which significantly reduced the mortality rate (Wain *et al.*, 2015). Due to inappropriate usage of drugs, drug resistant strains were reported in 1970s with emergence of "plasmid-medicated chloramphenicol resistance" (Mannan, *et al*., 2014) reducing the effectiveness of first line drugs. MDR strains resistant to all three first line drugs were reported by 1980s. Emergence of plasmid-mediated extended spectrum β-lactamases resulting in reports of third generation cephalosporin resistant *S. typhi* that shows resistance to multiple drugs. More studies exhibited increased third generation cephalosporin resistance in other organisms too. This increased resistance, being plasmid mediated can be transferred

to *S. typhi* from other organisms, for instance, gut commensal organisms (Park, 2014; Morita, et al., 2010).

The last resort of defense with increasing third generation cephalosporin resistance is 4th generation cephalosporins like Cefepime and Cefpirome due to their low rate of hydrolysis and ineffective ESBLs against these cephalosporins (Giamarellou, 1999). Recently, we isolated a highly resistant clinical isolate exhibiting resistance pattern never reported before.

RESEARCH AIM

The aim of the current study is **to identify the molecular basis for increased resistance to Cephalosporins and Fluroquinolones in the current *S. typhi* isolate.**

RESEARCH OBJECTIVES

The objective of the current research are:

1. To classify the current isolate (MDR or XDR or PDR) for antibiotic resistance by Antimicrobial Susceptibility Assays
2. To identify resistance determinants conferring resistance to antimicrobials via PCR and Whole Genome Sequencing.

2 LITERATURE REVIEW

2.1 ANTIBIOTICS

Discovery of penicillin by Fleming in 1929 followed by its initial clinical trials in 1941, marked an era in pharmaceutical industry which revolutionized the treatment for bacterial infections in the medical history (Ligon, 2004; Rao, 1998). Since then, numerous antibiotics and classes have been discovered and produced. Different classes of antibiotics act on various target sites within a bacterial cell, inhibiting the cell from its normal metabolism, eventually leading to death.

2.2 CLASSIFICATION OF ANTIBIOTICS

Antibiotics can be classified in two ways; on the basis of their biological function or on the basis of their chemical structure and characteristic(Korzybski, Gindifer, & Kurylowicz, 2013). Although chemical structures vary, there are three major classes of antibiotics based on mode of antibiotic action:

i. Inhibition of cell wall synthesis
ii. Inhibition of protein synthesis, and
iii. Inhibition of DNA synthesis.

Other than the above stated mode of actions, a few classes of antibiotics also either inhibit synthesis or cause damage to the cytoplasmic membrane. Still other antibiotics modify the energy metabolism within bacterial cells, eventually causing death of the cells. Fig. 1 below shows different classes of antibiotics affecting different target sites within a bacterial cell(Neu, 1992).

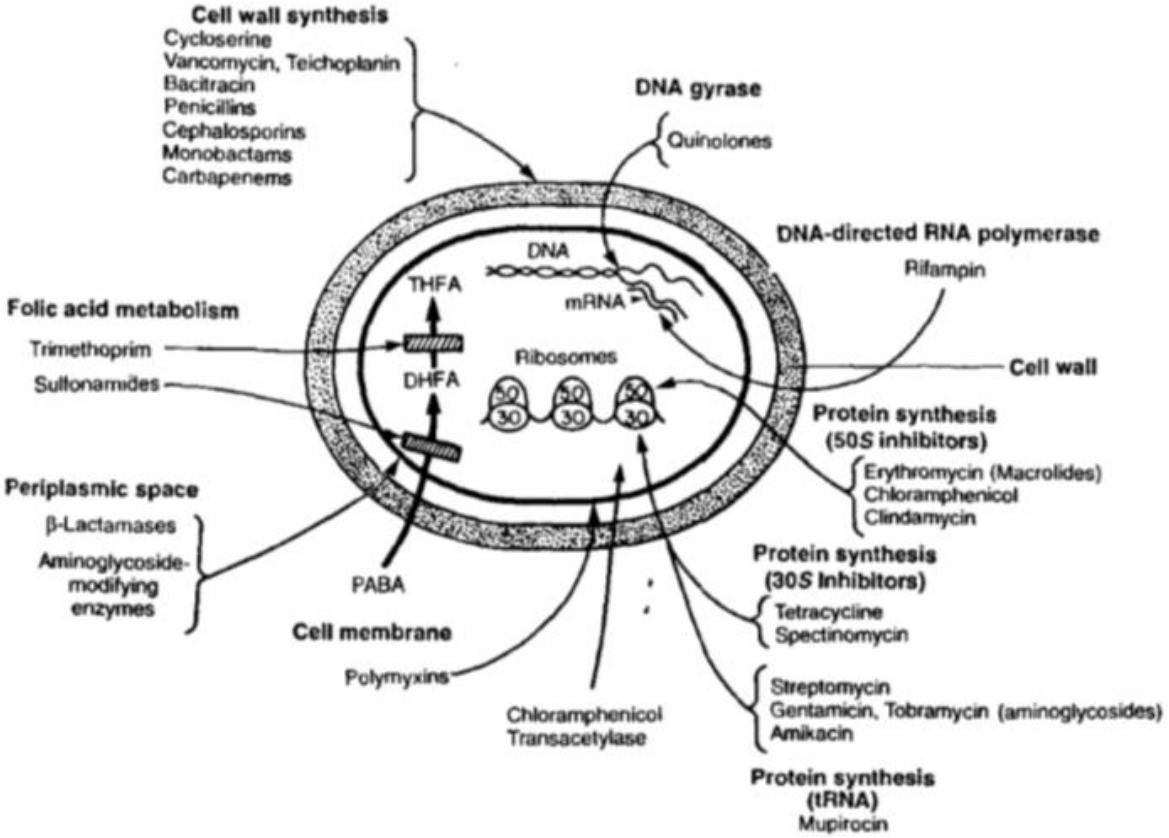


Figure. 1 Mode of action of different classes of antibiotics (Neu, 1992)

2.3 ANTIBIOTICS PRESCRIBED FOR TYPHOID INFECTION

Early reports for treatment of typhoid fever indicate the research on use of pure crystalline extract from liquid culture of Streptomyces spp., Chloromycetin, which showed "significant therapeutic effects(Woodward, Smadel, Ley Jr., Green, & Mankikar, 1948). Similarly, Woodward et al. (1950) reported chloramphenicol as a highly effective antibiotic in the treatment of typhoid fever with no fatalities and a decreased relapse of typhoid fever. The first line of treatment for typhoid was chloramphenicol, ampicillin and trimethoprim but the emergence of MDR strains made these antimicrobials ineffective causing numerous outbreaks in developing countries(Rowe, Ward, & Threlfall, 1997). Such was the case that early report of chloramphenicol resistance surfaced as early as 1950 and a chloramphenicol resistant typhoid fever outbreak occurred 2 decades later in 1970s (Shohel, Rajia, & Hasan, 2014; Wain & Kidgell, 2004).

Since the emergence of multi-drug resistant strains in 1989 exhibiting resistance to all the first line of drugs such as "chloramphenicol, ampicillin, trimethoprim-sulphamethoxazole (TMP-SMZ), streptomycin, sulfonamides and tetracycline", other antibiotics were prescribed for treatment of typhoid infections(WHO, 1996). Fluoroquinolones such as ciprofloxacin emerged as the effective choice for treatment of typhoid fever. The use of extended spectrum cephalosporins such as ceftriaxone and cefixime came forward with increased fluoroquinolone resistance(Sen, Bhattacharya, & Niyogi, 2008). Extended spectrum cephalosporin (third generation) antibiotics such as ceftriaxone and next generation Fluroquinolone such as Moxifloxacin are currently prescribed for the treatment of typhoid infections(Shohel et al., 2014).

2.4 RESISTANCE TO ANTIBIOTICS

As seen in the previous sections, resistance to antibiotics emerged after a few years of use, rendering the antibiotic ineffective for the treatment of that infection. Two mechanisms underlie the development of antibiotic resistance; Biochemical Aspects and Genotypic Aspects.

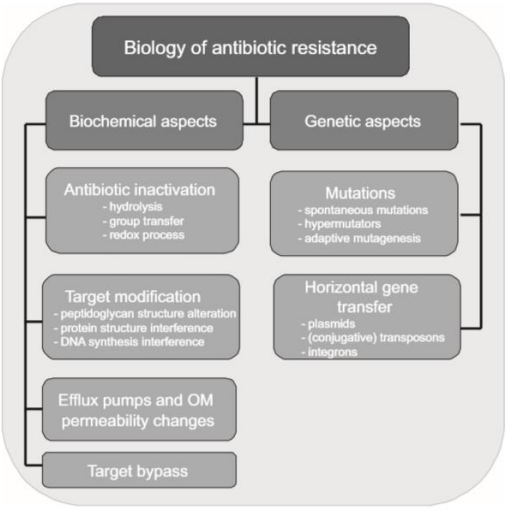


Figure. 2 Antibiotic Resistance Mechanisms: Biochemical and Genetic Aspects (Dzidic, Suskovic, & Kos, 2008)

2.4.1 Biochemical Aspects

Biochemical and functional mechanism which renders an antibiotic drug ineffective can be described on the basis of 4 mechanisms; antibiotic inactivation/modification, target inactivation, efflux pump and permeability barriers, and target bypass.

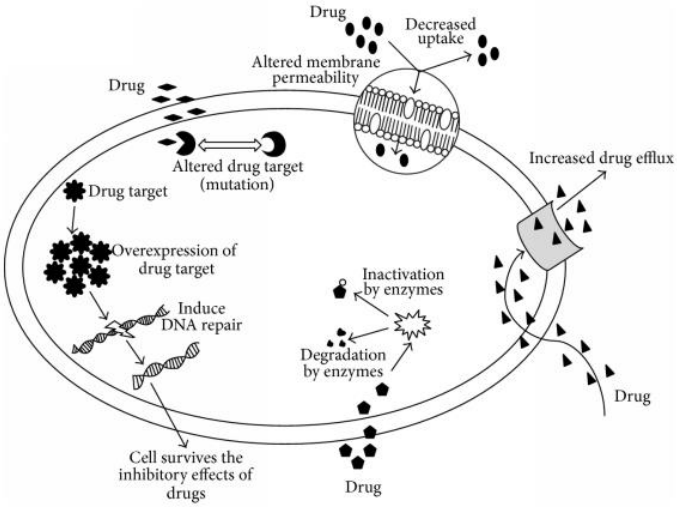


Figure 3 Biochemical mechanism of antibiotic resistance (Tanwar, 2014)

Antibiotic inactivation/Modification

Major class of resistance determinants involving degradation of antibiotics comprises the inactivation and degradation of β-lactams. These antibiotics are inactivated by hydrolysis of the β-lactam ring by β-lactamases. So far, more than a thousand individual resistance determinants belonging to β-lactamases have been reported (Munita & Arias, 2016). These are classified on two basis; molecular basis (amino acid sequences) and functional classification. Molecularly, β-lactamases are divided into four classes; class A, C and D have a serine amino acid for hydrolysis of β-lactam ring whereas class B comprises a metallo-β lactamase enzymes (Karen Bush & Jacoby, 2010). Recent years have revealed an expedited increase in the emergence of newer resistant determinants of β-lactamases with increased use of antibiotics as can be seen in the Figure 4 (Davies & Davies, 2010).

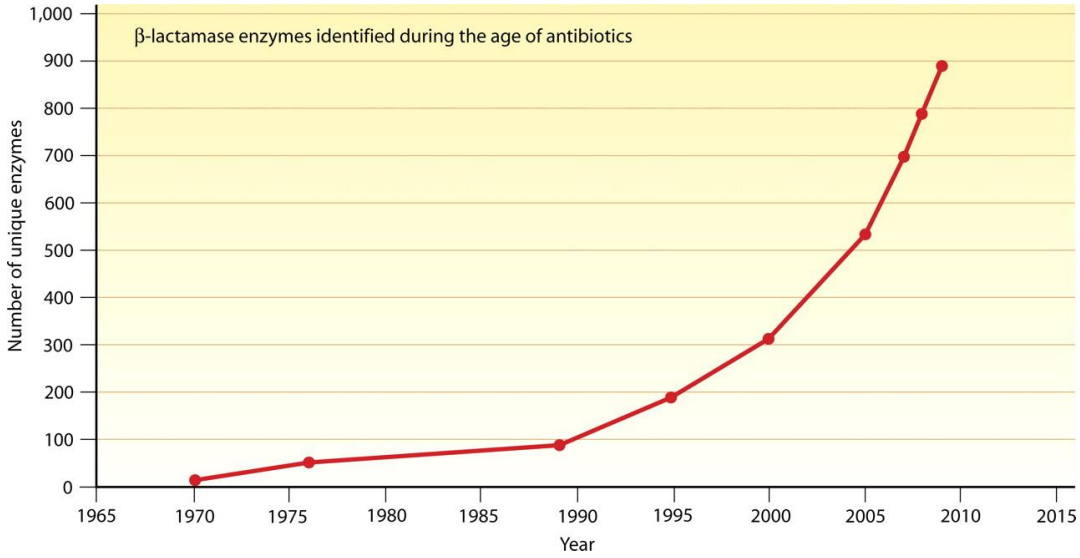


Figure 4 Trend between the number of beta-lactamases identified since 1970 (Davies & Davies, 2010)

Other determinants of resistance by inactivation/modification of antibiotics include enzymatic degradation of chloramphenicol by chloramphenicol acetyl transferase enzyme, fosfomycin inactivation by thioltransferases, and enzymatic modification of aminoglycosides by adenylation (*O*-nucelotidyltransferases), acetylation (N-acetyltransferases), and by phosphorylation (*O*-phosphotransferases) (Davies & Davies, 2010; Giedraitienė, 2011; Ramirez & Tolmasky, 2010).

2.4.2 Target modification

Antibiotics being specific in nature are affected by slightest of changes in the target thus rendering them ineffective. Fluroquinolone resistance has been one of the most well documented cases of resistance mediated by target modification. Fluroquinolone activity occurs by inhibition of DNA synthesis by inhibition of gyrase and topoisomerase. Mutations arising within gyrase (*gyrA* and *gyrB*) and in topoisomerase IV (*parC-parE*) affects the binding affinity between antibiotic molecule and the target site. These mutations, accumulated overtime will disrupt the binding enough to produce a significant clinical resistance to antibiotics (Giedraitienė, 2011; Munita & Arias, 2016).

2.4.3 Efflux pumps and permeability changes

Among other antibiotic resistance mechanisms, multidrug resistance efflux pumps is also deemed as an important resistance mechanism by exporting the antibiotic molecules from the cell to the outside environment, either into the periplasmic space, or to the outside environment. For instance, drug efflux is considered as one of the major tetracycline resistance mechanism in Gram-negative bacteria (Li & Nikaido, 2004). Ever since the earliest discovery of the efflux pump mediated resistance in *Escherichia coli* by Tet proteins, a growing number of drug efflux pumps have been discovered and reported for their significant role in contribution to antibiotic resistance(Levy, 1992).

Classification of efflux pumps is done on the basis of their phylogenetic classification by protein sequences. Transport proteins especially involved in conferring antibiotic resistance can be classified into 5 groups, RND, MFS (major facilitator superfamily), MATE(multidrug and toxic compound extrusion), SMR (small multidrug resistance), and ABC (ATP-binding cassette) superfamilies or families as represented in Figure 5 (Hernando-Amado et al., 2016; Li X-Z., 2015).

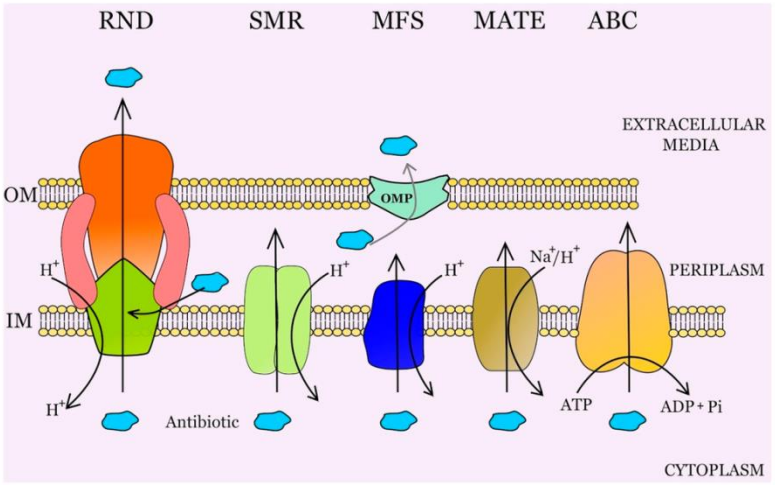


Figure 5 Schematic Representation of different MDR efflux pump and their mechanism of efflux (Blanco et al., 2016)

2.4.4 Target bypass

Target bypass involves replacing the original target site with another molecule that has the same biochemical function but cannot be inhibited by the antibiotic molecule. Such case was observed in resistance to β-lactam by altered peptidoglycan structure of Penicillin Binding Protein. This was observed in Methicillin Resistant *Staphylococcus aureus* (MRSA) where a variant PBP2A was also observed along with PBP. This variant has no/low affinity for binding to all β-lactam antibiotics and thus provides a bypass and resistance to antibiotics (11597450, (Giedraitienė, 2011; Munita & Arias, 2016).

2.5 GENETIC ASPECTS

This section is to determine the genetic insight into the development of resistance and transfer of resistance to other bacteria. Antibiotic resistance could either be intrinsic as a result of mutations occurring within the organism, or acquired by lateral gene transfer from other resistance microbes via phages, conjugation or transformation (Aminov & Mackie, 2007; Dzidic et al., 2008; Marti, Variatza, & Balcazar, 2014b). Such aspects will be viewed in detail below.

2.5.1 Horizontal gene transfer.

Phenomenon of gene transfer between bacteria was discovered around 70 years ago, the impact of which has been seen in full effect with the increasing resistance to antibiotics in pathogens. Although bacterial genomes are stable through generations, they maintain their plasticity for acquisition of genes. Horizontal gene transfer (HGT) is a term used to describe the movement of genetic material from one organism to another. Gene transfer could occur by three mechanism; Transformation, Transduction or Conjugation. (Arber, 2014; Burmeister, 2015; Darmon & Leach, 2014).

