CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE:

2.1 Evolution of antibiotic resistance:

The use of therapeutic agent is somewhat compromised from the time it is employed by the potential development of tolerance or resistance to that compound, wide range of biochemical and physiological factors are responsible for it. During Second World war Penicillin was first introduced by Alexander Fleming (1928) as amazing drug against bacterial infection (Figure 1) (Davies and Davies, 2010). Initially Penicillin and sulphonamides (1935) were the main backbones of antibiotic therapy (Davies and Davies, 2010). But the way of therapy not only requires longer and complex treatment, they also more significantly expensive to treat. In the last 80 years, a major improvement was observed in prophylaxis of infectious diseases and have resulted reduction in the morbidity and mortality associated with illness. As more antibiotic were discovered and newer formulations developed access to antibiotics eased considerably and their use become widespread. *In order to survive from lethal external agent's bacteria remarkably adapt themselves from new and old antimicrobial agents (Alanis, 2005). Consequence of this adaption makes them resistance to antimicrobial agents and rendering these drugs ineffective as treatments of choice for severe infections caused by these pathogens.* The incompetence of antibiotic treatment against bacterial infection has become a major healthcare problem in the 21 century. The list of bacteria those are surprisingly shows resistance against sulphonamide and penicillin-resistant *Staphylococcus aureus* in 1930s and 1940s (*Alanis, 2005*), beta lactamase-producing *Haemophililus influenza* in the 1970s, methicillin resistance

Staphylococcus aureus (MRSA) and resurgence of multi drug resistance in the late of 1980s (*Alanis, 2005*). Initially bacteria causing nosocomial infection in hospital settings mainly associated with critically ill and immune suppressed patients but now extended into the community which results worries to treat. The resistance genes are maintained in environment by host bacteria and there has to be a defence mechanism against the antibiotics.

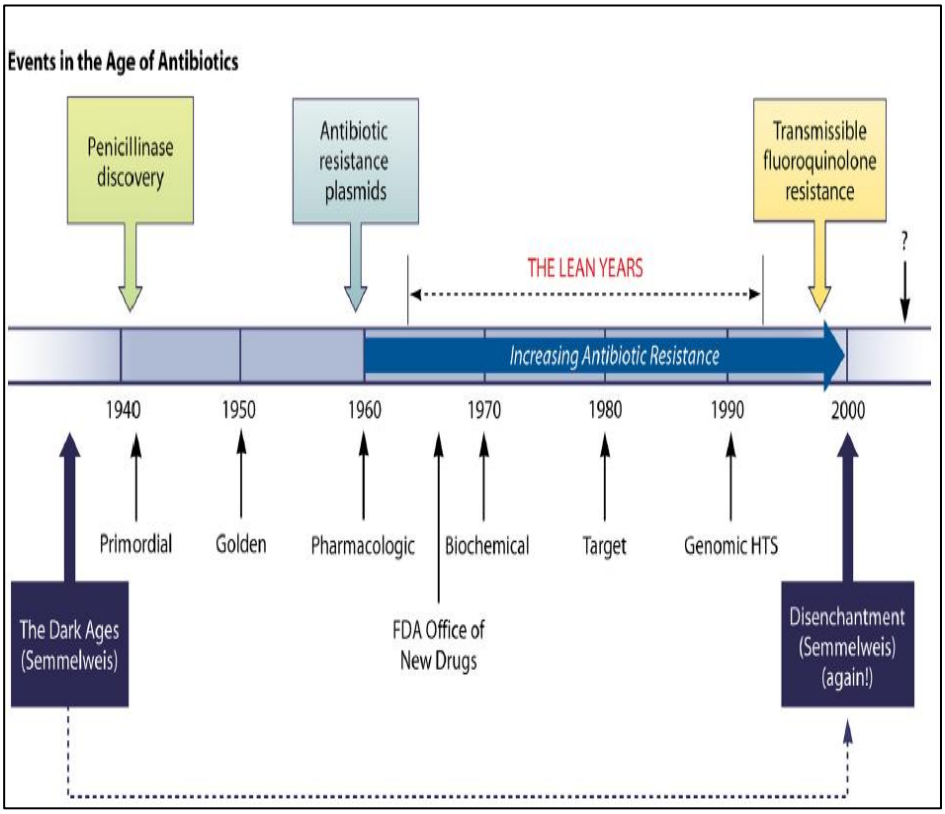

Figure 1: Evolution of antibiotic discovery and related development of antibiotic resistance. Source: Davies J and Davies D, 2010.

2.1.1 Multidrug resistance: The resistance against antibiotic in bacteria provides the most dramatic scenario in terms of treatment with bacterial infections. The means that microbes use to evade antibiotics certainly predate and outnumber the therapeutic interventions themselves (Alekshun and Levy, 2007). Antibiotics are not only used as a medicine but also for the prophylaxis

and growth promotion in animals and passed through to environment via food chain (Livermore, 2007). In the hospital settings there is more evidence where the antibiotic use maximum and remarkably bacteria become resistant against antibiotics (Kapil, 2005). In developing countries the main reason is to misuse and inappropriate practice in antibiotics policy. This problem is further compounded by the lack of education and "over the counter" availability of antibiotics (Kapil, 2005). Due to excessive use of antimicrobial agents results the emergence of bacterial resistant. Under the prolong use of antibiotics those bacteria become inherently resistant or have acquired resistance and expanded across the globe (Kapil, 2005 Livermore, 2007). Despite advance in medical technology for diagnosis and patient care a person can still die of an infection due to multidrug resistance.

In last one decade the incidence of multidrug resistance among microorganism increased worldwide. The management of infectious diseases with MDR microorganisms has become foremost challenge (Davies and Davies, 2010). Antibiotic resistance among bacteria is an innate physiological change which occurs through vertical or horizontal gene transfer (Wright and Sutherland, 2007). The combinatorial genetic shift helps in accumulation of multiple antibiotic resistant genes in bacteria (Wright and Sutherland, 2007). The Infectious Disease Society of America has identified Methicillin- Resistant *Staph. aureus* (MRSA), Vancomycin Resistant *Enterococcus faecium,* Extended Spectrum B-lactamase producing Enterobacteriaceae and Multidrug resistant *Acinatobacter baumanii are the example of those especially challenging* infectious diseases (Talbat et al., 2006).

2.1.1.1 Mechanism of Resistance: New antibiotics and new therapeutic strategies are responsible for the expanding chemical diversity which modifies adaptability in microorganism (Davies and Davies, 2010). Resistance mechanism can be of different types (Figure 2).

