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CHAPTER 7

Current Status of Antimicrobial Resistance in Enteric Bacterial Pathogens

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Abstract: Bacterial enteric pathogens are by far the most dominant scourge of mankind. There are more than 200 million cases and 3 million deaths caused by these bacteria every year. Before the antimicrobial era, there were pandemics of enteric diseases which sometimes swept away whole populations. Advent of antimicrobial era provided a tool in the hand of mankind to fight this menace. In the beginning the results were promising and there was optimism of a decisive victory against disease causing bacteria. But the reality dawned within a couple of decades when antimicrobial resistance started to emerge and every new antimicrobial was generally knocked out in a couple of years. It became apparent that these bacteria held a distinct advantage because of very fast evolution rate due to relatively simple and small genome and short generation time. Currently, we are always playing a catch up game because the enemy is always ahead. The emergence of multiple drug resistance (MDR) has aggravated the situation and there is a distinct possibility that some of these menacing bugs may get out of control and situation of pre-antimicrobial era may return. Recently, a new term extreme-drug resistance (XDR) has been coined. This refers to bacteria resistant to all available drugs. This aptly summarizes the situation we are facing today. This catastrophe can only be avoided by putting more efforts in developing new concepts and products. This chapter is an effort to encompass the properties of these pathogens, the antimicrobials currently in use and the mechanisms of drug resistance evolved by these formidable bacteria.

Keywords: Antimicrobial drug resistance, human enteric pathogens, molecular mechanisms.

INTRODUCTION TO BACTERIAL ENTERIC PATHOGENS

Most of the bacterial enteric pathogens belong to family *Enterobacteriaceae.* In

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addition, *V. cholerae*, and some microaerophilic/anaerobic bacteria especially *Campylobacter jejuni* are important enteric pathogens. *Although Enterobacteriaceae* includes nearly 50 genera, the significant members are *Salmonellae*, *Shigellae*, and pathogenic *Escherichia coli.* Members of the *Enterobacteriaceae* family are rod-shaped, and are typically 1-5 μm in length, Gram-negative, and facultative anaerobes. Many members of this family are a normal part of the gut flora found in intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants.

Members of *Enterobacteriaceae* family not only cause enteric diseases but are also important causes of urinary tract infections (UTIs), respiratory tract infections, bloodstream infections, hospital and healthcare associated pneumonias, and various intra-abdominal infections. Lower respiratory tract and bloodstream infections are the most lethal and UTIs are the most common [1]. The emergence and spread of resistance in *Enterobacteriaceae* are complicating the treatment of serious nosocomial infections and threatening to create strains resistant to all currently available agents [2].

Salmonellae --- Cause of Typhoidal Diseases

The majority of disease-associated *Salmonella* are serovars of *S. enterica* subspecies *enterica* that accounts for 99% of all human and animal infections [3]. *S. enterica* serovars *Typhi*, *Paratyphi A*, *Paratyphi B* and *Paratyphi C* are collectively referred to as typhoidal *Salmonella* serovars [4].

Typhoid fever is a potentially fatal bacterial infection caused primarily by *Salmonella enterica* serovar Typhi (hereafter referred to as *S. Typhi*). The estimated incidence is approximately 33 million cases each year. In the developed countries, the incidence is much lower, and most cases are usually from travelers returning from endemic areas. Humans are the natural host and reservoir for *S. Typhi* which can survive for days in groundwater or seawater and for months in contaminated eggs and frozen oysters. The infectious dose varies between 10^3-10^6 organisms when taken orally. Transmission of infection occurs by ingestion of food or water contaminated with feces.

In Asian countries, the incidence of typhoid fever among children (5-15 years) appears to be highest in South Asia (400-500 cases per 100,000 persons per year), intermediate in Southeast Asia (100-200 cases per 100,000 persons per year) and lowest in Northeast Asia (<100 cases per 100,000 persons per year). Similar high fever incidence rates have been reported from Bangladesh [5]. These data, together with the data from Nepal [6], seem to suggest that the sub-continental nations, including India, Pakistan, Bangladesh and Nepal, are at a very high risk for typhoid fever. These data are consistent with the previous observations [4].

Despite the role of the Vi antigen as a distinguishing feature of serovar *Typhi*, *Vi* negative isolates are not uncommon. Vi negative *S. Typhi* have been reported from various locations. In the 1970's Vi negative isolates were encountered in Jamaica [7], Indonesia [8], New Zealand, and Malaysia [9]. In 2000, there was a report from India showing prevelance of Vi negative strains [10]. We have also isolated and reported these strain from Faisalabad, Pakistan [11].

S. Paratyphi A is more prevalent in war torn and developing countries. A report from New Delhi, India, demonstrated a significant increase in *S. Paratyphi* A isolation from 1.7% in 2001 to 18% in 2005 to 2006. There was an increase of 3.8% in patients requiring hospitalization. One large sample size report from Nepal also indicated an increased incidence of *S. Paratyphi* A (from 23% during 1993 to 1998 to 34% during 1999 to 2003) [12]. Some reports from China also demonstrate a high incidence of *S. Paratyphi* A with infection rates up to 64% among all EF cases [13]. Our group has also reported high prevalence of *S. Paratyphi* A in Faisalabad, Pakistan [14].

Shigellae --- Major Cause of Bacillary Dysentery

Diarrheal diseases are the most common cause of morbidity and mortality and rank as fourth most common killer disease in the world and second as a cause of years of productive life lost due to premature mortality and morbidity [15]. Among diarrheal diseases, dysentery caused by *Shigella* is one of the most important contributors. Shigellosis is a major public health problem not only because of morbidity but also for growth retardation and malabsorption in children.

According to WHO, the annual number of *Shigella* episodes in developing countries throughout the world is 164.7 million. Out of these, 163.2 million (99%) are in developing countries (including 1.1 million mortality) and 1.5 million in industrialized countries [16]. High risk population for *Shigella* infection includes kids less than five years of age, and senior citizens. It has been found that 69% of all episodes and 61% of all deaths in shigellosis involve children under 5 years of age [17]. Improper personal hygiene and sanitation resulting in the contamination of food and drinking water are the major causes of spread of *Shigella* infection [18].

Shiga Toxin Producing *E. coli* (STEC)

Escherichia coli (E. coli) are predominately found in intestinal micro flora of humans and other mammals. These commensal *E. coli* are usually harmless but certain pathotypes are implicated in diarrhea and other enteric problems and called as "diarrheagenic *E. coli*". Diarrheagenic *E. coli* have been divided into six major pathotypes which include enteropathogenic *E. coli* (EPEC), atypical enteropathogenic *E. coli* (ATEC), enterohemorrhagic *E. coli* or Shiga toxin-producing *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) [19, 20].

STEC (EHEC) are important emerging pathogens and have been associated with number of complications like bloody diarrhea, hemorrhagic colitis and potentially fatal renal disease, hemolytic uremic syndrome (HUS). Year 1983 was momentous for microbiologists as first reconnaissance of STEC O157:H7 outbreak [21] and later its association with HUS was reported [22]. Since then STEC has been detected from an increasing number of food borne outbreaks of bloody diarrhea and HUS. The most notorious STEC serotype is O157:H7. However non-O157 STEC serotypes are also emerging as notable pathogens.

There are more than 250 different *E. coli* O serotypes implicated in Shiga toxin production and 100 of them have been found in various diarrheal outbreaks in humans [23]. Various studies indicate that among non-O157:H7, serotype O111, O26, O145 and 103 are more frequently associated with STEC outbreaks and HUS [24, 25]. The incidence of STEC mostly varies according to age group. In

USA, highest incidence (0.7 cases per 100,000) was observed in children under 15 year of age. In most of the cases (63-85%), the etiological agent is transmitted through food stuff [26].

Extraintestinal Pathogenic *E. coli* (ExPEC)

E. coli isolates capable of causing disease outside the gastrointestinal tract are known as extraintestinal *E. coli*. Extraintestinal pathogenic *E. coli* (ExPEC) can invade urinary tract, cerebrospinal fluid and blood stream. ExPEC are responsible for a variety of diseases such as urinary tract infections (UTIs), neonatal meningitis, septicemia, nosocomial pneumonia, intra abdominal infections, osteomyelitis and wound infections. These are major pathogens of UTIs in normal and unobstructed urinary tracts [27, 28].

Extraintestinal strains of *E. coli* can infect every organ, all age groups and all types of hosts. Severe illness and mortality can occur in normal, healthy hosts; however, adverse outcomes become increasingly prevalent in the presence of co-incidental disease and abnormalities in host defenses [29].