2.5.1.1 Transduction

Transduction describes the genetic exchange mediated by the transfer of genes by bacteriophages (Frost, Leplae, Summers, & Toussaint, 2005). Phages mediate this genetic transfer by carrying some portion of genetic traits from a donor bacteria to a recepient bacterial cell following lytic or lysogenic cycle of phage. The transfer could be random or generalized per se where any portion could be transferred to the recepient cell by a lytic or lysogenic bacteriophage. On the other hand, a specialized transduction occurs by activity of lysogenic (temperate) phages only. The specialized transfer of genetic material occurs for some specific genes of the bacterial chromosome. These specialized phages could induce change in the bacetrial phenotype, a process known as lysogenic conversion (Balcazar, 2014; Brabban, Hite, & Callaway, 2005).

Transduction has been seen to be a plausible source of spread of antibiotic resistance genes. Various studies have highlighed the presence of resistance determinants such as quinolone resistance determinants (*qnrA, qnrB, qnrS*), β-lactamases such as bla_{TEM}, $bla_{\text{CTX-M}}$ *and* bla_{SHV}, and *mecA* encoding PBP2 on phage DNA isolated from various sources such as fecally polluted waters, urban sewage samples and activated sludge (Balcazar, 2014; Marti, Variatza, & Balcazar, 2014a; Parsley et al., 2010)

2.5.1.2 Conjugation

Bacteria have an ability to share their genetic information with another bacterial cell by means of direct exchange of genetic material, a process referred to as conjugation, from a donor cell to a recepient cell via pilus, a tube-like structure. The genetic material exchanged could be the double stranded plasmids, or conjugative transposons (Chen, Christie, & Dubnau, 2005; Juhas et al., 2009).

Role of conjugation in transfer of antibiotic resistance has been well studied. Mammeri et al. (2005) reported the transfer of fluroquinolone resistance genes, by transfer of quinolone resistant determinant regions from a resistant bacteria to a previously fluoroquinolone susceptible bacteria.

Another study revealed the transfer of $bla_{\text{CTX-M-1}}$ from a clinical isolate of *E. coli* was able to transfer resistance to third generation cephalosporin to a previously susceptible *Salmonella* Typhimurium isolate. The plasmid profile did not alter between the susceptible and transconjugant host strain despite the transconjugant strains exhibiting resistant phenotypes, highlights the role of small low copy number plasmids in confering and transfering resistant phenotypes (Vo, van Duijkeren, Fluit, & Gaastra, 2007).

2.6 ANTIBIOTIC RESISTANCE TRENDS

Globally, antibiotic resistance to higher generation Cephalosporin and Fluoroquniolones has been on the rise. Increased resistance to the third generation cephalosporin has been reported recently. For instance, 87.5% of the clinical isolates of *Pseudomonas aeruginosa* were resistant to third generation cephalosporin antibiotics (Moremi, Claus, & Mshana, 2016).

2.7 RECENT RESISTANCE TRENDS IN *SALMONELLA TYPHI*

Resistance to the higher generation cephalosporin drugs is being highlighted in the recent years. A study done over a period of 7 years, an average of 22 isolates per year of *S. enterica* were recovered which were resistant to 3rd generation cephalosporins (Eller et al., 2013). Another recent study by Qamar et al., (2014) reviewed laboratory reports of typhoid between 2009 and 2011 in a Pakistani hospital where a few cases of *S. enterica spp.* were resistant to treatment by third generation cephalosporins such as ceftriaxone. It was also reported that the resistance to fluoroquinolone increased alarmingly from 84.7% to 91.7%.

A study by Gandra et al., (2015) elucidated an increase in resistance to ciprofloxacin from 13% to 22 % of the total isolates of *Salmonella sp.* Similarly, another study by Dutta et al., (2014) on *Salmonella enterica* ser. Typhi isolates tested between 2009 and 2013 revealed an increased resistance to fluroquinolone antibiotics, the current choice of treatment of typhoid fever. This resistance was mostly plasmid mediated with the presence of non-IncHl 1 plasmid in 71.4% of the MDR organisms incorporating various resistance determinants. In another study, 56.3% of clinical *Salmonella* isolates at a tertiary hospital in Tanzania showed resistance to higher generation cephalosporin drugs such as ceftriaxone (Moremi et al., 2016).

2.8 GENES CONFERRING RESISTANCE TO CEPHALOSPORIN

Intrinsic cephalosporinases, belonging to β-lactamases, class C. The group has AmpC or AmpC like enzymes encoded by CMY, DHA or ACC-1 genes but *Salmonella* lack structural AmpC gene. ESBLs belonging to β-lactamases A hydrolyze cephalosporins but are inhibited by commercially available β-lactamase inhibitors such as clavulanate, tazobactam etc. These include penicillinases encoded by SHV, TEM, CTX-M, and PER (V Miriagou, Tassios, Legakis, & Tzouvelekis, 2004).

AmpC penicillinases hydrolyze Aztreonam and are “clinically important cephalosporinases”. Their mode of action is similar to Cephalosporins, penicillins, oxyimino-β-cephalosporins and cephamycins with limited resistance to Cefepime, Ceftriaxone and Carbapenems (Georgopapadakou, Smith, & Skyes, 1982; Jacoby, 2009). Simlarly, CTX-M group penicillinases, with more than 50 variants, hydrolyze cefotaxime and ceftriaxone readily than ceftazidine (Miriagou, Tassios, Legakis, & Tzouvelekis, 2004; Naas, Oxacelay, & Nordmann, 2007).

Increased resistance among genes could be attributed to certain point mutations among genes encoding β-lactamses Naiemi et al., (2008) observed that 3 aminoacids

subsitutions in SHV-12 in Enterobacteriacae, caused an increase in hydrolysis of penicillins and extended spectrum cephalosporins, with susceptibility to amoxicillin-clavulanate, ciprofloxacin, meropenem, and piperacillin-tazobactam. Other than that, increased prevalence of CTX-M-15 and TEM-1 has been reported in *S. enterica* isolates conferring resistance to higher generation cephalosporin antibiotic across various regions of the world (Ahmed et al., 2012; Gonzalez-Lopez et al., 2014; Rotimi, Jamal, Pal, Sovenned, & Albert, 2008).

2.9 ANTIBIOTIC RESISTANT GENES INVOLVED IN FLUROQUINOLONE RESISTANCE

Fluoroquinolones have been widely used owing to their wide spectrum activity against Gram positive and Gram-negative bacteria. Widespread use has also caused development of increased antibiotic resistance such that number of fluoroquinolone resistant *Klebsiella pneumoniae* isolates rose from 11% to 50% in 7 years in a study conducted in Italy (Redgrave, Sutton, Webber, & Piddock, 2014)

The most notable mechanism of resistance to fluoroquinolones is by target modification of one or more of the target proteins. Changes in type II topoisomerases encoded by *gyrA, gyrB* (Gyrase), *parC and parE* (Topoisomerase IV) genes result in amino acid substitutions in the target proteins confer varying level of resistance (Aldred et al., 2013).

Mutations in *gyrA* gene conferred resistance to first generation drugs such as naldixic acid. This was dealt by using introduction of second generation fluroquinolone antibiotics such as Levofloxacin and Ciprofloxacin which target *parC* subunit of topoisomerase IV (Patel et al., 2011). By increased use of the second generation antibiotics, bacteria harbored mutations in *parC* and *par*E subunits of topoisomerase IV resulting in the development of resistance to these higher generation antibiotics. This increased resistance resulted was

countered with increased use of moxifloxacin, a 4th generation Fluroquinolone antibiotic that has broad specificity for both gyrase and topoisomerase IV with equal binding affinity. By use of moxifloxacin, either of the mutations in both of the target region could be countered (Bolon, 2009).

Although major mechanism of fluoroquinolone resistance occurs by changes in resistance encoded chromosomally as described above, plasmid mediated quinolone resistance has been widely reported since 1987, despite the idea being withdrawn in the beginning (Munshi et al., 1987; Poirel, Cattoir, & Nordmann, 2012) until another report of "true PMQR" was reported to be present in a *K. pneumoniae* isolate exhibiting resistance to naldixic acid, ciprofloxacin and other similar antibiotics (Martinez-Martinez, Pascual, & Jacoby, 1998).

Plasmid mediated quinolone resistance is conferred by three mechanisms; *qnr* gene, a variant of aminoglycoside acetyltransferase AAC(6')-Ib and lastly, via plasmid encoded efflux pumps QepAB and OqxAB (Hansen, Jensen, Sorensen, & Sorensen, 2007; Robicsek, Strahilevitz, et al., 2006).

We belive, that mutations or acquisition of novel resistant determinants has resulted in development of such higher resistance to not only third generation, but fourth generation cephalosporins and fluoroquinolones too such as cefepime and moxifoloxacin respectively. We aim to characterise all the resistant determinants present in the genome by identifying any novel resistance determinant which could explain such diverse resistance pattern.

3 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Ethical Review for carrying out the study was obtained from Institutional Review Board (IRB), Atta-ur-Rehman School of Applied Biosciences (ASAB), National University of Science and Technology, Islamabad. The highly resistant MDR *Salmonella enterica* subsp. enterica serovar Typhi strain was obtained from Pathology Lab, Army Medical College in February 2016.

The clinical isolate was cultured on an agar slant, incubated overnight and carried to lab in a sealed container, keeping in lieu with the biosafety guidelines for transport of biological substances.

3.2 PATIENT HISTORY

Patient was a 17 year old, previously healthy male, admitted to Military Hospital, Rawalpindi with a 14 day persistent fever of 39 °C. He was diagnosed with typhoid fever. The patient was initially treated with 2g IV of ceftriaxone but he did not respond to therapy. Blood culture reports indicated MDR *S. typhi* infection exhibiting resistance to Ampicillin, Cotrimoxazole, Chloramphenicol, Ciprofloxacin, Azithromycin, Aztreonam, Gentamicin and Ceftriaxone, was an ESBL producer and sensitive only to Meropenem. The patient was treated with 1g IV Meropenem for 10 days prior to discharge from hospital (Munir, Lodhi, Ansari, Andleeb, & Ahmed, 2016).

3.3 SAMPLE PROCESSING

The *S. typhi* clinical isolate was obtained from AFIP lab in the form of antibiotic susceptibility assay plate exhibiting contamination. Subculturing was performed on Salmonella-Shigella Agar for purification of *Salmonella typhi* isolate. The plates were

incubated for 36-48 hour at 37°C. Biochemical identification of *Salmonella enterica* subsp. enterica ser. *typhi* was done for further confirmation.

3.4 GLYCEROL STOCK PREPARATION

Glycerol stocks were prepared of the MDR isolate. Liquid cultures were prepared by touching 3-4 single centered colonies in LB Media followed by overnight incubation at 37 °C at 180 RPM in a rotary shaker. Growth was indicated by media becoming turbid.

The culture was centrifuged at 4500rpm for 5 min to obtain a pellet, followed by re-centrifugation in the same tubes. Supernatant was discarded and the pellet was resuspended in 1 ml fresh LB media.

The contents were transferred to cryovials containing 0.5ml of 80% sterile glycerol. After addition of resuspended culture, the tubes were vortexed at low speed for homogenization of glycerol. The tubes were then snap-freezed in liquid nitrogen before placing them at -80 °C.

3.5 ANTIMICROBIAL SUSCEPTIBILITY ASSAYS

After isolation and storage of *S.* typhi isolate, antibiotic susceptibility assays; Kirby-Bauer Disk Diffusion and Minimum Inhibitory Concentration assays were performed for different antibiotics using EUCAST guidelines to obtain an antibiogram of the MDR Isolate.

3.5.1 Kirby Bauer Disk Diffusion Assay

EUCAST guidelines for media selection and preparation, inoculum preparation, culturing, disk placement and measurement of zone of inhibitions were followed for this assay.

By touching a colony or two, isolated colonies from an overnight culture on a general purpose, non-specific agar were picked. The colony was suspended in 1ml of sterile normal saline to obtain turbidity equal to that of 0.5 McFarland. 100 µl of the prepared suspension

was plated on Muller-Hinton Agar plate. Once the plate dried out, the antibiotic disks were placed on the culture plate. Following antibiotic disks were used for this purpose.

Table 1 Antibiotic Disks and their concentration used for KB Disk Diffusion Antibiotic Susceptibility Assay

Antibiotic	Concentration (µg)
Penicillin	
Ampicillin	30
Amoxicillin	30
Augmentin	30
Fluroquinolones	
Levofloxacin	30
Moxifloxacin	30
Ciprofloxacin	30
Carbapenem	
Imipenem	30
Meropenem	30
Cephalosporins	
Cefotaxime	30
Cefepime	30
Cefoxitin	30
Ceftriazone	30
Cefpirome	30
Cefixime	30
Aztreonam	30

3.5.2 Minimum Inhibitory Concentration using Broth Microdilution

Minimum Inhibitory Concentration was adapted from the protocol published by Wiegand, Hilpert, and Hancock (2008).

Pure cultures of bacterial isolates were obtained on SS Agar. 4 or 5 colonies were touched and were inoculated in 2 ml LB media under aseptic conditions. The culture was grown for 2-3 hours at 225rpm at 37 °C to obtain turbidity equal to that of McFarland 0.5. MICs were determined for following antibiotics.

Table 2: Antibiotics used for determination of Minimum Inhibitory Concentration (MIC) Assay

Antibiotic	Solvent	Concentration (mg/mL)
Penicillin		
Ampicillin	Distilled water	0.5-256
Amoxicillin	Phosphate buffer pH 6.0	0.5-256
Augmentin	Distilled water	0.5-256
Fluroquinolone		
Levofloxacin	DMSO	0.5-256
Ciprofloxacin	DMSO	0.5-256
Moxifloxacin	DMSO	0.5-256
Carbapenem		
Imipenem	Distilled water	0.5-256
Meropenem	Distilled water	0.5-256
Cephalosporins		
Cephradine	Distilled water	0.5-256
Cefotetan	Distilled water	0.5-256
Cefixime	Phosphate Buffer (pH 7.0)	0.5-256
Ceftriazone	Distilled water	0.5-256
Cefepime	Distilled water	0.5-256

Meanwhile media supplemented with Antibiotic was prepared by using the following scheme illustrated in Table 3. Following preparation of antibiotic dilution, 50 µl of inoculum was inoculated with 50 µl of antibiotic substituted MH broth media in a 96-well plate. This halved the concentration to Final Concentration as is shown in Table 3.

After inoculation, the plate was placed in a zip-lock back with a moist paper towel and sealed to prevent the media in the wells from drying out. Culture was incubated for 24 hours at 37°C after which, turbidity was visually assessed and confirmed using a micro-titer plate reader for determination of MIC.

Table 3: Protocol followed for preparation of required Antibiotic Solutions. Final Concentration represents the antibiotic concentration in the reaction mixture

Stage	Stock	Source	Vol. of Antibiotic Solution (µL)	Vol. of Sterile Broth (µL)	Concentration Obtained (µg/mL)	Final Concentration (µg/mL)
1	10 mg/ml	Stock	100	900	1000	512
2	1 mg/ml	Stage 1	512	488	512	**256**
3	512 µg/ml	Stage 2	500	500	256	**128**
4	256 µg/ml	Stage 3	500	500	128	**64**
5	128 µg/ml	Stage 4	100	100	64	**32**
6	128 µg/ml	Stage 4	100	300	32	**16**
7	128 µg/ml	Stage 4	100	700	16	**8**
8	16 µg/ml	Stage 7	100	100	8	**4**
9	16 µg/ml	Stage 7	100	300	4	**2**
10	16 µg/ml	Stage 7	100	700	2	**1**
11	2 µg/ml	Stage 10	100	100	1	**0.5**

3.6 TOTAL DNA EXTRACTION BY BOILING METHOD

Total DNA extraction was done by boiling method. A single bacterial colony from overnight *S. typhi* culture grown on MH Agar was suspended in 100 µl NF (nuclease free) water. The suspension was then centrifuged using by SpinFuge microfuge for 1 min to wash the bacterial colonies. The supernatant was discarded and the colonies were again suspended in 100µl water. The suspension was then given a heat shock at 99 °C for 20 minutes in PCR machine. After 20 minutes, the tubes were quickly placed on ice and then centrifuged by SpinFuge microfuge for 2-3 minutes. The supernatant was then transferred to a new PCR tube and kept at -20°C for later use and the pellet was discarded.

3.7 DNA EXTRACTION

Whole genome DNA extraction was carried out by Salting Out method modified from He. (2011) protocol; 5M NaCl was used for protein precipitation instead of phenol-chloroform method.

1.5 ml of overnight bacteria culture grown in LB media was centrifuged at max speed for 1 min to pellet cells. The supernatant was discarded and cells were resuspended in 600 µl Lysis Buffer (9.34 ml lysis buffer, 600 µl of 10% SDS and 60 µl Proteinase K (20mg/ml)) and vortex. The mixture was incubated at 37 °C for 1 hour, preferably in a shaking water bath. The mixture became viscous followed by lysis.

1-1.5 vol of 5M NaCl was added to the mixture for protein precipitation and slow vortex for 15 sec was done followed by centrifugation at max speed for 5 min. After centrifugation, upper aqueous layer was aspirated and transferred to a new microfuge tube. This can be repeated until complete loss of white layer is seen.

DNA was precipitated by addition of 1ml absolute chilled ethanol, and left in refrigerator for 15-30 min followed by centrifugation at max speed at 4 °C for 15 min. The supernatant

was discarded and DNA pellet was rinsed with 1ml 70% ethanol and centrifugation at max speed for 2 min.

The supernatant was discarded and DNA pellet was air dried at room temperature by inverting it on a paper towel.

3.8 PCR FOR IDENTIFICATION OF RESISTANCE GENE

3.8.1 Primer Designing

Primers listed in the recent literature were used for identification of resistance determinants; *bla* CTX-M, *bla* TEM, and *bla* SHV.