2.1.1.1.1 Mutational alteration of the target protein: Virtually all the clinically important floroquinolone are unlikely become inactivated by mutation within the target enzymes (Nikaido, 2009), DNA topoisomerase and gyrase (Dalica and Malik, 2003). Mutations that give rise to the fluroquinolone resistance are predominantly found in Quinolone-Resistance-Determining-Region (QRDR). This multi-subunit composed of two subunits: GyrA and GryB for gyrase and ParC/Gal and ParE/ GrlB for topoisomerase which helps bacteria during DNA replication (Alekshun and Levy, 2007). The GryA and ParC/GrlA proteins contains the DNA binding functions and are targeted by the fluroquinolones whereas GryB and ParE/GrlB perform ATP binding and hydrolysis of target drugs.

2.1.1.1.2 Enzymatic drug modifications: In this mechanism microorganisms hydrolyse or modify the effect of drug target site that which leads them as a microbiologically inactive compound. Typical example of this type are; beta-lactam antibiotics (penicillins, cephalosporins and carbapenems such as imipenem) inactivated by hydrolysis of beta lactamases in the periplasm and others such as aminoglycosides (kanamycin, tobramycin and amikacin) inactivated by enzymatic phosphorylation (aminoglycoside phosphoryltransferase), acetylation (aminoglycoside acetyltransferase) and adenylation (aminoglycoside adenyltransferase or nucleotidyltransferase)

respectively and perform chemical transformations (Jacoby and Manoz- Price 2005, Nikaido 2010).

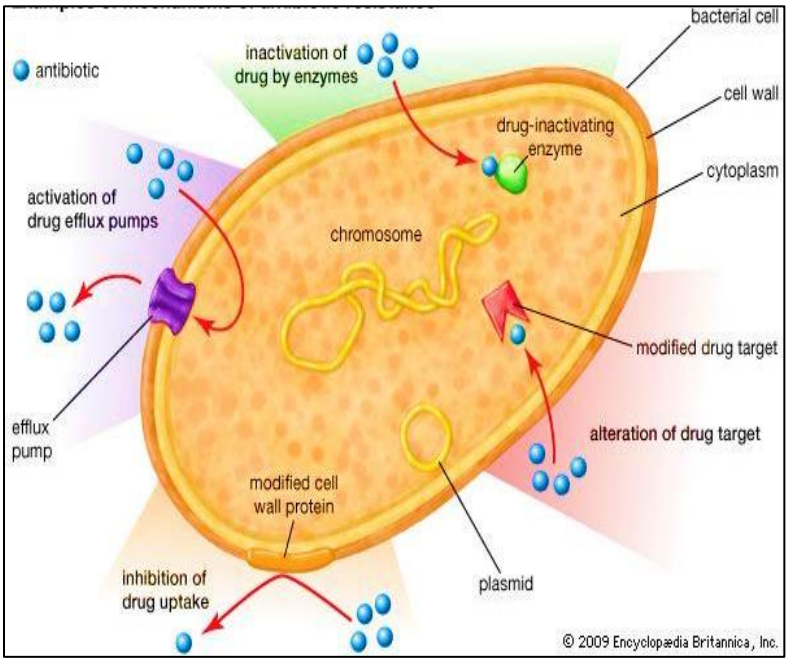

Figure 2: Mechanism of antibiotic resistance;

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2.1.1.1.3 Alteration of target molecules: In the mid of 1940's first penicillin resistant Staphylococcus.aureus was identified which was linked to the acquisition of a gene with an altered penicillin binding proteins (PBP). Methicillin is a derivative of penicillin and the alteration found to be resistant against beta-lactamas with a specified mecA gene (Alekshun and Levy, 2007). In normal condition Staphylococcus.aureus uses multiple PBP during cell wall biosynthesis, contains two kinds of bi-functional enzyme transpeptidase and transglycosylase. Removal of transglycosylase function of PBP imparts beta-lactam susceptible (Fuda et al., 2005).

2.1.1.1.4 Permeability: The outer membrane of the gram-negative cell envelope is a barrier to both hydrophobic and hydrophilic

compounds. Microorganism has evolved porin proteins (eg; OmpF in *E.coli* and OprD in *P.aeruginosa*) which helps in non-functional entry and exit points for antibiotics (Alekshun and Levy, 2007). Imipenem and other basic amino acid pass through the OprD that decrease expression of the porin contribute to clinical imipenem resistance.

2.1.1.1.5 ***Efflux systems:*** Another stunning expression of bacteria is efflux activity first introduced by McMurry et al., in 1980. In this mechanism drugs are pump out from the cell interior and preventing them to entry into the cell. Most of the antibiotics effectively penetrate into gram-negative organisms but fails to reach intracellular membrane due to efflux activity (Li and Nikaido, 2004). RND efflux system is an intrinsic mechanism which mainly dealing with different antibiotic classes using a single resistance determinant (Alekshun and Levy, 2007).

2.2 PAN drug resistance: The word "PAN" was originated from Greek language which implies "All" or "whole", initially in medical term this PAN word used to referred those organisms that are specifically resistance to 7 antimicrobial agents (cefepime, ceftazidime, imipenem, meropenem, piperacillin-tazobactam, ciprofloxacin and levofloxacin) (Falagas et al.,2008). But the increasing incidence of multidrug resistance in Gram-negative bacteria has introduced a term called PAN drug resistance. Pseudomonas aeruginosa, Acinetobacter baumannii and klebsiella pneumonia are the common nosocomial pathogens has led to the emergence of clinical isolates susceptible to only one class of antimicrobial agents and eventually to pan drug-resistance (Falagas and Bliziotis, 2006). Polymyxin is a group of antibiotics that was abandoned during 1980s and 1990s in the most part of the world but recently in some *clinical*

studies have reported the therapeutic use of polymixin in treatment of Pan Drug Resistance (Falagas and Bliziotis, 2006). The emergence of PAN drug resistance represents a fearful clinical situation in terms of treatment option leaving none or very limited treatment option (Falagas, 2008).

2.3 Transfer of antibiotic resistance: Bacterial genomes are extremely dynamic, entertaining rapid acquisition, deletion and arrangements of relevant information (Dutta and Pan, 2002). Two types of mechanisms may be involved in exchange of genetic information in bacterial world: (i) internal modification of genetic information and vertical transmission of existing genes that take place through accumulation of mutation (Whittam, 1996) and (ii) acquisition or loss of specific set of genes from another species through the process of horizontal gene transfer (Lawrence and Roth 1999, Dutta and Pan, 2002). Mutation is a very slow process but continuous modification can be occurs in bacterial genome and in case of horizontal gene transfer large scale of alteration and change in the organization of genome can occurs (Dutta and Pan, 2002). Initially, microbial evolution mainly focused on the vertical transmission and very less attention has been paid to the horizontal gene transfer of bacterial genetic materials.