Vibrios – Cause of Cholera

There is some debate regarding inclusion of cholera in enteric bacterial diseases because the causative organisms are quite different from other enteric pathogens. But we have included cholera because it is one of the most devastating diseases of enteric system. Cholera, caused by *Vibrio* is an acute diarrhoeal disease that can kill within hours if left untreated. There are estimated 3–5 million cholera cases and 100,000–120,000 deaths due to cholera every year. However, up to 80% of cases can be successfully treated with oral rehydration salts [30].

Cholera has smoldered in an endemic fashion on the Indian subcontinent for centuries. Epidemic cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. In 1961, the "El Tor" biotype (distinguished from classic biotypes by the production of hemolysins) reemerged and produced a major epidemic in the Philippines to initiate a pandemic. Since then, this biotype has spread across Asia, the Middle East, Africa, and parts of Europe [31].

The genus *Vibrio* consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum. The Family *Vibrionaceae* is distinct from Family *Enterobacteriaceae* although members of both families are described as facultatively anaerobic Gram-negative rods [32].

V. cholerae and *V. parahaemolyticus* are pathogens human. Both produce diarrhea, but in ways that are entirely different. *V. parahaemolyticus* is an invasive organism affecting primarily the colon; *V. cholerae* is noninvasive affecting the small intestine through secretion of an enterotoxin [31].

Campylobacter – Cause of Acute Diarrhea

Campylobacter like Vibrio are different from members of Enterobacteriacae. The main pathogenic species*, C. jejuni* and *C. coli* are microaerophilic, spiral-shaped bacteria that asymptomatically colonize birds, including chicken [33, 34]. But these bacteria cause serious disease in humans. *Campylobacter* species are a leading cause of acute infectious diarrhea resulting in up to 14% of cases worldwide. In a study of 100, 000 persons in Sweden, *C. jejuni* was isolated in 56% of enteritis cases [35]. Ingestion of undercooked poultry and cross-contaminated food stuffs results in a spectrum of acute diarrheal disease, ranging from watery diarrhea to dysentery and a mesenteric adenitis syndrome mimicking acute appendicitis [36].

INTRODUCTION TO ANTIMICROBIALS

What is an Antimicrobial?

An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, or protozoa. Antimicrobial drugs either kill microbes (microbiocidal) or prevent the growth of microbes (microbiostatic). Disinfectants are antimicrobial substances used on non-living objects or outside the body.

Scientifically, antibiotics are only those substances that are produced by one microorganism that kill or prevent the growth of another microorganism. However, in common usage, the term antibiotic is used to refer to almost any drug that attempts to rid our body of a bacterial infection. Antimicrobials include not just antibiotics, but synthetically formed compounds as well.

Before penicillin became a viable medical treatment in the early 1940s, no true cure for gonorrhea, throat infections, or pneumonia existed. Patients with infected wounds often had to have a wounded limb removed, or faced death from infection. Now, most of these infections can be cured easily with a short course of antimicrobials.

There are mainly two classes of antimicrobial drugs:

1. Those obtained from natural sources:

    a. β-lactam antibiotic (such as penicillins, cephalosporins)

    b. Protein synthesis inhibitors (such as aminoglycosides, macrolides, tetracyclines, chloramphenicol, polypeptides)

2. Synthetic agents:

    c. Sulphonamides, cotrimoxazole, quinolones

Antimicrobial Modes of Action

The antimicrobials act on different targets in a bacterial cell. The β-lactams which include penicillins, cephalosporins and several other groups act on cell wall by binding to and inhibiting enzymes needed for the synthesis of peptidoglycan. This creates breaches making the organism to burst in hypotonic surroundings. Quinolones inhibit DNA replication with the formation of double-stranded DNA breaks; treatment with rifamycins arrest DNA dependent RNA synthesis. Inhibitors of protein synthesis induce cell death or stop cell growth by affecting cellular energetics, ribosome binding and protein mistranslation, as tetracycline inhibit protein synthesis by binding to 30S ribosomal unit of ribosome and chloramphenicol by binding to 50S ribosomal subunit. These are reversible bindings, but aminoglycosides bind to 30S ribosomal unit irreversibly. Polymyxins behave as detergents increasing the permeability of the membranes which encase bacteria, and causing the contents of the bacterial cell to leak out. In addition, recent evidence points towards a common mechanism of cell death involving disadvantageous cell responses to drug induced stresses that are shared

by all classes of bactericidal antimicrobials (Fig. **1**), which ultimately contributes to killing by these drugs [37, 38].

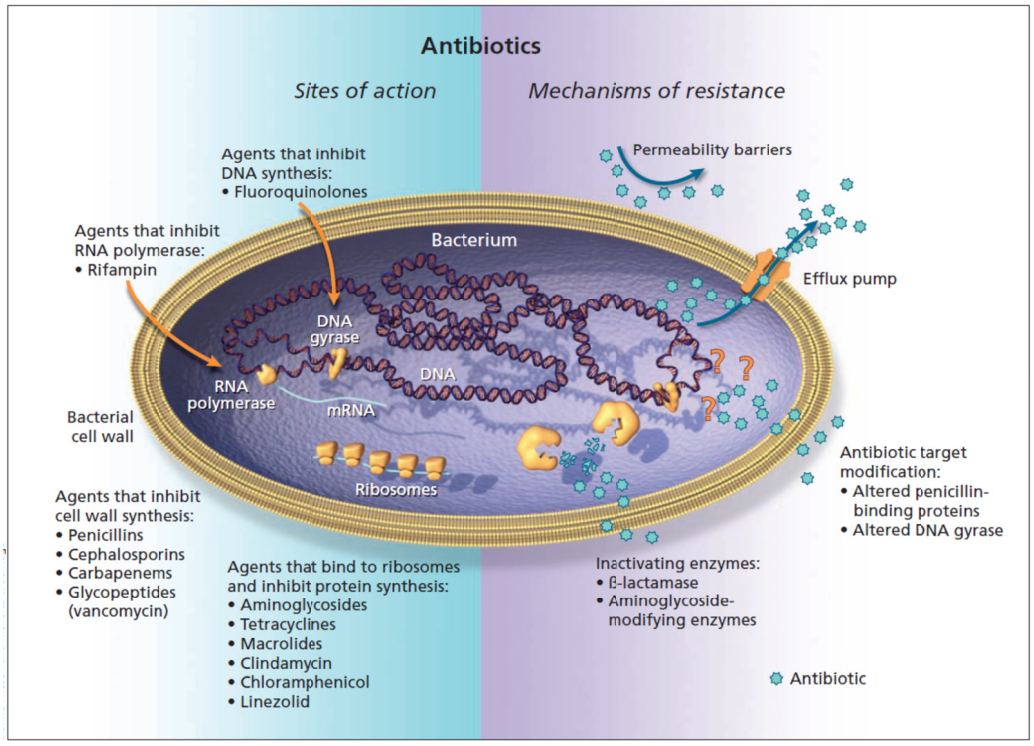

Figure 1: Sites of action and mechanisms of resistance development of various antimicrobial agents [39].

β-lactam Antibiotics

The β-lactam drugs are the most widely used antimicrobial agents exhibiting a rapid bactericidal effect and are well tolerated. All β-lactam drugs share a characteristic ring structure (the β-lactam ring) from which their name is deduced and on which antimicrobial activity of these drugs depends. These drugs target bacterial cell wall and are bactericidal. β-lactam drugs are divided into four major groups: penicillins, cephalosporins, monobactams, and carbapenems [40, 41].

Quinolones /Fluoroquinolones

Quinolones represent a group of synthetic chemotherapeutic antibacterial agents. Nalidixic acid was the first quinolone with antibacterial activity.

Fluoroquinolones are one of the several derivatives of quionolones [42]. The early quinolones such as nalidixic acid are considered first-generation quinolones; ciprofloxacin, and ofloxacin as second-generation; gatifloxacin, sparfloxacin, and temafloxacin are included in third -generation and trovafloxacin, moxifloxacin, and gemifloxacin are representatives of fourth-generation fluoroquinolones [43]. Quinolones are bactericidal and exert their antibacterial effects by inhibition of bacterial topoisomerase enzymes, namely DNA gyrase (bacterial topoisomerase II) and topoisomerase IV. These essential bacterial enzymes alter the topology of double-stranded DNA (dsDNA) within the cell.

The Antifolate Group

Trimethoprim, an antifolate is a synthetic antimicrobial agent, which interferes with folate synthesis in both Gram-negative and Gram-positive bacteria. It behaves bacteriostatically after competitive and strong binding to dihydrofolate reductase (DHFR) [44], which catalyses the formation of tetrahydrofolate from dihydrofolate. Although DHFRs from eukaryotic cells can also bind trimethoprim, the affinity of the drug to the bacterial enzymes is higher [45]. Sulfonamides are synthetic substances too, which inhibit the first step of bacterial folate synthesis pathway and work bacteriostatically [46]. Trimethoprim combined with sulfonamide have a bactericidal effect and this synergistic effect is the reason why most of the preparations on the market are a combination of trimethoprim and sulphonamides [47]. The combination is known as co-trimoxazole or trimethoprim-sulphamethoxazole.

