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Research Article

OCCURRENCE OF ANTIMICROBIAL RESISTANCE GENES PRIOR TO APPROVAL OF ANTIBIOTICS FOR CLINICAL USE: EVIDENCES FROM COMPARATIVE RESISTOME ANALYSIS OF *SALMONELLA ENTERICA* SPANNING FOUR DECADES

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ABSTRACT: Growing antimicrobial resistance (AMR) in *Salmonella* spp. is an evolving crisis exacerbated by antibiotic misuse. The present study was undertaken to delineate the temporal trends in the emergence of AMR among *Salmonella enterica* strains from India. Genome sequences and metadata of *Salmonella enterica* isolates were obtained from NCBI and were analyzed by the Resistance Gene Identifier tool (Comprehensive Antimicrobial Resistance Database), ABRicate (PlasmidFinderDB), BacAnt (IntegronDB, TransposonDB). Core-genome phylogeny was constructed with Roary and IQ-TREE. Antibiotics approval data were collected from the Central Drug Control Standards Organisation website and were analyzed with SPSS. Overall, AMR genes against 23 antibiotic classes were detected with cephalosporins and fluoroquinolones being at the top (17 genes each). For many antibiotics (monobactams, glycylcyclines, fosfomycins, elfamycins, edeine) occurrences of AMR genes preceded approval of the antibiotic for use. A statistically significant difference ($p<0.05$) was observed for resistance mechanisms, gene families, and SNP patterns, though no difference was noted for AMR gene carriage among salmonellae at decadal intervals. Our results deciphered the trends of AMR in Indian isolates of *Salmonella enterica*, highlighting occurrences of AMR genes and mechanisms before antibiotic approval and the usefulness of WGS-based AMR analysis for guiding future policy.

Key words: Antibiotic usage, Antimicrobial resistance epidemiology, Resistance genes, *Salmonella* spp.

INTRODUCTION

Salmonellae are important zoonotic food-borne pathogens accounting for a loss of 21.2 million Disability-adjusted Life years (DALYs) besides other economic losses (Kirk *et al*. 2015). The overwhelming majority of these infections are caused by different serovars of the species *Salmonella enterica* subsp. *enterica*. Among *Salmonella enterica* serovars, the serovar Typhi causes around 200,000 deaths annually worldwide (Klemm *et al*. 2018). The contribution of non-Typhoidal serovars to the global disease burden is around 94 million cases and 155,000 deaths annually (Phuong *et al*. 2017). Other serovars frequently associated with Non-typhoidal salmonellosis include Typhimurium and Enteritidis (Phuong *et al*. 2017, Taib and Abdulrahman 2022). In the United States, serovars Bareilly and Nchanga were reportedly associated with food-borne disease outbreaks (Hoffmann *et al*. 2016). However, in the Indian context, the distribution of Non-Typhoidal serovars remained dynamic over the years with Typhimurium currently being the most common, followed by Weltevreden, Bareilly, and Newport (Jacob *et al*. 2020).

Besides health implications, growing antimicrobial resistance (AMR) in *Salmonella* spp. is an evolving crisis with increasing reports of Multi-drug resistant (MDR) and Extremely Drug-resistant (XDR) *Salmonella* strains

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(Katiyar *et al*. 2020, Reis *et al*. 2018). Fluoroquinolone-resistant *Salmonella* strains were in the 'second highest priority' category in the WHO priority pathogens listing (Cuypers *et al*. 2018). Further, the increasing occurrence of MDR, especially in the haplotype H58 is of concern due to its global spread following its emergence from South and Southeast Asia (Klemm *et al*. 2018). MDR in H58 is associated with the possession of several genes such as *bla*TEM-1*, dfrA7, sul1, sul2, catA1,* and *strAB* in a transposon-mediated mechanism (Wong *et al*. 2015).

The history of AMR among *Salmonella* strains dates back to 1950 when resistance was first reported against chloramphenicol followed by resistance to ampicillin and co-trimoxazole in the late 1980s and then against fluoroquinolones. Following the emergence of fluoroquinolone-resistant strains, third-generation cephalosporins, and azithromycin were used as drugs of choice mostly in southeast Asia with decreasing efficacy over the years (Karkey *et al*. 2018, Katiyar *et al*. 2020). On the other hand India with endemic *Salmonella* infections is predicted to be one of the largest consumers of antibiotics in all sectors (Klein *et al*. 2018). In this milieu of high antibiotic usage in different sectors and easy animal transportation, zoonotic spread (Vineesha *et al.* 2021, Hoque *et al*. 2022, Taha 2022), plummeting susceptibility and scarcity of newer antimicrobials (Pattanayak 2022), etc. reasons, it is imperative to delineate the trend of AMR emergence in salmonellae for future actions.

Of late, whole genome sequencing (WGS) based AMR prediction has gained traction and was successfully attempted on several organisms (Katiyar *et al*. 2020, Rokney *et al*. 2020, Salih and Shafeek 2019). With the increasing availability of genomic data, sequence-based determination of AMR offers a precise and detailed methodology to understand the co-carriage of genes and temporal trends and thus can help in delineating the potential drivers of antimicrobial resistance development and their persistence (Hendriksen *et al*. 2019, Banerjee and Acharyya 2021, Arun *et al.* 2022).

Therefore, in this study, we have attempted to use genomic tools to compare the resistomes of *Salmonella enterica* strains isolated in India to apprehend the temporal trends in the emergence of antimicrobial resistance genes concerning the approval of different classes of antibiotics for clinical use.

MATERIALS AND METHODS

Study Design

The study was conducted on the genomes of *Salmonella enterica* strains isolated from India available in the NCBI Genome database till date 26th December 2020. Data analysis was undertaken at the Computational Biology Unit of the Division of Animal and Fisheries Sciences, Indian Council of Agricultural Research - Research Complex for North Eastern Hill Region, Umiam, India.

Genome data of *Salmonella enterica*

A preliminary search was made in the NCBI Genome database with the keywords "Salmonella enterica" to ascertain the range of sequence lengths for the organism. Subsequently, the NCBI Nucleotide database was searched with "Salmonella enterica"[Organism] AND India [All Fields] AND ("4482090"[SLEN]: "5219420"[SLEN]) on 26th December 2020 which yielded 246 hits and their sequence data was downloaded in FASTA format. Since NCBI is a constantly growing database with new genomes being submitted by authors each day. Therefore, we fixed 26 th December 2020 as the cut-off date to obtain a definite dataset to complete the downstream processing.

