Tracking of Novel Plasmid mediated Azithromycin's resistant & virulence genes in Salmonella Typhi

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This work is submitted as a MS thesis in partial fulfillment of the requirement of the degree of

MS in Biomedical Sciences

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

Dedicated to my incomparable parents and adored siblings whose marvelous support and backing led me to this wonderful triumph

Declaration

I certify that this research work titled "*Tracking of Novel Plasmid mediated Azithromycin's resistant & virulence genes in Salmonella Typhi"* is my own work. The work has not been presented elsewhere for consideration. The objects that has been used from other sources it has been appropriately acknowledged / referred.

Signature of Student

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Copyright Statement

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Abstract

Typhoid fever is a serious public health threat for which the needs of antimicrobial treatment are increasing. This acute illness coupled with fever caused by the virulent and pathogenic multi-drug resistance plus extensively-drug resistant *salmonella enterica typhi* bacteria. A total of 85 fresh human clinical *Salmonella Enterica Serovar typhi* isolates were recovered from patients in different hospitals of Pakistan during the period of 2020-2021. Of the total 85 samples, 35 (40.4%) strains were phenotypic azithromycin-resistant while, 24 (28.5%) were genotypic azithromycin-resistant strains of S.typhi. The PCR analysis publicized that 59 isolates (69.5%) contained acrB gene expression and 46 isolates (54.7%) carried rplV gene expression and all the isolates carried rplV gene expression were also positive for acrB gene. Similarly, the virulotyping of three specified genes such as; pefA, sopE1 and gipA by polymerase chain reaction (PCR) of salmonella typhi isolates was also done in this study. By using Pearson's chi-square test, a significance differences were observed between MDR and XDR status with specified virulence genes. However, no significant differences were identified among any virulence gene and antibiotic resistance patterns at less than p value ($p<.05$). In addition, the final product of two-way factorial ANOVA identifies significant differences between both factors i.e., virulence genes and AR patterns, with F calculated is bigger than F-critical value. However, the interaction effect between virulent and AR-status was not significant, as F-calculated was smaller (1.014) than F-critical (3.84). This study is designed to determine whether there is a complex relationship exists between AR status and virulence genes of S-typhi. However, the study of rplV gene expression is Novel in Pakistan and is conducting for the first time in this study. The findings from this study will help the scientific community to permit a better perceptive of pathogenic character of salmonella typhi and to develop a modified-treatment strategy that will help in the management, cure and prevention from salmonellosis.

Key Words: *Typhoid fever, Antimicrobial resistance, Salmonella Typhi, Azithromycin, Salmonellosis, virule*

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INTRODUCTION

Food borne diseases encompass a spacious range of illnesses from diarrhoea to cancers. Diseases causing diarrhoea are a major dilemma around the world, even if the burden is carried disproportionately by low and middle-income countries and by children less than 5 years of age. The recent report from the Food borne Diseases Active Surveillance Network, [Foodnet] showed that Salmonella has become a leading cause of death as food-borne bacterial pathogens in the US [Behravesh., 2011]. S typh is one of the predominant serotypes among, 2500 serotypes of salmonella in many developed and developing countries which causes Typhoid fever, and the global outbreak of food-borne diseases due to infection by S.Typhi is inspiring [Wang., 2017]. The typhoid fever ranges between 11-21 million cases and roughly 128,000-161,000 deaths annually, according to recent report released by World Health Organization [2018]. S Typhi infection has also been often reported in Pakistan and was found to be the most prevalent serotype and is now one of the serious health problems in Pakistan.

Salmonellosis is a major intestinal epithelium infection caused by genus salmonella that infects humans from a variety of different food products includes salmonella Typhimurium and salmonella Enteritidis [Yang et al., 2016]. The occurrence of salmonellosis in humans is still lofty due to the rate of virulence factors in *Salmonella enterica* which take part in the process of infection in the host and the spread of disease in humans and animals [Andesfha et al., 2019].

Antimicrobial Resistance (AMR) is an ongoing issue in the treatment of typhoid fever. The MDR strains of S.Typhi have been endemic in Pakistan for the last two decades. In 1990s, the rise of multidrug resistance (MDR) was followed by flouoroquinolones resistance, resulted in limited cure options. At the end of 2016, the first outbreak of extensively drug resistant (XDR) strains of typhoid was observed in Sindh, Pakistan which was caused by MDR S.Typhi that had become resistant to ciprofloxacin and third generation cephalosporin [Nizamuddin et al., 2021]. These XDR variants of S. Typhii are resistant to ampicillin, fluoroquinolones, chloramphenicol, co-trimoxazole, streptomycin, and third generation cephalosporin [Iqbal et al., 2020]. The spread and emergence of extensively drug resistance (XDR) S.Typhi serotype in Pakistan, has left azithromycin as the only realistic option for typhoid treatment in Pakistan [M Levine et al., 2018]. With the increasing use to this

Antimicrobial agent in Pakistan has left specific concerns, is the emerging resistance against azithomycin, the only remaining oral drug to treat XDR typhoid cases [Sajib et al., 2021].

The pathogenesis of salmonella typhi, which causes typhoid fever in humans, is mainly endorsed by the acquisition of horizontally transferred DNA elements. The salmonella pathogenicity islands (SPIs) are the major form of horizontally acquired DNA with respect to pathogenesis of this bacterium. The mutations in any of these transferable SPI may have impact on virulence potential of salmonella typhi [Liaquat et al., 2018]. Similarly, the virulence factors of salmonella contribute to systematic infections. This pathogenesis of bacterial strains has been related to various virulence genes presents on chromosomal salmonella pathogenicity islands [Nayak et al., 2004]. Every gene has its own function for example; genes such as invA presents on SPI, allows salmonella typhi to invade epithelial cells. While, the salmonella outer protein (sops), which is encoded by sop gene have relevance to salmonella virulence [Huehn et al., 2010]. Similarly, another gene contributes to the adhesion of S.typhi to epithelial cells are known as plasmid encoded fimbriae (pefA) gene [Murugkar et al., 2003]. There are numerous other chromosomal genes like stn, which encodes enterotoxin production, found to be a causative agent of diarrhoea and spv operon (salmonella plasmid virulence), a plasmid carring virulence gene contributes to the colonization of deeper tissues among other functions [Thung et al., 2018].

The antibiotic resistance (AR) and virulence are the two most significant characteristics of salmonella, and their relationship is complex. It is not known to date if the increase to antibiotic resistance in salmonella has increased the virulence potential of this bacterium and vice versa. Few researches have been conducted to study the relationship between virulence and AR, with different conclusions [Osman et al., 2014 and Yue et al., 2020]. It is observed that phenotypic changes that give antibiotic resistance may be coupled with decreased virulence, but some reports of the opposite that resistance can boost virulence [Roux et al., 2015]. Similarly, one set of data has found that the acquisition of AR has no cost to S.typhi due to compensatory mutations [Andersson et al., 2010].

As the number of reports of azithromycin-resistance (Azm-R) S.Typhi in Pakistan is on the rise. So, this study illustrated the underlying molecular mechanisms in multiple variants of Azm-R salmonella typhi and one Azm-R Paratyphi strain. Here, we developed a PCR based study to detect

the mutations in two most common Azm-R genes i.e., AcrB and rplV in typhoidal salmonella and three highly observed virulence genes known as gipA, pefA and sopE1, which is less costly and undemanding tool to rapidly detect this mutation. This study also determines the relationship between three most dominant virulence genes of S.typhi with drug resistance phenotypic and genotypic salmonella of clinical human isolates. Then, the data sets were analyzed by using a series of statistical and computational methods to find the co-relationship between AR and virulence, the two important characteristics of salmonella. Seven azithromycin resistance strains of S. Typhi have been sent to Finland and further processed for Whole genome sequencing (WGS). We also analysed the upgrading Azithromycin- Resistance profiles of S.Typhi in Pakistan to elucidate the molecular mechanisms underlying the emergence of XDR and specially Azithromycin resistivity in these isolates. Our findings will surely highlights the importance of rising surveillance on these samples with Azm-R, the only last orally administered drug in Pakistan, which will definitely help pharmaceutical companies to discover and develop a new medicine, as alternate to azithromycin. These findings will also provide methodical evidence to help understand the pathogenicity of salmonellosis and its treatment.

METHODS & MATERIALS

This study was permitted by joint association of National Institute of Health, Islamabad and department of Biomedical Sciences and Engineering, National University of Science and Technology Islamabad. This study focuses on identification, isolation and the Resistive Azithromycin and virulence genes profiling of Salmonella Typhi from fresh human clinical isolates.