Aminoglycosides

Aminoglycosides are among the oldest and powerful bactericidal drugs, characterized by the presence of an aminocyclitol ring linked to amino sugars in their structure. They have a broad spectrum of activity against Gram-positive and Gram-negative bacteria, mycobacteria and protozoa. These drugs act by binding irreversibly to the ribosomal acceptor (A) site and inhibiting bacterial protein synthesis. Examples of these drugs include those derived from *Streptomyces* spp. (streptomycin, neomycin and tobramycin) or *Micromonospora* spp. (gentamicin) or synthesized *in vitro* (netilmicin, amikacin, arbekacin and isepamicin) [48].

Tetracyclines

Tetracyclines were discovered in 1940s. Broad spectrum activity, low toxicity and low cost had made tetracyclines perfect therapeutic agents but their efficacy reduced by passage of time [49]. Tetracyclines act by penetrating bacterial cells by passive diffusion and binding reversibly to the ribosome, thereby preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site and inhibiting bacterial protein synthesis [50].

Chloramphenicol

Chloramphenicol binds reversibly to the 50S subunit of the bacterial ribosome and inhibits peptidyl transferase reaction, which forms the peptide bonds between the amino acids, and thereby suppress bacterial protein synthesis. Chloramphenicol has broad spectrum activity and act bacteriostatically on Gram-negative and Gram-positive bacteria [51].

ANTIMICROBIAL DRUG RESISTANCE

Antimicrobial resistance is ability of the microorganisms to with stand the dose of antimicrobial that was effective in the past due to the repeated exposure of a microbe to a particular drug. Bacterial drug resistance can be attained through intrinsic properties, mutation acquired mechanisms, biochemical alterations, selective pressure or physical barriers such as biofilm formation.

Intrinsic Mechanisms

Intrinsic resistance may naturally occur as a result of the bacteria's genetic makeup. It is either due to the inaccessibility of the targets by the drug. *e.g.,* aminoglycoside resistance in strict anaerobes is due to multidrug efflux systems or drug inactivation by the bacteria. *E. coli* is intrinsically resistant to vancomycin because vancomycin is too large to pass through porin channels in outer membrane. Gram-positive bacteria, on the other hand, do not possess an outer membrane, thus are not intrinsically resistant to vancomycin. Drug resistance may also be due to naturally occurring genes found on the host’s chromosome, such as, AmpC β-lactamase of Gram-negative bacteria and many efflux systems.

Mutational Resistance

Mutations in the genome results in the target site modification or reduced permeability or uptake of the drug by organism (Fig. **2**). Mutations can also cause metabolic bypass or derepression of multidrug efflux systems.

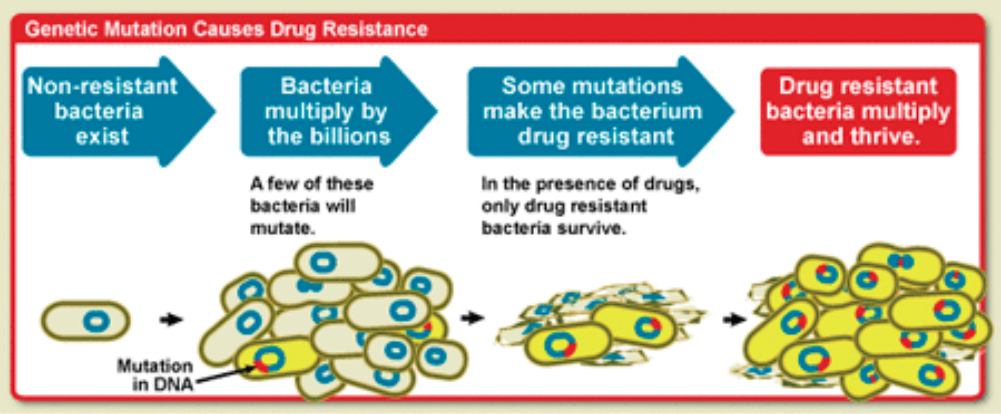

Figure 2: Genetic mutation causes drug resistance [52].

Extrachromosomal or Acquired Resistance

This type of drug resistance is disseminated by plasmids or transposones resulting in either drug inactivation, drug efflux, target site modification or metabolic bypass [53]. Among the horizontal gene transfer mechanisms, conjugation (*via* plasmids and conjugative transposons) is thought to play the most significant role in the spread of resistance genes [54]. But other mechanisms including transduction (*via* bacteriophages) and transformation (*via* incorporation of chromosomal DNA, plasmids, and transfer of DNAs from dying organisms into the chromosome) are also important [54] (Fig. **3**).

Selective Pressure

In the presence of an antimicrobial, microbes are either killed or, if they carry resistance genes, survive. These survivors will replicate, and their progeny will quickly become the dominant type throughout the microbial population.

Biochemical Mechanisms of Drug Resistance

Antimicrobial agents are rendered inactive by different mechanisms that include:

- Enzymatic drug modification and destruction
- Mutational alteration of the target protein
- Substitution and protection of drug targets
- Reduced drug accumulation due to efflux systems, porins and outer membrane proteins [56].

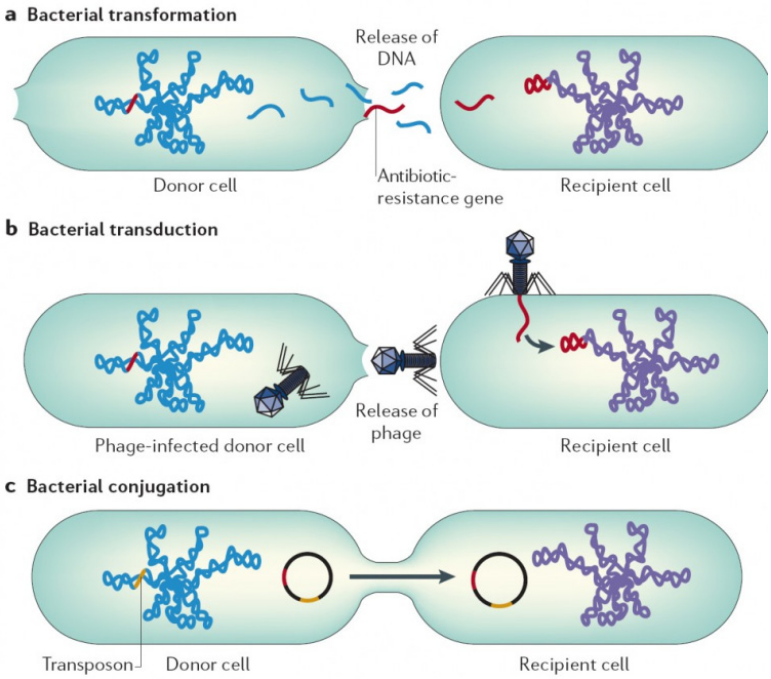

Figure 3: Horizontal gene transfer between bacteria [55].

TOOLS OF GENETIC MOBILITY

The physical movement of DNA relies on a number of molecular 'cut and paste' mechanisms that are able to control and translocate DNA fragments. Enzymes with these potentialities include recombinases, transposases, integrases and resolvases which are encoded by an assortment of selfish mobile genetic elements (Fig. **4**) insertion sequences, transposons and integrons). These genetic elements can facilitate gene deletion or capture, and accretion of genetic elements on higher order mobile elements such as conjugative plasmids [57].