Genome inclusion criteria

Downloaded sequence data were included in the study based on their metadata. Thirty-six entries were excluded due to ambiguity in the metadata (viz., ambiguity in serovar identification, same strain with multiple accession numbers, ambiguity in the year of isolation, and report of non-Indian isolates published in Indian journals). For genomes with multiple accessions for a particular strain, the latest version was included. Altogether, 210 strains along with their sequences and metadata were analyzed. To ensure genome quality, we undertook a Quality assessment of the downloaded assemblies through the QUAST tool (Gurevich *et al*. 2013). For ease of interpretation, we assigned codes to each genome in the format W_XXX_YY_ZZ, where 'W' indicated isolation source ('C' – Clinical origin; 'A' – Animal origin; 'S' – Spice origin; 'U' – Unknown origin), 'XXX' indicated the serovars ('BAR' – Bareilly; 'H58' – Typhi Haplotype H58; 'TYP' – Typhi; 'VIR' – Virchow; 'OSL' – Oslo; 'WEL' – Weltevreden; 'BER' – Berlin; 'SEN' – Senftenberg; 'ENT' – Enteritidis; 'TYM' – Typhimurium), 'YY' indicated the last two digits of the isolation year (*eg.* 11 for the year 2011, 00 for the year 2000, 77 for the year 1977) and 'ZZ' indicated the isolate number.

Serovar determination

Two strains with unidentified serovars were identified as serovar Senftenberg or Dessau and serovar Typhi with

Seqsero 1.2 available at https://cge.cbs.dtu.dk/services/SeqSero/ choosing "Assembled genome/ contigs" option (Zhang *et al*. 2015).

Prediction of resistance determinants

Sequence data were uploaded in FASTA format onto the RGI 5.1.1, CARD 3.1.1 web portal with "Perfect and Strict hits only", "High-quality coverage" and "Exclude nudge" parameters. "Perfect" hits indicated 100% sequence similarity, while "Strict" hits indicated more than 95% sequence similarity in the CARD database (Alcock *et al*. 2020, https://github.com/arpcard/rgi)

Screening for mobile genetic elements

Salmonellae genomes were screened for the presence of plasmids with ABRicate tool v1.0.1 using the PlasmidFinder database updated till 10 May 2022 (https://github.com/tseemann/abricate) using 12 threads. Using BacAnt v3.3.1 tool (Hua *et al*. 2021), all genomes of Salmonellae were screened for integrons and transposons employing two databases, IntegronDB and TransposonDB updated to version 2.0 (11 May 2021). During the run, coverage was set at 80% and identity was set at 90%.

Core Genome alignment and Phylogenetic analysis

Salmonellae genomes (n=210) were subjected to analysis by Roary v3.13.0 tool which uses a probabilistic multiple alignment program (PRANK) (www.wasabiapp.org/software/prank) for building a core genome alignment (Page *et al*. 2015). Before analysis by Roary, all genomes were re-annotated using the Prokka tool v1.14.5 (https://github.com/tseemann/prokka) for use as input to Roary. Core genome alignment was curated using GBlocks v0.91b with default settings (Castresana 2000). Curated blocks were further analyzed by IQ-TREE v1.6.12 (Nguyen *et al*. 2015) run with ModelFinder (Kalyaanamoorthy *et al*. 2017) for constructing the phylogenetic tree. The resulting tree was visualized and annotated in FigTree v.1.4.4 (https://github.com/rambaut/figtree/releases).

Collection of antimicrobial approval data

The clinical usage approval data were obtained from the Central Drug Control Standards Organisation website (https://cdsco.gov.in/opencms/opencms/en/Approval_new/Approved-New-Drugs/).

Analysis and interpretation

All data were tabulated and collated in MS-ExcelR. Venn diagrams for graphical representation were created with the help of VennPainter V1.2.0 (Lin *et al*. 2016). Statistical analysis was undertaken with the help of IBM SPSS Statistics Subscription Build 1.0.0.1447.

RESULTS AND DISCUSSION

Genome features

Antimicrobial resistance in *Salmonella* spp. is an important problem in human medicine and the livestock sector. Our study focussed on understanding the temporal trends of the occurrence of resistance genes concerning antibiotic approval for clinical use in India. Genome quality assessment data indicated no major errors. Genome sizes of the *Salmonella enterica* ranged from 4571935 bp to 4958561 bp. The earliest and the latest strain included in this study were strains of *Salmonella enterica* subsp. *enterica* serovar Typhi isolated in the years 1977 (strain 77-303) and 2019 (strain R19.2839), respectively. The details of the strains included in this study are given in Tables 1A, 1B, 1C, and Fig.1. We identified that H58 was the most prevalent entry (123/221, 55.65%) over 42 years (1977-2019). Other studies too established the dominance of the H58 haplotype (also termed genotype 4.3.1) among *Salmonella* Typhi strains (Wong *et al*. 2015, Divyashree *et al*. 2016, Britto *et al*. 2020). Same type of findings is also reported by some other workers (Katiyar *et al*. 2020, Shin *et al*. 2021). H58 haplotype is associated with two different lineages, responsible for fluoroquinolone-resistant infections and MDR-*Salmonella* infections, which originated in southern Asia and caused a serious impact in Africa (Wong *et al*.

Table 1A. Number of Salmonellae genomes classified into Group A (years up to 2000).

Serovars	Upto year 2000 (Group A)			
	Clinical	Spices	Animal origin foods	Unknown
Typhi	-	-	-	5
Typhi H58	-	-	-	1
Bareilly	-	2	-	-
Typhimurium	-	-	-	-
Nchanga	-	-	-	-
Oslo	-	-	-	-
Weltevreden	-	-	-	-
Berlin	-	-	-	-
Enteritidis	-	-	-	-
Newport	-	-	-	-
Senftenberg	-	-	-	-
Virchow	-	-	-	-

Table 1B. Number of Salmonellae genomes classified into Group B (years 2001-2010).