2.3.1 Vertical gene transfer: The resistance genes are transferred into bacteria and these genes travel not singly but in tandem arrays of several resistant determinants to chemically distinct antibiotics. The DNA sequence of bacterial chromosome can endow that cell with a new property and all subsequent progeny will inherit the property because bacteria are haploid (Figure 3). All successful antibiotics are directed at some single, essential

function, typically carried out by a chromosomally encoded protein (Summers, 2006). A mutation that renders such an essential protein resistant to the antibiotic will in most cases also render the protein slightly in its normal function. When no antibiotic is present, such cells will be a disadvantage in the wild and will not persist or spread. In an environment where exposure to lethal levels of antibiotics is periodic rather than continuous it is unlikely that antibiotic resistance could become an epidemic problem if single chromosomal mutations were its sole genetic basis (Summers, 2006). While vertical inheritance of single resistance mutations does take place, it is an enormously incomplete picture of the genetic basis of antibiotic resistance in the real world.

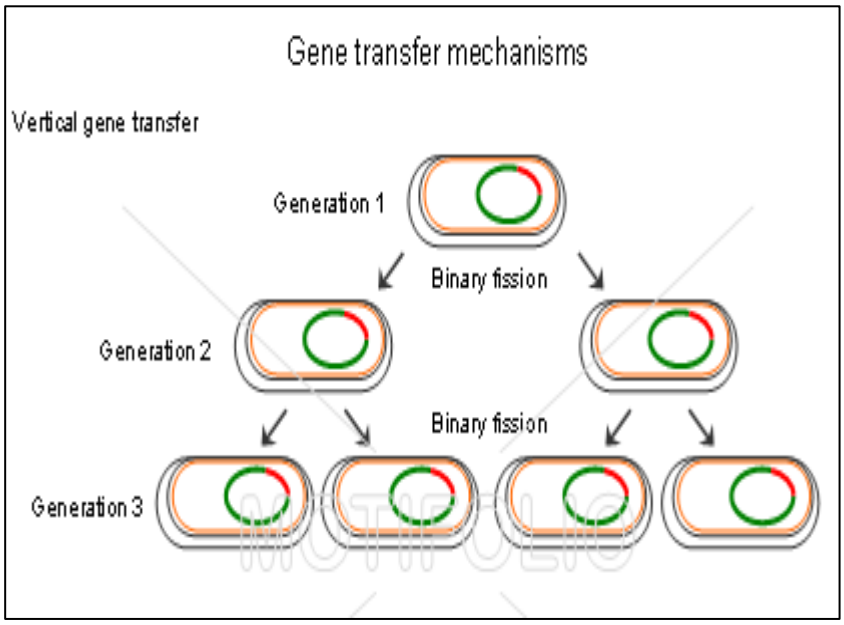

Figure 3: Schematic diagram of Vertical gene transfer.

Source: *http://site.motifolio.com/images/Gene-transfer-mechanisms-1021148.png*.

2.3.2 Horizontal gene transfer: The significance of horizontal gene transfer (HGT) for bacterial evolution was not recognized until the 1950s, when the multidrug resistance emerged on a world wide scale (David, 1996). For successful HGT the transferred gene must persist in the host chromosome and

should be provide a selective benefit to the recipient organism (Lawrence, 1999). Resistance gene acquired through HGT carries significant phenotypic properties in specific lineages (Dutta and Pan, 2002).

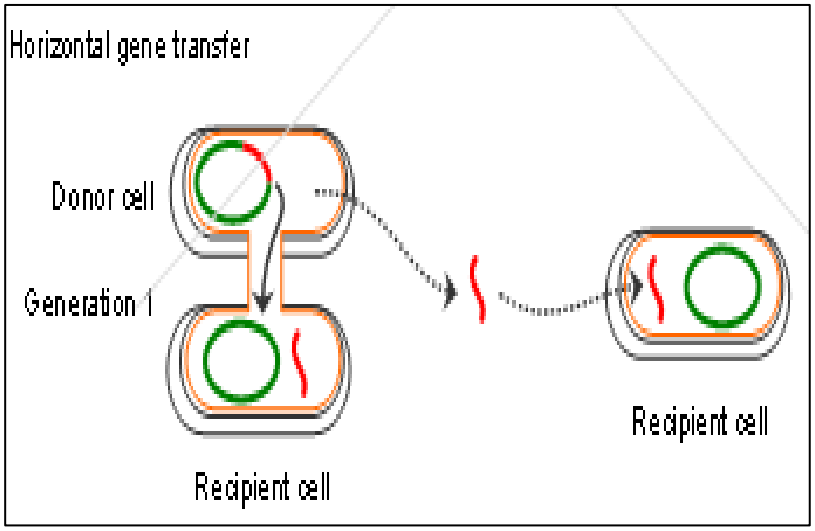

Figure 4: Horizontal gene transfer.

Source: ***http://site.motifolio.com/images/Gene-transfer-mechanisms-1021148.png***

The lateral transmission of resistance gene is accompanied by one of the three distinct transfer mechanisms; transduction, transformation or conjugation and each mechanism is characterize by its specific host range (Dorge et al.,1998) (Figure 2,3,4):

2.2.2.1 Transformation: In this mechanism naked DNA can be transferred between two distantly related organisms and provide the donor and recipient cells present at the same lace at the same time (Ochaman, 2000). Bacillus subtilis and Streptococcus pneumoniae can exhibit high level of transformation, become competent upon a certain physiological stage in their life cycle (Ochaman et al., 2000).

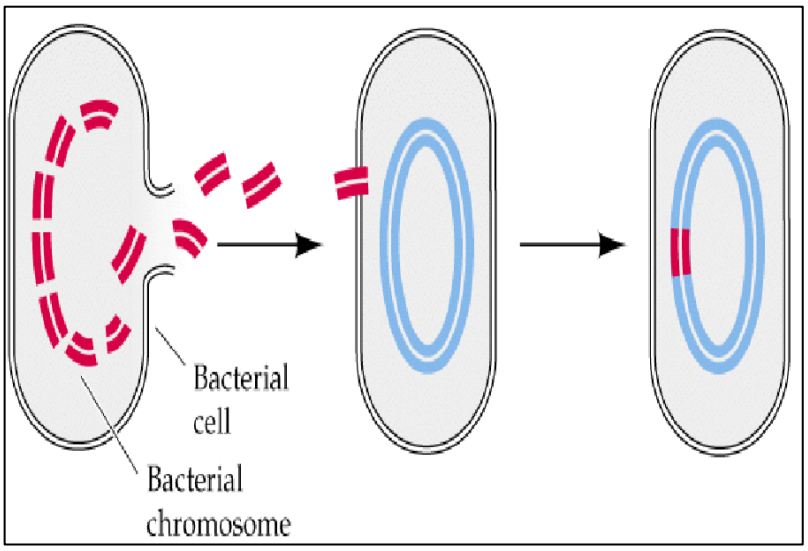

Figure 5: Transformation of bacterial horizontal gene transfer

Source: http://figures.boundless.com/12699/full/fig2.gif

But the organisms like Neisseria.gonorrhoeae and *Haemophillus influenzae* are constantly competent to take up foreign DNA, as their proficiency is enhanced by the presence of specific sequences (5´ GCCGTCTGAA-3´ and 5´ AAGTGCGGT 3´) in their genome (Dutta and Pan, 2002).