2.1. Study Design and Specimens Collection

A cross sectional study was conducted at different cities across Pakistan from January to October 2021. Fresh human blood culture isolates were obtained from Typhoid fever patients who had visited five different tertiary care hospitals across Pakistan i.e., PIMS and NIH in Islamabad, Khyber teaching hospital in Peshawar, Jinnah Hospital Lahore and Agha khan Hospital, Karachi. Over eighty five bacterial isolates were arbitrarily selected by different hospitals and sent to the public health and laboratory division, National institute of health Islamabad for further analysis. After cross examination of strains they were then stored at -80 ◦C until use. The S.typhi strains were routinely cultured in nutrient rich broth i.e; brain heart infusion broth and were then streaked on SS- agar plates at 37◦C with an incubation time of 24 h. The blood culturing and isolation method were carried out as per standard protocol.

2.2. Samples conformation Tests

Transparent and translucent colonies with black centers were seen on SS agar surface after incubation of 24 hours. Presumptive colonies were further processed to identify all isolates on the basis of gram staining test and biochemical tests i.e., Indole test and oxidase test. Single presumptive colony from each isolate was stored for one year at -20°C for future research purpose. Identification of samples was also done by API 20E test strips (bioMerieux Vitek, Marcy-l'Etoile, France.).All isolates were then serotyped by slide agglutination with trade antiserum (S&A Reagents Laboratory, Thailand). The identification of isolates was finally confirmed by MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time OF Flight Mass Spectrometry), to cross check all the above described methods, with 99% accurate detection of salmonella enterica serovar typhi.

2.3. Media preparation

Salmonella- shigella (SS, OXOID CM0099) agar were used for the isolation of salmonella species in this study. SS agar is highly selective and differential medium for isolation, differentiation and cultivation of salmonella species and highly recommended for testing clinical specimens [Gray., 1995]. We re-suspended 500 g of powder in 900 ml distilled water and were continuously mixed until the powder becomes dissolved. Then heat to boil shacked frequently, until dissolved completely. Then put them in water bath and cooled to 45-50 °C. After cooling, the freshly prepared media were then poured in Petri dishes in aseptic conditions by using BSC-II (Bio safety Cabinet- class 2). Then let the media to solidify with the lids of plates partially removed. The Petri plates were then incubated at 37 °C for 24 hours and were then stored in refrigerator for one week. After streaking the samples on to the agar surface, the pure salmonella colonies were obtained with opaque, translucent and transparent colonies with or without black centre **(Figure 1)**.

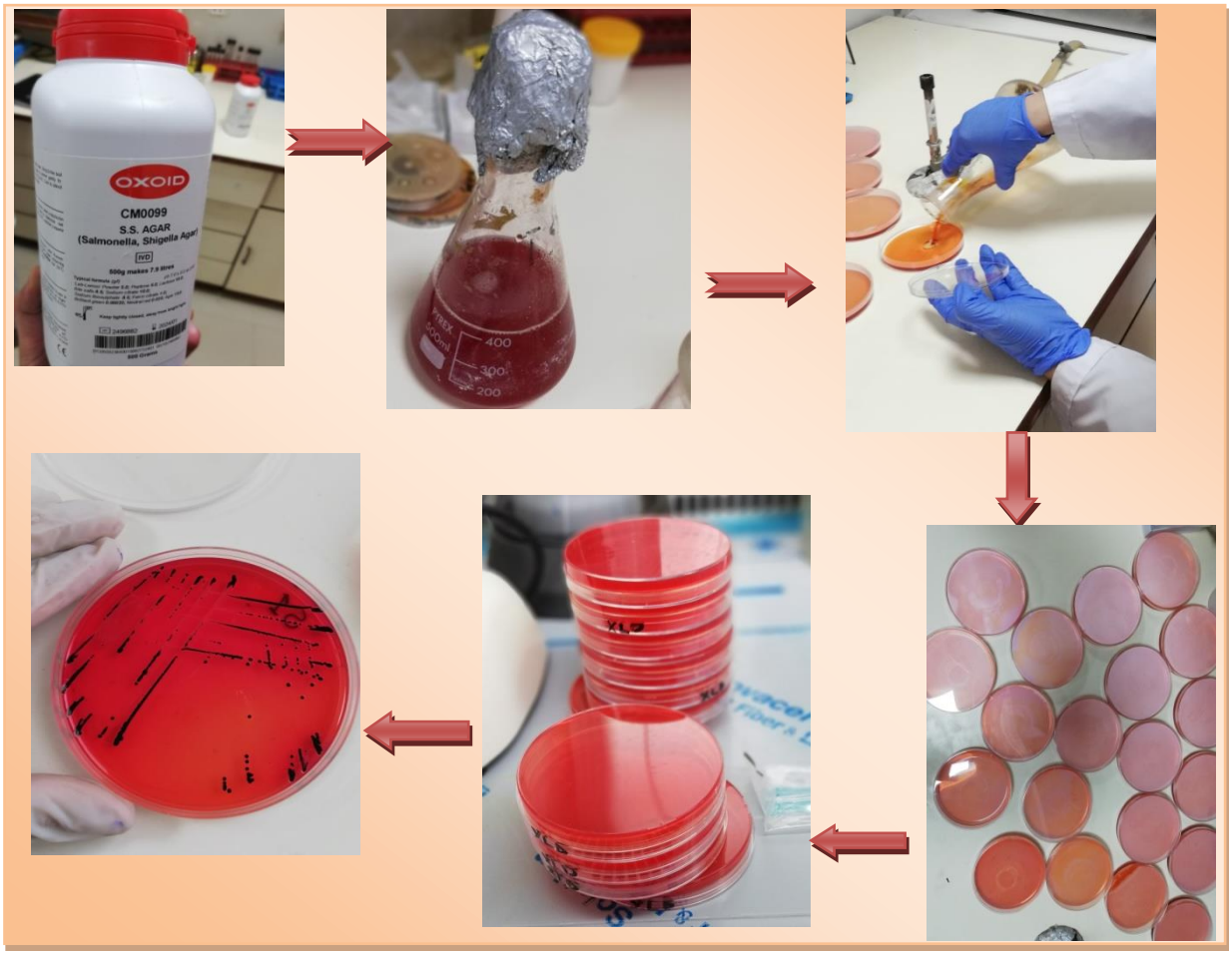

Figure 1: Illustration of Media Preparation

Figure 2: A Graphical representation of Bio Safety cabinet (BSL-II), where a Scientist working in Aseptic conditions

2.4. Kit and Reagents

A variety of reagents and equipment was used in this study. The list of chemical reagents and tools are as following: XLD medium i.e; salmonella shigella (SS) agar plate's specific for salmonella culturing, brain heart infusion broth used as a nutrient rich medium, Oxidase strips, API 20E test strips by Vitek, Marcy-l'Etoile, France, AST discs (Oxoid, UK), microbial detection system such as MALDI-TOF MS (biomerieux, vitek MS) which gives rapid and accurate identification of bacterial strains with 99% accuracy, primers (synthesized by molecular biology products Co., Ltd.) , DNA extraction kit (Qiagen Valencia CA), water bath, centrifuge machine (Eppendorf), DNA Engine PCR amplifier (Bio Rad), gel electrophoresis system; Gel Doc XR and gel imaging system (supplied by Alpha Innotech), 2 × HSTaq Master Mix and 1000 bp DNA marker.

2.5. Disc Diffusion Method

The determination of antibiotic resistivity was conducted by using disc diffusion method (Oxoid, Ltd UK), to test the patterns of susceptibility of S.typhi isolates in concord with the guidelines of clinical and laboratory standards institute (CLSI). For all 85 isolates, a list of 13 different antibiotics was tested in this procedure. The drugs used here in antimicrobial susceptibility test (AST) are as follow; Azithromycin(15 µg) , Meropenum (10 µg), Chloramphenicol (10 µg), Cefotaxime (30 µg), Co-trimoxole (30 µg) , Ampicillin (10 µg), Cefepime (30 µg) , Ciprofloxacin(5 µg), Piperacillin/ Tazobactam (10 µg), Ceftrixone (35 µg), Imipenum (10 µg) , Cefixime (5 µg), Cefoperazone/Sulbactum (30 µg). By performing discs diffusion technique, freshly 24 hours incubated bacterial cultures were inoculated into test tubes with the help of culture loops, while each test tube contains 10ml of nutrient broth agar. The test tubes were then incubated at 37oC for 24 hours. After 24 hours, turbidity of salmonella cultures was adjusted with McFarland solution. The antibiotics discs were gently placed on uniformly prepared lawns with the help of sterilized forceps, against each isolate on nutrient agar media plate at equal distances. After placement of discs on nutrient agar media, all the Petri dishes were incubated at 37c for 24 hours. Next day, different band size zones were created against their respective antibiotics which were then measured. For all azithromycin resistance (AzmR) isolates, the zone of inhibition was measured at < 13mm.