Serovars	2001-2010 (Group B)			
	Clinical	Spices	Animal origin foods	Unknown
Typhi	1	-	-	16
Typhi H58	-	-	-	37
Bareilly	-	7	5	-
Typhimurium	-	-	-	-
Nchanga	-	-	-	-
Oslo	-	-	2	-
Weltevreden	-	-	-	-
Berlin	-	-	-	-
Enteritidis	-	-	-	-
Newport	-	-	-	-
Senftenberg	-	-	-	-
Virchow	-	1	-	-

Table 1C. Number of Salmonellae genomes classified into Group C (years 2011-2019).

Serovars	2011-2019 (Group C)			
	Clinical	Spices	Animal origin foods	Unknown
Typhi	3	-	-	27
Typhi H58	-	-	-	85
Bareilly	1	2	8	-
Typhimurium	3	-	-	-
Nchanga	-	-	-	-
Oslo	-	-	-	-
Weltevreden	-	-	1	-
Berlin	-	-	1	-
Enteritidis	-	-	1	-
Newport	-	-	-	-
Senftenberg	-	-	1	-
Virchow	-	-	-	-

2015). Of late, fluoroquinolone-resistant lineage has become predominant (Britto *et al*. 2019) and the emergence of XDR *Salmonella* cases in Pakistan has been reported (Klemm *et al*. 2018, Leggiadro 2019, Rasheed *et al*. 2020).

Identification of resistance determinants

A total of 51 best-hit Antibiotic Resistance Ontologies (AROs) were identified in the genomes included in this study conferring resistance to 23 classes of antibiotics. The highest number of genes responsible for resistance against a particular class was found to be cephalosporins and fluoroquinolones (17 each), followed by penams (16) and tetracyclines (15). However, there were a few classes for which only one ARO was identified (aminocoumarins, elfamycin, benzalkonium chloride, nitroimidazole, and rhodamine). We identified the highest numbers of genes conferring resistance to these antibiotics, which aligns with recent reports (Britto *et al*. 2020, Rasheed *et al*. 2020).

Screening for mobile genetic elements

A total of 12 plasmid types were identified among the 210 genomes included in the analysis (Fig. 2). IncFIB(pHCM2)_1 was the most commonly occurring plasmid type, followed by plasmid type IncFII(S)_1. The genomes which harbored IncFIB(pHCM2)_1 plasmid were all typhoidal serovars whereas, the plasmid type IncFII(S)_1 was identified only from non-typhoidal serovars. Plasmids have been regarded as formidable agents of horizontal spread of antibiotic resistance in various taxonomic groups, especially *Enterobacteriaceae* (McMillan *et al*. 2020). Plasmidome analysis of Salmonellae genomes revealed the predominance of IncFIB(pHCM2)_1 plasmid type which is a cryptic plasmid initially identified from an MDR *Salmonella* Typhi in the year 1993. The sequence was found to be homologous to pFRA, the virulence-associated plasmid of *Yersinia pestis* (Kidgell *et al*. 2002). The presence of this plasmid in genomes of *Salmonella* Typhi isolated in India has been documented in a previous study (Katiyar *et al*. 2020). The second most commonly observed plasmid type was IncFII(S)_1. The FIB and FII replicon type plasmids in *Salmonella enterica* are usually associated with resistance to aminoglycosides, chloramphenicols, fluoroquinolones, β-lactams, tetracyclines, trimethoprim and sulphonamides (McMillan *et al*. 2020). On screening 210 genomes for the presence of integrons, 27 genomes (12.9%) were found to harbor integrons. The majority of these genomes (n = 24) were *Salmonella* Typhi H58 haplotypes and the rest were of serovar Typhi (n = 2) and serovar Bareilly (n=1). Based on their source, only 2 genomes of clinical origin were found to harbor integrons while the rest were of unknown origin. The occurrence of integrons among H58 haplotype genomes was found to be 19.51%. Interestingly, 4 of 27 (14.81%) integron-carrying genomes were found to harbor AMR genes (*dfr* allelic variants, *sul1*, *sul2*, *bla*TEM-1, *strA*, *strB*, and AAC) which are known to be an integral part of class-1

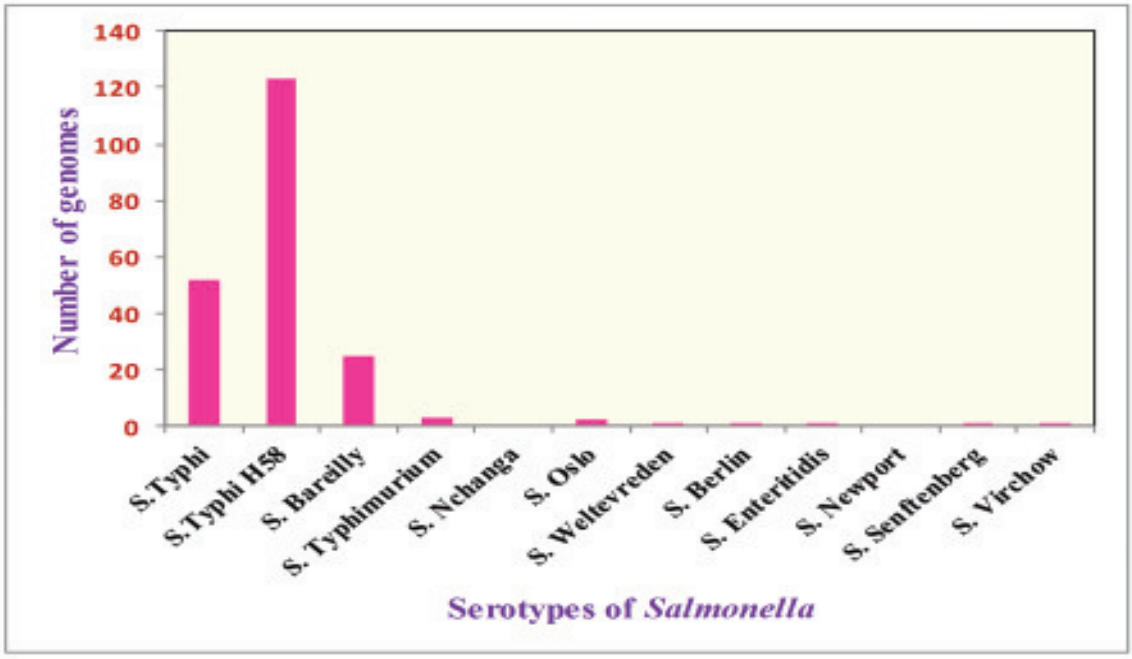

Fig. 1. Salmonellae genomes and serotypes analysed in the current study.