2.2.2.2 Transduction: In this mechanism new genetic material introduced into a bacterium through bacteriophage and replicate within a donor microorganism and packaged random DNA fragments or DNA adjacent to the phage attachment site (Ochaman et al., 2000) (Figure 6). The amount of DNA that can be transferred is limited with the size of phage capsid. Phages are prevalent in the environment but the transduction depends upon the spectrum of microorganisms that can be transduced by the bacteriophage (Ochaman et al., 2000). In this mechanism, phage encoded proteins can promote the integration of

the transferred sequence into the host chromosome to protect it from degradation by host restriction endonucleases (Dutta and Pan, 2002).

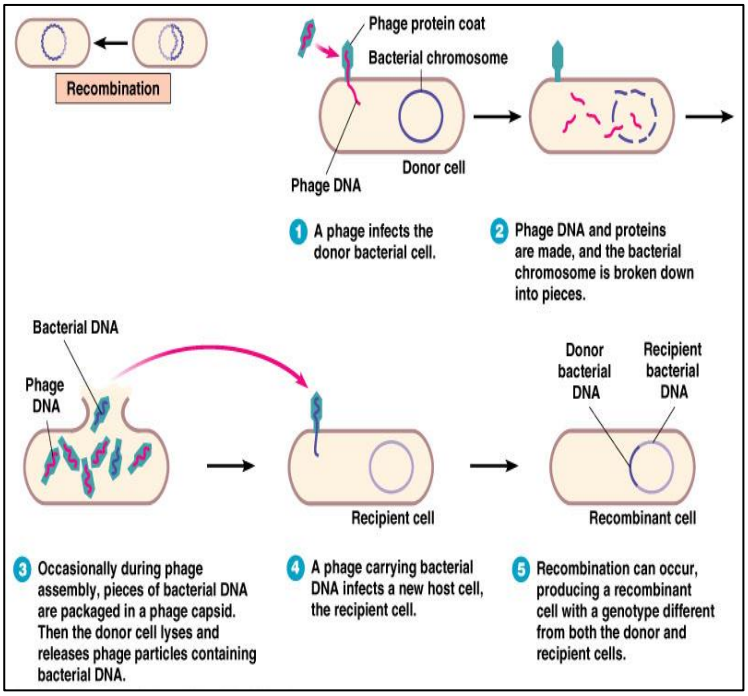

Figure 6: Horizontal gene transfer through transduction process.

Source:http://classes.midlandstech.edu/carterp/Courses/bio225/chap08/0828.

2.2.1.3 Conjugation: Conjugation process requires a physical contact between donor and recipient cells and can be mediated by a plasmid which is self-replicated or mobilizable or integrated into the chromosome and formed Hfr strain (Wollaston et al., 1987 and Dutta and Pan, 2002). It can take place through conjugation transposons which encoded protein for their excision from donor chromosome and forming Conjugation Bridge (Figure 7). Genetic material can be exchanged through conjugation but recipient cytoplasm does not ensure until the newly transformed sequence remains stable in the host chromosome. For

stable incorporation of foreign DNA into bacterial genomes can be mediated by: (Dutta and Pan, 2002).

I. Homologous recombination, between closely related organisms.
II. Persistence as an episome, through natural selection.
III. Integration mediated by mobile genetic elements, and
IV. Illegitimate incorporation through change double strand break repair.

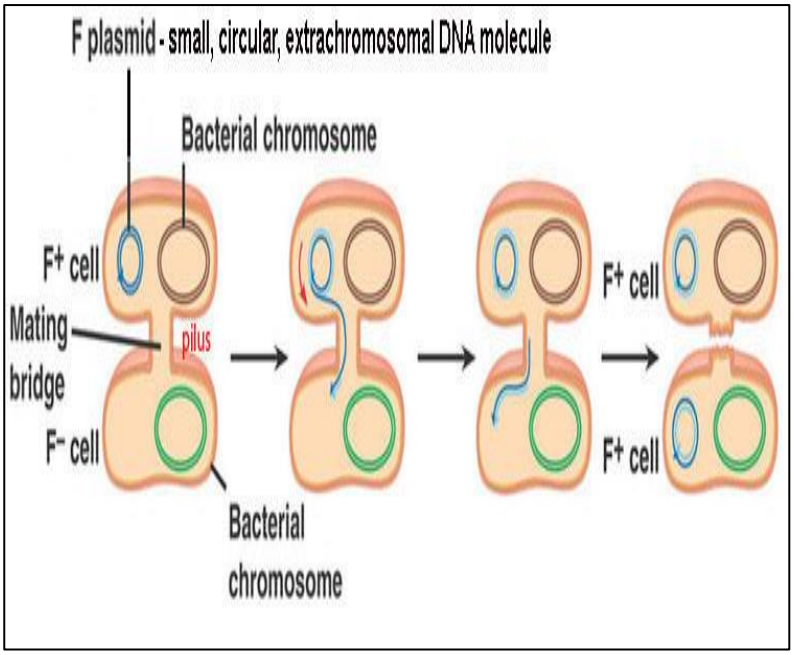

Figure 7: Conjugation and transfer of bacterial F plasmid.

Source: https://www.withfriendship.com/images/i/40591/Bacterial-conjugation-wallpaper.jpg

Horizontal gene transfer now appears to be a common practice in organisms and probably due to the ability of shaping the architecture of microbial genomes, conferring novel metabolic capabilities to recipient genome and enabling an organism to explore new ecological niches. (Dutta and Pan,

2002). Immediately after the incorporation following novel physiological traits can introduced through HGT:

I. **Antibiotic resistance**: Microorganism can acquire resistant determinants and associated with mobile genetic elements like; transposons, plasmid and integrons. (Mazel et al., 1998, Dutta and Pan, 2002). For example: dfr and sul gene conferring resistance to trimethoprim and sulphonamide respectively.