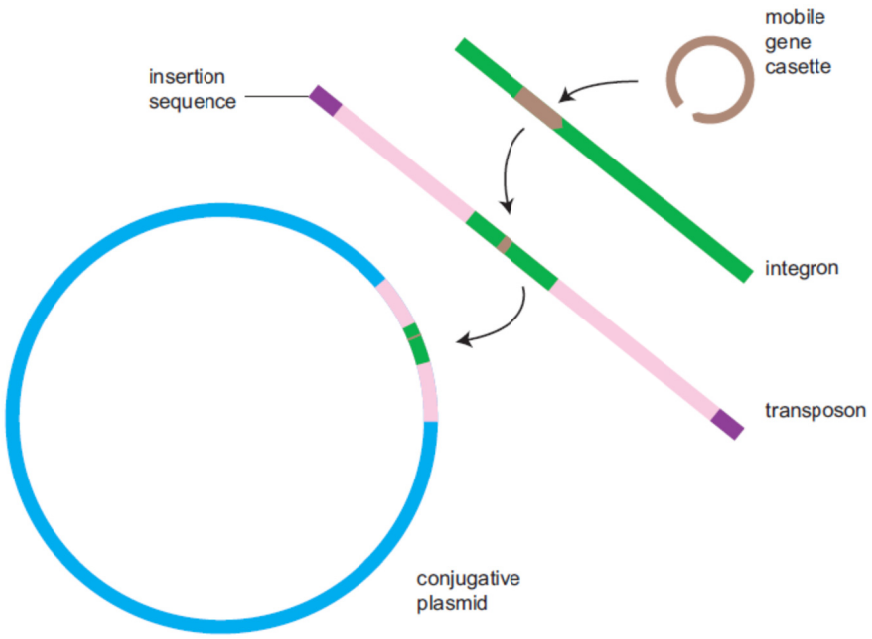

Figure 4: The schematic composition of mobile genetic elements [57].

Integrons

Integrons are "assembly platforms that incorporate exogenous open reading frames by site-specific recombination and convert them to functional genes by ensuring their correct expression". An integron includes two parts: the gene cassette and the recombination platform. The recombination platform also called the 'core' integron includes a site-specific recombinase (integrase) gene (*intI*), a recombination site (attI) and an outward- orientated promoter (Pc) that directs transcription of the captured genes (Fig. **5**). The gene cassette usually consists of one or more genes and a second type of recombination site which was originally termed the 59-base element by Hall and colleagues [58], but is now called *attC* (attachment site associated with cassettes). Although not independently mobile, integrons are widespread versatile DNA elements and can be divided into two distinct subsets: the mobile integrons and the chromosomal integrons [58, 59].

Mobile Integrons

These are primarily involved in the spread of antimicrobial resistance genes and are linked to mobile DNA elements. Five classes of mobile integrons are known to have

a role in the dissemination of antibmicrobial resistance genes. Class 1, 2 and 3 integrons which are involved in multiple- antibmicrobial -resistance phenotype, belongs to 'historical' classes of mobile integrons. The other two classes of mobile integrons, class 4 and class 5, have been identified in *Vibrio* species through their involvement in the development of trimethoprim resistance [59].

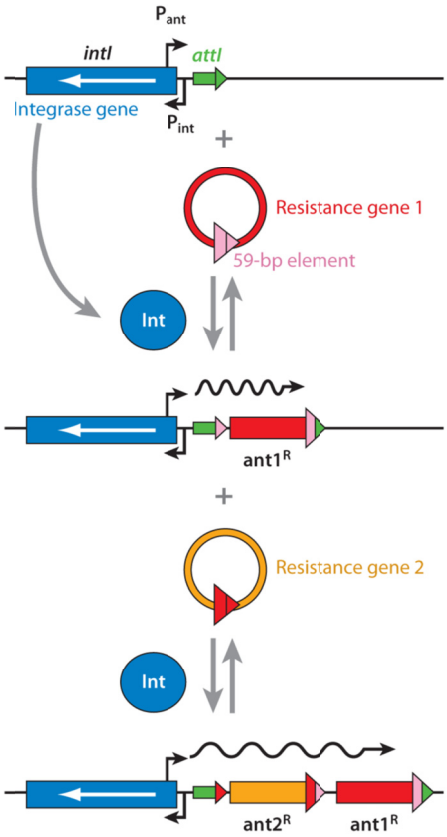

Figure 5: Mechanism of intake of resistance genes by integrons [60].

Chromosomal Integrons

These were first identified on chromosome 2 of the *V. cholerae* genome as an organization of cluster of repeated DNA sequences. The key features that define this subset include encoding of a specific integrase, VchIntIA, which is related to the integrases encoded by mobile integrons but has two characteristics that distinguish it from known mobile integrons. Firstly, large number of gene

cassettes are associated with the integron and secondly, being immobile, it is located on the chromosome and not associated with mobile DNA elements [61].

Transposons

Transposons are self directed elements that code for transposases. Transposases are enzymes that bind to the ends of a transposon and catalyze its movement to another part of the genome by a cut and paste mechanism or a replicative transposition mechanism. Different types include unit, composite, conjugative and mobilizable transposons [62].

Conjugative transposons also known as integrative conjugational elements (ICEs) play a substantial role in the dispersal of antibiotic resistance genes amongst pathogenic bacteria and can only maintain themselves stably by integrating into the chromosome [63, 64]. They have an enormously broad host range (Gram-negative and Gram-positive bacteria) and confer resistance to a wide range of antimicrobials (ampicillin/penicillin, cefoxitin, chloramphenicol, erythromycin, mercuric chloride, gentamycin, kanamycin, streptomycin, tetracycline-minocycline, and vancomycin) [65].

Plasmids

Plasmids can be classified by several criteria as conjugative or mobilizable, and on the basis of incompatibility groups copy number and host range [66]. Plasmids of Gram negative bacteria are either conjugative or mobilizable depending upon the presence or absence of three different elements. These three elements are present on all conjugative plasmids and include a cis-element called *oriT* (origin of transfer), one or more *mob* (mobilizing) gene and the *tra* (transfer) gene which codes for the pilus gene anchored in the two membranes and responsible for making contact with the recipient cells [67].

Plasmids are most frequently classified into incompatibility groups according to their mode of replication and maintenance in a bacterial cell [68]. Two different plasmids are said to be compatible with each other, if they can stably coexist without selective pressure [67].

Based on the copy number per bacterial chromosome, plasmids can be arranged into four different groups: low-copy-number (1-2), medium-copy-number (5-10),

high-copy-number (20-25) and very-high-copy-number (100-500). Host range is another feature for characterizing plasmids [67].

Drug Efflux Pumps

Efflux pumps are transport proteins involved in the expulsion of toxic substrates (including virtually all classes of clinically relevant drugs) into the external environment from inside of cells. These proteins are found in both Gram-positive and Gram-negative bacteria as well as in eukaryotic organisms [69]. Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including drugs of multiple classes); such pumps can be associated with multiple drug resistance (MDR). Drug efflux pumps are now recognized as significant contributors to both innate and acquired bacterial resistance to many of these agents because of the very broad variety of substrates they recognize [70, 71].

Pathogenicity Islands

Bacterial species can frequently exchange 5-10 kb regions of genomic DNA. These regions are generally designated as ‘islands’ due to their large size. These genomic islands sometimes carry virulence associated genes or drug resistance genes. Such genomic islands are referred to as pathogenicity islands (PAI or PI). PAIs are commonly found in pathogenic strains whereas in non pathogenic strains they are absent or rarely found [72]. The unstable regions are of > 30 kb size carrying bacterial virulence genes. These regions are also called plasticity zones with atypical G+C contents relative to the rest of the genome and such DNA segments are originated from a different organism through horizontal gene transfer. PAIs are associated with tRNA genes, which act as integration sites for foreign DNA. Insertion sequences or direct repeats often flank these PAIs, while transposases, origins of plasmid replication, and integrases are often found within these PAIs [73].

BIOFILMS - THE PHYSICAL BARRIERS

A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also

referred to as slime (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings [74, 75] (Fig. **6**). The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium.

Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibmicrobials [76, 77]. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated [78].

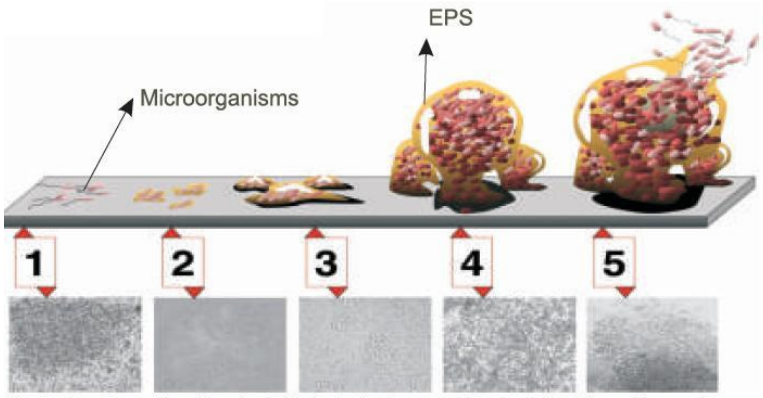

Figure 6: Steps in biofilm production [79].

BIOFILMS AND INFECTIOUS DISEASES

Biofilms are important survival mechanisms for bacterial cells. It is difficult for phagocytic cells to engulf bacteria in biofilms. Also, biofilms are much more resistant than planktonic cells to antimicrobial agents. These are highly developed colonies with bacteria at different stages of life placed in separate segments with intercommunication facilities *via* water channels. An antimicrobial has to be able to reach its target bacteria before it is inactivated during its journey through a biofilm. There are some bacteria in biofilms called “persistors” which are most

difficult to kill. Flouroquinolones are effective against biofilms because of their fast penetration rate.

Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections [80]. Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis [81], coating contact lenses [82], and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves [83]. More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds [84].

SPECIFIC MECHANISMS OF DRUG RESISTANCE AGAINST MAJOR DRUG GROUPS

β-lactams

Bacteria show resistance to β-lactam antibiotics due to the hydrolysis of antibiotic by β-lactamase enzymes, the most common resistance mechanism in Gram-negative bacteria [85]. Other mechanisms of β-lactam resistance described include cellular permeability which occurs through changes in outer membrane proteins leading to a lowered permeability for the enzyme (porin deficiencies), or the export of β-lactams *via* multi-drug transporters [40]. Alterations or modification in penicillin binding proteins (PBPs) are described in some Gram-positive bacteria [86].

Quinolones /Fluoroquinolones

Mechanisms of bacterial resistance to quinolones fall into three categories: 1) alterations in target of quinolones, 2) decreased accumulation of quinolones due to impermeability of the membrane or 3) due to an over expression of efflux pump systems [91].

Quinolones target alterations occur predominately in domains near the enzyme active sites, which are known as the quinolone-resistance determining region

(QRDR). QRDR is a portion of the DNA-binding surface of the topoisomerase at which amino acid substitutions can diminish quinolone binding and subsequently cause resistance to quinolones. Quinolone resistance generally results from stepwise chromosomal point mutations mainly in the *gyrA* and *parC* genes due to amino acid substitution. Amino acid substitutions within the quinolone resistance-determining region (QRDR) mostly involve the replacement of a hydroxyl group with a bulky hydrophobic residue [92].

The initial mutations in *gyrA* result in resistance to nalidixic acid and afterwards, additional mutations lead to fluoroquinolone resistance [93]. Plasmid-mediated quinolone resistance (PMQR) is associated with low level resistance to fluoroquinolones and represent the production of Qnr proteins protecting the targets against the effects of quinolones [94].

Antifolates

So far, more than 30 different trimethoprim resistance mediating dihydrofolate reductase (*dfr*) genes have been identified [87]. These are subdivided on the basis of their structure into two major types 1 and 2 [88], which nowadays are referred to as *dfrA* and *dfrB*. A second trimethoprim resistance mechanism is to use alternative folate pathways either by usage of external supply of thymidine or by the use of other thymidylate synthases [89].

Sulfonamide resistance can result either from mutations in the chromosomal DHPS gene (*folP*), which decreases DHPS affinity for the sulfonamide inhibitors or, more frequently, from the acquisition of genes encoding alternative drug-resistant variants of the DHPS enzymes which are plasmid borne [46]. Only three genes *sul1, sul2, sul3* are currently known to code for sulfonamide-resistant DHPS [90].

Aminolycosides

Most frequently encountered aminoglycoside resistance is mediated by modifying enzymes which attach certain groups to the aminoglycoside molecule thereby destroying its antibacterial activity. These enzymes are classified as aminoglycoside N-acetyltransferases (AAC), aminoglycoside O-adenyltrans-

ferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside O-phosphotransferases (APH) depending on their type of modification. For each of these three classes, numerous members are known which differ more or less extensively in their structure [95, 96].

In addition to aminoglycoside-modifying enzymes other mechanisms that confer resistance against these agents include decreased accumulation of the drug due to expression of efflux systems, and methylation of the 16S rRNA within the 30S subunit [97].

Tetracyclines

Bacterial resistance is mediated mainly by three mechanisms: 1) efflux of antibiotic to reduce intracellular concentration, 2) ribosome protection of the antibiotic target and 3) modification of antibiotic making it inactive [50]. The most common resistance mechanism against tetracyclines in Gram-negative bacteria is efflux of these drugs. Different classes of tetracycline specific exporters have been identified [98].

Chloramphenicol

The most common resistance mechanism to chloramphenicol in Gram-negative bacteria is the expression of a chloramphenicol acetyltransferase (CAT), which mediates O-acetylation of chloramphenicol, destroying its affinity for bacterial ribosomes and thus its ability to inhibit bacterial growth [99]. There are two separate families of CAT enzymes in bacteria, CATA and CATB [100].

DRUG RESISTANCE IN ENTERIC PATHOGENS

The global problem of antimicrobial resistance is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents. Gastrointestinal, respiratory, sexually transmitted, and nosocomial infections are leading causes of disease and death in the developing world, and management of all these conditions has been critically compromised by the appearance and rapid spread of drug resistance. Even though surveillance of resistance in many developing countries is suboptimal, the general picture is one of accelerating rates

of resistance spurred by antimicrobial misuse and shortfalls in infection control and public health. Reservoirs for resistance may be present in healthy human and animal populations. Considerable economic and health burdens emanate from bacterial resistance, and research is needed to accurately quantify the problem and propose and evaluate practicable solutions. In this section, we will discuss the emerging drug resistance against most of the previously and also currently popular antimicrobials specifically with reference to enteric pathogens.

Typhoidal Bacteria

For over 60 years, drugs have been used to treat typhoid and the first drug introduced in 1948 for this purpose was chloramphenicol. It was followed by ampicillin and co-trimoxazole. These three drugs are called the first line antityphoidal drugs. Along with emergence of resistance against these first line antimicrobials, additional resistance to streptomycin and tetracyclines has been reported in many developing countries, especially Pakistan and India. Such strains are called multidrug-resistant (MDR) [101]. To cope with this situation, fluoroquinolones became the treatment of choice along with third generation cepaholsporins and azithromycin as alternative for resistant isolates [102].

Chloramphenicol was recognized as the drug of choice to treat typhoid fever when introduced in 1948 [103]. Two years later cases of chloramphenicol resistant typhoid fever were reported [104], but chloramphenicol resistance took a long time to become established in *S. Typhi* population. In May 1972 in Kerala, India the first reported antibiotic resistant typhoid fever outbreak occurred [105], which was proved to be plasmid borne. Two other chloramphenicol resistant outbreaks were also documented in the same year in Mexico and Vietnam; both were caused by IncH plasmids carrying *S. Typhi* [106, 107]. Some recent reports show the re-emergence of sensitivity in high proportions to chloramphenicol along with ampicillin and co-trimoxazole in *S. Typhi* strains [108]. Our recent findings provide support to these observations as we have found that resistance level against chloramphenicol is midway between ampicillin and co-trimoxazole [109].

Mechanisms conferring chloramphenicol resistance described so far in *Salmonella* are enzymatic inactivation and the export of chloramphenicol by specific efflux

proteins. The most common resistance mechanism to chloramphenicol in Gram-negative bacteria is the expression of a chloramphenicol acetyltransferase (CAT) which mediates O-acetylation of chloramphenicol, destroying its affinity for bacterial ribosomes and thus its ability to inhibit bacterial growth [99]. In a long duration study in Pakistan, it was unexpectedly found that the ratio of MDR isolates decreased gradually from 1995 to 2001 (Fig. **7**).