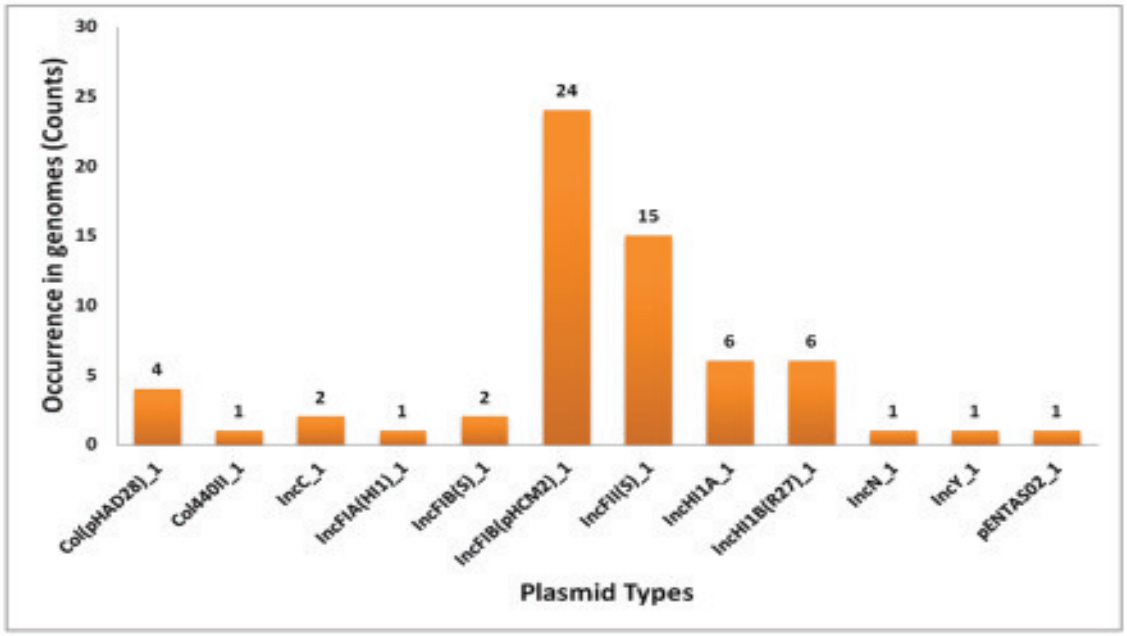

Fig. 2. Occurrences of various plasmid types in *Salmonellae enterica* genomes (n=210) isolated from India.

integrons. However, phylogenetic analysis (Fig. 3), did not reveal much phylogenetic relatedness except for two isolates (U_H58_12_01 and C_TYP_19_02). Integrons play an important role in integrating gene cassettes onto the genetic makeup of the organism and subsequently mediate the dissemination of antimicrobial resistance. In our study, 12.86% of all genomes and 19.51% of H58 genomes were found to be positive for integrons, which is comparatively higher than the general prokaryotic average of 10 to 17% (Domingues *et al*. 2012). The vast majority of integron-bearing genomes also harbored class 1 integron-associated AMR genes (*dfrA7, sul1, sul2*). The clustering of these genes might have been due to the influence of class 1 integrons, which are the most predominant class of integrons among Gram-negative bacteria (Domingues *et al*. 2012). Transposon detection