II. **Pathogenicity islands:** The sporadic distribution of pathogenic organisms has occurs by horizontal acquisition of pathogenicity determinants ((Dutta and Pan, 2002). Recent studies revealed that, virulence genes often exist in the bacterial chromosome as discrete gene clusters and referred as "Virulence cassettes" or "Pathogenicity islands" (Groisman and Ochman, 1996, Hacker et al., 1997).

2.3 Genetic environment of drug resistance determinant:

2.3.1 Plasmids: Plasmids are the platforms on which resistance gene arrays are assembled and reasserted, can move from one bacterial cell to another by so called horizontal gene transfer. Bacterial plasmids are mainly small, auxiliary, conjugative and dispensable chromosome (Bennett, 2005) (Figure 8). Most of the plasmids are circular, double-stranded, DNA molecules and ranges in size from 2-3 kb. They are capable to replicate independently and majority of replication functions are provided by the host cell and they do not accommodate any set of core genes needed for their growth and multiplication (Selimovic et al., 2007). When it can be integrated into the chromosome then it is called episomes and big bacterial plasmids called "Co-intregrated", which sometimes fails to reversible dissociation onto eperated units (Brunton et al., 1986). Plasmid

carries genes which confers antibiotic resistance genes and other number of toxic elements provided enzymes and explore periodically depends upon the environmental situations like to survive or thrive in the presence of potentially lethal antibiotics.

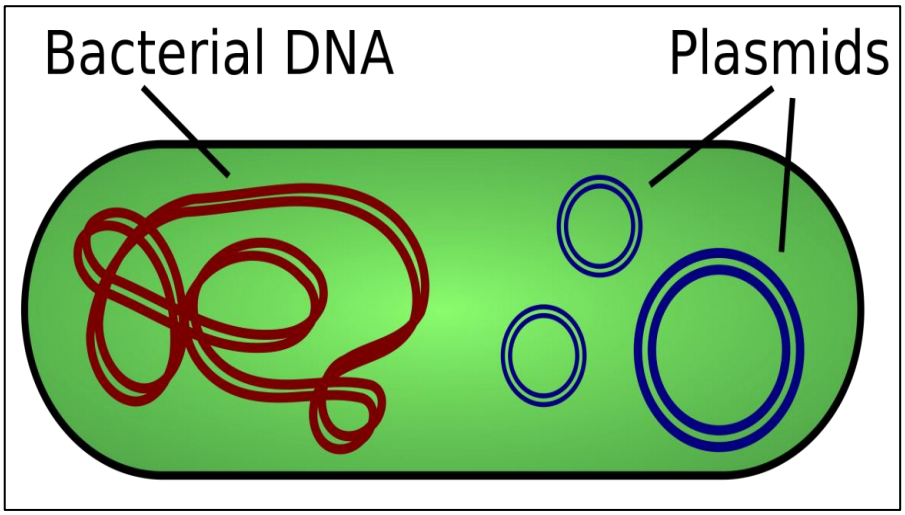

Figure 8: Bacterial plasmid

Source:**http://upload.wikimedia.org/wikipedia/commons/thumb/c/cf/Plasmid_(english).svg/2000px-Plasmid_(english).svg.png.**

A resistance plasmid may carry one or more antibiotic resistance genes, a metabolic plasmid because it encodes a metabolic function or a virulence characteristic because it possesses one or more virulence genes (Bennett, 2008) (Figure 9). Antibiotic resistance genes are often present in a single R plasmid and resistance can be transferred susceptible bacterium into a single conjugation event (Nikaido, 2009). Resistance plasmids are discovered in Japan in the 1950s with resistance profiling contains aminoglycosides, tetracyclines, chloramphenicol and sulphonamides (Nikaido, 2009).

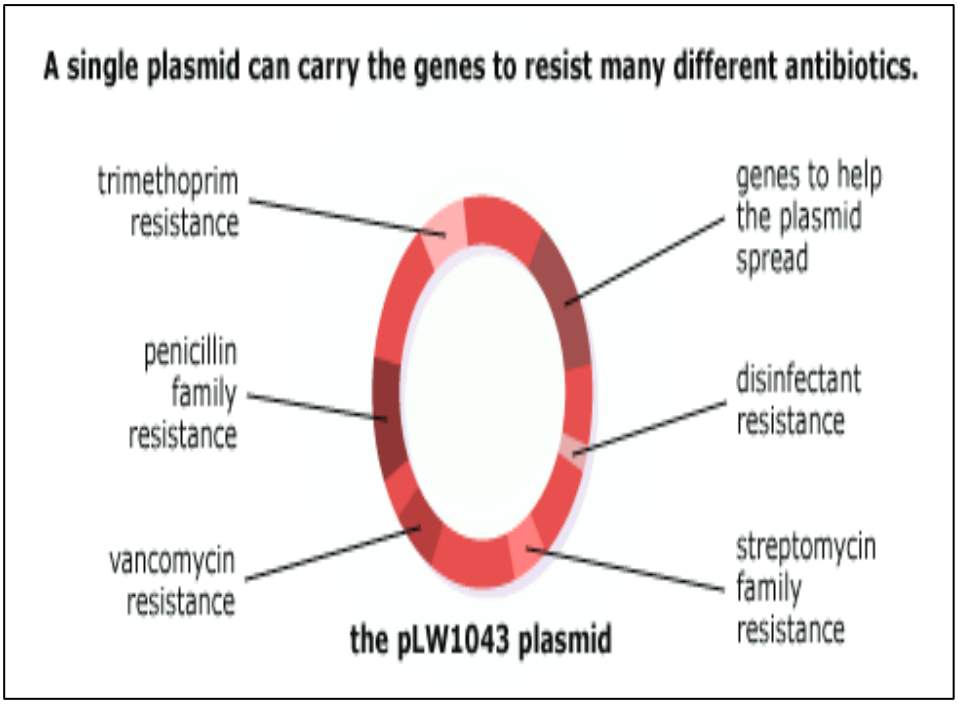

Figure 9: Involvement of plasmid in evolution of antibiotic resistance.

Source: http://evolution.berkeley.edu/evolibrary/images/news/plasmid.gif.