Table 4 Primers used for identification of β-lactamases

Primer	Primer Sequence	Amplicon Size (bp)	Reference
TEM			
TEM-F	5′-TTCTTGAAGACGAAAGGGC-3′	1150	Brinas, Zarazaga, Saenz, Ruiz-Larrea, and Torres (2002)
TEM-R	5′-ACGCTCAGTGGAACGAAAAC-3′		
SHV			
SHV-F	5′-CACTCAAGGATGTATTGTG-3’	885	(Brinas et al., 2002)
SHV-R	5′-TTAGCGTTGCCAGTGCTC-3′		
CTX-M			
CTX-MUF	5' -CGA TGT GCA GTA CCA GTA A-3'	592	(Batchelor et al., 2005)
CTX-MUR	5'-TTA GTG ACC AGAATC AGC GG-3'		

3.8.2 PCR reaction mixture for identification of β-lactamases

PCR was performed for identification of antibiotic resistance genes; CTX-M, TEM, and SHV at standard PCR conditions.

Table 5 PCR reaction mixture and concentrations used

Reagent	**Volume used (µl)**	**Concentration**
Taq Buffer	2.5	10X
dNTP Mix	2.5	0.2 mM
Forward Primer	1	1 mM
Reverse Primer	1	1mM
Template	5	-
Taq DNA Pol	0.25	5 U/µl
NF Water	12.75	-
Total Reaction Mixture (µL)	**25**	

3.9 PLASMID CURING

Plasmid Curing Assay was performed by using EtBr and SDS as the plasmid curing agent. The concentration of EtBr was tested at 75 µg/ml, 100 µg/ml and 125 µg/ml substituted in Muller Hinton Broth (prepared as per manufacturer's guidelines). Similarly, SDS concentration tested were 0.5 % (*w/v*), 0.75% (*w/v*) and 1% (*w/v*).

100 µl of overnight culture was used as an inoculum in 10 ml each of EtBr and SDS substituted media. Culturing in SDS and EtBr substituted media were done at 37°C and 43 °C. The cultures were subcultured for 3 days.

After 3 days of sub-culturing in the media supplemented with same concentration of curing agent, cultures were serially diluted from 10^{-4} to 10^{-7} followed by plating on Nutrient Agar. After overnight growth, colonies from plates having 5-100 colonies were replica-plated on MH media substituted with 128µg/ml cefepime.

3.10 WHOLE GENOME SEQUENCING

Whole Genome Sequencing was performed by isolation of whole genomic DNA from 10 colonies using MoBio Bacteremia DNA Kit followed by preparation of library preparation using Illumina Nextera XT kit (Baym et al., 2015; Schmieder & Edwards, 2011). Prepared libraries were sequenced by Illumina NextSeq 2500 which were processed at High Throughput Computing Facility at Washington University School of Medicine sequenced the prepared DNA libraries, St. Louis. Illumina adapters and potentially contaminating human reads were removed using trimmomatic and deconseq (Bolger, Lohse, & Usadel, 2014; Schmieder & Edwards, 2011). The paired end reads were assembled using SPAdes 3.10.0 (Bankevich et al., 2012). The contigs were analyzed by QUAST (Gurevich, Saveliev, Vyahhi, & Tesler, 2013). Generated contigs were then used for further processing.

3.11 GENOME ALIGNMENT

3.11.1 Obtaining Reference Sequence

Reference FASTA sequence for *Salmonella enterica* subsp. enterica ser. Typhi str. CT18 (NC_003198) with its pHCM1 (NC_003384.1) and pHCM2 (NC_003385.1) plasmids was retrieved from ncbi.nlm.nih.gov.

3.11.2 Contig Rearrangement using Mauve

Using the "Move Contigs" tool in Mauve, the file containing contigs was rearranged using the *S. typhi* str. CT18 as the reference. This generated a multi-fasta file with contigs in

the specified order as that of the reference (A. C. Darling, Mau, Blattner, & Perna, 2004; A. E. Darling, Mau, & Perna, 2010).

3.11.3 Creating single FASTA file using Artemis

The generated .FNA sequence containing reordered contigs was retrieved from the alignment folder and opened on Artemis. "Write All Bases" function was then used to create a single sequence FASTA file for further analysis (Carver et al., 2005).

3.12 GENOME ANNOTATION

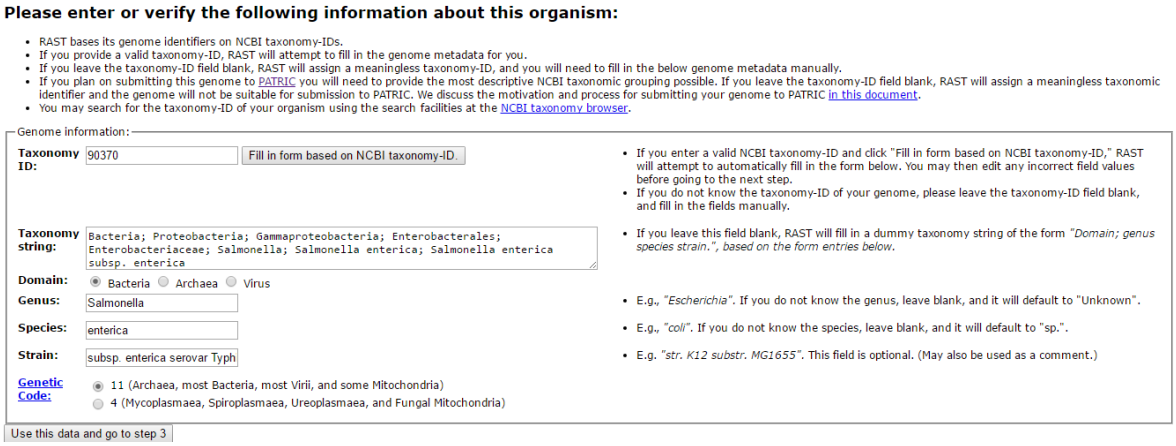
3.12.1 Genome Annotation Using Prokka

The single FASTA file was annotated using Prokka by using the command line prokka/home/asab/filename.fna. The output files were saved for further analysis (Seemann, 2014).

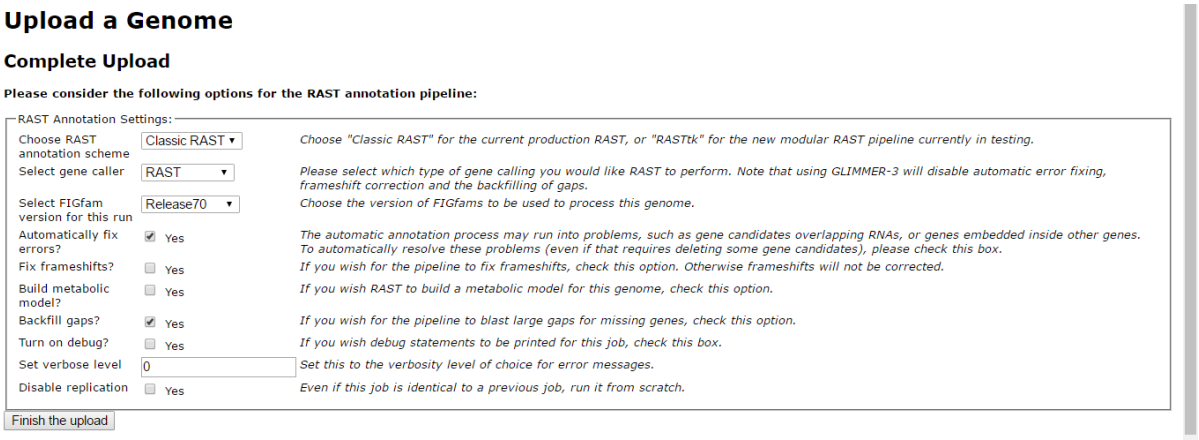
3.12.2 Genome Annotation using Rapid Annotation Subsystem Technology (RAST)

Single .FASTA file containing *S. typhi* sequence was also annotated using Rapid Annotation Subsytem Technology 2.0 online server available at rast.nmpdr.org (Aziz et al., 2008; Overbeek et al., 2014). FASTA file containing arranged contig was uploaded on the server after registering for an account.

After the file upload was complete, the genome information of the organism was using the Taxonomy ID: 90370. Following that, the rest of the information was made available by "Fill in form based on NCBI taxonomy-ID." button.



After step 2, the settings for Annotation were selected as shown in the figure before finally submitting the genome for annotation.



Afterwards, RAST annotation settings page was displayed. The genome was annotated using default settings i.e. Classic RAST was used as annotation scheme, RAST was used as gene caller and FIGfam Release 70 was used for this job. The options “Automatically fix errors” and “Backfill gaps” were also checked. Verbose level was also kept to 0.

3.12.3 Genome Annotation using PATRIC ver 3.3.5

Genome annotation was also done using PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center ver. 3.3.5. PATRIC employs RASTtk for genome annotation which is an updated RAST server which is still under trial. The RASTtk server allowed calling of CRISPR sequences and also uses PhiSpy algorithm for identifying prophage insertion sequences (Brettin et al., 2015; Wattam et al., 2017).

3.12.4 Genome Annotation using Prokaryotic Genome Annotation Pipeline (PGAP)

Genome Annotation was also performed using Prokaryotic Genome Annotation Pipeline (PGAP) hosted by NCBI database when the draft genome sequence was submitted in GenBank.

3.13 GENOTYPIC CHARACTERIZATION USING CENTER FOR GENOMIC EPIDEMIOLOGY

The Center for Genomic Epidemiology offers various tools for phenotyping and typing such as Identification of acquired antibiotic resistance genes Multi Locus Sequence Typing, PlasmidFinder, serovar identification using SeqSero, and SNP analysis using available at http://www.genomicepidemiology.org/.

3.13.1 Multi Locus Sequence Typing using CGE

Multilocus Sequence Typing was also performed using MLST 1.8 tool available at https://cge.cbs.dtu.dk/services/MLST/. MLST configuration was used for *Salmonella enterica* and data type was Assembled Genome/Contigs in .FASTA format (Larsen et al., 2012).

3.13.2 PlasmidFinder-CGE

PlasmidFinder 1.3 available at https://cge.cbs.dtu.dk/services/PlasmidFinder/ was used to identify plasmid sequences in the assembled genome annotation. Database Plasmid-Enterobacteriaceae was selected as the database with a threshold for ID% set at 95%. FNA file was uploaded to identify the plasmid sequences (Carattoli et al., 2014).

3.13.3 SNP Determination

Single Nucleotide Polymorphism (SNP) were detected against three genomes using . The genomes are available at NCBI. *S. typhi* CT18 (AL513382) was used as the reference

genome and two other Strains, Ty2 (AE014613)and P-stx-12 (CP003278) were used along with sequenced isolate to identify the SNPs present within the genome. CSI Phylogeny hosted by Center of Genomic Epidemiology was used to identify the SNP presents within genome (Kaas, Leekitcharoenphon, Aarestrup, & Lund, 2014) Following Input Parameters were used for computation of SNP:

Minimum depth at SNP positions: 10

Relative depth at SNP positions: 10

Minimum distance between SNPs (prune): 10

Minimum SNP quality: 30

Minimum read mapping quality: 25

Minimum Z-score: 1.96

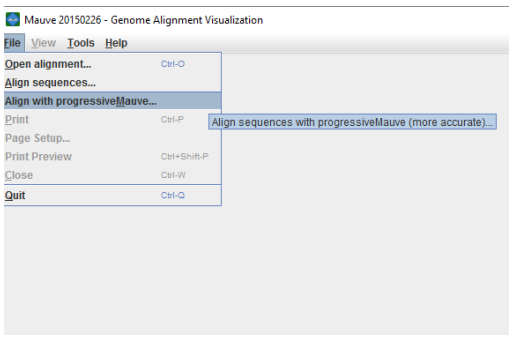
The SNP data was downloaded in Newick format and was opened in MEGA 7 to obtain a phylogenetic tree. The alignment file in .FASTA format was obtained from SNP calling was opened in MEGA 7 and realigned using ClustalW algorithm. The alignment file was then used to determine the evolutionary relationship of the taxa by constructing UPGMA tree with bootstrap value of 500(Kumar, Stecher, & Tamura, 2016).

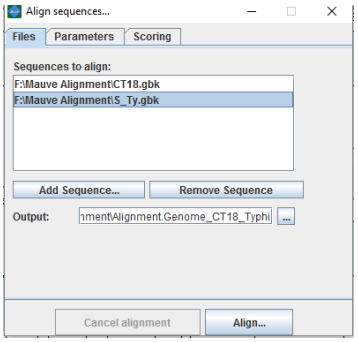
SNP analysis was also done using RealPhy with default settings. The input included *S. typhi* str. P-Stx-12 as reference, *S. typhi* str. RWP1_PK1 and 44 Whole Genome Sequences available on PATRIC of *S. typhi* genomes reported from Pakistan. The output was available in the form of a phylogenetic tree.

3.14 WHOLE GENOME COMPARISON

3.14.1.1 Whole Genome Comparison by using Mauve

Annotated *S. typhi* genome was also aligned with *S. typhi* str. CT18 and *S. typhi* P-Stx-12 using Mauve Multiple Genome Alignment Visualization ver. 20150226. Using "Align with Progressive Mauve" function, the .gbk files were aligned.





The log file along with alignment files was saved. DCJ, GAP and SNP analysis was also performed for alignment using Mauve Matrix Alignment.

3.14.1.2 Whole Genome Comparison with BRIG v0.95

Genome was also aligned using BRIG v0.95 (Alikhan, Petty, Ben Zakour, & Beatson, 2011) using NCBI BLAST+ (v. 2.6.0) with three complete *S. typhi* genomes available: str. CT18 (NC_003198.1, NC_0033384, NC_003385); str. Ty2 (NC_004631); str. P-Stx-12 (NC_016832.1, NC_016825)**.** Upper and Lower BLAST threshold value was placed at 90% and 70% respectively.

3.15 IDENTIFICATION OF ANTIBIOTIC RESISTANCE GENES (ARGS)

3.15.1 Identification of ARGs by Antibiotic Resistance Database (ARDB)

Antibiotic Resistance Genes were also identified using Antibiotic Resistance Database available at http://ardb.cbcb.umd.edu/index.html by using the Genome Annotation and Comparison tool. Blastx program was used with 40% identity and E-Value of 0.0001.

The Multiple Sequences Annotation and Resistance Profile Comparison tool was used along with the Multiple Sequence Annotation program. *S.* Ty str. CT18 and *S.* Ty 2 were used for comparison of resistance profiles (Liu & Pop, 2009).

3.15.2 Identification of ARGs by ResFinder

ResFinder 2.1 was also used for identifying acquired antimicrobial resistance genes. 90% threshold was used for identification of resistance genes with a minimum length of 60%. The type of reads used were Assembled Genome/Contigs (Zankari et al., 2012).

3.15.3 Identification of ARGs by Comprehensive Antibiotic Resistance Database (CARD)

The Comprehensive resistance database is available at https://card.mcmaster.ca/. By using the Resistance Gene Identifier (RGI) tool available at the server, all the resistance determinants in the genome were identified. The input file was .FNA, DNA sequence was the data type and Selection criteria was “Discovery-Perfect, Strict and Loose hits” (Jia et al., 2017; McArthur et al., 2013).

3.15.4 Identification of ARGs by ResFams

ResFams was also used for identifying the antibiotic resistance determinants. Using Hidden Markov Model, the putative resistance determinants were identified from the ResFams database (Gibson, Forsberg, & Dantas, 2015).

3.16 GENOMIC ISLAND PREDICTION BY ISLANDVIEWER 4

Genomic Islands in the *S. typhi* genome were predicted using IslandViewer 4. Genomic Islands were predicted using Island Viewer 4 against various *S. typhi* genomes available on the database (Bertelli et al., 2017). Input file for the analysis was the FASTA file containing contigs as IslandViewer4 can reassemble and arrange contigs using the reference genome. Genomic islands were predicted by using 6 complete genomes of *S. typhi* as a reference separately: str. CT18 (NC_003198.1, NC_0033384, NC_003385); str. Ty2 (NC_004631); str. Ty21a (NC_021176.1), str. P-Stx-12 (NC_016832.1, NC_016825); str. B/SF/13/03/195 (CP012151.1) and str. PM016/13 (NZ_CP012091.1).

4 RESULTS

4.1 *S. TYPHI* ISOLATION AND PURIFICATION

After incubation at 37°C for 24 hours, round colonies were observed on the SS agar plates. Some colonies were pink in color and some were opaque. On further incubation for 48 hours, *S. typhi* colonies became opaque with black centers. The isolated colonies were further confirmed using biochemical identification listed in Table 6.

Table 6 Biochemical Characteristics of *S. typhi* Isolate

Test	**+ve/-ve**
Gram Staining	-ve rods
Catalase	+ve
H_2S	+ve
Oxidase	-ve

4.2 ANTIMICROBIAL SUSCEPTIBILITY ASSAYS

4.2.1 Kirby-Bauer Disk Diffusion Assay

Antibiotic susceptibility using disk diffusion assay revealed the strain to be resistant to all major β-lactam antibiotics and Fluroquinolones. The isolate exhibited susceptibility only to Carbapenem and Cephamycin class of antibiotics such as meropenem and Cefoxitin respectively. The mean zone of inhibition diameters are listed in Table 7.

Table 7 Average zone diameters of antibiotics and their susceptibility based on EUCAST Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017. * represents Cephamycin; + indicates susceptibility; - indicates resistance

Antibiotic	Zone Diameter (mm)			Average (mm)	EUCAST	Susceptibility
Fluroquinolones						
Ciprofloxacin	11.00	11.00	10.00	10.67	**<24**	-
Levofloxacin	14.00	14.00	14.00	14.00	**<19**	-
Moxifloxacin	13.00	12.00	10.00	11.67	**<22**	-
Carbapenem						
Meropenem	26.00	24.00	26.00	**25.33**	**<16**	+
Imipenem	31.00	32.00	33.00	**32.00**	**<16**	+
Cephalosporin						
Cefixime	0.00	0.00	0.00	0.00	**<17**	-
Cefotaxime	0.00	0.00	0.00	0.00	**<17**	-
Ceftriazone	0.00	0.00	0.00	0.00	**<22**	-
Cefpirome	8.00	0.00	0.00	2.67		-
Cefepime	0.00	0.00	0.00	0.00	**<21**	-
Cefoxitin*	25.00	26.00	21.00	24.00	**<19**	+
Monobactam						
Aztreonam	0.00	0.00	0.00	0.00	**<21**	-

4.2.2 Minimum Inhibitory Concentration (MIC) using Broth Microdilution Assay

Minimum Inhibitory Concentration was determined using the Broth Microdilution Assay. The results exhibited high level of Minimum Inhibitory Concentration for all the higher generation of antibiotics (Table 8). All antibiotics proved ineffective at inhibiting the growth of bacterial cells except those belonging to Carbapenem class.