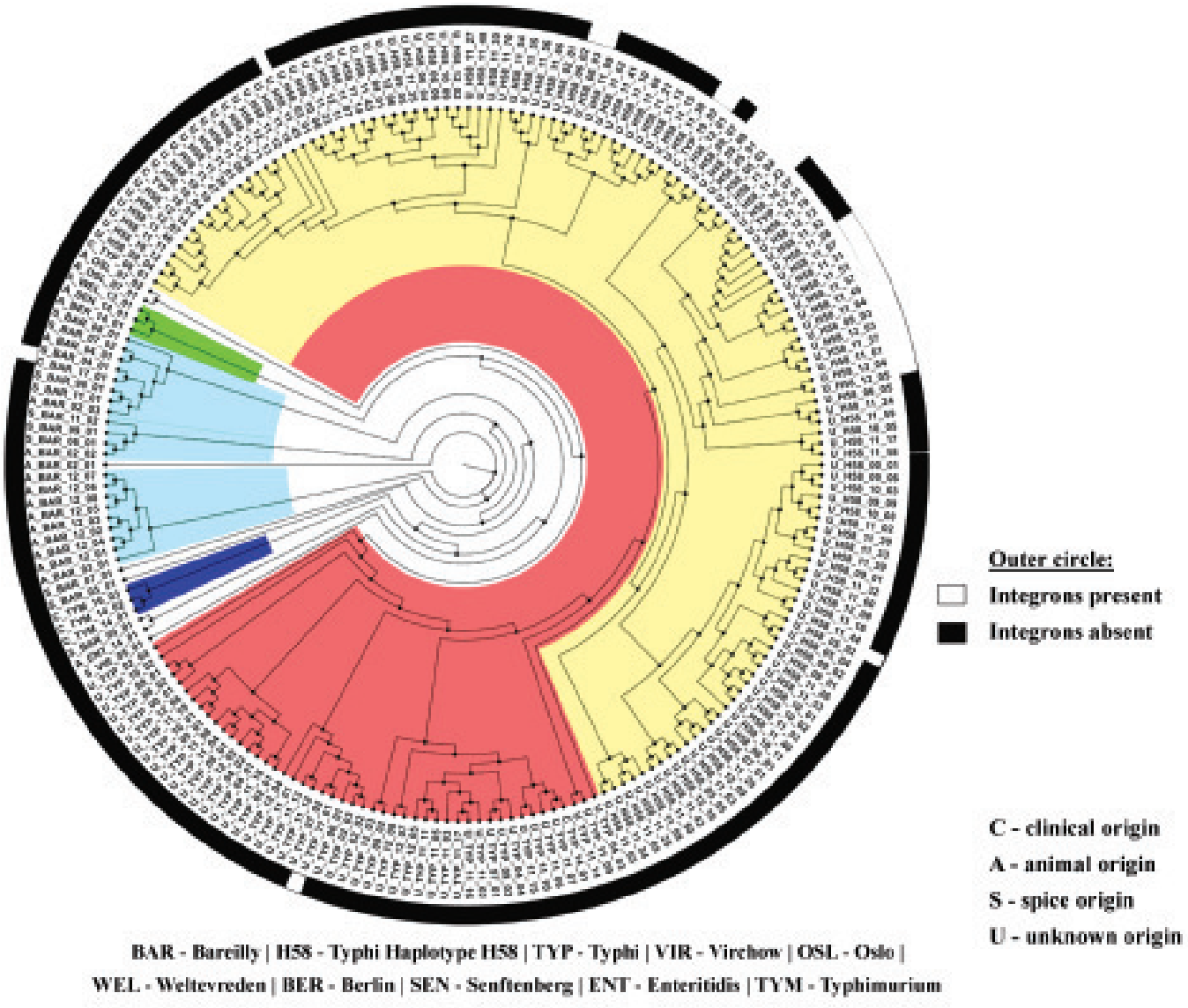

Fig. 3. Core genome phylogeny of *Salmonella enterica* genomes (n=210) isolated from India.

using BacAnt tool revealed that few transposons (Tn*10*, Tn*1721*, Tn*1722*, Tn*2003*, Tn*2555.3*, Tn*2921*, Tn*4655*, Tn*5041*-like, Tn*5394*, Tn*5542*, Tn*6097*, Tn*6178*, Tn*6181*, Tn*6211*, Tn*6286*, Tn*6290*, Tn*6291*, Tn*6934*, Tn*7*-like) were present in all 210 genomes. Other commonly observed transposons included Tn*2610*, Tn*6019*, Tn*6171*, Tn*6183*, Tn*6302*, Tn*7*, Tn*6127*, Tn*4676*, Tn*5422*, Tn*6126*, Tn*6027*, Tn*6187*, Tn*6214* and Tn*9*-like. The transposons Tn*602*, Tn*1000*, Tn*2012*, Tn*5044*/Tn*5046*, Tn*6273*, Tn*6276*, Tn*1207.1*, Tn*2009_2*, Tn*2010*, Tn*2020*, Tn*558*, Tn*6272* and Tn*aphA6* were sparsely identified among the 210 genomes. The transposons associated with multidrug resistance such as Tn*21* and Tn*10* were identified in 31 and 210 genomes, respectively. Transposons are jumping genes capable of shuttling between genetic elements in the bacteria (Babakhani and Oloomi 2018). In the present study, transposons were found in all the Salmonellae genomes (n=210). A total of 31 genomes carried Tn*21* transposon which, is a non-composite transposon belonging to the Tn*3* family, usually harboring resistance genes against cephalosporins and sulphonamides (Babakhani and Oloomi 2018). In contrast to our study, a Tn*2670*-like complex transposable element was identified in the majority of H58 genomes in a previous study (Wong *et al*. 2015). However, the sub-component transposon Tn*6029* which is known to carry *bla*TEM-1, *strAB*, and *sul2* (Wong *et al*. 2015) was identified in 32 genomes of the present study.

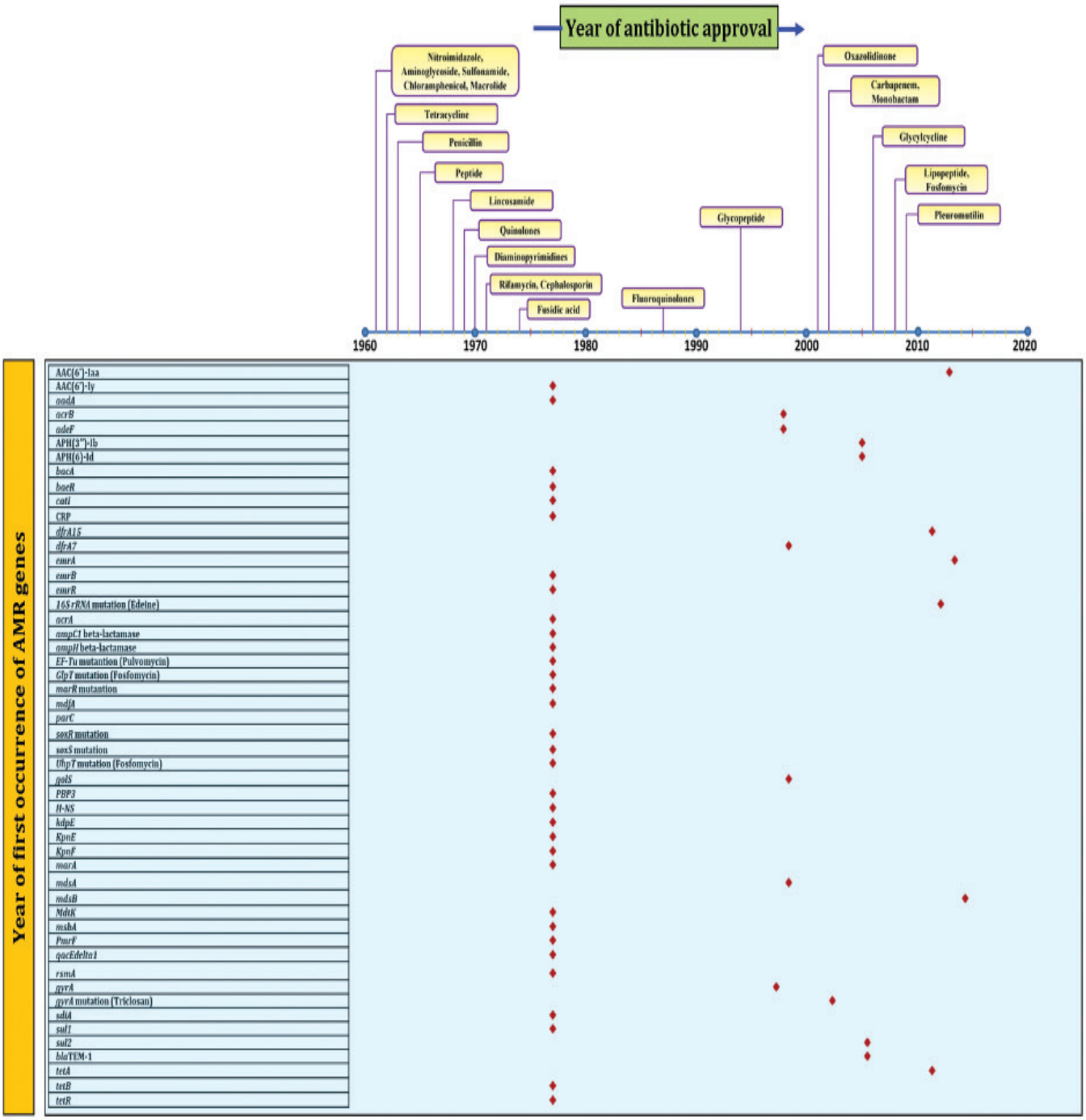

Fig. 4. Time-line of antibiotic approval in India and the year of first report of AMR genes.