2.6. Genomic DNA Mining

A microbial DNA extraction kit, Qiagen plasmid midi prep kit (Qiagen, Valencia, CA) was used to withdrawal genomic DNA from each sample following the manufacturer's protocol. The purity of DNA was assessed by NanoDrop TM Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and calculates the concentration of DNA for each isolate. Two sets of DNAs were extracted by using this method, one set was stored at -20 ◦C for later use and one set was utilized for virulotyping by PCR.

2.7. PCR Assay

The azithromycin resistance genes and virulotyping (virulence gene profile) by polymerase chain reaction was done for each S.typhi isolate targeting five selected genes i.e, acrB, rplV, gipA , sopE1 and pefA gene. A PCR examine using subsequently described primers was used to screen for the following Azithromycin resistance genes and virulence genes i.e. acrB, rplV and gipA,

pefA and sopE1. The resistance genes primers used for this study were designed at NCBI primer designing tool and the specificity of these primers were checked on primer-BLAST. While, the virulence genes primers used in this study were reported in the literature [Huehn et al., 2010 and Cortez et al., 2006 and Figueiredo et al., 2015]. The primers used for PCR amplification are shown in **Table 1**.

Table 1: Targeted genes, their primers and PCR conditions

Virulence/ Resistance Genes	**Location**	**Primer sequences (5'-3')**	**Base pair size (bp)**	**Annealing temp (°C)**	**References**
gipA	Gifsy-1 bacteriophage	ACGACTGAGCAGGCTGAG TTGGAAATGGTGACGGTAGAC	518	56	[Huehn et al., 2010]
SopE1	Cryptic bacteriophage	CGGGCAGTGTTGACAAATAAAG TGTTGGAATTGCTGTGGAGTC	422	58	[Huehn et al., 2010]
pefA	Plasmid encoded major fimbrilal subunit	TTCCATTATTGCACTGGGTG GGCATCTTTCGCTGTGGCTT	497	61	[Cortez et al., 2006 and Figueiredo et al., 2015]
acrB	Cytoplasmic memberane	TACAATTGCGTGAAAGCCGC AACCGACGGCATTACTGGTT	250	65	This study
rplV	Polypetide exit tunnel outside of memberane	TGACCCATTGCTAGTCTCCAG AGCGAAGAAGAAATAAGGTAGG	388	65	This study

The PCR cyclic conditions (**Table 2**) required against specific genes are given as: 95 °C for 10 mints, followed by 30 cycles of 95 °C for 1 mint, 56 °C for 1 mint (i.e; gipA annealing temp, every gene has specific annealing temperature), 72 °C for 1 minute, ending with final elongation step at 72 °C for 5 mints and followed by infinite at 4 °C.

Table 2: PCR conditions required in Thermal cycle for amplification of targeted genes

Steps	temperature (°C)	Time	no of cycles
Initial denaturation	95	10 mints	1
Final denaturation	95	1 mint	30
Annealing	56	1 mint	30
Initial extension	72	1 mint	35
Final extension	72	5 mint	
Incubate	4	∞	-

2.8. Data Analysis

The data were examined by using both descriptive statistics and computational methods. The purpose of this study was to conduct the analysis that how the virulence genes are linked with AR patterns across S.typhi isolates. To find out the association between the two above described factors, a Pearson's chi-square analysis was used for the correlation scrutiny. Firstly, all the records were incorporated and managed by MS-2016 Excel sheets for arithmetical study. The significance level was measured by p value ($p < 0.05$). Secondly, the outcome of individual factors (virulence genes and AR status), and their interaction effect on the treatment strategy of typhoidal salmonella patient's were tested by Factorial Two-way ANOVA (variance analysis).

RESULTS

3.1. Characteristics of isolates distribution

A total of 85 fresh human clinical isolates of Salmonella typhi were collected randomly from different hospitals across Pakistan and were analysed through presumptive identification methods. After analysing, they were categorized into two groups' i.e; male and females on the basis of age period **(figure 3.)**. Of total 85 isolates, 37 (44%) were female patients while, 48 (56%) accounts male patients. Both male and female patients were further classified on the basis of age periods such as: Childs, Adolescence and Adults. The female child patient's accounts only 7 cases of the total 37 isolates, while adolescence contains the highest cases which are 19 isolates and adults patients contain 11 cases. However, the adult males have the highest infection rates which are 23 cases of the total 48 male patients. Similarly, the child and adolescence male patients contain 15 and 10 isolates of the total samples. So, the total percentages of salmonella typhi isolates in Adults, Adolescence and Childs are as followed as 40%, 34% and 23% respectively. While, the infection rate was noted higher mainly in autumn and summers **(figure 4)**.

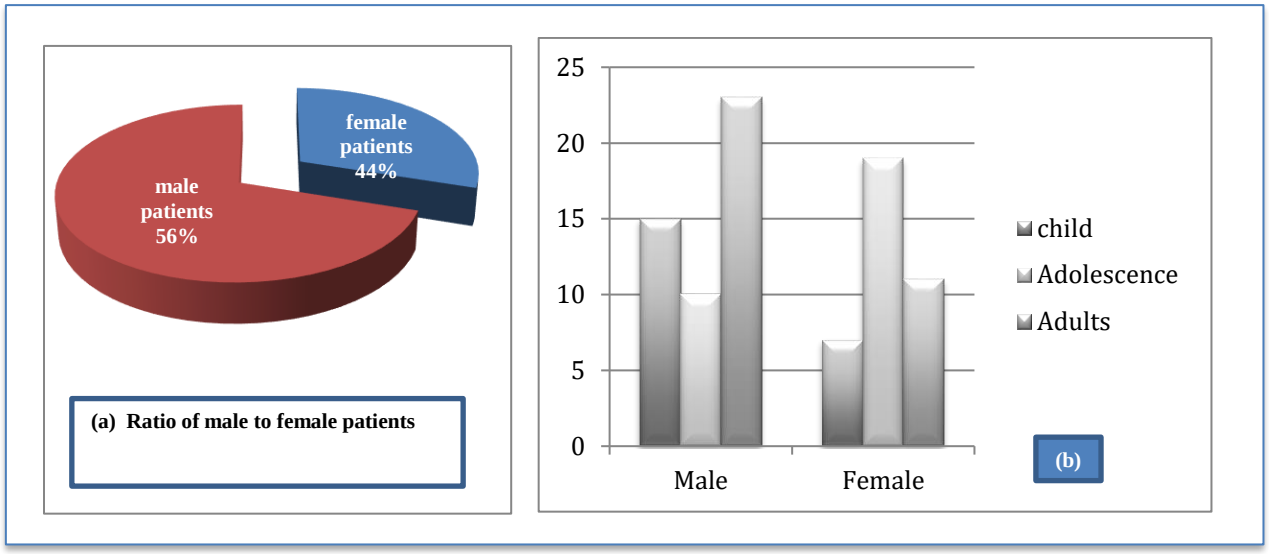

Figure 3: Distribution of salmonella typhi isolates (a) on basis of Gender (b) Age groups

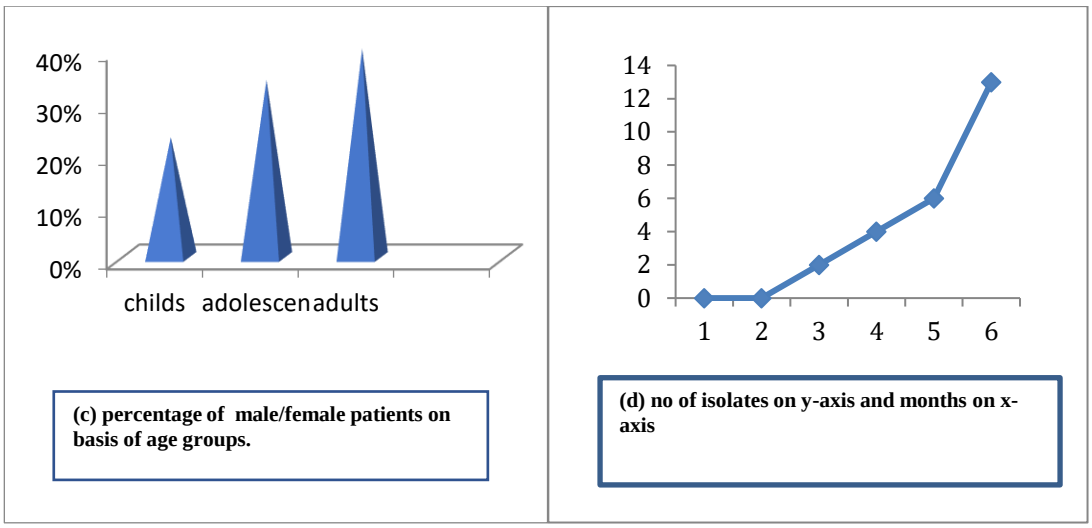

Figure 4: Distribution of clinical isolates on basis of (a) the total percentage of male & female patients in each group (b) infection rate in different time period

3.2. Screening of human S.Typhi Isolates

Among 85 human clinical positive blood isolates, salmonella typhi were secluded by culturing and biochemical analysis. After samples streaking on Salmonella- Shigella (SS) agar surface, pure salmonella colonies were observed with opaque, translucent and transparent colonies with or without black centre **(Figure 5.).** Strains of S. Typhi were also identified by biochemical tests i.e. Oxidase, Catalase and Indole tests. Similarly, the Oxidase tests were noted negative and Catalase tests were found positive for the bacterium. In addition, the isolates of typhoidal salmonella's were confirmed by API 20E **(Figure 6.)** test strips kit (bioMerieux Vitek, Marcy-l'Etoile, France.).