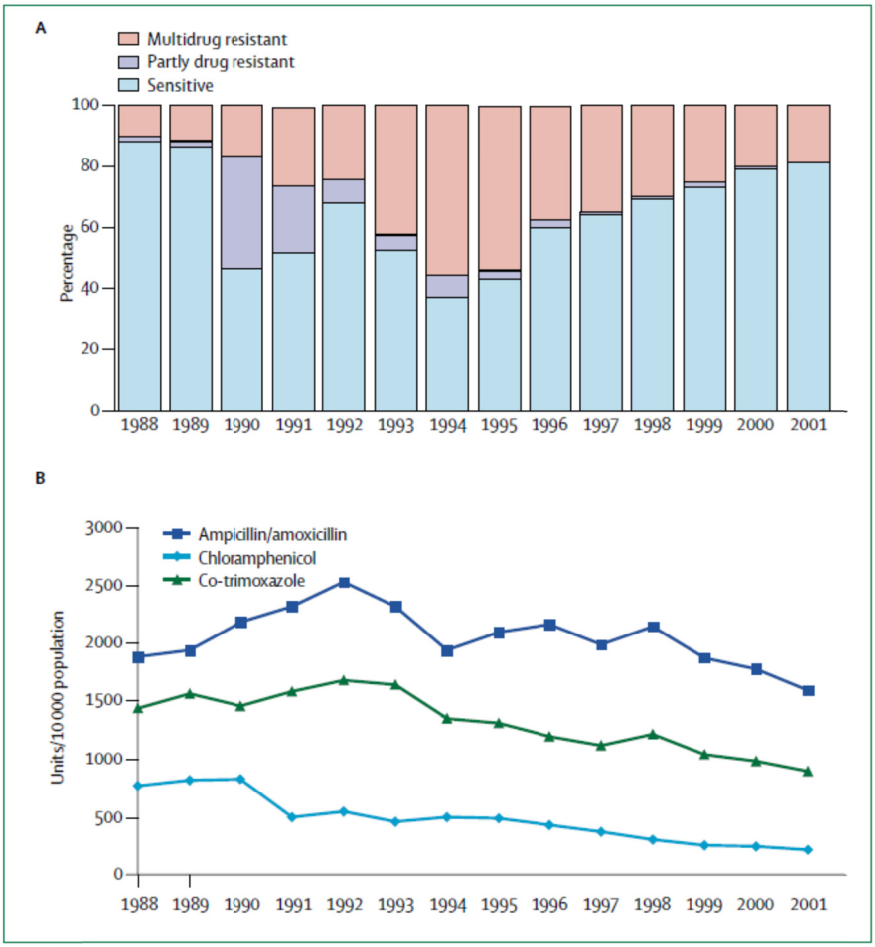

Figure 7: (A) Antimicrobial resistance patterns among *S. Typhi* isolates from children presenting at the Aga Khan University Hospital, Karachi, Pakistan (1988-2001); and (B) antimicrobial sales data for Karachi (units/10,000 population) in the same period [110].

During a large epidemic in Mexico in 1972, isolates resistant to both chloramphenicol and ampicillin were reported. However, resistance to these two

drugs was transferred independently by two separate plasmids [111]. The plasmids conferring resistance to chloramphenicol were later identified as incompatibility group H where as the ampicillin resistance plasmids were of the incompatibility group I or A/C [112]. The usefulness in treatment has however decreased as a consequence of increasing resistance, mainly due to β-lactamases like TEM and SHV. The action of these enzymes can in most cases be overcome with the addition of a β -lactamase inhibitor like clavulanic acid [113].

Genes encoding for β-lactamases are known as *bla* genes and a considerable number of *bla* genes have been identified in *Salmonella* while an ESBL-producing *S. Paratyphi* A was reported in India [114]. Among the TEM-type β-lactamases class 2b includes those encoded by the genes $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$ which are broad-spectrum penicillinases. Other bla_{TEM} genes, from class 2b include $bla_{\text{TEM-3}}$, $bla_{\text{TEM-4}}$, $bla_{\text{TEM-20}}$, $bla_{\text{TEM-27}}$, $bla_{\text{TEM-52}}$, $bla_{\text{TEM-63}}$, and $bla_{\text{TEM-131}}$ and code for extended spectrum β -lactamases (ESBLs) which can also inactivate oxyiminocephalosporins and monobactams. Among SHV-and OXA type β-lactamases found in *Salmonella* are those encoded by $bla_{\text{SHV-2}}$, $bla_{\text{SHV-2a}}$, $bla_{\text{SHV-5}}$, $bla_{\text{SHV-9}}$, $bla_{\text{SHV-12}}$ $bla_{\text{OXA-30}}$and $bla_{\text{OXA-53}}$ [115, 116]. But fortunately we have found in an ongoing study that ESBL production is not detectable in local isolates in Pakistan at present [109].

After the development of ciprofloxacin resistance in *S. Typhi* and *S. Paratyphi* A, cephalosporins (ceftriaxone and cefixime) were one of the few choices left for the treatment of enteric fever. The first reported trial for the use of ceftriaxone to treat typhoid fever was conducted in Bangladesh in 1988 [117]. Although resistance to third generation cephalosporins in non-typhoidal *Salmonella*e had been reported as early as 1989 [118], resistance in *S. Typhi* remains rare. The first cases of reduced susceptibility or resistance to ceftriaxone were documented in Bangladesh and Kuwait in 2008 [119, 120]. A recent case of ceftriaxone resistant *S. Typhi* was reported from an Iraqi woman who returned to Germany after a month's long visit in Iraq. Interestingly, this resistance was encoded on an IncN plasmid of ~50 kb carrying the $bla_{\text{CTX-M-15}}$ and *qnrB2* genes [121]. Fortunately, in Pakistan, ceftriaxone resistance is still very rare and it is considered as one of the most effective drugs against typhoid [109].

Until the 1980s, there was no report of single isolates harboring resistance to all three first line drugs (chloramphenicol, ampicillin and cotrimoxazole). In 1980, resistance to these first line drugs was described in Bangkok [122]. Resistance to antifolates is mediated through different mechanisms including: (1) the permeability barrier and/or efflux pumps, (2) naturally insensitive target enzymes, (3) regulational changes in the target enzymes, (4) mutational or recombinational changes in the target enzymes, and (5) acquired resistance by drug-resistant target enzymes. The most common resistance mechanism to trimethoprim is the expression of a trimethoprim-resistant DHFR. This DHFR is expressed additionally to the original enzyme and the gene coding for this additional enzyme is very often located on mobile genetic elements, like plasmids, transposons or gene cassettes [45, 87, 123]. High-level trimethoprim resistance in Enterobacteriaceae is mainly due to the replacement of a trimethoprim-sensitive dihydrofolate reductase by a plasmid-, transposon- or cassette-borne trimethoprim resistant dihydrofolate reductase whose configuration escapes the action of TMP. In Pakistani isolates of *S. Typhi*, resistance against ampicillin and cotrimoxazole is very high whereas it is comparatively lower against chloramphenicol according to our findings [109]. It has been reported that this MDR resistance type found in our isolates is encoded by large plasmids belonging to H1 incompatibility group [124].

After the emergence of MDR *S. Typhi*, fluoroquinolones became the treatment of choice for typhoid treatment. However, there have been many reports of nalidixic acid resistant (NA^R) *S. Typhi* which exhibit decreased susceptibility to ciprofloxacin and show poor clinical response to fluoroquinolones [125, 126]. A major outbreak of MDR *S. Typhi* was encountered in Tajikistan in 1997, by consuming contaminated drinking water that affected nearly 9,000 individuals leading to 95 deaths. This epidemic MDR *S. Typhi* strain developed resistance to nalidixic acid and reduced susceptibility to ciprofloxacin [127] during the outbreak.

Mandal and colleagues reported a simultaneous increase in resistance levels to fluoroquinolones and a decline in the percentage of MDR in the *S. Typhi* population under fluoroquinolone treatment [128]. However, the emergence of high-level ciprofloxacin resistance in *S. Typhi* and *S. Paratyphi* A has been

reported in India [129, 130]. The treatment for resistant typhoid fever now depends on third generation cephalosporins and azithromycin [131]. Ciprofloxacin, ofloxacin and gatifloxacin are still considered effective in Pakistani isolates, and very low level of resistance is observed against these florouquinolones according to our findings [109]. Ciprofloxacin is currently a suitable empirical choice in presumed enteric fever cases in Pakistan [132].

Aminoglycosides are not commonly used for the treatment of typhoid fever because they face difficulty in penetrating tissue or cells so are less likely to be effective for facultative intracellular pathogens such as *S. enterica* serovar Typhi. Even so, many authors admit the use of aminoglycoside antimicrobials such as gentamicin and amikacin in susceptibility testing in order to create a treatment regimen for MDR typhoid fever when it is urgently required. Furthermore treatment failure with ciprofloxacin and third generation cephalosporins in treating typhoid fever is also reported. In such settings, there is a need to find a cost-effective treatment regimen for typhoid fever [133-135].

S. Typhi, the causative agent of typhoid in humans, is also capable of producing biofilms which contribute to its resistance and persistence in the host. *S. Typhi* is transmitted through the fecal-oral route by contaminated water and food. Typhoid is communicable for as long as the infected person is capable of excreting bacteria in stool. These bacteria usually disappear from the stool about a week after symptoms of illness have resolved. However, a percentage of these infections can result in asymptomatic carriage of *salmonellae* possibly due to formation of biofilms as a mechanism that contributes to the development of the carrier state [136].

Bacteria in biofilms are generally considered well protected against environmental stresses, antimicrobials [137], disinfectants and the host immune system [138], and as a consequence are extremely difficult to eradicate [139]. Planktonic *Salmonella* populations are found to be sensitive to different antimicrobials as compared to biofilms. We have recently reported that most of the biofilm producing bacteria show MDR pattern of drug resistance hence delaying their clearance from the body [140]. It is reported that *S. Typhimurium* biofilms pre-formed on microplates are up to 2000-fold more resistant to ciprofloxacin as

compared to planktonic cells [141]. This is of particular concern as ciprofloxacin is commonly used to treat *Salmonella* infections [142].