Table 8 Average Minimum Inhibitor Concentration of antibiotics and their susceptibility based on EUCAST Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017. + indicates susceptibility; - indicates resistance

Antibiotic	Conc. (mg/mL)	MIC (mg/l)			Avg. MIC (mg/L)	EUCAST resistance breakpoint (mg/L)	Susceptibility
		1	2	3			
Pencillin							
Amoxicillin	0.5-256	>256	>256	>256	>256	>8	-
Augmentin	0.5-256	16	16	16	16	>8	-
Clavulanate	0.5-256	>256	>256	>256	>256	N/A	N/A
Piperacillin + Tazobactam	0.5-256	128	128	128	128		-
Fluroquinolones							
Levofloxacin	0.5-256	4	4	4	4	>1	-
Ciprofloxacin	0.5-256	2	2	2	2	>.06	-
Moxifloxacin	0.5-256	4	4	4	4	>0.25	-
Carbapenem							
Imipenem	0.5-256	<0.5	<0.5	<0.5	<0.5	>8	+
Meropenem	0.5-256	<0.5	<0.5	<0.5	<0.5	>8	+
Cephalosporins							
Cephradine	0.5-256	>256	>256	>256	>256	>2	-
Cefotetan	0.5-256	>256	>256	>256	>256	>2	-
Cephazolin	0.5-256	>256	>256	>256	>256	>8	-
Cefixime	0.5-256	>256	>256	>256	>256	>1	-
Ceftriazone	0.5-256	>256	>256	>256	>256	>2	-
Cefepime	0.5-256	256	256	256	256	>4	-

4.3 PCR FOR IDENTIFICATION OF B-LACTAMASES

PCR was carried out for bla CTX-M using universal primers and CTX-M class specific primers; CTM-M1, CTX-M2, CTX-M8, CTX-M9, and CTX-M25. All except CTX-M using universal primer showed a result with an amplicon size of 592 bp. PCR for amplification of bla-TEM and bla-SHV showed negative results

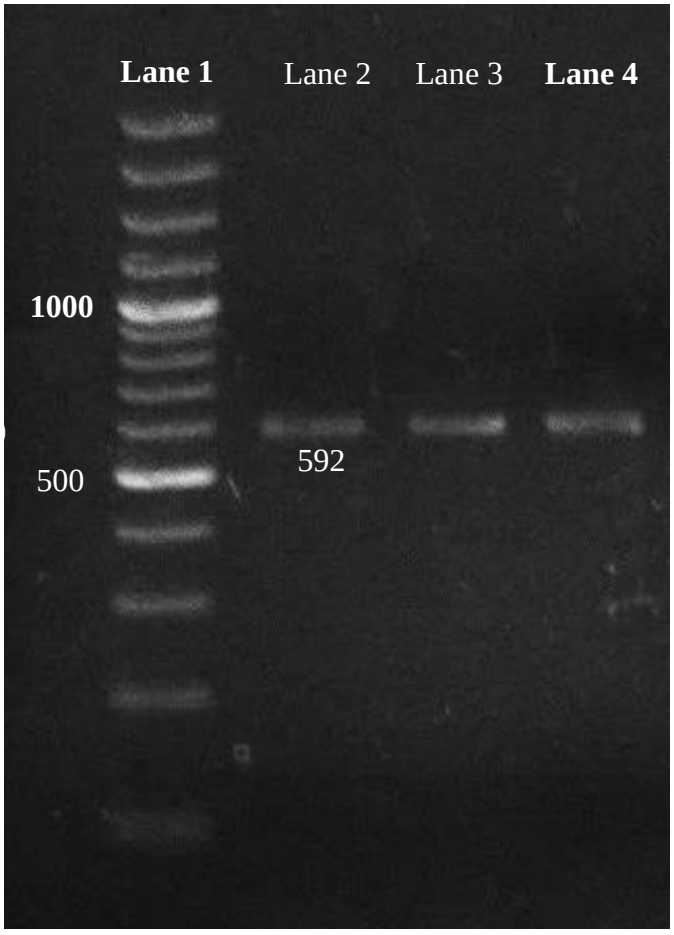


Figure 6: Gel Electrophoresis for PCR to detect *bla*$_{\text{CTX-M15}}$. Lane 1 shows 100bp+ DNA Ladder; Lane 2, 3 and 4 show CTX-M positive results (592 bp) using CTX-MU primer.

4.4 PLASMID CURING ASSAY

It was observed that no growth occurred in media grown at 37 °C in SDS at 0.5 % w/v SDS substituted Muller Hinton media. Plasmid Curing Assay revealed no loss of plasmids in any of the colonies after 3 days of subculturing and plating on antibiotic containing media in

EtBr substituted media and SDS substituted media grown at both 37 °C and 43 °C.The results are as shown in Table 9 and Table 10.

Table 9 Plasmid Curing at 43 °C. D represents the dilution factor at which colonies were suitable for replica plating to an antibiotic susbsituted media. (a) CFU at the dilution factor from which it was subsequently plated; (b) CFU on the cefepime (128 µg/ml) substituted media.

S. No	**SDS (%w/v)**	***D***	**a**	**B**
1	0.1	10^{-4}	6	6
2	0.25	10^{-5}	105	105
3	0.50	10^{-3}	97	97
S. No	**EtBr (µg/ml)**	***D***	**a**	**B**
1	75	10^{-5}	3	3
2	100	10^{-5}	4	4
3	125	10^{-3}	58	58

Table 10 Plasmid Curing at 37 °C. D represents the dilution factor at which colonies were suitable for replica plating to an antibiotic susbsituted media. (a) CFU at the dilution factor from which it was subsequently plated; (b) CFU on the cefepime (128 µg/ml)

S. No	**SDS (%w/v)**	***D***	**a**	**B**
1	0.1	10^{-4}	24	24
2	0.25	10^{-4}	79	79
3	0.5	-	-	-
S. No	**EtBr (%w/v)**	***D***	**a**	**B**
1	075	10^{-5}	7	7
2	100	10^{-3}	13	13
3	125	10^{-4}	20	20

4.5 WHOLE GENOME SEQUENCING

10,307,350 paired-end 150bp reads were generated from an Illumina NextSeq 2500 and processed using the High Throughput Computing Facility at Washington University School of Medicine (St. Louis, USA). Removal of Illumina adapters and potentially contaminating human reads using trimmomatic and deconseq generated 10,288,264 paired-end reads which were assembled into scaffolds using SPAdes 3.10.0. Contig Analysis by QUAST revealed that the assembly produced 95 contigs > 500 bp, with an N50 of 144,739 bp. The longest contig was 393,347 bp. The genome coverage was 161X.

4.6 ANNOTATION OVERVIEW

Whole Genome sequencing was done using 4 different genome annotation tools: , Genome was annotated using Prokka (Seemann, 2014), RAST, Rapid Annotation Subsystem Technology ver 2.0 (Aziz et al., 2008; Overbeek et al., 2014), and PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center ver. 3.3.5 (Wattam et al., 2017). Overview of the genome annotation using different annotation tools is is shown in Table 11.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank and as *Salmonella enterica* subsp. enterica serovar Typhi str. RWP1_PK1 under the accession NIFP00000000. The version described in this thesis is version NIFP01000000.

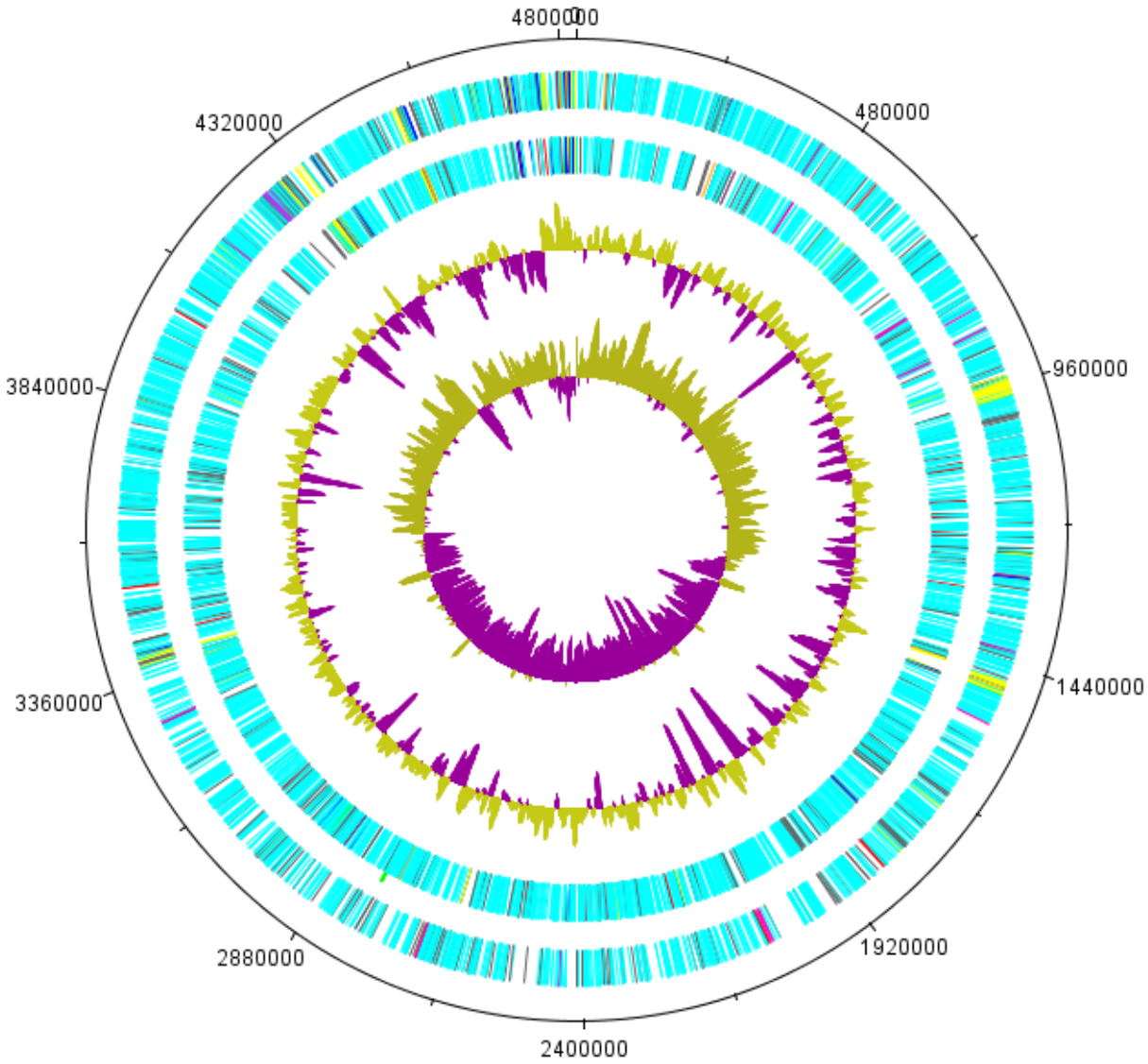


Figure 7 Circular plot of *S. typhi* RWP1_PK1 Genome (Accession No. NIFP01000000) depicting annotation using PATRIC, The All-Bacterial Bioinformatics Database and Analysis Resource Center v.3.3.5. Image drawn using DNA Plotter Release 1.11. Track Order outwards toward the center of the circular genome depiction is: Forward Strand CDS; Reverse Strand CDS; G-C Plot (%); G-C Skew (%); Above Average; Below Average

Table 11 Overview of genome annotation using different annotation servers. ***CDS- coding sequences; tRNA- transfer RNA genes; rRNA- rRNA genes; CRISPR- Clustered Regularly interspaced palindromic repeats***

	Genome Length	**CDS**	**G-C %**	**tRNA**	**rRNA**	**Repeat region**	**CRISPR_ repeat**	**CRISPR_ spacer**	**CRISPR_array**
Prokka	4826797	4,661	52	69	3	1	-	-	-
RAST	4826797	4,988	52	75	8	-	-	-	-
PATRIC	4826797	5,074	52.02	74	4	112	7	6	1
PGAP	4826797	4,975	52	69	4	-	-	-	1

4.7 WHOLE GENOME COMPARISON

Alignment by mapping contigs to CT18 reference assembly yielded an alignment comprising of 4809037 bp of which 29421 bases were extra in assembly while 141490 bases were missed in the reference nucleotide sequence. Data yielded by alignment revealed that 61 contigs were extra in the sequenced assembly.

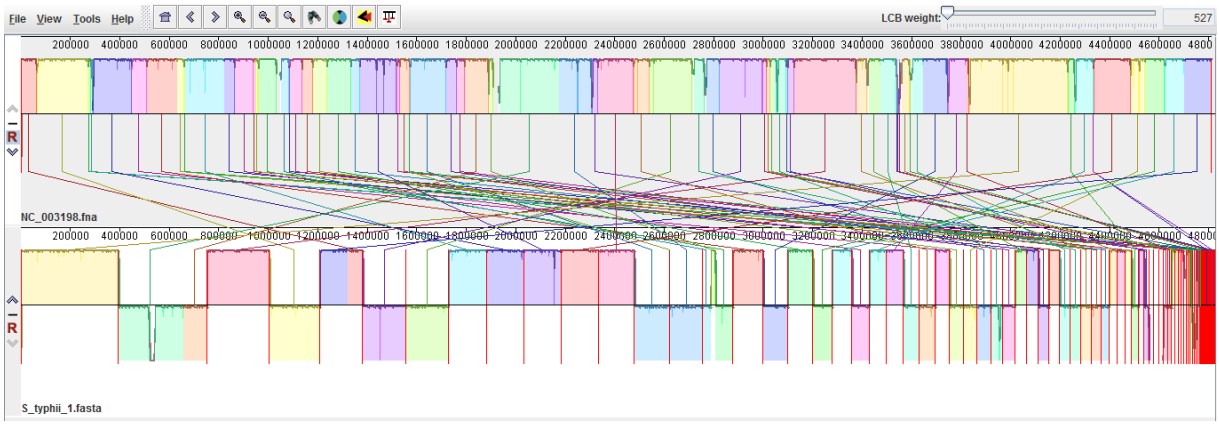


Figure 8: Alignment file using FNA files of Reference and Contig files.

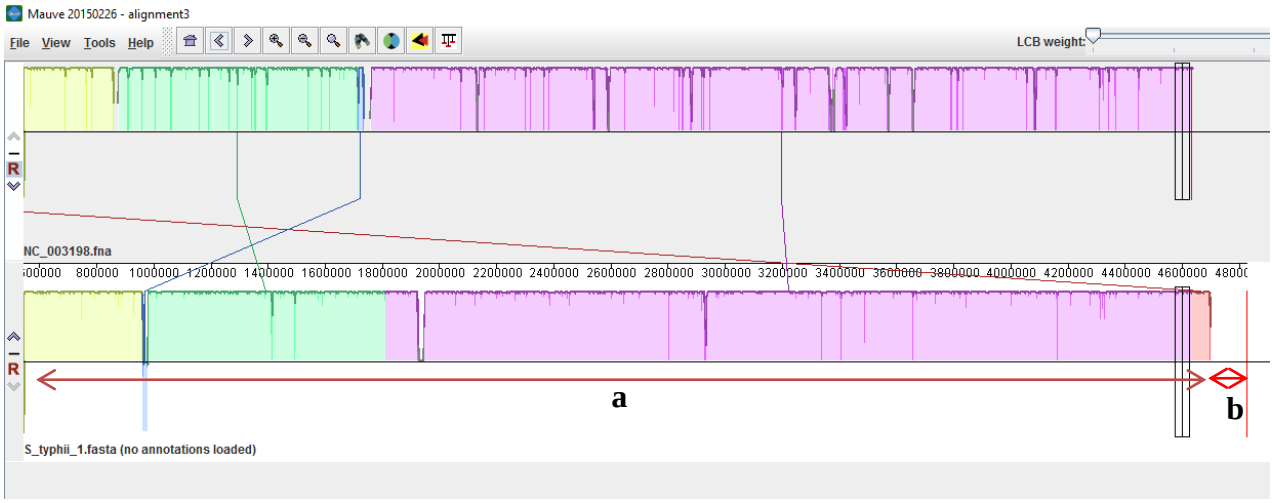


Figure 9 Alignment file showing the contig file rearranged and aligned with reference genome. "a" shows the aligned region; b Region not present in the reference genome

PATRIC which uses RASTtk gave a highly detailed annotation and identified more regions within the genome whereas annotation using Prokka identified fewer regions as compared to the rest two servers. The difference between annotation using RAST and RASTtk was observed in the identification of CRISP elements such as CRISPR Repeats, CRISPR Spacer and CRISPR Array in the latter.