The detection of isolates was finally confirmed by MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time OF Flight Mass Spectrometry), with 99% accuracy of salmonella enterica serovar typhi **(Figure 7.).** This technique is a rapid, cost –effective and rapid method for bacterial characterization and identification, which generates distinctive mass spectral fingerprints that are unique for each organism and are thus ideal for an accurate microbial identification at the genus and species levels [Croxatto., 2011].

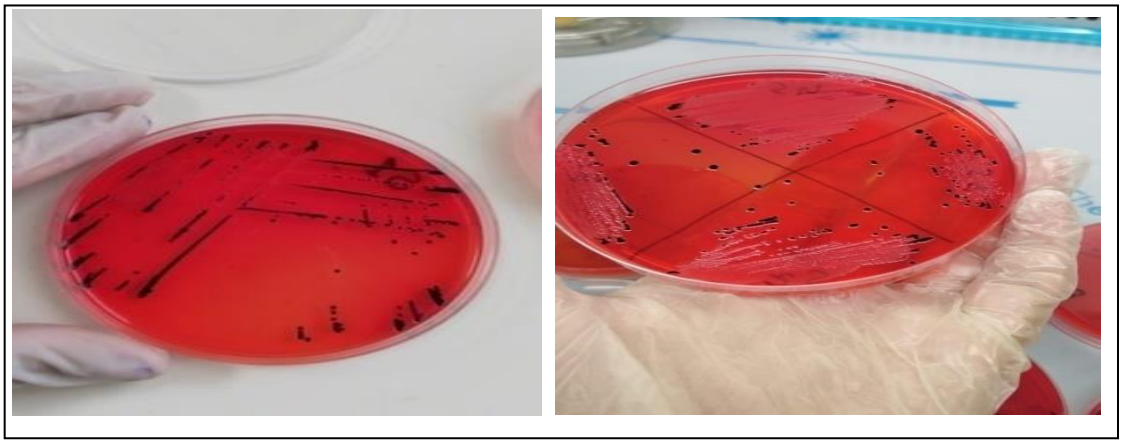

Figure 5: Morphology of salmonella typhi on SS agar plates

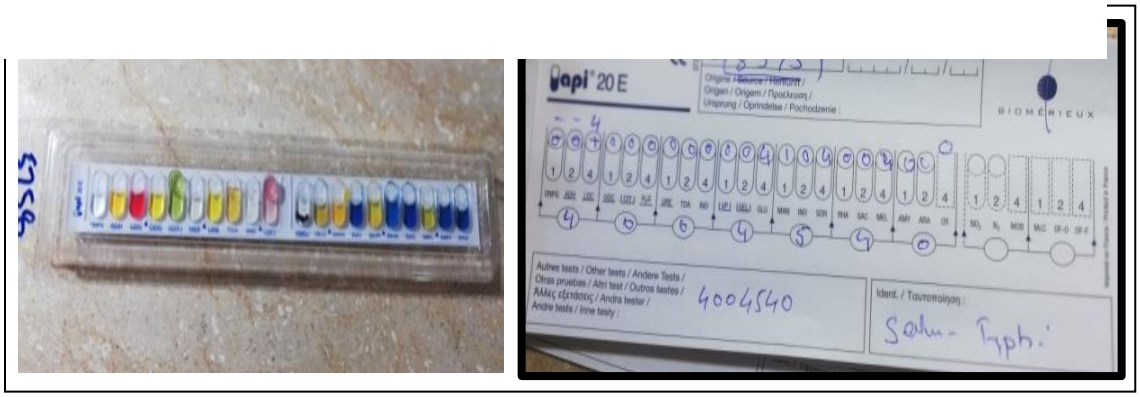

Figure 6: Identification of S. Typhi by API 29E test strips

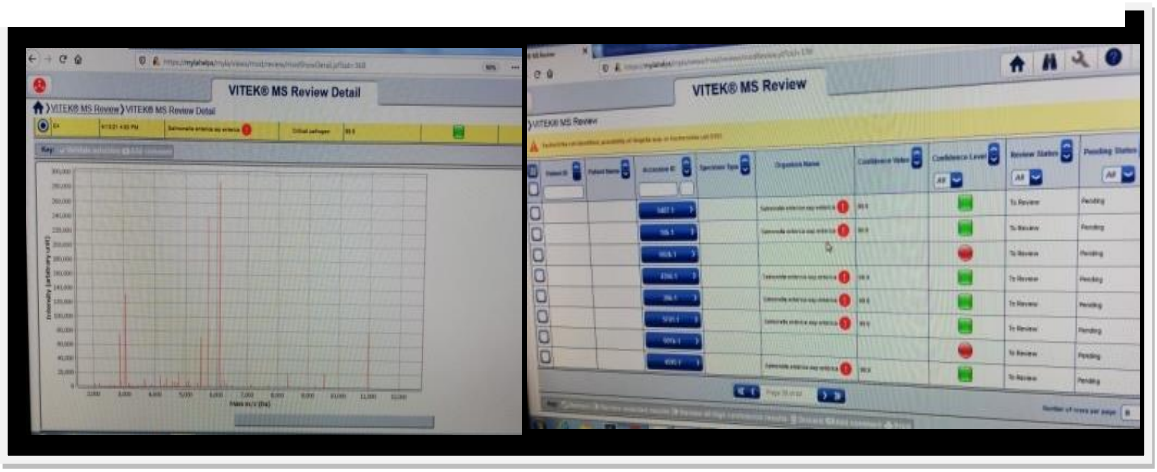

Figure 7: Examination of S.Typhi by using MALDI-TOF (MS)

3.3. Antimicrobial sensitivity Testing

AST was checked on Muller- Hinton agar by using 13 different types of antibiotics discs such as Azithromycin(15 µg) , Ampicillin (10 µg) , Co-trimoxole (30 µg) , Cefotaxime (30 µg) , Cefixime (5 µg) , Ceftrixone (35 µg), Ciprofloxacin(5 µg), Cefepime (30 µg), Chloramphenicol (10 µg) , Tazobactam (10 µg) , Imipenum (10 µg) , Meropenum (10 µg) , Cefoperazone/Sulbactum (30 µg). The sensitivity patterns were characterized as Resistant, Intermediate resistant and susceptible on the basis of zone of inhibition measured in mm **(Table 3).**

Table 3: Antibiotics resistance profiles of salmonella typhi isolates

Drugs	Resistance	Intermediate resistance	Susceptible
Ampicillin	**60**	**5**	**20**
Co-trimoxole	**52**	**11**	**22**
Cefotaxime	**50**	**15**	**20**
Cefixime	**40**	**25**	**20**
Ceftriaxone	**40**	**29**	**16**
Ciprofloxacin	**64**	**13**	**8**
Azithromycin	**34**	**25**	**26**
Chloramphenicol	**68**	**7**	**10**
Tazobactum	**16**	**25**	**44**
Imipenum	**8**	**9**	**68**
Meropenum	**6**	**11**	**68**
Sulbactum	**30**	**27**	**28**
Cefepime	**44**	**17**	**24**
Total no of Isolates		**85**	