Core-genome alignment and phylogeny

To infer the relatedness among genomes a phylogenetic tree was constructed using the best-fit model - GTR+F+R3 as identified by the ModelFinder algorithm of IQTREE tool based on Bayesian information criterion (BIC) scores. Visualization of the phylogenetic tree (Fig. 3) revealed that all H58 haplotypes formed a tight cluster with four instances of intermingling by non-H58 Salmonellae of clinical and unknown origin (C_TYP_08_01, C_TYP_18_01, C_TYP_19_02, U_TYP_12_01) (Fig. 3, yellow shaded area). Similarly, most of the Salmonellae genomes of serovar Typhi agglomerated into a single cluster (Fig. 3, pink-shaded

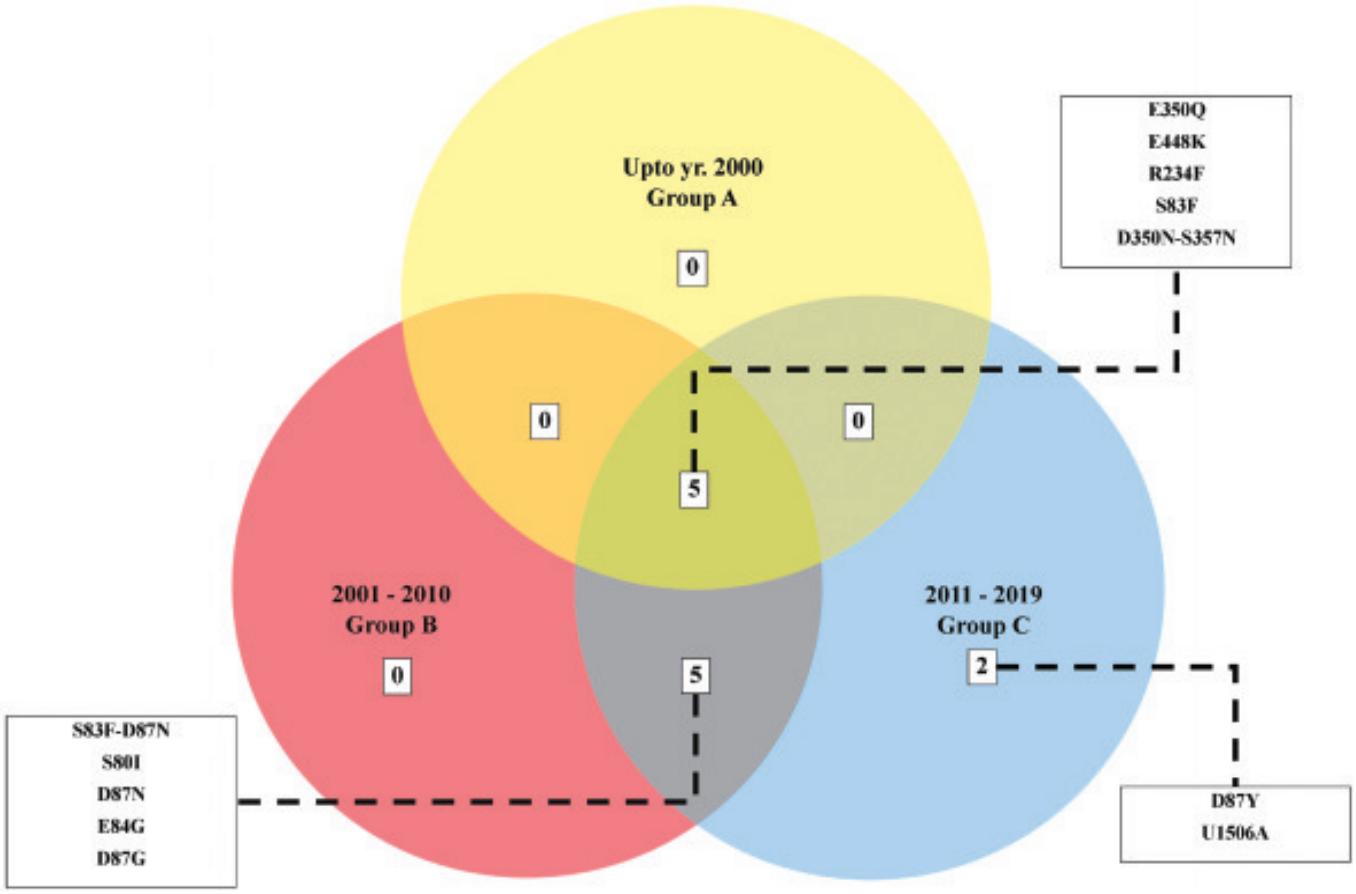

Fig. 5. SNP patterns identified in *Salmonella enterica* genomes shared among the three decadal groups (Group A: Upto yr. 2000; Group B: 2001-2010; Group C: 2011-2019).

area). Following the serovar-conformed clustering, isolates of serovar Bareilly formed two clusters (faint aqua-shaded area, Fig. 3), serovar Typhimurium formed a small yet prominent cluster (blue-shaded area, Fig.3), and a mixed serovar cluster comprising of isolates belonging to serovar Berlin, serovar Senftenberg, serovar Weltevreden. Core genome alignment-based phylogenetic analysis revealed clustering patterns conforming to the serovar distribution among the isolates barring a few exceptions. In the absence of attributable metadata (such as source, host disease/condition, and outbreak information) such a clustering pattern is not unexpected as was also observed previously (Bloomfield *et al*. 2022).