Whole genome of rearranged contig was also aligned using BRIG v0.95 against three complete genomes: str. CT18 (NC_003198.1, NC_0033384, NC_003385; str. Ty2 (NC_004631; and str. P-Stx-12 (NC_016832.1, NC_016825)

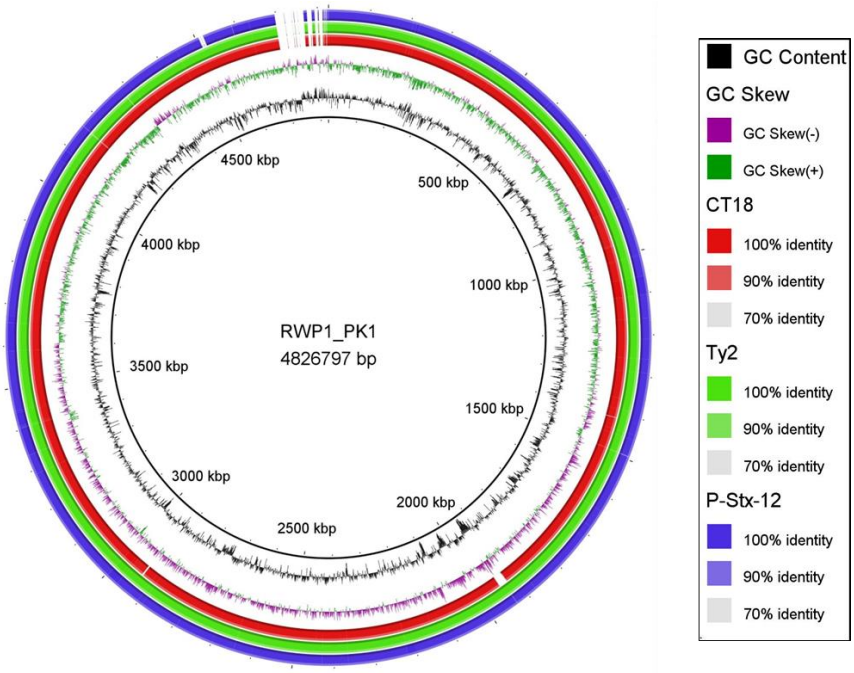


Figure 10: Whole Genome sequence comparison of *S. typhi* RWP1_PK1 with 3 reference strains using Brig v0.95: str. CT18 (NC_003198.1, NC_0033384, NC_003385); str. Ty2 (NC_004631); str. P-Stx-12 (NC_016832.1, NC_016825).

4.8 GENOTYPIC CHARACTERIZATION USING CGE

4.8.1 Multi Locus Sequence Typing (MLST)

MLST Typing by MLST-1.8 revealed the sequence type to be ST-1. The output was revealed in alignment and tabular format. The results are as follows:

Table 12 Alleles and sequence types of *S. typhi* RWP1_PK1 revealed by MLST-1.8 hosted by CGE.

Locus	% Identity	HSP Length	Allele Length	Gaps	Allele
Aroc	100.00	501	501	0	*aroc-1*
Dnan	100.00	501	501	0	*dnan-1*
hemd	100.00	432	432	0	*hemd-1*
Hisd	100.00	501	501	0	*hisd-1*
Pure	100.00	399	399	0	*pure-1*
Suca	100.00	501	501	0	*suca-1*
Thra	100.00	501	501	0	*thra-5*

4.8.2 SNP Determination

The SNP analysis revealed that 97.33% of the reference *S. typhi* CT18 genome was covered by all three subject sequences; including P-Stx-12, Ty2 and the clinical isolate. The clinical isolate had 4779606 valid positions as compared to CT18 reference sequence. The SNP Matrix index is shown in Table 13.

Table 13 SNP score index obtained from CSI Phylogeny tool available at CGE. The highest number of SNPs was observed between *S. typhi* isolate and *S. typhi* str. CT18 sequence while the minimum number of SNPs was observed between *S. typhi* RWP1_PK1 and *S. typhi* P-Stx-12 strain.

	CT18 (ref)	RWP1_PK1	P-Stx-12	Ty2
CT18 (ref)	0	367	354	332
RWP1_PK1	367	0	35	195
P-Stx-12	354	35	0	178
Ty2	332	195	178	0
min: 35 max: 367				

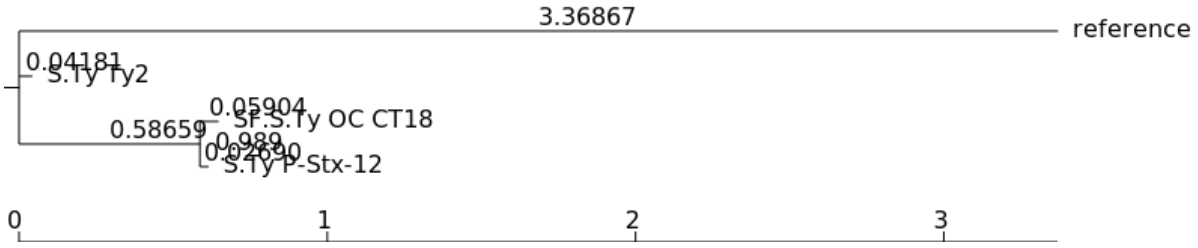


Figure 11: Evolutionary Relationship based on SNP calling by Center of Genomic Epidemiology. Reference used for generation of Phylogenetic tree was *S. typhi* CT18. The clinical isolate was related to S. Ty P-Stx-12 as can be inferred from the phylogenetic tree.

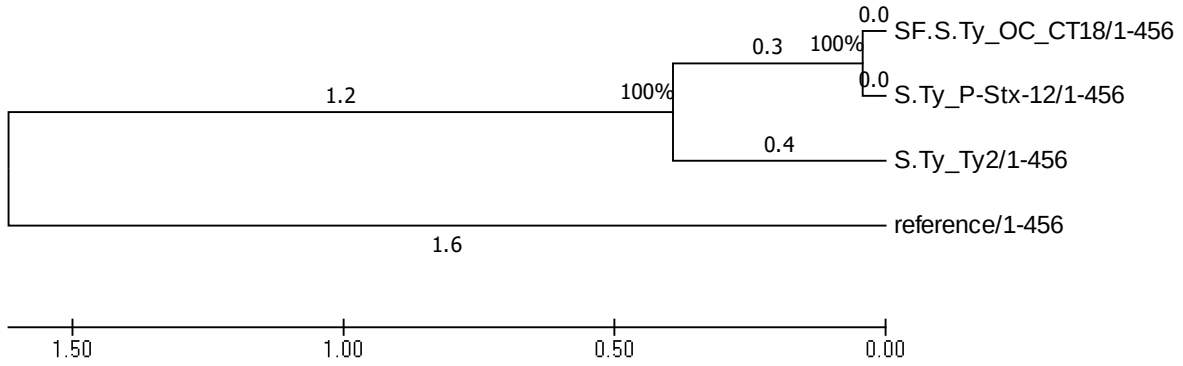


Figure 12 Evolutionary relationships of taxa. The evolutionary history was inferred using the UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length = 3.67056819 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 456 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016); *S. typhi* RWP_PK1 represented by SF.S. Ty_OC_CT18/1-456

SNP analysis was also performed against 44 *S. typhi* genome sequences from Pakistan available at Patric. *S. typhi* isolate is more phylogenetically related to *S. typhi* MDUST 156 (GID: 90370.1801) based on SNP analysis than *S. typhi* P-Stx-12.

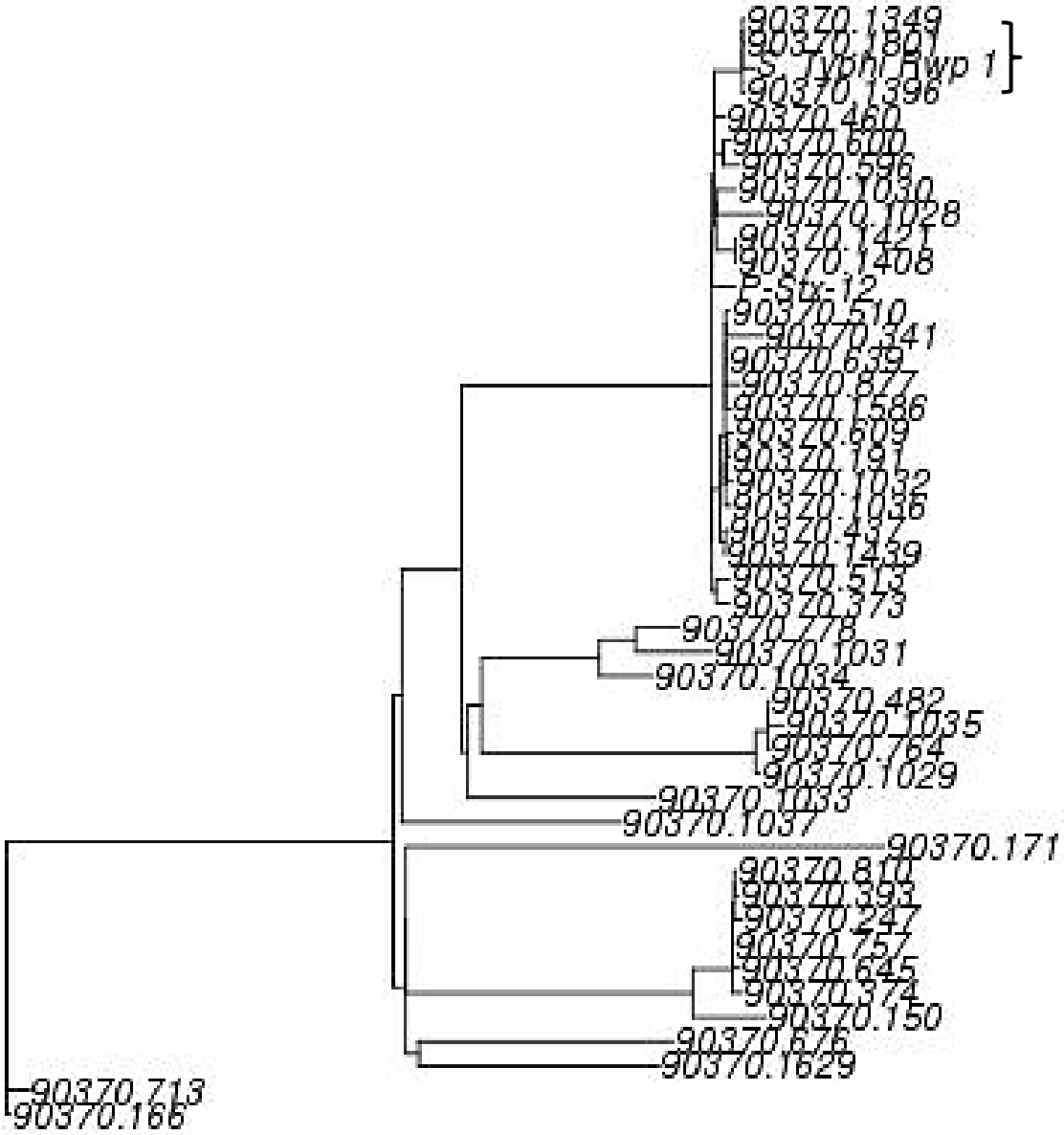


Figure 13 Evolutionary Relationship based on SNP calling with 44 whole genome sequences of *S. typhi* RWP1_PK1 from Pakistan predicted by RealPhy. Reference used for generation of Phylogenetic tree was *S. typhi* P-Stx-12. The clinical isolate was related to S. Ty P-Stx-12 as can be inferred from the phylogenetic tree.

4.9 IDENTIFICATION OF ANTIBIOTIC RESISTANCE DETERMINANTS

Different databases were used for identification of Antibiotic Resistance Determinants present within the annotated assembly. The results of the antibiotic determinants identified by different databases are shown in the subsequent sections.

4.9.1 Antibiotic Resistance genes identified by ARDB

24 anitbiotic Resistance genes were identified by Antibiotic resistance database. The genes included all resistance determinants that had a cut-off value above 0.0001 (Liu & Pop, 2009). Results of the ARDB database search are shown in Table 14.

Table 14Antibiotic Resistance determinants identified by Antibiotic Resistance Database (ARDB). Resistance genes were identifed by blastx algorithm with a cutoff value of 0.0001 and percent identity of 40% (Liu & Pop, 2009).

Type	Definition	Resistance	Query Genome
acra	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	aminoglycoside glycylcycline macrolide beta_lactam acriflavin	fig|90370.2042.peg.439
acrb	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	aminoglycoside glycylcycline macrolide beta_lactam acriflavin	fig|90370.2042.peg.438 fig|90370.2042.peg.3393
ant3ia	Aminoglycoside O-nucleotidylyltransferase, which modifies aminoglycosides by adenylylation.	spectinomycin streptomycin	fig|90370.2042.peg.4914
aph33ib	Aminoglycoside O-phosphotransferase, which modifies aminoglycosides by phosphorylation.	Streptomycin	fig|90370.2042.peg.4954
aph6id	Aminoglycoside O-phosphotransferase, which modifies aminoglycosides by phosphorylation.	Streptomycin	fig|90370.2042.peg.4955
baca	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	Bacitracin	fig|90370.2042.peg.3202
bl2be_ctxm	Class A beta-lactamase. This enzyme breaks the beta-lactam antibiotic ring open and deactivates the molecule's antibacterial properites.	monobactam penicillin cephalosporin_iii ceftazidime cephalosporin_ii cephalosporin_i	fig|90370.2042.peg.4872
cata1	Group A chloramphenicol acetyltransferase, which can inactivate chloramphenicol.	Chloramphenicol	fig|90370.2042.peg.4919
emrd	Multidrug resistance efflux pump.	NA	fig|90370.2042.peg.3803
ksga	Specifically dimethylates two adjacent adenosines in the loop of a conserved hairpin near the 3'-end of 16S rRNA in the 30S particle. Its inactivation leads to kasugamycin resistance.	Kasugamycin	fig|90370.2042.peg.38

maca	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump. Macrolide-specific efflux system.	Macrolide	fig|90370.2042.peg.849
macb	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump. Macrolide-specific efflux system.	Macrolide	fig|90370.2042.peg.850
mdtg	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	deoxycholate fosfomycin	fig|90370.2042.peg.1082
mdth	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	deoxycholate fosfomycin	fig|90370.2042.peg.1096
mdtk	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	enoxacin norfloxacin	fig|90370.2042.peg.1578
mdtl	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	Chloramphenicol	fig|90370.2042.peg.3758
mdtm	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	chloramphenicol acriflavine norfloxacin	fig|90370.2042.peg.4716
pbp2	The enzyme has a penicillin-insensitive transglycosylase N-terminal domain (formation of linear glycan strands) and a penicillin-sensitive transpeptidase C-terminal domain (cross-linking of the peptide subunits)	Penicillin	fig|90370.2042.peg.1919 fig|90370.2042.peg.1918
qnrs	Pentapeptide repeat family, which protects DNA gyrase from the inhibition of quinolones.	Fluoroquinolone	fig|90370.2042.peg.4866
sul1	Sulfonamide-resistant dihydropteroate synthase, which can not be inhibited by sulfonamide.	Sulfonamide	fig|90370.2042.peg.4913
sul2	Sulfonamide-resistant dihydropteroate synthase, which can not be inhibited by sulfonamide.	Sulfonamide	fig|90370.2042.peg.4953
tolc	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	aminoglycoside glycylcycline macrolide beta_lactam acriflavin	fig|90370.2042.peg.3183

4.9.2 Antibiotic resistance genes identified by CARD

Resistance Gene Identifier was used to identify resistance determinants present within the assembled and annotated genome. Various Antibiotic Efflux pumps were also identified in the sequence which could be contributing to the increased antibiotic resistance (Jia et al., 2017; McArthur et al., 2013; McArthur & Wright, 2015). Table 15 summarizes the genes involved in conferring antibiotic resistance in the isolate as identified by CARD database. The resistance genes identified are also shown in the form of an RGI wheel and heatmap as shown in the Figure 14 and Figure 15.

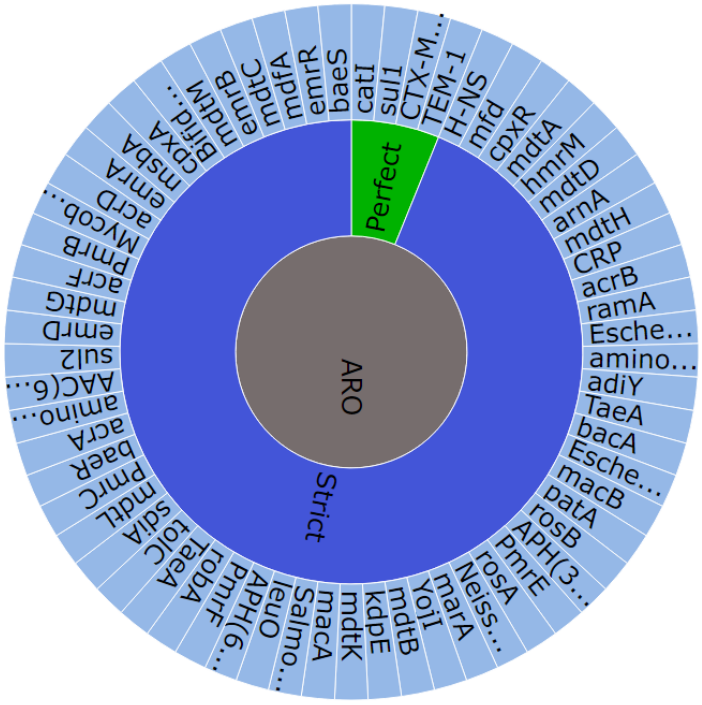


Figure 14 RGI Wheel showing Antibiotic Resistance genes identified in the sequence with Perfect and Loose Hits