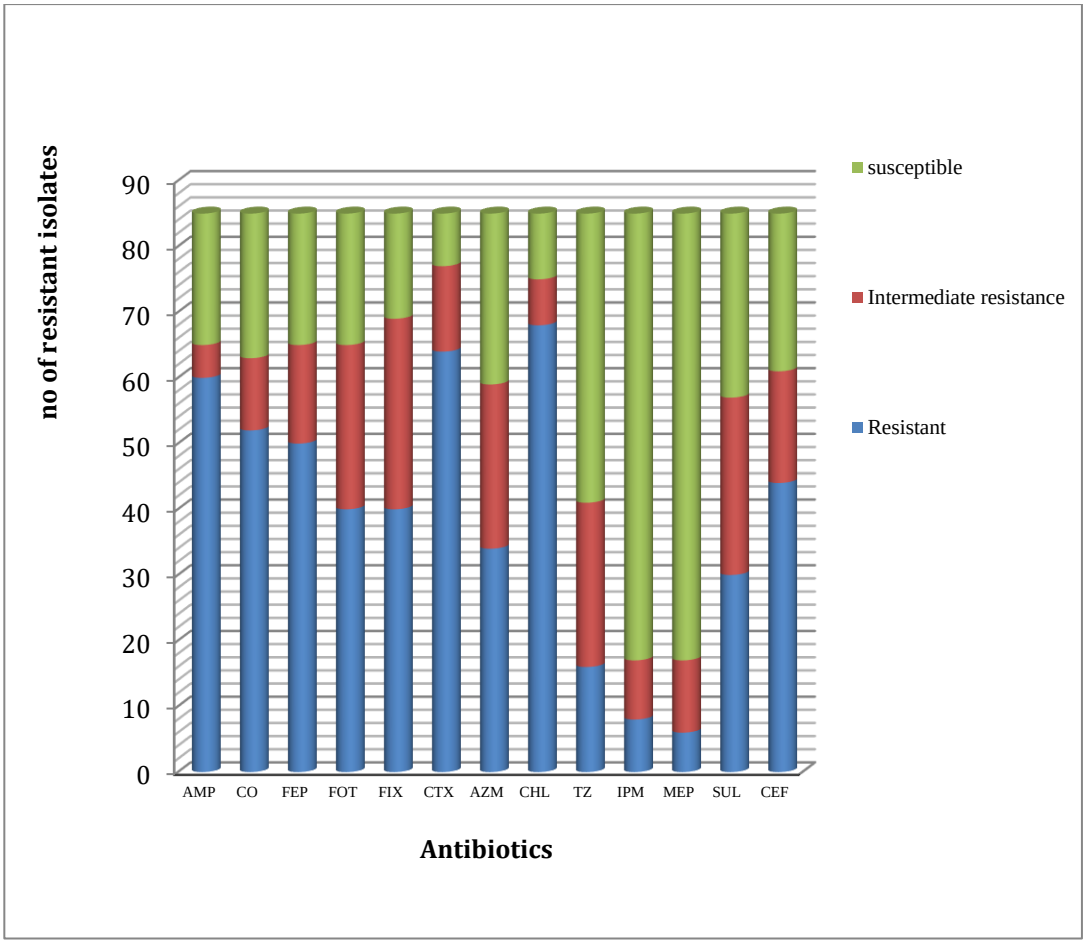

Figure 8: Graphical representation of Antibiotic sensitivity profiles

The above **(Figure 8)** shows that the resistivity to chloramphenicol was the most common (80.25%), followed by ciprofloxacin (76.1%), ampicillin (71.4%), co-trimoxole (61.9%), cefotaxime (59.5%), cefixime and ceftriaxone (47.6%), and azithromycin (40.4%). In addition, resistance to tazobactum, sulbactum, imipenum and meropenum was found 8%, 15%, 2% and 1% of isolates, respectively. Among 34 (40.4%) azithromycin resistant isolates, 14 samples showed

resistivity to almost all classes of drugs and were referred as Extensively Drug Resistant (XDR) salmonella's.

3.4. Occurrence of Antibiotic Resistivity patterns and Virulotypes

The salmonella typhi antibiotics resistivity (AR) patterns were broadly classified into two main groups such as sensitive antibiotics and resistance antibiotics. Both MDR and XDR strains comes under antibiotics resistance patterns of salmonella typhi. In this study, of the total 85 clinicaly human isolates the resistance antibiotics contains 36 (42%) XDR strains and 27 (31%) MDR strains of S.typhi. The highest antibiotic resistance was found against chloramphenicols 61 isolates (80.25%), followed by ciprofloxacin (76%), ampicillin (76%), co-trioxone (61.9%), cefotaxime (59%), ceptriaxone and cefixime (47%), azithromycin (40%), as the azithromycin is considered the only last orally administered drug for typhoid patients in Pakistan **(figure 9).**

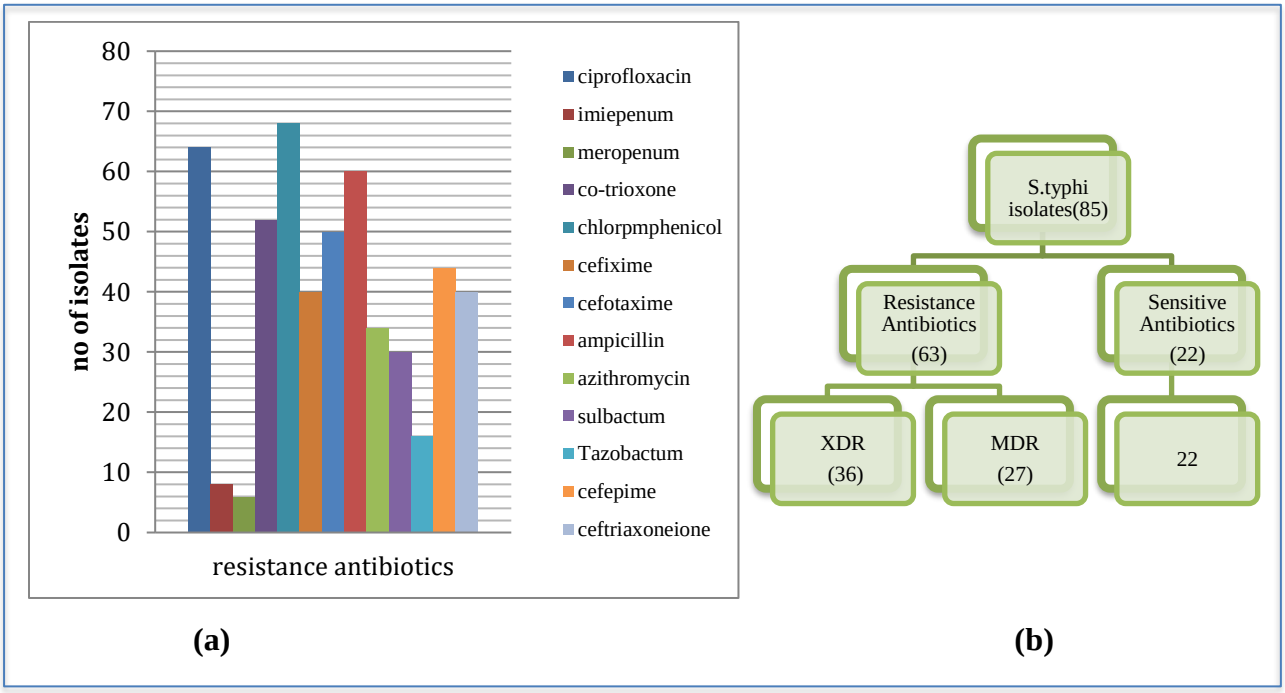

Figure 9: Shows the (a) graphical representation of the frequencies of resistance antibiotic in typhoidal salmonella and (b) the division of typhoidal isolates.

Resistance genes including acrB And rplV of Azithromycin were detected by using PCR. PCR screening publicized that 59 isolates contained acrB gene expression and 46 isolates were positive for rplV gene, respectively **(Figure 10).** In addition, PCR results showed that the 24 isolates which were Phenotypically sensitive to Azithromycin are actually Genotypically resistant to Azithromycin. However, 46 isolates carried both acrB and rplV genes expressions. These results indicate that the synergic effect of both acrB and rplV genes may lead to an increase azithromycin resistant strains of typhoidal salmonella in Pakistan. In addition, the resistant azithromycin strains which are positive for both acrB and rplV genes have been sent for whole genome sequencing to Finland for further analysis. This study is conducted for the first time in Pakistan to study the rplV gene expression in Pakistani population.