Antimicrobial resistance of *S. Paratyphi* A appears to be an emerging problem. The 1996 outbreak of Paratyphoid fever in India showed that the isolates were sensitive to all antimicrobials including chloramphenicol, ciprofloxacin, and ceftriaxone [143]. Two years later, there was a report from New Delhi, India, describing drug-resistant *S. Paratyphi* A. The incidence of resistance to ciprofloxacin increased to 24%, and 32% of isolates had decreased susceptibility to ciprofloxacin (minimum inhibitory concentration (MIC) >2 mg/mL), the drug of choice for enteric fever (EF) in India [144]. Reports from a north Indian tertiary care hospital showed increasing multidrug-resistant *S. Paratyphi* A strains [145]. In an outbreak of paratyphoid fever in 2001 in Nepal, 84% of the isolated strains were reported as resistant to nalidixic acid, which is considered the best predictor of clinical response to fluoroquinolones [146].

One of the largest prospective studies of EF in recent years reported a worrisome result: *S. Paratyphi* A was significantly more likely to be resistant to nalidixic acid (75.25% *vs.* 50.5%) and ofloxacin (3.6% *vs.* 0.5%) than *S. Typhi.* Moreover, MICs of other antibacterials were also higher in *S. Paratyphi* A. A high-level ciprofloxacin-resistant strain has been reported in India (MIC 8 mg/mL) and Japan (MIC 128 mg/mL) [147].

This tendency is not limited to Asian strains. A study from 10 European countries showed an increasing incidence of multidrug-resistant *S. Paratyphi* A. It rose from 9% in 1999 to 25% in 2001, and the incidence of decreased susceptibility to ciprofloxacin also increased from 6% to 18% [148].

Shigellae

Antimicrobial treatment is recommended for moderate to severe shigellosis [149] aimed at resolving the symptoms of diarrhea or reducing its duration and its transmission to close contacts. The antimicrobials are helpful in limiting the duration and shedding of bacteria [150].

Due to the misuse of antimicrobials, resistance has developed against the commonly used trimethoprim sulphamethoxazole, ampicillin and chloramphenicol.

Quinolones and fluoroquinolones are relatively effective in the treatment but resistance has started to emerge [150]. Antimicrobial resistance demands repeated reevaluation of treatment recommendation. According to a study conducted on antimicrobial resistance in *Shigella* in eight Asian countries it was reported that there was an increasing trend in multidrug resistance strains in Asia. Calculated percentage was 78%. The main cause might be the overuse of antimicrobial in these countries among humans and animals [149] and the knowledge on the epidemiology and molecular mechanisms of antimicrobial resistance is important to implement intervention strategies [151].

Resistance towards antimicrobial drugs originate either by mutations in chromosomal genes or acquisition of exogenous material carrying the resistance genes. Mutation in the chromosome may cause phenotypic mutants which enhance the likelihood of acquisition of resistance mutation. A multidrug resistance regulatory locus is widespread among *Salmonella, Shigella, E.coli, Klebsiella* and a multidrug resistance locus on chromosome in *S. flexneri 2a* strain shares homology with resistance region of *Shigella* R plasmid, NR1 [152].

Transferable drug resistance was discovered in the late 1950's and since then many types of plasmids and transposones have been discovered [153]. *Shigella* was among the first organisms shown to harbor transferable antimicrobial resistance patterns [152]. It has a tendency to acquire drug resistance frequently by mobile genetic elements, including the R plasmids, transposones, integrons and genomic islands, on the bacterial genome [154].

Antimicrobial resistance frequency among *Shigella spp.* has increased globally [155] showing great geographical variation [156] and is creating problems in the medical community. *Shigella* isolates resistant to first line drugs are present throughout the world [157]. Multidrug resistance strains occur in Europe, Africa, Asia and South America but they are more prevalent in India and China [151]. They are also increasing significantly in other parts of Asia [158]. Very little data are available describing the distribution of resistant strains of *Shigella* in Pakistan. Data of antimicrobial susceptibility of routinely used antibiotics including ampicillin, tetracycline, chloramphenicol, co-trimoxazole and nalidixic acid from different areas of Pakistan show high resistance to co-trimoxazole (87.75%),

ampicillin (55.5%), nalidixic acid (39%) but interestingly chloramphenicol (11.25%), is reemerging as a useful drug [155]. Quinolones such as norfloxacin and ciprofloxacin are reported to be effective against shigellosis [151]. Our recent studies present a gloomy picture as we have found rapidly emerging resistance to ceftriaxzone (cephalosporins) in our local isolates, which is unique and alarming as it has not been reported earlier from Asia [159]. We have also found higher resistance against ciprofloxacin as compared to other Asian and Middle East countries.

According to WHO recommendations, ciprofloxacin is the drug of choice in patients with bloody diarrhea irrespective of the age [15]. These broad spectrum antimicrobials are found to be safe in the treatment of shigellosis [160]. Quinolones are contraindicated in children because they cause the bone marrow depression [157] and are considered to be an important threat to treatment of shigellosis especially in children [15]. However, some clinical studies have shown that they are safe in children and adults [160] because the risk of joint damage in children appears to be minimal with the short term courses of fluoroquinolones [161].

Drug resistance against third generation cephlosporins and flouroquinqlones is an emerging problem especially in children. In 2008, 50% of *Shigella* strains were resistant to nalidixic acid in Bangladesh and 29% in India. But no *Shigella* strain was resistant to nalidixic acid in a study conducted in Central Africa. These drugs are no longer recommended because of the risk of development of quinolones and flouroquinolones resistance and poor efficacay [161]. In India 30% of the strains were resistant to fluoroquinolones because of their extensive and indiscriminate use [160]. This trend has been increasing since 2002 [162]. The resistance to fluoroquinolones has also been found in other Asian countries. Fluoroquinolone resistant strains isolated from India were found to be susceptible to azithromycin and ceftriaxzone. In addition to this, cephalosporin resistance strains were also identified from Spain and Argentina [160]. Cephalosporin and quinolone resistance has also been reported from Pakistan [157].

A recent study has indicated that biofilm phenomenon may be present in *Shigella* species. An increase of the salts concentration enhances the ability of *Shigella*

species to attach and to invade the tissue culture cells. The percentage of adherence increased to 15% and the invasion to 90% at 6% salt concentration [163].

Shiga Toxin Producing *E. coli* (STEC)

Tetracycline, sulphonamides, ampicillin and streptomycin are the major antimicrobial agents to have conferred resistance by *E.coli*. However resistance to frontline antimicrobial agents like fluoroquinolones, expanded-spectrum ß-lactams, and third-generation cephalosporins have also become a serious problem, particularly in STEC serotype O157, O26, O103, O111, O128, and O145 [164]. Initially STEC, particularly O157:H7 were found to be sensitive to many commonly used antimicrobials [165], but now both O157 and non-O157 STEC have been implicated in antimicrobial resistance. Zhao and coworkers found that 39 out of 50 (78%) STEC isolates showed resistance to at least two or more antimicrobial classes and multiple resistance to streptomycin, sulfamethoxazole, and tetracycline was frequently observed as well. Class I integrons were present in nine of the STEC strains [166]. Similar observations were made in another study of 141 STEC O157:H7 strains isolated from cattle, sheep, humans and food. Antimicrobial resistance was frequently observed against sulphisoxazole, tetracycline and streptomycin [167].

The use of antimicrobials is often mandatory in severe enteric diseases like cholera, typhoid fever and shigellosis. Unfortunately in case of STEC infection the use of antimicrobials remains controversial. Administration of antimicrobial agents for STEC infection is equivocal because of the risk pertinent with an increased release of Shiga toxin in response to various antimicrobials [168, 169].

It was reported for the first time in 1989 during an outbreak of *E. coli* O157:H7 that antimicrobial therapy of patients with diarrhea due to *E. coli* O157:H7 might be a risk factor for development of HUS [170].

In 1997, Yoh and colleagues reported that fosfomycin which was a drug of choice in Japan exacerbated the Stx1 production from *E. coli* O157:H7 *in vitro*. A seven fold increase in Stx1 release was observed in the culture exposed to fosfomycin while other antimicrobial agents like minocycline, cefazolin, gentamicin and doxycycline caused slight increase in Stx1 and had no effect on Stx2 release [171].

In another study the utility of 13 antimicrobial agents on the production and release of Stx from three different STEC O157 strains was evaluated. Culture exposed to sub-inhibitory concentration of cotrimoxazole, trimethoprim, azithromycin and gentamicin were associated with increased Stx production. It was also observed that increase in Stx production with different antimicrobials was strain specific; moreover increase in toxin production under different antimicrobials was attributed more to the strains producing Stx2 alone [172]. Bacteriostatic agents like roxithromycin, rokitamycin and clindamycin were found to suppress the release of Stx *in vitro* but not the number of viable strains, while exposure to cefdinir, fosfomycin or levofloxacin stimulated Stx release with the destruction of bacterial cells [173].