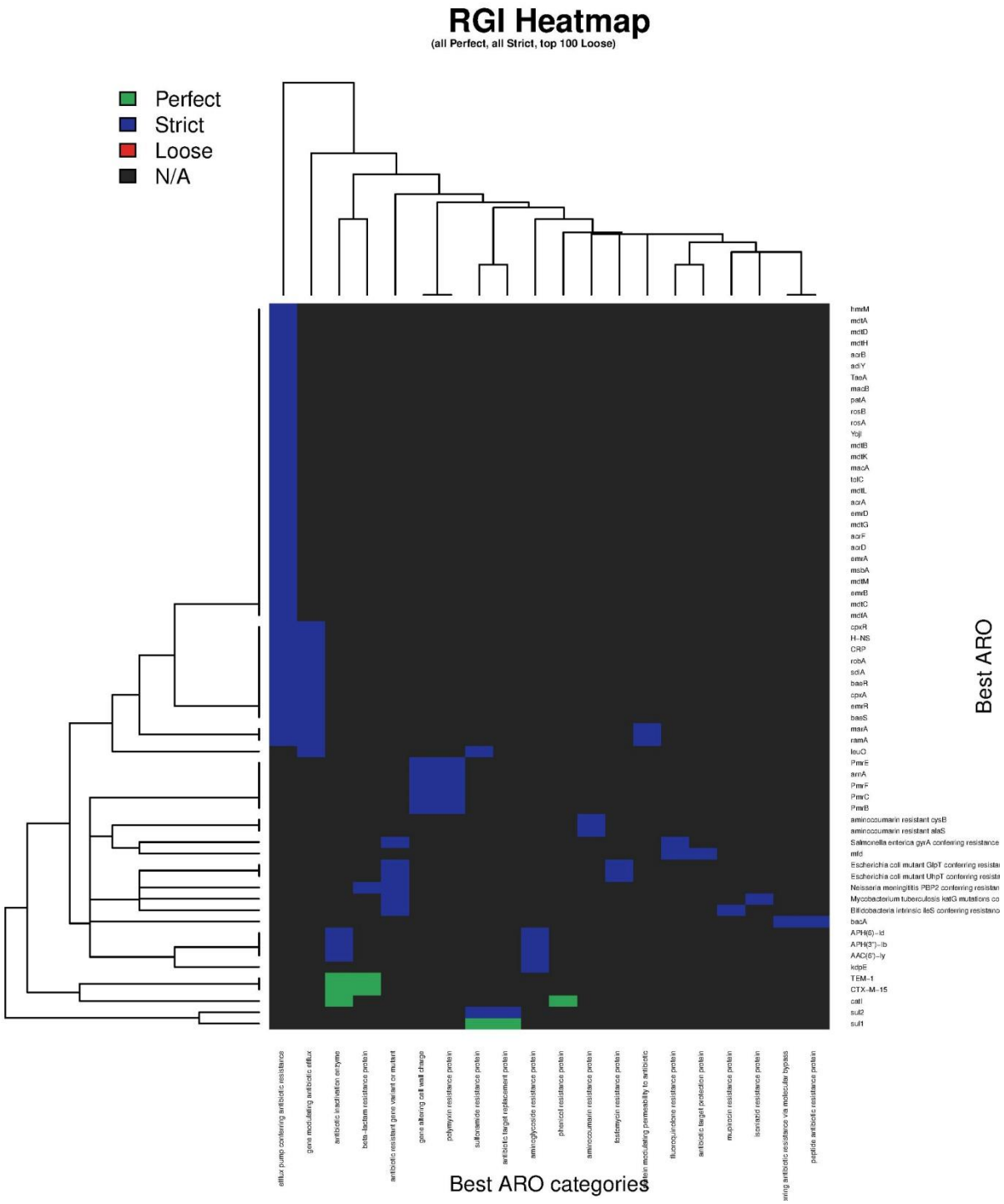


Figure 15 Heatmap showing antibiotic resistance genes hit against dataset hosted by CARD

Table 15: Antibiotic resistance genes identified by RGI by using DNA sequence as the data type with default Perfect and Strict hits only.

Perfect hits

PEG ID	ARO_name	Model_type	SNP	AR0_category	bit_score	Best_Hit_evalue	Best_Id
peg.4919	catI	protein homolog model	n/a	antibiotic inactivation enzyme; phenicol resistance protein	465.692	4.27E-169	100
peg.4913	sul1	protein homolog model	n/a	antibiotic target replacement protein; sulfonamide resistance protein	553.132	0	100
peg.4872	CTX-M-15	protein homolog model	n/a	antibiotic inactivation enzyme; beta-lactam resistance protein	593.578	0	100
peg.4968	TEM-1	protein homolog model	n/a	antibiotic inactivation enzyme; beta-lactam resistance protein	591.267	0	100

Strict hits

PEG ID	ARO_name	Model_type	SNP	AR0_category	bit_score	Best_Hit_evalue	Best_Id
peg.1146	mfd	protein homolog model	n/a	antibiotic target protection protein; fluoroquinolone resistance protein	2258.41	0	95
peg.3815	E. coli mutant UhpT	protein variant model	E350Q	antibiotic resistant gene variant or mutant; fosfomycin resistance protein	868.226	0	95
peg.2773	alaS	protein homolog model	n/a	aminocoumarin resistance protein	1723.75	0	95
peg.3202	bacA	protein homolog model	n/a	gene conferring antibiotic resistance via molecular bypass; peptide antibiotic resistance protein	532.717	0	97
peg.2328	GlpT	protein variant model	E448K	antibiotic resistant gene variant or mutant; fosfomycin resistance protein	887.486	0	96
peg.4954	APH(3")-Ib	protein homolog model	n/a	aminoglycoside resistance protein; antibiotic inactivation enzyme	537.339	0	98

peg.2114	PmrE	protein homolog model	n/a	gene altering cell wall charge; polymyxin resistance protein	709.909	0	88
peg.1838	PBP2	protein variant model	A510V	antibiotic resistant gene variant or mutant; beta-lactam resistance protein	351.673	7.11E-114	36
peg.2316	*gyrA*	protein variant model	S83F	antibiotic resistant gene variant or mutant; fluoroquinolone resistance protein in *S. enterica*	1766.9	0	99
peg.67	leuO	protein homolog model	n/a	gene modulating antibiotic efflux; sulfonamide resistance protein	584.719	0	87
peg.4955	APH(6)-Id	protein homolog model	n/a	aminoglycoside resistance protein; antibiotic inactivation enzyme	563.148	0	99
peg.2343	PmrF	protein homolog model	n/a	gene altering cell wall charge; polymyxin resistance protein	590.497	0	87
peg.4332	PmrC	protein homolog model	n/a	gene altering cell wall charge; polymyxin resistance protein	937.947	0	82
peg.1228	cysB	protein homolog model	n/a	aminocoumarin resistance protein	565.844	0	94
peg.1331	AAC(6')-Iy	protein homolog model	n/a	aminoglycoside resistance protein; antibiotic inactivation enzyme	300.442	7.46E-106	97
peg.4953	sul2	protein homolog model	n/a	antibiotic target replacement protein; sulfonamide resistance protein	526.554	0	99
peg.4330	PmrB	protein homolog model	n/a	gene altering cell wall charge; polymyxin resistance protein	630.172	0	85
peg.3573	M. tuberculosis katG	protein variant model	Q224EA139VA243S	antibiotic resistant gene variant or mutant; isoniazid resistance protein	761.911, 761.911, 761.911	0	56

peg.662	kdpE	protein homolog model	n/a	aminoglycoside resistance protein	421.779	1.08E-151	91
Peg.4846	ileS	protein homolog model	n/a	antibiotic resistant gene variant or mutant; mupirocin resistance protein	228.409	4.64E-63	24
Efflux Pumps							
peg.1189	H-NS	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	232.261	1.02E-79	94
peg.983	TaeA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	352.058	1.78E-112	32
peg.849	macA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	186.422	2.02E-55	35
peg.4773	TaeA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	359.377	2.18E-116	39
peg.1578	hmrM	protein homolog model	n/a	efflux pump conferring antibiotic resistance	404.831	1.17E-137	46
peg.850	macB	protein homolog model	n/a	efflux pump conferring antibiotic resistance	650.203	0	50
peg.458	rosA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	512.301	0	70
peg.3758	mdtL	protein homolog model	n/a	efflux pump conferring antibiotic resistance	610.527	0	78
peg.2344	arnA	protein homolog model	n/a	gene altering cell wall charge; polymyxin resistance protein	1125.15	0	79
peg.4334	adiY	protein homolog model	n/a	efflux pump conferring antibiotic resistance	409.068	5.91E-146	79
peg.4778	robA	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	508.834	0	82

peg.2170	mdtD	protein homolog model	n/a	efflux pump conferring antibiotic resistance	774.622	0	85
peg.540	ramA	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux; protein modulating permeability to antibiotic	212.231	1.66E-72	85
peg.439	acrA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	666.766	0	85
peg.2167	mdtA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	624.78	0	86
peg.4716	mdtM	protein homolog model	n/a	efflux pump conferring antibiotic resistance	708.753	0	86
peg.811	mdfA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	726.087	0	87
peg.2171	baeS	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	828.165	0	88
peg.2762	emrA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	702.205	0	89
peg.3392	acrF	protein homolog model	n/a	efflux pump conferring antibiotic resistance	1684.46	0	89
peg.3183	tolC	protein homolog model	n/a	efflux pump conferring antibiotic resistance	863.988	0	89
peg.1082	mdtG	protein homolog model	n/a	efflux pump conferring antibiotic resistance	697.967	0	90
peg.2308	YojI	protein homolog model	n/a	efflux pump conferring antibiotic resistance	1009.21	0	90
peg.1096	mdtH	protein homolog model	n/a	efflux pump conferring antibiotic resistance	717.613	0	91
peg.2169	mdtC	protein homolog model	n/a	efflux pump conferring antibiotic resistance	1857.03	0	91
peg.2168	mdtB	protein homolog model	n/a	efflux pump conferring antibiotic resistance	1870.9	0	91
peg.3803	emrD	protein homolog model	n/a	efflux pump conferring antibiotic resistance	645.58	0	92

peg.438	acrB	protein homolog model	n/a	efflux pump conferring antibiotic resistance	1975.67	0	93
peg.2761	emrR	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	337.806	4.46E-120	93
peg.2536	acrD	protein homolog model	n/a	efflux pump conferring antibiotic resistance	2020.74	0	94
peg.2763	emrB	protein homolog model	n/a	efflux pump conferring antibiotic resistance	944.495	0	95
peg.1425	marA	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux; protein modulating permeability to antibiotic	251.136	1.37E-87	95
peg.3629	cpxR	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	456.062	5.71E-165	96
peg.890	msbA	protein homolog model	n/a	efflux pump conferring antibiotic resistance	1160.59	0	96
peg.3630	cpxA	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	908.286	0	96
peg.2172	baeR	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	467.233	3.92E-169	96
peg.4163	CRP	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	431.409	6.08E-156	98
peg.1959	sdiA	protein homolog model	n/a	efflux pump conferring antibiotic resistance; gene modulating antibiotic efflux	499.59	0	98
peg.2041	mdtK	protein homolog model	n/a	efflux pump conferring antibiotic resistance	927.932	0	99

4.9.3 Resistance Genes identified by ResFinder-2.1 Server (CGE)

Antibiotic resistance genes were also identified by ResFinder 2.1 hosted by Center of Genomic Epidemiology identified Resistance determinants are as listed in Table 16.

Table 16: Antibiotic Resistance Determinants identified by ResFinder 2.1 (CGE); Threshold for %ID was 90 % with 60% minimum length.

Resistance gene	Identity	Query/HSP	Contig	Position in contig	Phenotype	Accession no.
catA1	99.85	660/660	peg.4919	1..660	Phenicol resistance	V00622
strA	99.88	804/804	peg.4954	1..804	Aminoglycoside resistance	AF321551
sul2	100	816/816	peg.4953	1..816	Sulphonamide resistance	FN995456
strB	100	837/831	peg.4955	1..831	Aminoglycoside resistance	M96392
sul1	100	840/840	peg.4913	1..840	Sulphonamide resistance	AY224185
blaTEM-1B	100	861/861	peg.4968	1..861	Beta-lactam resistance	JF910132
blaCTX-M-15	100	876/876	peg.4872	1..876	Beta-lactam resistance	DQ302097

4.9.4 Antibiotic Resistance genes identified by ResFams

Antibiotic resistance genes were also identified using ResFams using Hidden Markov Model. The antibiotic resistance genes identified include class A and Class B β-lactamases, aminoglycoside resistance enzymes along with quinolone resistance protein and antibiotic efflux genese like RND, Mex, and ABC efflux genes. The detailed description of antibiotic resistance determinants is listed in Table 17.

Table 17 Antibiotic Resistance genes identified by ResFams using Hidden Markov Model (HMM). The Accession number of sequence along with it's E-value, score and bias is shown in the table along with the Target name and description

Target Name	Accession	E-value	score	bias	description of target
RND_efflux	RF0115	0	1053.7	35.3	resistance-nodulation-cell division (RND) antibiotic efflux pump [ARO:0010004]
RND_efflux	RF0115	0	1024.5	19.8	resistance-nodulation-cell division (RND) antibiotic efflux pump [ARO:0010004]
SubclassB3	RF0125	8.30E-107	349.4	0	Subclass B3 (metallo-) beta-lactamase hydrolize penicillins, cephalosporins and carbapenems [ARO:3000571]
ClassB	RF0054	7.40E-55	178.9	0	Class B beta-lactamase [ARO:3000004]
tolC	RF0147	5.60E-195	641	28.8	tolC: subunit of efflux pump conferring antibiotic resistance [ARO:3000237]
RND_efflux	RF0115	0	1211.6	9.4	resistance-nodulation-cell division (RND) antibiotic efflux pump [ARO:0010004]
soxR	RF0121	5.20E-72	233.3	0	soxR: mutant efflux regulatory protein conferring antibiotic resistance [ARO:3000836]
ABC_efflux	RF0007	1.40E-77	254.3	1	ATP-binding cassette (ABC) antibiotic efflux pump [ARO:0010001]
RND_efflux	RF0115	0	1133.0	14	resistance-nodulation-cell division (RND) antibiotic efflux pump [ARO:0010004]
MexX	RF0101	5.70E-84	275	11.2	mexX: subunit of efflux pump conferring antibiotic resistance [ARO:3001214]
MexE	RF0098	2.20E-56	184.3	7.3	mexE: membrane fusion protein of the MexEF-OprN multidrug efflux complex [ARO:3000803]
msbA	RF0107	8.00E-189	621.3	9.8	msbA: ATP-binding cassette (ABC) antibiotic efflux pump [ARO:3000460]
ABC_efflux	RF0007	9.20E-125	410	1.6	ATP-binding cassette (ABC) antibiotic efflux pump [ARO:0010001]
macB	RF0089	6.20E-213	701	6.8	macB: subunit of efflux pump conferring antibiotic resistance [ARO:3000535]
phoQ	RF0112	1.90E-262	863.8	1	phoQ: subunit of gene modulating antibiotic efflux [ARO:3000835]

emrB	RF0065	1.60E-200	660	19.8	emrB: subunit of efflux pump conferring antibiotic resistance [ARO:3000074]
msbA	RF0107	3.60E-213	701.7	6.5	msbA: ATP-binding cassette (ABC) antibiotic efflux pump [ARO:3000460]
ABC_efflux	RF0007	1.90E-141	465	0.1	ATP-binding cassette (ABC) antibiotic efflux pump [ARO:0010001]
Qnr	RF0113	5.90E-123	401.4	8.6	quninolone resistance protein (Qnr): antibiotic target protection protein [ARO:3000419]
CTXM	RF0059	1.50E-192	631.1	4.4	CTX-M beta-lactamase (class a) [ARO:3000016]
ClassA	RF0053	6.40E-129	421.9	0.4	Class A beta-lactamase [ARO:3000078]
Chlor_Acetyltrans_CAT	RF0050	2.20E-85	278.6	5.8	chloramphenicol acetyltransferase (CAT) [ARO:3000122]
APH3	RF0033	2.50E-43	141.2	0	aminoglycoside phosphotransferase (APH3) [ARO:3000126]
APH6	RF0034	2.40E-150	492.5	1.6	aminoglycoside phosphotransferase (APH3) [ARO:3000151]
TEM	RF0126	1.80E-205	673.4	0	TEM beta-lactamase (class a) [ARO:3000014]
ClassA	RF0053	1.70E-130	427.1	0	Class A beta-lactamase [ARO:3000078]

4.9.5 Genes conferring resistance to β-lactams

All 4 databases, namely ARDB, CARD, ResFams and CGE were able to identify the resistance determinants which confer resistance to β-lactam antibiotics. The resistance determinants were namely β-lactamase CTX-M-15 and β-lactamase TEM-1B.

The identified genes exhibited 100% similarity to the already reported sequences in other organisms.