Antibiotic approval vs antibiotic resistance and temporal trends

The drug approval data obtained from the Central Drug Standards Control Organization of India was used to construct a timeline of antibiotics approved in India as shown in Fig. 4. The timeline comprised of 23 antimicrobial classes. Among the Anti-TB drugs, only rifamycins were included in the timeline preparation. Best-hit AROs were plotted on their first occurrence (year). For ease of analysis, *Salmonella* strains were grouped into A (up to year 2000), B (2001-10), and C (2011-19).

Monobactams, carbapenems, beta-lactams, and aminoglycosides

Monobactams and carbapenems were approved for clinical use in India in 2002. However, genes for monobactam resistance (*marA* and *Escherichia coli soxS* with mutation) could be detected in a strain of *S. enterica* serovar Typhi (77-803) isolated in 1977. Two more genes (*golS* and *mdsA*) were detected in two *Salmonella enterica* subsp. *enterica* serovar Bareilly strains isolated from cumin powder and coriander powder in the year 1998 and 1999, respectively. The gene *bla*TEM-1, which confers resistance to

monobactams but not carbapenems could be detected from 2005 onwards and was mostly carried by *S. enterica* serovar Typhi H58 haplotype. Out of the 16 cephalosporin resistance genes, 14 were common in all three groups (decades). Beta-lactamase genes identified in our study included *bla*TEM-1, *Escherichia coli AmpC1*, and *Escherichia coli AmpH* of which *bla*TEM-1 and *bla*TEM-2 reportedly serve as the starting point of ESBL derivatives (Bush 2018). It took 34 years for *bla*TEM-1 to first occur in 2005 after the entry of cephalosporins in the Indian market in 1971. *AmpC* β-lactamases are clinically important cephalosporinases (Gupta *et al*. 2014) and the corresponding genes were not identified in Typhoidal salmonellae until 2010 when the first report of *bla*ACC-1 harboring *Salmonella* Typhi was isolated in India (Harish *et al*. 2010). However, we could detect *the Escherichia coli ampC1* beta-lactamase gene in all the strains included. While the possible explanation may be due to the cryptic resistance potential of *ampC* (Salipante *et al*. 2003), this finding also highlights the advantages of WGS-based AMR screening. Lately, the CTX-M family of â-lactamases has emerged as the dominant MDR contributor in gram-negative bacteria (Bush 2018). Though studies have reported the occurrence of *the bla*CTX-M gene among *Salmonella* isolates during 2001-10 (Jin and Ling 2006, Rotimi *et al*. 2008), our analysis could not detect *bla*CTX-M gene despite an increase in the usage of cephalosporins (Klein *et al*. 2018). A similar absence of cephalosporin resistance determinants in *Salmonella* strains was also reported earlier (Katiyar *et al*. 2020). Literature from India indicated that the occurrence of *the bla*CTX-M gene among Typhoidal serovars was comparatively less than among Non-typhoidal serovars (Divyashree *et al*. 2016, Jacob *et al*. 2021, Pfeifer *et al*. 2009). To our knowledge, the first Indian report of *bla*CTX-M among Paratyphi and Typhi was in 2015 (Roy *et al*. 2015) and 2017 (Ramachandran *et al*. 2017), respectively. As our study included a large number of Typhoidal strains (depending on availability) and only a few post-2017 Typhoidal strains, perhaps this led to the non-detection of *bla*CTX-M in the current analysis. Of the 10 aminoglycoside resistance genes identified, 5 were present in all three decadal groups. Of these, APH(3")-Ib (*strA*) and APH(6)-Id (*strB*) started appearing in the year 2005 in *Salmonella enterica* subsp. *enterica* serovar Typhi H58, while AAC(6')-Iaa, was detected initially from a strain of *Salmonella* enterica serovar Weltevreden isolated from imported frozen meat in the year 2013 and was subsequently identified from clinical isolates of serovar Typhimurium and *Salmonella enterica* subsp. *salamae*. Interestingly, *the aadA* gene was identified in one strain each in Groups A (1977) and C (2011), being missed during 2001-10. When we compared plasmid carriage rates among the genomes belonging to three groups (Group A – the upto year 2000; Group B – 2001 to 2010; Group C – 2011 to 2019) there was no significant difference (ANOVA, p=0.106) among these groups.

Fluoroquinolones, Tetracyclines, and Diaminopyrimidines

Fluoroquinolones (norfloxacin) were first approved in 1987 for treating urinary tract infections and quinolones were introduced 18 years previously. Among all AMR genes, fluoroquinolone resistance genes were the majority (17). *Escherichia coli parC* gene first surfaced in 2003 and was identified only in strains of serovar Typhi H58 and a few strains of serovars Senftenberg/Dessau and Typhi. *emrA* gene appeared later (2010) and was identified in serovars Weltevreden and Senftenberg isolated from imported frozen meat. Resistance to tetracyclines in salmonellae was mediated by 15 genes. *tetA* gene was identified in *S. enterica* serovar Typhi H58 isolated in the year 2011; Meanwhile, *tetB* and *tetR* were co-occurring in one strain of serovar Typhi isolated in 1977 (Group A) and three strains of serovar Typhi H58 of Group B (isolated in 2005 and 2006). Resistance to tetracycline antibiotics was mostly mediated by efflux pumps (*tetA* and *tetB*). However, in our investigation, *the tetA* gene was not detected until 2011, though lymecycline (tetracycline) was approved in 1962 and the co-occurrence of *tetB* and *tetR* was already there before 2011. Similar co-occurrence was also reported previously (Katiyar *et al*. 2020). Three genes namely, *rsmA*, *dfrA7*, and *dfrA15* conferred resistance to trimethoprim. The gene *rsmA* codes for resistance against multiple classes of antibiotics, whereas *dfrA7* and *dfrA15* are specific to diaminopyrimidines. A total of 5 strains in Group B and 11 strains in Group C were harboring *the dfrA7* gene.

Phenicols

Resistance towards phenicols was identified across all three decadal groups and contributed by 12 genes, except *mdsB* which was identified in 3 strains of serovar Typhimurium of Group C. Only two genes namely, *sul1* and *sul2* were responsible for making sulphonamides ineffective. The former was detected in all three groups, whereas the latter was identified from 2005 onwards. The genes *bla*TEM-1 and *sul2* co-occurred in the same 34 strains except for one strain of serovar Typhi H58 which harbored only *the bla*TEM-1

gene and not *sul2*.