In case of Pakistani isolates, *in vitro* experimental data suggest that cefotaxime and gentamicin are safe at MIC level. However, we found that there is increase in toxin release and cytotoxicity at sub- MIC levels of ampicillin [174]. It is, therefore, suggested to avoid its use in STEC-related illness, and if proper diagnosis is not available, in all diarrhea cases. These findings are especially relevant to developing countries where, because of financial constrains, inadequate, low-dose self-treatment for insufficient period is common and usually the treatment is stopped as soon as the severity of symptoms subsides.

E. coli O157:H7 is known to produce exopolysaccharides (EPS) [175], which can provide a physical barrier to protect cells against environmental stresses. EPS is also involved in cell adhesion and biofilm formation [176]. EPS can serve as a conditioning film on inert surfaces, affect cell attachment by functioning as an adhesive or antiadhesive [177], and influence the formation of three-dimensional biofilm structures [178].

Extraintestinal *E. coli* (ExPEC)

Community acquired urinary tract infections (UTI) are highly prevalent in developing countries and are usually difficult to eradicate because the pathogenic bacteria have developed resistance to most of the drugs. UTI has been shown to be an independent risk factor for both bladder cancer and renal cell carcinoma [179]. Women are more likely to experience UTI than men. UTIs affect a large

proportion of the world population and are responsible for significant morbidity and high medical costs [180, 181]. Uropathogenic *E. coli* (UPEC) cause 90% of urinary tract infections [28]. The frequent use of antimicrobials is considered the most important factor which promotes multiple drug resistance (MDR) in UPEC in both veterinary and human medicine [182].

Different pathotypes of UPEC can be identified by phylogenetic analysis. Phylogenetic studies have revealed that the UPEC are not of very diverse origins and fall into four main groups A, B1, B2, and D [183, 184]. Picard *et al*. [19] found that UPEC which correspond to phylogenetic group B2 were more susceptible to antimicrobials than those falling in A, B1 and D. Moreno and colleagues investigated that among human UPEC isolates, resistance to quinolones, fluoroquinolones and trimethoprim/sulfamethoxazole showed shift from phylogenetic group B2 towards groups A, B1 and/or D [185]. In a recent study, we have reported that among Pakistani isolates, group D isolates were highly drug resistant as compared to phylogenetic groups A, B1 and B2 which is contrary to the previous reports. This group was also found the most hemotoxic [186].

Uropathogenic strains of *Escherichia coli* (UPEC) account for 70-95% of the UTIs. Bacteria that invade the bladder cells and form biofilms may be responsible for many recurrent UTIs. Significant production of biofilm has been reported with some reports showing nearly 2/3rd UPEC to produce biofilm [187] whereas others [188] claim biofilm production in more than 90.0%.

Vibrio

Antimicrobial treatments for one to three days shorten the course of the disease and reduce the severity of the symptoms. Patients recover without antimicrobial use if sufficient hydration is maintained [189]. Doxycycline is typically used as first line drug although some strains of *V. cholerae* have shown resistance. Testing for resistance during an outbreak can help determine appropriate future choices. Other antimicrobial proven to be effective include cotrimoxazole, erythromycin, tetracycline, chloramphenicol, and furazolidone. Fluoroquinolones, such as norfloxacin may also be used, but resistance has been reported [190].

Multiple antimicrobial resistant (MAR) *V. cholerae* with epidemic outbreaks (both classical and El Tor biotypes) have been reported in Bangladesh [191, 192].

Even though the reservations about the use of ciprofloxacin as a first line of treatment in such cases of MAR cholera have been expressed in developing countries [193], the high level resistance to nalidixic acid has led to the use of ciprofloxacin in pediatric cases. Subsequent reports of relapses and treatment failure led to the determination of MIC of ciprofloxacin, which was found to be high. This was responsible for the emergence of ciprofloxacin resistance in *V. cholerae* O1 Inaba [194]. Such resistance can be due to spontaneous mutation in *V. cholerae* or transfer of resistance from other co-inhabiting microbes, which are fluroquinolone resistant. The profiles of major MAR *V. cholerae* as documented in Kolkata and other parts of India and Bangladesh are: AFZ (Ampicilllin, Furazolidone), AFZN (Ampicillin, Furuzolidone, Neomycin), AFZ NS (Ampicillin, Furazolidone, Neomycin, Streptomycin) [195]. The antimicrobial resistance pattern of epidemic strains have changed frequently with the emergence of different *V. cholerae* O1 or O139 strains. Therefore, selection of such drug resistant clones can lead to seasonal epidemics of cholera with emergence of new clones replacing the existing clones.

V. cholerae become drug resistant by exporting drugs through efflux pumps, chromosomal mutations or developing genetic resistance *via* the exchange of conjugative plasmids, conjugative transposons, integrons or self transmissible chromosomally integrating SXT elements. *V. cholerae* use multidrug efflux pumps to export a broad range of antimicrobials, detergents and dyes that are chemically and structurally unrelated [70]. The two major groups of *V. cholerae* efflux pumps are distinguished by their energy sources: ATP hydrolysis, or the proton-motive force (PMF) of transmembrane H+ or Na+ gradients [196]. PMF pump families include MATE (multidrug and toxic compound extrusion), MFS (major facilitator superfamily), RND (resistance–nodulation–cell division) and SMR (small multidrug resistance) [70].

The spread of antimicrobial -resistant *V. cholerae* is also facilitated by horizontal gene transfer *via* self-transmissible mobile genetic elements, including SXT elements – mobile DNA elements belonging to the class of integrative conjugating elements (ICEs). Besides conferring antimicrobial resistance, SXT elements have the capacity to mobilize conjugative plasmids and genomic islands in trans [197], providing alternative mechanisms for antimicrobial resistance gene transfer.

Dissemination of antimicrobial resistance genes is also facilitated when V. cholerae cells share mobile integrons with other bacterial cells. All *V. cholerae* isolates harbor large chromosomal integrons, giving them the capacity to rapidly transfer gene cassettes containing antimicrobial resistance genes [59]. In addition, clinical and environmental *V. cholerae* can also contain mobile integrons, which are smaller (0–10 cassettes), but are embedded within mobile elements such as conjugative plasmids and transposons [59] and can disseminate horizontally.

Although virulence of Vibrios is mainly due to toxin production, the foothold to the bacteria is provided by biofilm formation. It also enables *V. cholerae* to survive in nutrient-poor conditions outside of the host [198]. Biofilm formation also increases the infectivity of *V. cholerae*, but, importantly, dispersal of biofilms is thought to occur once the bacteria colonize the host [198, 199].

Campylobacter

Campylobacter infections are among the most common causes of bacterial diarrhea in humans worldwide [200]. A recent study on illness and death due to foodborne infections in France estimated an isolation rate of 27–37/100,000 persons/year for Campylobacter infection [201]. Although the genus Campylobacter is composed of 18 described species [202], human illness is associated with thermophilic *Campylobacter*, primarily with *C. jejuni* and *C. coli* and infrequently with *C. upsaliensis, C. lari,* and *C. fetus*.

Drugs of choice for treating campylobacteriosis are erythromycin, quinolones, tetracycline, ampicillin, chloramphenicol and gentamicin. Nowadays there is a compelling evidence regarding an alarming increase in resistance of *Campylobacter* to antimicrobials administered in human treatment [203-205]. *Campylobacter* resistant strains have mainly emerged as a consequence of the use of antimicrobial agents in animal food production. Most of the strains are resistant to cloxacillin, nafcillin, oxacillin, sulfamethoxazole/trimethoprim, trimethoprim, and vancomycin [206].

In this scenario, fluoroquinolones have emerged as alternative therapy [207]. However, resistance to fluoroquinolones is also emerging [208]. Combined

studies in humans and poultry have implicated the use of fluoroquinolones in poultry in the emergence of drug resistance [209].

It has been suggested that *C. jejuni* maintains itself in the environment by forming a biofilm [210]. *C. jejuni* has been found in preformed biofilms of other bacterial species [211]. *C. jejuni* in monoculture can attach to surfaces and form a biofilm, and can form a pellicle at both 37° and 30°C. It also forms a biofilm growing unattached and this aggregate biofilm has increased resistance to environmental stress. This may be relevant to the survival of the organism in the environment and in the epidemiology of *C. jejuni* infection [212].

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CONFLICT OF INTEREST

The author(s) has confirmed that there is no conflict of interest.

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