4.9.6 Fluroquinolone Resistance Genes

Resistance determinants known to cause fluroquinolone resistance were identified, namely *qnr*S1, *mfd* and *gyrA*. *gyrA* encoding gyrase exhibited 99% identity to the already reported *S. enterica* with S83F mutation detected in the sequenced genome. *Mfd* gene encodes antibiotic target protection protein; providing protection from fluroquinolone antibiotics. *qnr*S1 was also identified in the sequence. Various efflux pumps that confer resistance to fluroquinolone antibiotics were identified in the sequence. These *included mdtK, acrD, acrB, tolC, acrF* and *acrA.*

4.9.7 Genetic Environment of Antibiotic Resistance Genes

Antimicrobial resistance elements including *qnrS1*, $bla_{\text{CTX-M15}}$, bla_{TEM1}, *catA1*, *dfrA7*, *sul1*, *sul2*, *strA*, and *str* were concentrated in one region within the genome. In this region, the resistance genes were flanked by various mobile elements including Transposases, Transposons, Insertion sequences, Recombinases,

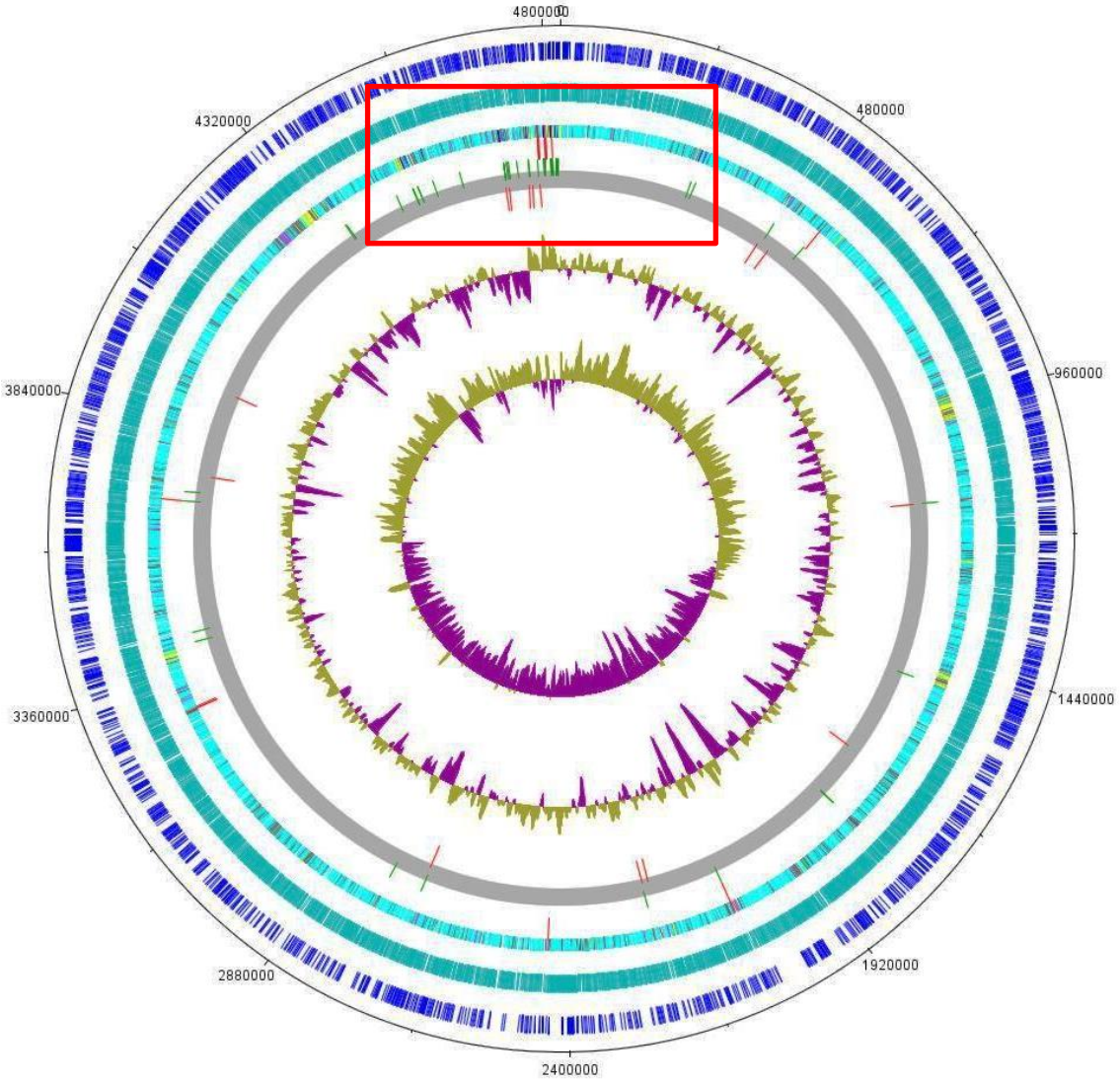


Figure 16: CDS plot of *S. typhi* isolate as presented by DNA Plotter 1.11. The plot shows location of various Antibiotic Resistance genes and mobile element proteins along with other CDSs. This could be a putative resistance island. *Track Order: CDS; Forward Strand; CDS Reverse Strand; All CDSs* (*Mobile Element Proteins;* *Antibiotic Resistance Genes*); *GC Content (%); GC-Skew (%).* Above Average; Below Average

4.9.8 Genetic Environment of β-lactamases

Genetic environment of β-lactamases as assessed by manual visualization on Artmeis revealed the presence of various mobile elements flanking β-lactamases genes. TEM 1 was flanked by Insertion Sequence, ISPa38, Tn3, and TnAs1 along withvarious prophage sequences including prophage insertion sequences. The genetic environment of TEM-1 gene is shown in Figure 17.

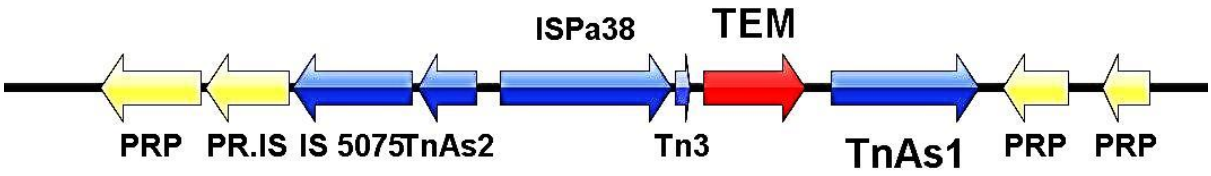


Figure 17 Genetic environment of TEM-1B gene in Genome; TnAs1, TnAs2 – Transposases; IsPa38, IS 5075- Insertion Sequence; PRP – Prophage, PRP.IS –Prophage Insertion Sequence

Similarly, *bla*$_{\text{CTX-M15}}$ was also flanked by various transposes and recombinases. Quinolone resistance protein, *qnrS1*, was also present on the same coding frame downstream of *bla*$_{\text{CTX-M15}}$ gene (Figure 18).

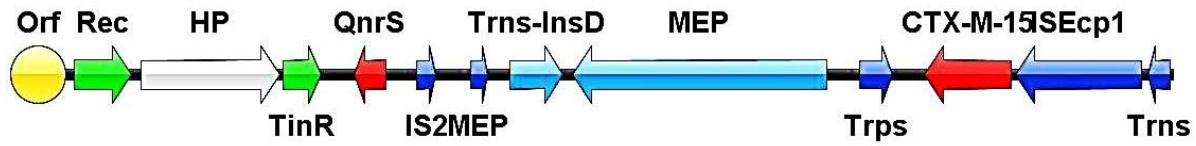


Figure 18 Genetic environment of blaCTX-M-15 gene within the genome: Orf -Orf 477; QnrS- Quniolone Resistance Determinant; MEP –Mobile Element Protein; TinR – Resolvase/Integrase; Trns-InsD - Transposon, Trps – Transposases, ISEcp1, IS2 – Insertion Sequence; Trns –Transposons, Rec – Recombinase

4.10 GENOMIC ISLAND PREDICTION

Genomic Islands were predicted using IslandViewer4 with 6 reference complete genomes: : str. CT18 (NC_003198.1, NC_0033384, NC_003385); str. Ty2 (NC_004631); str. Ty21a (NC_021176.1), str. P-Stx-12 (NC_016832.1, NC_016825); str. B/SF/13/03/195 (CP012151.1) and str. PM016/13 (NZ_CP012091.1). The analysis revealed the presence of a ~300Kb genomic island that was not present in either of the 6 reference genomes (Figure 19 and Figure 20).

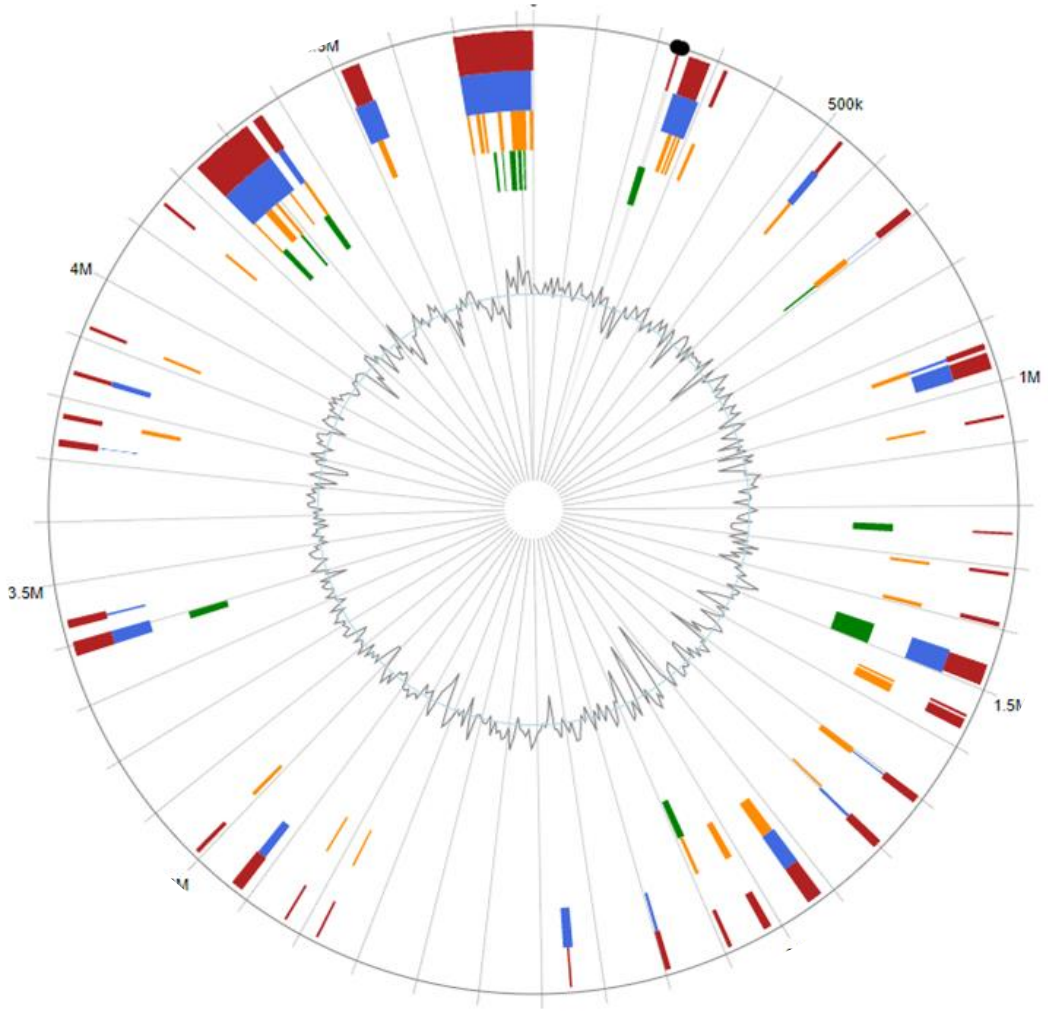


Figure 19 Genomic islands for *S. typhi* RWP1_PK1 as predicted by IslandViewer4 using Integrated, IslandPath-DIMOB, SIGI-HMM, IslandPick, and Islander programs as depicted by different colors. The innermost cirlcle shows GC Skew the outermost circle solid grey line represents the total genome length.

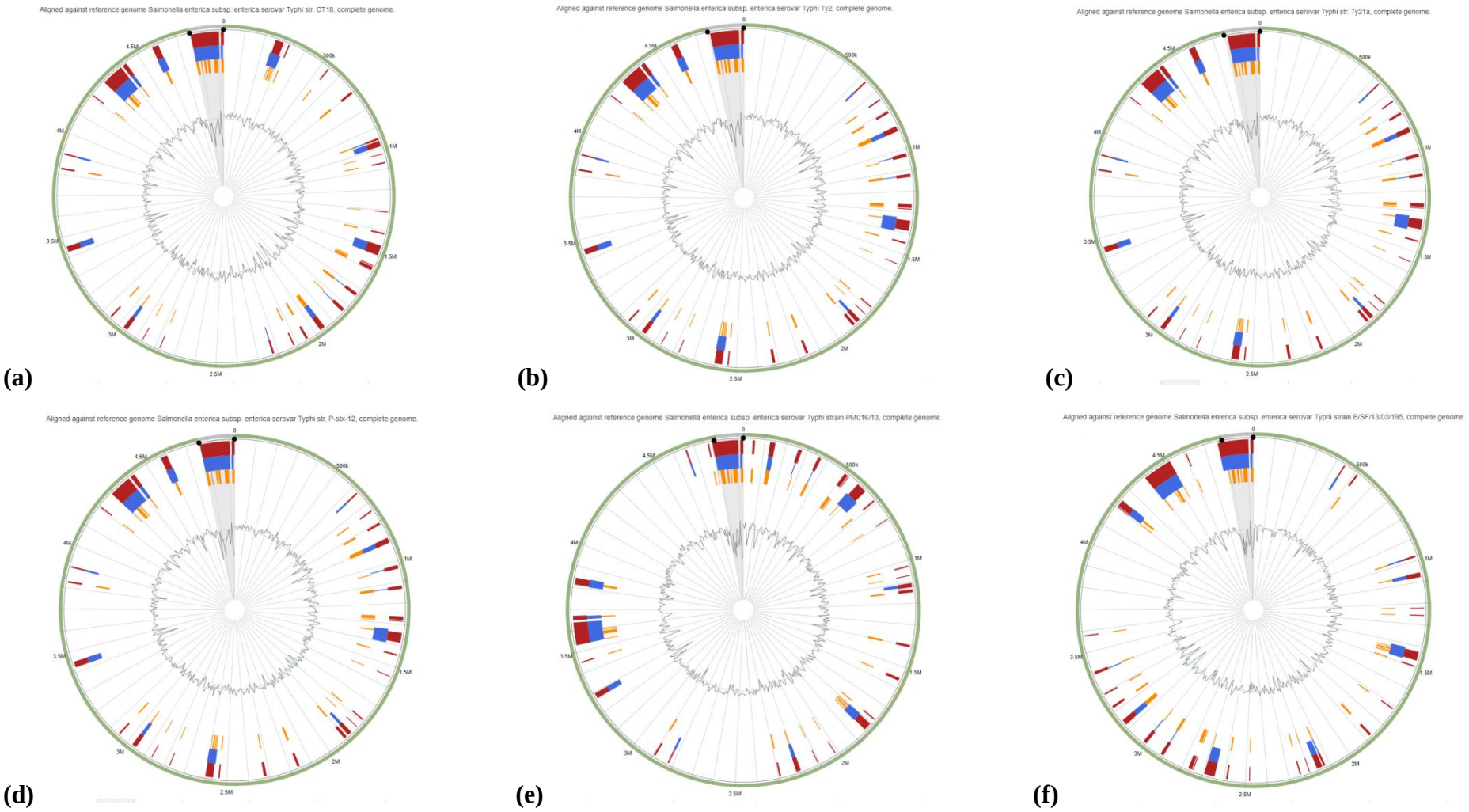


Figure 20Position of putative genomic islands predicted for *S. typhi*. RWP1-PK1 against 6 complete genome sequences of *S. typhi* using IslandViewer4. Alignment against 6 compete genome sequences show a putative resistance island (represented in grey highlight). (a) str. CT18 (NC_003198.1, NC_0033384, NC_003385) (b) str. Ty2 (NC_004631); (c) str. Ty21a (NC_021176.1), (d) str. P-Stx-12 (NC_016832.1, NC_016825); (e) str. B/SF/13/03/195 (CP012151.1) (f) str. PM016/13 (NZ_CP012091.1)

5 DISCUSSION

The present work aimed at identifying various resistance determinants present in the highly resistant *Salmonella enterica* subsp. enterica ser. Tyhi isolate confering high level of antibiotic resistance.

The clinical isolate was recovered from a previously healthy patient, who showed no imporvement in symptoms by the administration of 3rd generation cephalosporin (ceftriaxone) and 2nd generation fluroquinolone (ciprofloxacin, levofloxacin) antibiotics. The blood culture report not only reported resistance to 3rd, but the 4th generation (cefepime and moxifloxacin) of the above mentioned classes of antibiotics, which was alarming since ceftriaxone, ciprofloxacin and levofloxacin are the antibiotic empirically and commonly used for the treatment of *Salmonella typhi* infection. This limited the treatment option to only Carbapenem class of antibiotics, resistance to which has been seen to be rising due to rapid spread of genes imparting carbapenem resistance among pathogens which are globally spread (Hsu et al., 2017; Pesesky et al., 2015).

Antibiotic susceptibilty assays, KB Disk Diffusion and Minimum Inhibitory Concentraiton Assay of the anitbiotics especially, to the cephalosporin and fluoroquinolone antibiotics were performed. MIC assay was performed since it provides a quantitative information for resistance as compared to simple classfication by KB disk diffusion into susceptible, intermediate or resistant phenotypes. Broth Microdilution assay was performed for MIC determination to test the maximum number of anitbiotcs in minimum time using minimum volumes to make the procedure cost, time and labor efficient as compared to agar dilution and broth macrodilution method (Jorgensen & Ferraro, 2009; Mayrhofer et al., 2008; Wiegand et al., 2008). Similar approach to determine the extent of reistance in a clinical isolate exhibiting, rather

high level of resistance has been done by Sjolund-Karlsson, Howie, Crump, and Whichard (2014) where *S. typhi* isolate exhibited resistance to fluoroquinlone. Another research by Morita et al. (2010) also used broth microdilution assay to determine extent of cephalosporin resistance among 48 *S. typhi* clinical isolates.

Antimicrobial susceptibility assasys are also a used to clasifiy an organism as either Multidrug Resistance, Extensively Drug Resistant or Pan Drug Resistant depeneding on the number of classes of antibiotics to which the bacterial isolate is resistant (Magiorakos et al., 2012). The current isolate was resistant to more than three classes of antibiotics tested i.e. Penicllins, Cephalosporins, Monobactams and Fluoroquinolones.while it showed susceptibility only to Carbapenem class of antibiotics. This highlights the first incidence of the emergence of an extensively drug resistance *Salmonella enterica subsp. enterica* ser. Typhi.

High minimum inhibitory concentration also highlights the inefficacy of currently tested antibiotics even at a higher dose (Andrews, 2001). The mean plasma concentration after a 1g dose of cefepime reaches a maximum of 243 mg/L (µg/mL) after 3 days of 2g IV infusion every 12 hours in burn patients. In another study, the concentration of cefepime reached a maximum of 3.2 mg/L (3.2µg/mL) in critically ill patients with normal serum creatinine clearance (Endimiani, Perez, & Bonomo, 2008; Lipman, Wallis, & Rickard, 1999; Sampol et al., 2000). Compared to the current MIC results of the isolate which were 256µg/ml for cefepime and higher than 256 µg/ml for the rest of the cephalosporin antibiotics from the 1^{st}, 2^{nd} and 3^{rd} generation, all the cephalosporin drug could be referred to as entirely ineffective for treatment of Typhoid infection, if infected by a similar microbe. The situation is also alarming since using the higher dose of the IV antibiotic might be able to suppress the infection in limited run but it will not prove effective in reducing the emergence of anitbiotic resistance since the plasma concentration of the

antibiotic in blood should exceed by 8-10 times the MIC value in order to suppress the development of antibiotic resistance (Levison & Levison, 2009).