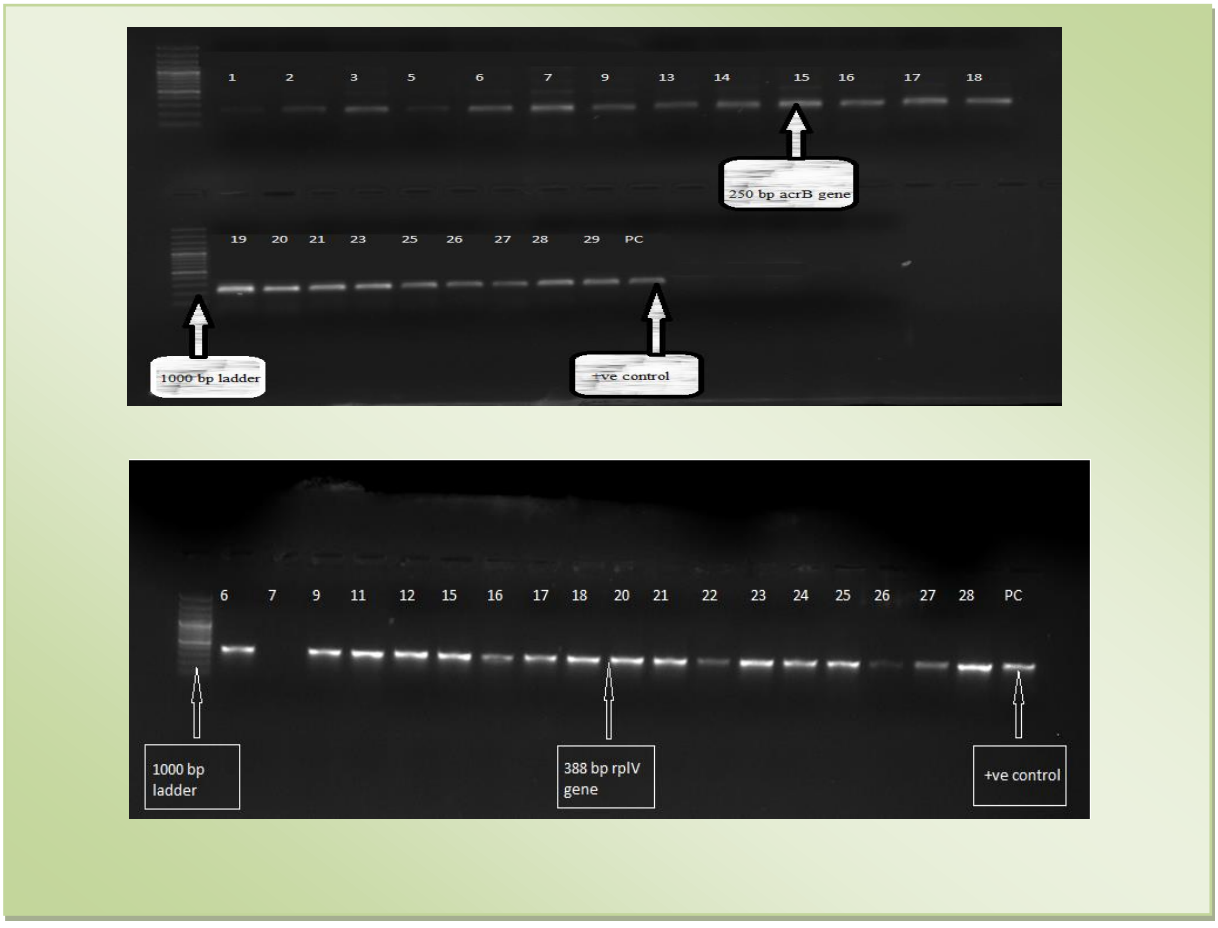

Figure 10: PCR assay for rapid detection of Azithromycin- resistant genes for S.typhi isolates from human clinical patients' (a) PCR reactions for acrB gene, (b) PCR reaction for rplV gene

The PCR testing was also used for virulotyping (virulence profiling), to identify the targeted virulence genes i.e; gipA, sopE1, and pefA in 85 isolates from fresh human clinical typhoid patients **(figure 11)**. The detection rate of sopE1 gene was relatively high i.e; 84 isolates (98%), irrespective of the strains that were phenotypically sensitive to antibiotics. Similarly, the second highest discovery rate is followed by gipA virulence gene which contains 64 (75.2%) isolates. However, the detection rate of pefA (3.5%) was relatively low as compared to gipA, and sopE1 virulence genes, and was detected in only three resistant isolates of salmonella typhoi

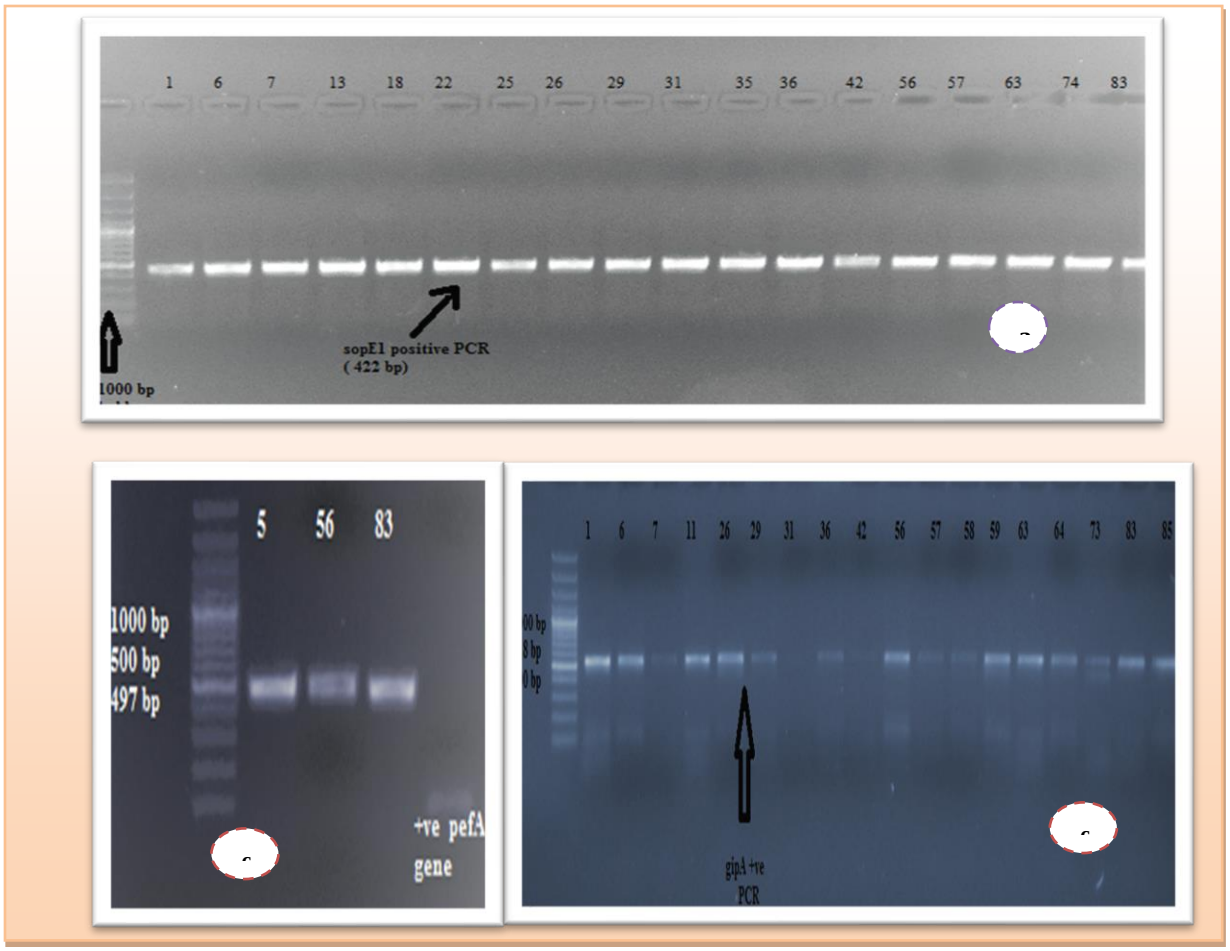

Figure 11: Virulence profile by PCR analysis of S,typhi strains from human clinical isolates of typhoid patients. (a) Prevalence of isolates positive for sopE1 virulence gene by PCR. (b) Occurrence of gipA positive isolates by PCR. (c) pefA positive for isolates by PCR.

The virulence genes such as sopE1 and gipA were present in both drug resistance and drug susceptible isolates **(figure 12).** While, the pefA virulence gene is present in only three drug resistance isolates. Of the total 85 typhoidal salmonella samples, 62drug resistance isolates contain sopE1 gene and almost all drug susceptible isolates (n = 22) have sopE1 virulence gene. A similar pattern was observed for gipA virulence gene, which were present in 55 drug resistance isolates and found in only nine (n =9, 14%) drug susceptible isolates of typhoid patients. Of the total 85 isolates, only two isolates carried the entire specified targeted virulence gene such as sopE1, pefA and gipA.