The conjugative resistance plasmids not only maintain their stability but also they can transfer with high efficiency. Mobilizable plasmids are relatively small whereas conjugative plasmids tend to be somewhat larger and reflecting the sizable amount of DNA which needed to encode the conjugation functions that permit cell-to-cell coupling, require less genetic information. Such coupling particularly occurs between gram negative bacteria and mediated by an external filamentous appendage called a sex pillus (Bennett, 2008). It actually acts like a grapping hook to join donor and recipient cells and then retracted into the donor to effect envelop contact and then transfer the DNA through pore forms to bridge the cytoplasmic compartments of the co-joined cells (Wilkins, 1995). Initiation is catalysed most frequently by one or few plasmid- encoded initiation

proteins which recognize plasmid-specific DNA sequences and determine the point from which replication starts (Del Solar et al., 1998). The migration develops due the enzyme DNA helicase I and transfer of single chain plasmid-specific DNA is associated with DNA polymerase III. The creation of the transferred DNA circle from is based on endonuclease enzymes activities (Willetts et al., 1984). Plasmid incompatibility is inability of two plasmids to carry them in stable manner in the same cell line (Couturier et al., 1988). The molecular mechanism is mainly studied on E.coli and in the plasmid incompatibility group F1 (F or sex plasmid), P (RP4) and W (Nikaido, 2009).Technique of "Plasmid replicon typing" identification of plasmids based upon recognition of genotype of different replicons rather than their incompatible phenotypes (Selimovic et al., 2007). Plasmid has broad range as well as narrow host range and depends on the surface receptor of the potential recipient cell by the conjugation process to maintain a large pool of genetic information.

2.3.2 Insertion Sequence: Resistance transposons, incorporating the resistance gene within the element by jumping gene systems and have an ability to move both intra and inter-molecularly (Bennett, 2004) (Figure 10). These mechanisms generally do not require DNA homology but they need an insertion site where it can incorporate (Carig, 2008). The transposons belong to the set of mobile elements which contains small cryptic elements called insertion sequence (Bennett, 2008). Bacterial insertion sequence was discovered in 1989 with an approx. 50 different types and now it become more than 500 types (Mahillon and Chandler, 1998; Bennett PM, 2008). Insertion sequence are small, phenotypic cryptic segments of DNA with a simple genetic organization and

capable of inserting at multiple sites in a target molecule and have a capacity to generate mutations of their translocation (Mahillon and Chandler, 1998; Bennett PM, 2008). The important role of this mobile element in dissemination of resistance genes and promotion of gene acquisition, clustered in an "islands" within plasmid genomes (Bukhari, 1977 and Bennett PM, 2008). Structure of insertion sequence are found to be flanked by DNA segment either as direct or inverted repeats to the central section that contains the genes confers antibiotic resistance, termed as composite or compound transposons (Berg and Howe, 1989; Bennett PM, 2008). The inverted arrangement of IS elements is more stable genetically and offers opportunity to migrate to another site (Bennett, 2008).

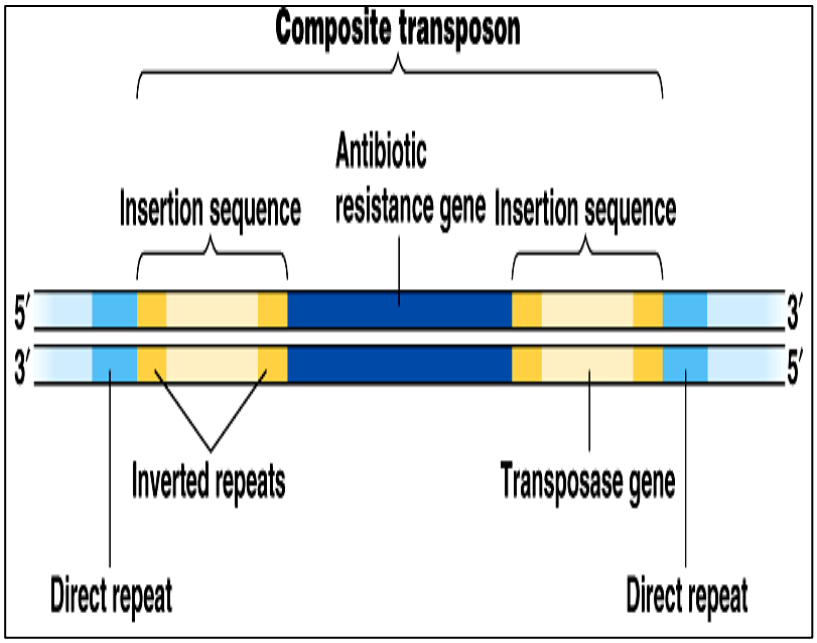

Figure 10: Schematic diagram of composite type insertion sequence.

Source:*http://www.zo.utexas.edu/faculty/sjasper/images/18.18.gif*.

The migration occurs by a two stage homologous recombination process where the composite structure is first excised from its existing site by a single crossover between the copies of the IS element releasing a circular double stranded DNA composed of central section of the composite resistance transposon and one copy of the IS element, other remaining at the original genetic location (Figure 11).

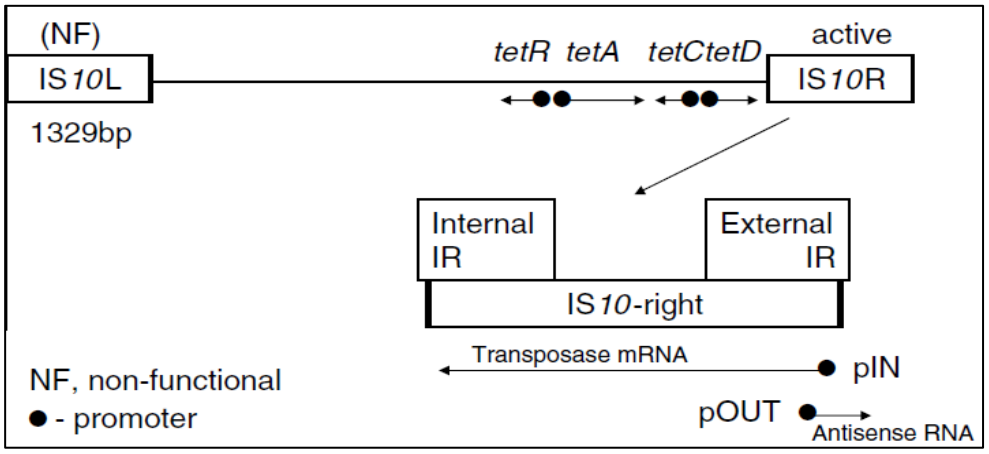

Figure 11: A diagrammatic representation of composite resistance transposons.

Source: Bennett PM, 2008.