Fewer studies have reported a higher MIC value to cefepime in *S. typhi* isolate have been reported before (Ahmed et al., 2012; Gonzalez-Lopez et al., 2014; Pfeifer, Matten, & Rabsch, 2009; Rotimi et al., 2008). Resistance to cefepime has been widely reported for clinical isolates of nosocomial infectious agents such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinteobacter baumanii* and *Klebsiella pneumonia*, but fewer instances have been observed in infection by *S. typhi*, a strictly human pathogen (Akhabue, Synnestvedt, Weiner, Bilker, & Lautenbach, 2011; Chong, Yakushiji, Ito, & Kamimura, 2010).

Most of the acquired genes are present on the plasmids especially in the case of *Salmonella typhi* since it carries no intrinsic resistance β-lactamse determinants unlike other *Salmonella enterica* serovars . Antibiotic resistance determinants are mostly acquired via three mechanisms, transduction, transformation or conjugation where most of the gene acquisiton occurs via transduction and conjugation (Bennett, 2008; Verraes et al., 2013). Conjugation experiments have shown how plasmids and other resistance determinants are shared between closely resistant bacterial species such as *E. coli* and *S. enterica*; with transconjugant exhibiting similar resistance as that of donor (Morita et al., 2010; Van Meervenne et al., 2012)

. Our hypothesis was based on this horizontal or vertical gene transfer due to which the clinical isolate has acquired a resistance determinant/s causing an increased resistance to antibiotics. Efforts to isolate plasmid via alkaline lysis method did not yield any genetic material, i.e. the plasmid could not be isolated. A possible explanation lies in that the bacterial isolate does not harbor the plasmid as separate genetic molecules, unlike CT18, and P-Stx-12 strains ((Ong et

al., 2012; Parkhill et al., 2001) but the plasmid is integrated within the chromosome, carrying along the resistance determinants .

To further elucidate this incorportation of plasmid carrying resistance determinants in the chromosome, Plasmid curing assays were performed. Plasmid curing assay was done to see if loss of plasmid by curing agents such as Ethidium Bromide, SDS or high temperature causes a change in susceptibility to antibiotic (Zaman, Pasha, & Akhter, 2010). A similar approach was used by Barman et al. (2010) to distinguish between the resistance determinants carried by chromosome and plasmid in a clinicla isolate of *Shigella flexneri*. It was seen that the loss of a 6.3 kb plasmid an increase in susceptibility and loss of resistance to streptomycin and sulfamethoxazole while the intrinsic resistance genes, which are not carried by plamid, continuted to confer resistance to trimethoprim, tetracycline, ampicillin and chloramphenicol. Another study by (Maheshwari, Yaser, Naz, Fatima, & Ahmad, 2016) revealed the loss of resistance simultaneously to ciprofloxacin and cefotaxime by loss of plasmid curing in ESBL positive *E. coli* isolates. On the other hand, no loss of plasmid based on loss of antibiotic resistance was observed in plasmid curing experiments with using SDS, EtBr and higher growth temperature as plasmid curing agents. This could also explain why no plasmids could be isolated during plasmid isolation.

To identify the resistant determinants, we performed PCR for detection of betalactamase resistance genes. PCR was performed for identification of $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. Only $bla_{\text{CTX-M}}$ showed positive PCR results. PCR identification of only $bla_{\text{CTX-M}}$ gene coincides with the previous reports of β-lactamase CTX-M causing resistance to higher generation cephalosporins (K. Bush & Fisher, 2011; Canton, Gonzalez-Alba, & Galan, 2012; Rawat & Nair, 2010).

Whole Genome sequencing was performed by using Illumina/Solexa to better identify the resistance determinants that could possibly explain the extended antimicrobial resistance in the clinical isolate. We obtained 128 contigs that were annotated using four different annotaion systems, Prokka, RAST, PATRIC and PGAP.

Using WGS data, *insilico* Multi Locus Sequence Typing (MLST) was performed using MLST 1.8. to identify the sequence type present in the isolate by identification of the alleles of 7 house keeping gene. The allelic forms reveal that the sequence type of the clinical isolate belongs to ST1 clonal MLST group of *Salmonella typhi*. ST1 clonal group has been attriubted to be prevalent in endemic region of South East Asia. This could also means that the the current isolate has evolved to exhibit such resistance pattern by acquisition of genes from within the endemic region since no allelic variation was observed as compared to the reported MLST sequences for *S. typhi*.

PlasmidFinder-1.3 server hosted by Center of Genomic Epidemiology also found plasmid sequences for IncY and IncQ1 plasmid within the WGS data provided. The IncQ plasmid has been reported as a self-transmissible, highly mobile plasmid among pathogenic bacteira as a vector for transfer of resistance genes with a broad host range (Rawlings & Tietze, 2001). Genomic signature of a recent outbreak in 2012 of typhoid fever was molecularly studied by Hendriksen et al. (2015) in Zambia between 2010 and 2012 also reported the presence of chromosomally translocated plasmids in 4 isolates carrying similar resistance determinants "*catA1*, $bla_{\text{TEM-1}}$, *dfrA7*, *sul1*, *sul2*, *strA*, and *str*" along with IncQ1 plasmid sequences.

SNP analysis was also done in order to determine the closest phylogenetic relative of the *S. typhi* RWP1_PK1 when compared with complete genomes from different regions. SNP

analysis revealed the isolate to be closesly related to *S. typhi* str. P-Stx-12, an MDR *S. typhi* sequence reported from India (Ong et al., 2012). Using these results, SNP analysis using RealPhy was done with 44 complete genomes from Pakistan with *S. typhi* str. RWP1_PKQ and by using P-Stx-12 as a reference since that was the closest relative of the complete genomes. Analysis revealed the closest relative to be *S. typhi* str. MDUST 156, a genome sequence of another MDR *S. typhi* isolate from Pakistan. This analysis thereby confirmed that *S. typhi* str. RWP1_PK1 developed its XDR characteristic within the endemic region of Pakistan. A study by Bakker et al. (2011) carried out SNP analysis to study the outbreak of a food borne pathogen, *Salmonella enterica* serovar Montevideo, using NGS analysis. The results confirmed the outbreak to be background cases, rather than food borne. Similarly, another study by Makendi et al. (2016) geographically segregated *Salmonella enterica* servoar Weltevreden by SNP analysis into two phylogentic clusters; one predominant within South East Asia, while the other was globally disseminated.

Afterwards, various antibiotic resistance determinants were identified within the genome contributing to the high level of resistance to cephalosporin and fluoroquinolone antibiotics via various antibiotic resistance databases. The genes were identified using ResFinder, Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017; McArthur et al., 2013), Antibiotic Resistance Database (ARDB) and ResFams. It was seen that CARD was more efficient than the rest of the databases in this particular senario, where it not only identified all the acquired genes buts also showed drug resistant genes that are chormosomally encoded. CARD also identified the presence of Mercury resistance operon which holds significant value in terms of its co-existence with other antibiotic resistance genes carried by IncQ plasmid. ARDB

and ResFinder also did not identify the chormosomal mutations. Other than that, CARD also identified multiple efflux pump that could confer increased resistance to antibiotics.

Genomic analysis revealed presence of CTX-M-15 and TEM-1 β-lactamase determinants by all antibiotic resistance databases except ARDB which failed to identify TEM-1. ResFams also identified a Subclass B3 metallo-β-lactamase which hydrolizes penicillins, cephalosporins and carbapenems. When the sequence was further confirmed using blastx, the results reported the gene to be 100% identical with no known function in imparting resistance to antibiotics. Other than that, resistance to cephalosporins but not to carbpenem antibiotic (MEM <0.5 mg/L, IMI 1mg/L) confirms the gene to be putative with no function.

The isolate exhibited resistance to cefepime, a 4th generation antibiotic, and all lower generation antibiotics like ceftriaxone (3rd generation cephalosporin) and cefotaxime (2nd generation cephlaospoirn) while showing suceptibility to carbapenems. Presence of CTX-M-15 has been reported to impart higher level of resistance in microbes to 4th generation cephalosporin like cefepime. CTX-M-15 and cefepime resistance. This was also reported by Barguigua et al. (2011) where presence of CTX-M-15 imparted MICs higher than 64 µg/ml in *K. pneumoniae* and *E. coli* isolates from Moroccan community. A similar study showed that most of the enterobacteriaceae isolates harboring a similar β-lactamase, CTX-M-14, were resistant to cefepime were also resistant to all lower generation cephalosporins but susceptible to carbapenems only (Chong et al., 2010).

Cefepime resistance has also been highlighed in a few studies previously in clinical isolates of *S. typhi*. Rotimi et al. (2008) conducted a wide surveillance of β-lactamase producting *Salmonella* spp. in Kuwait and UAE. Their results reported the presence of CTX-M type

extended spectrum β-lactamases in numerous of the 407 studied isolates. 14 of the isolates had $bla_{\text{CTX-M-15}}$, 2 of which were *S. typhi* isolates. These 2 isolates exhibited higher level of resistance as compared to the rest of the CTX-M-15 positive isolates.

Another study by Gonzalez-Lopez et al. (2014) reported a similar higher level of resistance to cephalosporin and β-lactam antibiotics in a clinical isolate in Guatemala. The patient was treated with an IV of entrapenem till complete recovery. Phenotypic and genotypic analysis revelaed the isolate to be resistant to cefepime (>256 mg/mL) and carrying $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ gene. The study by Gonzalez-Lopez et al. (2014) therefore reported the 4th case with a CTX-M-15 positive isolated *S. typhi* with Asian origin previously reported in Iraq, India and Bangladesh (Ahmed et al., 2012; Pfeifer et al., 2009; Rotimi et al., 2008). To our best knowledge, our study reports the 5th case in Asia and the first case in Pakistan of $bla_{\text{CTX-M-15}}$ positive isolate exhibiting resistance to Cefepime 4th generation cephalosporin.

The MIC values of 2nd generation fluroquinolone (ciprofloxacin, levofloxacin, and 4th generation fluroquinlone (Moxifloxacin) were higher than the clinical breakpoint standard set by EUCAST. To our knowledge, resistance to Moxifloxacin has not been reported before at all in *S. enterica*. Various resistance elements were identified that could explain the reduced susceptibilty of the isolate to fluroqinolones. Computational analysis reveals that a single point mutation in QRDR, S83F, was present in the *gyrA* gene. No point mutations in *parC* in the QRDR was noted although point mutation were observed at other regions in *gyrA* and *parC* genes. Mutations in *gyrA* and *parC* (drug targets of fluroquinolone antibiotics) are the widely attributed cause of resistance/reduced susceptibility to the concernred antibiotic (Ruiz, 2003).

Changes in *gyrA* gene leads to reduced susceptibility for 2nd generation fluroquinolone antibiotics. For instance, a single point mutation of S83F causes an increased MIC value (Biedenbach, Toleman, Walsh, & Jones, 2006). It has been reported that isolates with double mutations occurring at codon 83 and 87 in *gyrA* have higher MICs for quinolone antibiotics. Multiple mutations occurring in either a single or both of the target genes are known to cause significantly higher resistance to the ciprofloxacin and levofloxacin. This was also observed in a study conducted in Korean hospitals where all ciprofloxacin resistant *E. coli* isolates carried double mutations in *gyrA* and a minimum of single mutation in *parC* gene(Komp Lindgren, Karlsson, & Hughes, 2003; Moon et al., 2010; Morgan-Linnell & Zechiedrich, 2007). A study by (Gopal et al., 2016) highlights the presence of Ser83 mutation in 94 % of the *S. typhi* isolates studied which causes a reduced susceptibility to ciprofloxacin antibiotics. Since, a single mutation was observed in this isolate, this points out to the presence of other resistance genes conferring such significantly high resistance level.

Presence of a variant of aminoglycoside acetyltransferase allele, AAC(6')-Ib-cr has also been reported to impart resistance to hydrophilic fluroqinolones such as ciprofloxacin and norfloxacin but not to others such as levofloxacin and moxifloxacin. The mechanism of action relies on modification and reduction of activity of hydrophilic fluroquinolone (Luzzaro, 2008; Robicsek, Jacoby, & Hooper, 2006). This allelic form of aminoglycoside acetyltransferase has often been associated with increased level of resistance but no instance of this resistant gene was identified in our isolate. This also confirms as to why the MIC value of ciprofloxacin, a hydrophilic fluroquinolone antibiotic, was lower in our isolate as compare to levofloxacin and moxifloxacin.

qnrS1 was also identified to be present in the sequence, which confers resistance to fluoroquinolone antibiotics by binding to target site on *gyrA,* thus protecting *gyrA* binding of antibiotic molecule. It was identified as the first fluroquinolone resistance determinant to be transferred by plasmid causing reduced susceptibility to the concerned group (PMC4626314). A transconjugant study where an *E. coli* isolate was transformed with *qnrS* and *qnrB* caused an 8 fold increase in MIC of ciprofloxacin (Cattoir, Poirel, & Nordmann, 2007; Strahilevitz, Jacoby, Hooper, & Robicsek, 2009). In another study, transformation of *Shigella flexneri* 2b strain with *qnrS* caused an 8-fold increase in MIC from 1µg/mL to 8 µg/mL (Hata et al., 2005). Therefore, the increased resistance to fluroquinolone antibiotics could be attributed to the presence of *qnrS* within the genome.

Various other resistance elements were also identified that can explain this enhanced resistance to fluoroquinolone. These include resistance gene making up the AcrAB-TolC efflux pump such as Another study highlights that increased expression of AcrAB-TolC efflux pump with or without single or double mutations in *gyrA* and *parC* mutation were found to be causing ciprofloxacin resistance (Zhang et al., 2017). It is highly probable, that the increased expression of AcrAB-TolC could be the contributing factor in the increased fluroquinolone resistance.

Genome comparison revealed presence of a ~300 Kb of unaligned region when compared with 3 complete genomes of *S. typhi*; str. CT18, str. Ty2, and P-Stx-12. This same region also contained most of the antimicrobial resistance genes identified by various databases including but not limited to $bla_{\text{CTX-M15}}$, bla_{TEM}, *qnrS1* along with various mobile elements such as insertion sequences, transposons, transposases, and recombinases. This elucidate that the isolate carries a novel putative resistance island (Juhas et al., 2009; V. Miriagou, Carattoli, & Fanning, 2006).

Further downstream analysis using IslandViewer4 when compared with 6 complete reference genomes available on the database confirmed the presence of an unaligned region harboring antimicrobial resistance genes as highlighted in Figure 19. The region can be classified as a Resistance island because it carries various antimicrobial resistance genes such as $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *qnr*S1 along with *catA1*, *dfrA7*, *sul1*, *sul2*, *strA*, *strB* along with vairous mobile elements such as insertion sequences, transposons, and recombinases (Davies & Davies, 2010; V. Miriagou et al., 2006). The IncQ1 and IncY plasmid seqeueces identified by PlasmidFinder were also present within the same genomic island. Thus, signifying the incorporation of the island from plasmids within the chromosome of the bacterial isolate. The results coincide with earler findings where incorporation of a pathogenomic island within the genome of *Acinetobacter* caused an increase in resistance to various antibiotics (Nigro, Farrugia, Paulsen, & Hall, 2013).

The results have highlighted and elucidated the increased resistance to various contributing factors acquired by the *Salmonella enterica* subsp. enterica serovar Typhi str. RWP1_PK1. These results therefore characterize the strain to be an extensively drug resistant strain of a clonal subtype of *S. typhi* prevalent within the endemic region of Pakistan.

6 CONCLUSION

The present study was focused on characterizing a clinical isolate of highly antibiotic resistant *Salmonella enterica* subsp. enterica ser. *typhi* isolated from a previously healthy patient. The isolate was characterized phenotypically by biochemical testing and antibiotic susceptibility assay using commonly used antibiotics which revealed the isolate to be highly resistant. Susceptibility assays against all commonly used antibiotic belonging to different classes of antibiotics revealed the strain to be resistant to all antibiotics including the higher generation β-lactam and fluroquinolone antibiotics. The resistance level was significantly high for cephalosporin antibiotics, with MIC of 256 µg/mL for Cefepime and >256 µg/ml for other antibiotics of this generation. Similarly, higher MICs for Ciprofloxacin, Levofloxacin and especially Moxifloxacin (2 µg/mL, 4µg/mL and 4µg/mL respectively) was also alarming.

Genotypic characterization was also done by PCR identification of ESBL genes and Whole Genome Sequencing. Plasmid curing assay was also performed to determine the presence of any plasmid carrying antibiotic resistance determinant which were negative for loss of plasmids. WGS data was annotated using Prokka, RAST and PATRIC. The genomic data was subjected to *in silico* MLST analysis, serovar identification and SNP analysis using tools available at CGE. Antibiotic resistance determinants were identified using ARDB, CARD, and ResFams. Various antibiotic resistance genes including β-lactamase and quinolone resistance genes were also present.

7 FUTURE PROSPECTS

Salmonella enterica subsp. enterica serovar Typhi, the etiological agent of Typhoid, is a highly infectious pathogen that is prevalent in developing nations. The current study reports the emergence of a first extensively drug resistant *Salmonella enterica subsp. enterica* serovar Typhi isolate that showed resistance to all clinically prescribed antibiotics. Resistance to more than three classes of antibiotics, lack of plasmids and presence of a novel antimicrobial resistance island harboring various antibiotic resistance genes were the key highlights of the present study

The present research work can be furthered in various ways. Firstly, the isolate showed resistance to all clinically prescribed antibiotic for treatment of Typhoid. This calls for an extensive surveillance of antimicrobial resistance especially for resistance to higher generation cephalosporin and fluroquinolone drugs in *Salmonella spp.* within the endemic region. Increased resistance or reduced susceptibility to these antimicrobial agents could also indicate presence of novel antimicrobial resistance genes harbored within the genome of *Salmonella spp.* alongside novel virulence determinants.

Secondly lack of plasmid within the genome signifies the shift of dependence from carrying an antibiotic resistance conferring plasmid to chromosomally encoded resistance island. Therefore, the study also signifies conducting a molecular epidemiological surveillance by genotyping plasmids, pathogenicity islands and resistance islands within the *S. typhi* genome.

Most importantly, decreasing antimicrobial options for treatment of such highly infectious pathogens with high mortality also calls for importance of designing appropriate combination therapies for curtailing development of further antimicrobial resistance.

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