Other antimicrobial classes

Seven genes were identified in all three decadal groups encoding glycylcycline resistance, of which six genes were identified in the earliest strain (strain 77-303) isolated in 1977. Fosfomycin resistance genes (mutated genes *Escherichia coli GlpT* and *UhpT*) were identified in all three groups. Resistance to peptide antibiotics was mediated by 5 genes. Except for one (*Escherichia coli* 16S rRNA mutation conferring resistance to edeine), the remaining genes were identified in all three decades. Similarly, triclosan resistance was also primarily mediated by 6 genes that were identified in all three decadal groups.

Resistance-associated SNP patterns

A total of 14 point mutation patterns (protein variant models) were identified across the decadal groups (Fig. 5). Co-occurrence was identified among two sets of SNPs (D350N- S357N and S83F-D87N). 5 SNPs were identified in group A whereas 10 and 12 were identified in group B and group C, respectively. R234F was the most predominant SNP identified followed by E350Q and co-occurring pair of D350N and S357N. Of the 14 patterns, 6 patterns (S80I, S83F–D87N, S83F, D87N, D87Y, E84G) led to fluoroquinolone resistance and 2 patterns (E350Q and E48K) mediated fosfomycin resistance. Resistance to beta-lactams, elfamycin, triclosan, and edeine were contributed by the double mutant (D350N–S357N), R234F, D87G, and U1506A, respectively. Clinically important triple mutant pattern *i.e.* mutations at codons 83 and 87 of *gyrA* region and codon 80 of *parC* region which contribute to fluoroquinolone resistance were identified in 4 strains of Group B and 14 strains of Group C. Two new SNPs (D87Y, U1506A) were observed in group C indicating evolving mechanisms of AMR (Fig. 5). The most important SNPs in *Salmonella* are mutations at codon 83 and 87 of *gyrA* gene and codon 80 of *parC* gene called triple mutants known for their notoriety for resistance to fluoroquinolones (Britto *et al*. 2018). In this study, we also identified triple mutants in line with an earlier report from India (Katiyar *et al*. 2020). However, we could not detect the gatifloxacin-resistant triple mutant pattern (gyrA-S83F, gyrA-D87G and parC-E84G) reported from Nepal (Thanh *et al*. 2016) despite the presence of E84G-*parC* in 4 strains in our study. Interestingly, the most abundant SNP, R234F which confers resistance to pulvomycin belonged to the elfamycin group of antibiotics yet to be approved in India (Yarlagadda *et al*. 2020).

Statistical analysis

Kruskal-Wallis and Bonferroni tests on AMR gene carriage rates in different groups indicated significant differences among three predominant serovars (Typhi, Typhi H58, Bareilly) at $p<0.05$. However, Kruskal-Wallis' analysis of gene carriage among the three decadal groups revealed no significant difference. Antibiotic efflux was identified as the most predominant ($p<0.05$) mechanism in all three groups. Of the 28 gene families recorded in our study of 210 genomes of *Salmonella enterica*, the resistance-nodulation-cell division (RND) efflux pump was observed to be the most predominant across the groups followed by the major facilitator superfamily (MFS) efflux pump. Kruskal-Wallis and Bonferroni comparison of SNP abundance indicated a significant difference between groups A and C ($p<0.05$) (Fig. 5). We compared the average carriage rate of AMR genes per genome between the H58 haplotype and other serovars of Salmonellae with no significant difference (t-test; $p = 0.913$). However, we did notice qualitative differences among these two sub-sets of isolates. While AMR genes such as APH(6)-Id, *bla*TEM-1, *catI, dfrA7, parC, qacEdelta1, sul1,* and *sul2* were observed in H58 haplotype, they were absent in the other serovars. Conversely, AMR genes such as AAC(6')-Iaa, *acrB, adeF, emrA, golS, gyrA* mutation (Triclosan), *mdsA*, and *mdsB* were absent in H58 haplotypes but identifiable in other serovars. Statistical analysis on temporally grouped strains revealed that there was no direct consequential effect on the magnitude of gene carriage despite the availability of more variety of antibiotics over the years. Among various serovars, the H58 haplotype was found to carry a significantly higher proportion of AMR genes in line with previous reports (Wong *et al*. 2015). We observed that efflux and targ*et al*teration were major mechanisms that contributed towards antimicrobial resistance in *Salmonella enterica* as was also highlighted earlier (Martins *et al*. 2011). We noted that the RND efflux pump was the most commonly observed AMR gene family in our study, which validated their importance in effecting multi-drug resistance among Gram-negative bacteria (Fernando and Kumar 2013). Though, our results indicated no significant difference between the H58 haplotype and other serovars in terms of carriage rate of AMR genes (per genome basis) there was a qualitative difference in the gene distribution among these two groups of organisms. The gene distribution among isolates of the H58 haplotype indicated that genes carried by these were part of class-1 integron as was identified previously by

Wong *et al*. (2015) also. Nonetheless, our data needs to be interpreted with a safety margin considering the limited number of genomes that could be recruited for the study due to availability constraints.

While our study shone new light on the AMR landscape of *Salmonellae* from India over four decades, certain limitations were beyond our control. These included the absence of genomic data before 1977, a larger share of Typhoidal strains in the dataset, and a lack of sufficient representation from allied sectors (veterinary, foods, etc.). Further, not all AMR strains of *Salmonellae* are sequenced and submitted to public databases.

CONCLUSION

Taken together, our study offered new insights into the larger picture of the AMR among *Salmonella enterica* genomes from India which is considered a pivotal country for AMR emergence. Our results, based on available genomes in a public database (NCBI), highlighted that occurrences of AMR genes and mechanisms could precede antibiotic approval and usage (monobactams, carbapenems, glycylcyclines, fosfomycin, elfamycins, and edeine antibiotics). Moreover, there was no significant increase in the resistance gene carriage over the past four decades despite the availability of an increasing variety of antibiotics. Our study also reasserted the usefulness of WGS-based AMR detection for delineating AMR trends even for the isolates predating the WGS era. However, due to the paucity of appropriate metadata of the isolates, further studies are required to extrapolate the outcomes of our study to the generalized picture of AMR in salmonellae.

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