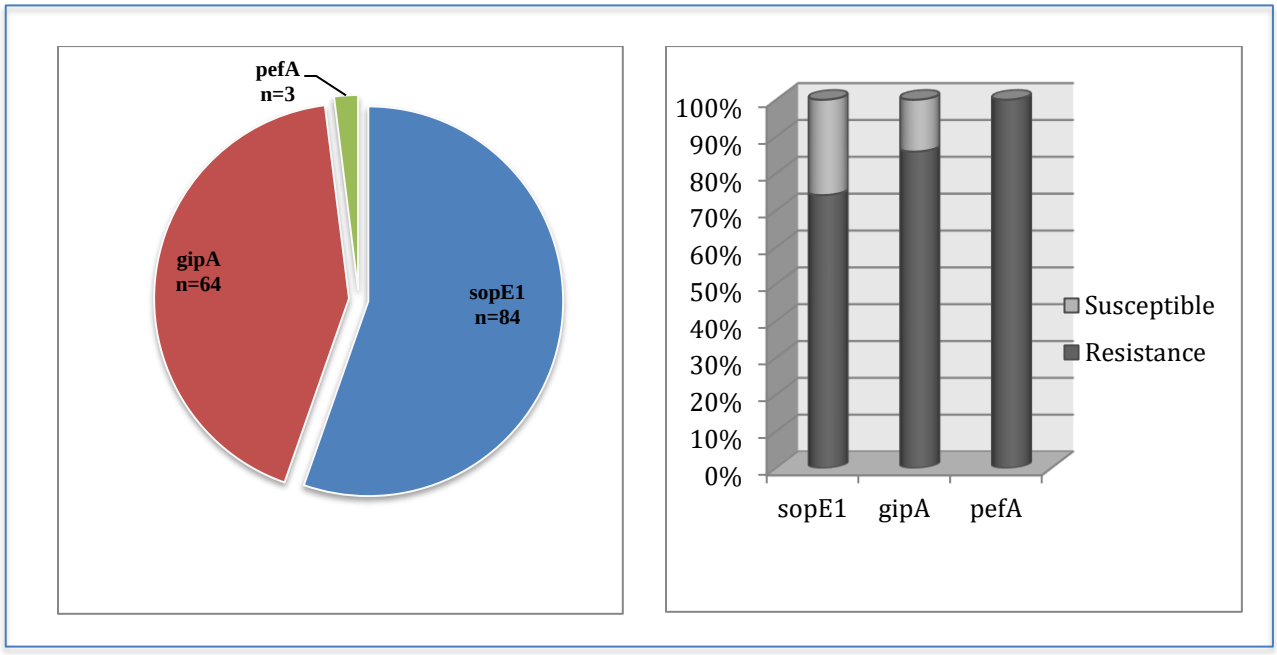

Figure 12: Illustrated the (left) Frequency distribution of virulence genes in S.typhi isolates, and (right) the percentage of virulence genes (sopE1, gipA, and pefA) in both drug – resistance and drug-susceptible islolates in typhoidal salmonella.

3.6. Association between virulence genes and AR patterns by arithmetical analysis

The correlation analysis between virulence genes and antibiotic resistance was conducted by Pearson's chi square (x^2) or Fischer's exact test. The relationship between 3 virulence genes (i.e., sopE1, pefA and gipA), and antibiotics resistance status such as; susceptible antibiotics, multi-drug resistance (MDR) and extensively drug resistance (XDR), by chi square test shows a momentous association between the two above described variables. As the virulence genes such as sopE1 and gipA were present in almost all drug resistance and drug susceptible isolates, but the significant analysis of sopE1[x^2 (df = 4) = 0.094787, $p < 0.04$] and gipA [x^2 (df = 4) = 0.145, $p < 0.05$] by Pearson's chi square were observed with MDR status simply. In the same way, the virulence gene pefA [x^2 (df = 4) = 0.0240, $p < 0.022$] were extensively correlated with only XDR status (**Table 4)**.

However, there was no such significant association observed between virulence genes and antibiotic susceptible pattern. The p values greater than 0.05 (significance level), representing no significance correlation between all other virulence genes and antibiotic status. So, the majority of multi-drug resistance isolates were found positive for gipA and sopE1genes, respectively and extensively drug-resistant isolates were positive for pefA gene.

Table 4: Association of S. typhi Virulence genes with Antibiotic resistivity by chi-square test

Variables (Virulence genes/ antibiotics status)	**Pearsons chi sq/ Fischer value**	***P* value**
SopE1/ MDR status	0.0947	0.04
GipA/ MDR status	0.1458	0.05
PefA / XDR status	0.0240	0.02

Table 4 only lists the correlated virulence genes and antibiotic resistance status. Of them, a test of independence was calculated comparing the virulence genes with antimicrobial status. X^2[(df=4, N=85) = 0.0947, 0.1458, and 0.0240; $p < 0.05$]. So, here there is a statistically significant association observed between antibiotic resistance drugs and virulence genes.

To investigate the individual effects of both variables (i.e. virulence genes and Antibiotics resistance pattern) and their level of interaction across salmonella typhi isolates, a two-way factorial analysis of variance (ANOVA) was conducted. The study of factorial ANOVA found that the individual effects of antibiotic resistance status and virulence genes on treatment strategies of typhoid patients were statistically significant at α 0.05. However, the interaction effect of both factors was not statistically significant at the 0.05 significance level **(Table 5)**.

The results of factorial two-way ANOVA showed a significant difference between virulence genes with; [$F(2, 8) = 7.25$ $p < .05$], as the rate of F calculated (7.25) is greater than F critical (4.46) value. Similarly, the antibiotic resistance patterns with significant difference; [$F(2, 8) = 24.08$ $p < .05$], as the F score is bigger (24.04) as compared to F critical which is 4.46. In both cases described above, we reject the null hypothesis ($H_{\circ}$) which means there is significance variance observed in both virulence genes and antibiotic resistance status.

However, the interaction effect was not significant [$F(4, 8) = 1.014$ $p < .05$], indicating that there was no combined effect for virulence genes and antibiotic resistivity pattern on the treatment plane of typhoidal salmonella patients. So, in this case the $H_{\circ}$ is accepted which means no significance or the sum of means of all groups are same. For interaction effect the calculated F score is less (1.014), than the F critical (3.84) value.

Table 5: A two -way factorial ANOVA measures significance difference between variables

	Sum of squares (SS)	Degree of freedom (DF)	Mean squares (MS)	F- Calculated	F- Critical @5%	Status of null hypothesis ($H_{\circ}$)
Factor A (virulence genes)	**357.5**	2	178.7	**7.25**	4.46 *	**reject**
Factor B (Antibiotic patterns)	**1186.8**	2	593.4	**24.08**	4.46*****	**reject**
Interaction (A* B)	**0.1**	4	0.025	**1.014**	3.84	*accept*
Residual/ Errors	**197.1**	8	24.64			
Total	**1741.5**	16				

***Significant at α 0.05**

- At the 0.05 level, the means of virulence genes of S.typhi are significantly different.
- At α 0.05, the Salmonella typhi isolates means of AR status are significantly variable.
- At the p =0.05, the interaction effect between Virulence genes and AR pattern is not significant

DISCUSSION

Extensively drug resistance strains of S.typhi and especially Azithromycin resistivity in typhoid patients is a serious major health problem in Pakistan. In present study, we found that S.typhi isolates exhibited a high rate of resistivity to traditional antibiotic i.e., Azithromycin which is left as the only last orally administered drug in Pakistan. This study found the noval resistance genes of azithromycin for the first time in Pakistani population. The findings from this swot recommend that the association between specified virulence genes and antibiotic resistance patterns may be influenced by the ability of salmonella typhi to correspond through quorum sensing plus two component systems both directly and indirectly. This study reveals that the status of specific virulence genes are significantly differs between MDR and XDR isolates of salmonella typhi. There was an evidence of a statistically significant effect of virulence genes on the antibiotic resistance behaviour of salmonella typhi. Nonetheless, there was no distinct trend for the microorganisms' resistive drug patterns in relation to the virulence genes.

Azithromycin is recommended as the last orally administered drug in Pakistan for typhoid patients. This study identified the azithomycin resistivity in typhoidal patients. The unremitting use of azithromycin in patients contaminated with strains having reduced susceptibility could have been liable for treatment failures reported may leading to the emergence of azithromycin-resistant strains [Molloy et al., 2010; Manesh et al., 2017; Kobayashi., 2014]. Similarly, few other studies have been reported which explains resistance against azithromycin in salmonella typhi [Choudhary et al., 2013; Patel et al., 2017].

In this study, the genotypic specificity for azithromycin's was surprisingly different than phenotypic specificity. The phenotypically resistant strains of azithromycin was just 40.4% of the total cases while, the genotypically resistant strains of Azithromycin accounts for 69% of the total strains. In contrast, only one study from Cambodia showed that no macrolide resistance strains were identified and all isolates were phenotypically susceptible to azithromycin and the genotypic specificity for azithromycin was 100% [Schawan et al., 2021]. However, no other such studies have been found to correlate the genotypic and phenotypic specificity patterns for azithromycin's in typhoid patients.

In our study, gene expressions associated with azithromycin resistance were found in *acrB* and *rplV* genes. Of the total 85 isolates, over expression of acrB gene was observed in 59 isolates. In these 59 isolates, 25 samples were phenotypically sensitive to azithromycin but were genotypically resistant. Similarly, over expression of rplV gene were also found in 46 isolates while 38 isolates were negative for rplV gene. In addition, 46 isolates carried both acrB and rplV genes which show that both genes have a strong relationship with each other to cause mutations and may lead to an increasing number of azithromycin resistant cases in typhoid patients. In contrast to our study, there is lack of data on phenotypic and genotypic expression of resistance genes of azithromycin in salmonella typhi. Prior to this study, there have been sporadic reports of azithromycin resistance genes of salmonella typhi in other parts of the world. Similarly, the expression of rplV gene by using PCR is novel in Pakistan. However, a study conducted by (Sharma et al., 2019) is similar to our findings which showed that azithromycin resistant strains of S. typhi were positive for rplV gene which was amplified through PCR [Sharma et al., 2019].