The transposons Tn5, encoding resistance to aminoglycosides such as kanamycin and neomycin, Tn10 encoding resistance to tetracyclin, Tn3 encoding resistance to a number of beta-lactam antibiotics, including ampicillin and Tn21 encoding resistance to streptomycin, spectinomycin and sulphonamide those are the common example of IS element associated resistance mechanism found in Enterobacteriaceae (Figure 12).

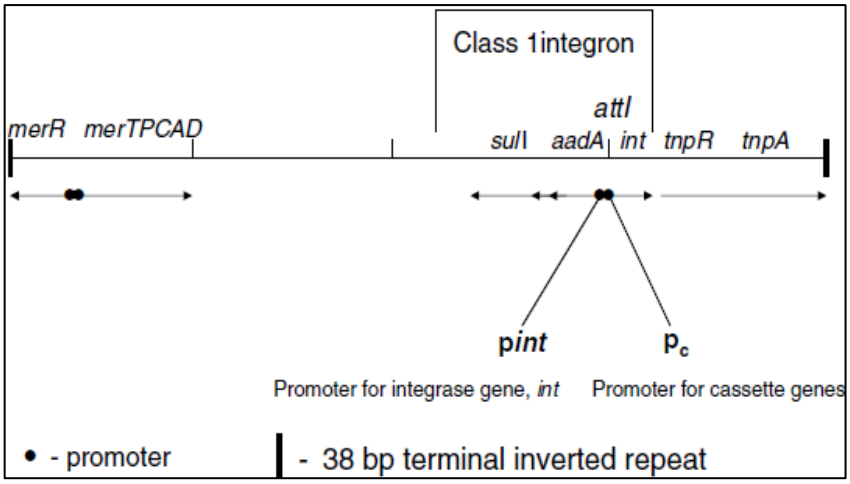

Figure 12: Schematic representation of the complex resistance transposon Tn21: (that confers resistance to streptomycin, spectinomycin, sulphonamide)**. Source: Bennett PM et al., 2008.**

2.3.3 Integrons: Integron is a genetic element which includes the determinants of site specific recombination systems that can capture gene cassette (Hall and Collis, 1995) (Figure 13). Gene cassettes are composed of smallest known mobilizable units of DNA and recombination site essential for the site specific recombination (Chowdhury et al., 2013). Although the site specific recombination reaction is catalyse by integrase protein and a member of tyrosine family (Chowdhury et al., 2013). Integrons provide their hosts suitable natural cloning machinery which helps them to incorporate new genes in cassettes (Hall, 2006). The essential components of an integron are intI gene and adjacent attI recombination site (Hall, and Collis, 1995). IntI gene and attI site are adjacent to the one another and attI site presence on the upstream of intI gene (Hall, 2006).

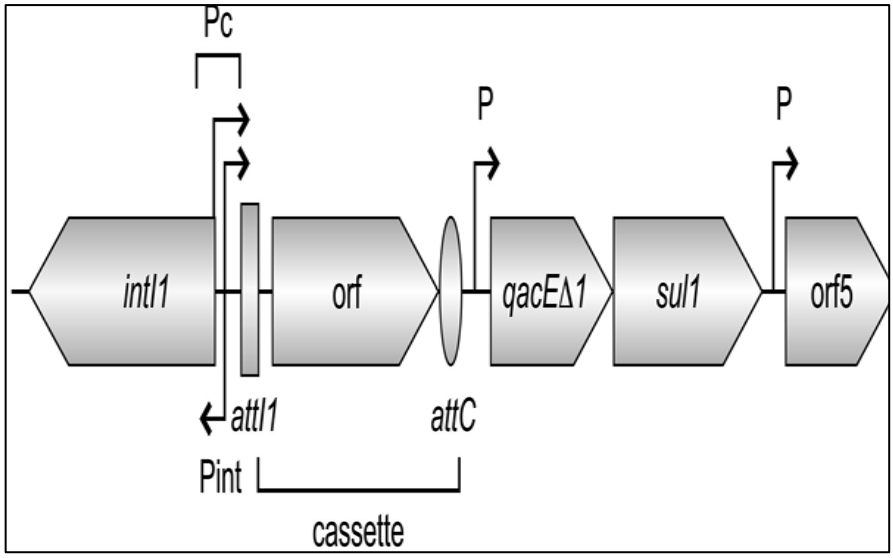

Figure 13: Generalised structure of class I integrons.

Source: *http://www.mobilednajournal.com/content/2/1/3*.

The gene cassettes are replicative mobile elements and generally couple an open reading frame (ORF) with an attC site. Gene cassettes are integrated or excised from the functional platform by IntI integrase. Two types of recombination can occur : i) between attI and attC site, resulting insertion of gene cassettes at the attI site, ii) between two attC sites, leading to excision of the gene cassettes of the gene cassettes (Stalder, 2012). Gene cassette can be found in a linear form or a closed circular free intermediate form. Expression of gene cassette mainly depends upon the promoter in the integron Pc which is presence in the 5´CS region of integron and regulates the transcription process of resistance genes. Gene cassettes are promoter less and require the Pc promoter for their expression in an operon (Collis and Hall, 1992). The consequence of this system is that the last integrated cassette is the closest to the Pc promoter (Collis et al., 1992 and Collis and Hall, 2004), leading to the highest level of expression in the integron.

2.3.4 Insertion Element Common Region (ISCR): Microorganisms possess a unique set of genetic makeup those help them to adjust with blueprint of the cell, the bacterial genome, enabling the cell to alter, add or loss genetic information (Toleman et al., 2006). Antibiotic resistance among bacterial population now become high on the clinical and scientific agenda (Toleman et al., 2006). Drug resistance genes are disseminated through site-specific and homologous recombination and other genetic structures such as plasmids, insertion sequences (IS element), transposons (Tn elements) and integrons with their gene cassettes. Other complex module structures, such as integrative conjugative, mobilizable elements (ICEs and IMEs respectively) and genomic islands are also involved in the process (Ilyina, 2012). These systems participate in gene transfer both in the same DNA molecule and between different molecules situated in the same cell that can further distributed intracellular by conjugation, transformation or transduction (Ilyina, 2012). It has become evident that, one more system of recombination takes part in creation of blocks of genes including antibiotic resistance genes containing extended-spectrum classes A, C and D beta-lactamases; metallo-betalactamases (MBL), quinolones, trimethoprim, cholamphenicol, aminoglycosides, tetracycline, macrolides etc (Ilyina, 2012). This aspect of plasmid evolution is based on a set of mobile genetic elements called ISCR elements (Toleman et al., 2006). They are small cryptic sequences, predicted as transpose like IS elements and the resistance mechanism is called rolling circle (RC) transposition which couples with RC replication (Tavakoli, 2000) (Figure 14). ISCR elements were first detected as sequences associated with a class I integrons and the same sequence is found in common region or CR (Stokes et al., 1993). CR sequence found in the Integron

are contains open reading frame (ORF) encoding a product of 513 amino acids. CR sequences could transpose not only themselves but also DNA sequences adjacent to their terminal end and this transposition can results a huge blocks of resistance genes (Ilyina, 2012). Transposition mechanism of CR elements can mobilize any region of the adjacent DNA and responsible for the fast emergence and spread of whole gene.