Somewhat surprisingly, the virulotyping by PCR in this study identified that the specified virulence genes such as gipA and sopE1 are found not only on drug resistive antibiotics but also on drug susceptible antibiotics. Indeed, over 98% isolates carried sopE1 genes although 22 isolates of them have a drug sensitive pattern, and was followed by gipA virulence gene with 75.2% isolates. However, the availability of pefA gene on S.typhi isolates were relatively low i.e., 3.5 % as compared to other two virulence genes. The statistics in this study showed that all specified virulence genes were detected, but their discovery rate was different across salmonella typhi isolates on the basis of AR status. Research suggests that pefA (49%) were found positive for drug resistant antibiotics and 51% for drug susceptible salmonella isolates. Similarly, the gipA contains 56.9% AR isolates of salmonella and 43% drug susceptible isolates. In addition, the virulence gene such as sopE1 was present in 59.7% of AR isolates of salmonella and 40.2% of the sensitive antibiotic isolates of salmonella [Higgins et al., 2020]. Another study suggests that the detection rate of pefA genes in salmonella isolates is zero [Yue et al., 2020].

Here in this study, we found that salmonella typhi exhibited a high rate of typhoid patients in male as compared to female patients. The positivity rates of male patients were 56% for typhoidal fever from salmonella and only 44% for female patients. Similarly, this ratio of male and female patient varies between age groups of patients. The highest sensitivity ratio of typhoidal salmonella were

observed in adults i.e., 40% of the total patients, followed by adolescence with 34% and than 23% ratio with child's. Contradictory to these findings, other studies suggest that the female patients' ratio were relatively high (52.6 %) for typhoid fever compared to male patients (47.3%). The similar study also reveals that the patients who were highly exposed to typhoid fever ranges in age group between 21-30 years [Rasool et al., 2017]. One study from US reported that there was a high female to male ratio of typhoidal salmonella patients and the relative trouble of salmonellosis has amplified in women [Reller et al., 2008].

In this study, 42% of salmonella typhi isolates were extensively drug resistant, a serious growing threat while, 31% of the isolates were multi-drug resistant and 25.9% of isolates showed sensitivity against different classes of antibiotics. Research indicates that 81% of XDR- typhoid fever was treated inpatient and more than half of them were male patients in Pakistan [Qureshi et al., 2020]. Similarly, 84.4% of salmonella typhi isolates were found positive for multi-drug resistant which was significantly higher than the percentage observed from surveillance data during 2005- 2009 in shanghai, China [Wang et al., 2017]. A survey conducted in five different Asian countries on typhoid fever revealed that MDR rates as capricious as 65% in Pakistan, 22% in Vietnam, 7% in India and 0% in China/Indonesia [Ochiai et al., 2008]. This study suggests the highest resistivity rate of S typhi isolates against ciprofloxacin (100%), chloramphenicols (80.25%), follow by ciprofloxacin (76%), ampicillin (71%), and Co-trioxone (61.9%),ceftriaxone (47.6%), and azithromycin (40.4%).

The majority of results in this study were similar to those reported from China [Wong et al., 2014], that most of the salmonella typhi isolates having developed resistance against ciprofloxacin and chloramphenicols (100%, and 97%). Prior to this study, there have been random reports of isolates of S typhi with azithromycin resistance in other parts of the world, where salmonellosis have been endemic [Molloy et al., 2010, and Hassing et al., 2014]. Contradictory to this study, other suggests the highest resistivity of S.typhi against different classes of antibiotics such as tetracycline (35%), streptomycin (32%), macrolides (43%) and 33% aminoglycosides [Higgins et al., 2020]. The findings in this study highlights the rising and spontaneous occurrence of azithromycin resistivity is S.typhi isolates. The danger of azithromycin resistance in future could cause a greater economic and emotional burden on typhoid fever patients and our healthcare system.

The correspondence between the two important features i.e. the virulence and drug resistance of salmonella typhi is complex. So, only few researches have been conducted to study the liaison between antibiotic resistance status and the virulence factor of S typhi in typhoid patients. In this piece of paper, three highly observed virulence genes (sopE1, pefA, and gipA) were tested among the three different patterns of antibiotic resistance such as susceptible, MDR and XDR- antibiotics across salmonella typhi isolates by using Pearson's chi-squ analysis. Fascinatingly, of the three most prominent virulence genes, only pefA was found to be significantly associated with XDR-salmonella isolates. While, gipA and sopE1 were significantly associated with MDR –salmonella typhi isolates using chi-suqare test with p values less than 0.05. The significant analysis of virulence genes of salmonella isolates by chi-square experiment are as followed as; sopE1 [x^2 (df = 4) = 0.094787, $p < 0.04$], pefA [x^2 (df = 4) = 0.0240, $p < 0.022$] and gipA [x^2 (df = 4) = 0.145, $p < 0.05$]. However, no significant association observed between specified virulence genes and Antibiotic susceptible patterns in this cram. Recent studies have reported a non-significant association among AR status and virulence genes by Pearson's chi-square, but a significant difference were found amid AR and a set of virulence genes when only MDR isolates were measured. Similarly, the isolates resistant to more than 5 drugs were significantly coupled with sopE1 [$\chi 2$ (df=1) =4.39, p=0.036], and pefA [$\chi 2$ (df=1) =6.75, p = 0.010] virulence gene [Higgins et al., 2020]. In this study, it is noted that the apparent absence of pefA genes in mostly isolates may be due to the disturbance in the phylogenetic distribution of fimbriae genes in salmonella enteric. A study has reported the phylogenetic distribution of fimbrial genes, that the *pef* operon offered two distantly related lineages; one that hybridize and the other did not hybridize with the pefA gene [Baumler et al., 1997].

Moreover, a two-way factorial variance analysis ANOVA were tested in this study to find out the individual effects of virulence genes and antibiotic resistance pattern and their interaction effect across *salmonella typhim* isolates. The statistics of ANOVA test demonstrates that the individual effects of both factors (AR status and Virulence genes) are significantly variable and their F-critical values are (4.46* and 4.4*, respectively) smaller than the calculated F-scores (7.25 and 24.08). In addition, the significant differences measured in virulence genes are [$F (2, 8) = 7.25$ $p < .05$] and in antibiotics pattern was; [$F (2, 8) = 24.08$ $P < .05$]. So, in this case the null hypothesis was rejected and alternate hypothesis was accepted which signify that the sum of all the means are not same and a significance variances observed in both factors. However, for interaction effect of both

factors the F-calculated value is 1.01 which is smaller than the F-critical value i.e., 3.84, and in this case no significant disparity observed were [F (4, 8) = 1.014 P < .05] so, null hypothesis was accepted which means the results are not significant at 0.05 level and hence proved that the interaction between virulence genes *and* antibiotics resistance is weak. No other study have been identified the significance difference between the interaction effect of virulence genes and antibiotic resistance.

One of the possible precincts of this study is the limited number of *salmonella typhi* isolates availability. Secondly, we analysed only 13 different antibiotics and only three virulence genes in this cram. Similarly, an increase no of isolates, virulence genes and the antibiotic resistance would provide us a more in depth organization between antibiotic resistance and virulence genes for future studies.

CONCLUSION

In conclusion, we report high ratio infected azithromycin-resistance isolates of S.typhi in Pakistan. We found that the strains which were phenotypically sensitive to azithromycin were genotypically resistive. The increase emergence of genotypic azithromycin-resistant strains of S.typhi in typhoid patients is a worrying phenomenon. The significant association between virulence genes and the pathogenic MDR and XDR *salmonella typhi* from human typhoidal patients is extremely alarming and has led us to a serious public health concern. Our results depict the correlation between virulence genes and antibiotic resistance patterns of salmonella typhi by using descriptive statistics and computational analysis. Similarly, the virulence genes and the MDR and XDR status of salmonella isolates are significantly different but, it did not differ between virulence genes and antibiotic susceptible patterns. This association deserves clinical attention and further research is required in this field. Such research will permit a better perceptive of the pathogenic characteristics and evolutionary process of *Salmonella typhim* as well as the purpose of how Salmonella spreads between hosts. In clinical exercise, a modified treatment arrangement can be developed after the characteristics of virulence genes and antibiotic resistance pattern in S typhi are fully tacit, which will be enter to the prevention, manage, and cure of salmonellosis. The findings of this study highlight the need for continuous monitoring of antimicrobial susceptibility of typhoidal salmonellae and investigating the mechanisms underlying azithromycin resistance

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