Insertion sequence (IS) of ISCR family unusual and commonly differ from typical IS elements in a manner that;

1. One ends of terminal sequences of IS elements have a function as origin of replication (oriIS), another one plays a role of the site termination of replication (terIS).

2. Elements of IS element specifically penetrate into 3´-end of the target sequence represented by the 5´-CTTG or 5´-GTTC tetra nucleotides.

3. When penetrating the target they do not from direct duplications of the target sequence.

4. Transpositions of IS elements differs from transposition of typical IS and includes RC replication, formation of non-replicative autonomous closed circular structure.

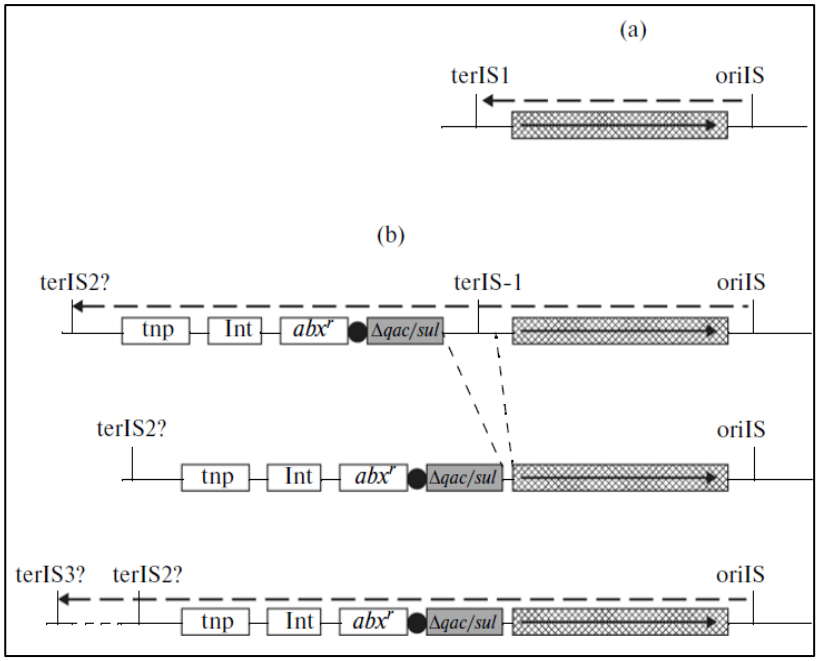

Figure 14: Mobilization of class I complex integrons by ISCR. terIS and oriIS are the initiation and termination and arrow indicate the way of transcription. Source : Ilyina TS, 2012.

The oriIS sequence is 5´-GxTTTxAAATTCCTAT-3´ and is located in the downstream from the transposase gene and terIS sequence is contains a perfect six-nuecleotide punctuated inverted repeat. Deletion of terIS cause one-ended transposition which flanked by ori at one end. The sequence of terIS of CR elements are not precisely determined due to the complicated determination of the site of confluence with the nucleotide sequence of the host DNA. The 23 types of ISCR elements are found today and they are closely related to the each other (Ilyina, 2012).

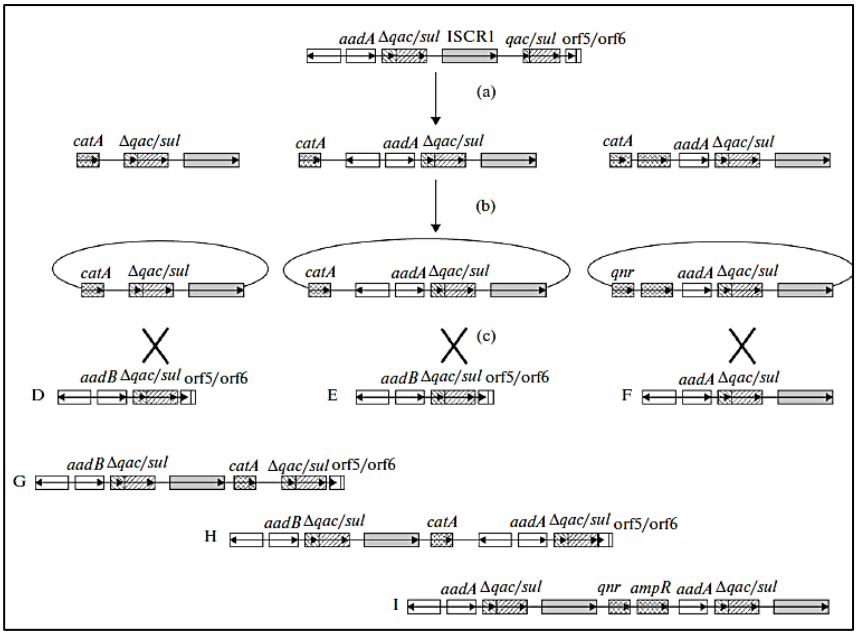

Figure 15: Model of ISCR-mediated contraction of complex class I integrons; showing the rolling circle replication of the ICSR1 element inserted into the integron generates transposition intermediates and formation of complex integron by homologous recombination. **Source: Ilyina TS, 2012.**

ISCR1 elements differ from other groups as they are localized in complex class I integrons containing duplication of the 3´-CS region with its only shortened copy and deletion of the 3´-CS includes cassettes and non-cassette genes, could transpose into other genetic structures due to the secondary transpositions (Ilyina, 2012).

ISCR2 was discovered in the SXT (Co-tromoxazole) integrative conjugative elements and harboured transposon like structure which includes several antibiotics like sulfamethoxazole, trimethoprim (Bennett, 2008).

ISCR3 groups contains several elements- ISCR3, ISCR4, ISCR5, ISCR14 and ISCR16 with identical GC contents and consists of flop and terR bracketed from the both side . They are associated with groEL genes in their terminal ends (Toleman and Walsh, 2010) (Figure 16).

The fourth subgroup of ISCR elements has been associated with the MBL gene $bla_{SPM-1,}$ confers resistance to all beta-1actam antibiotics except from aztreonam (Toleman, 2006). The sequence upstream of **ISCR4** encodes a predicted amino acid sequence with GroEL proteins (Poirel, 2004).

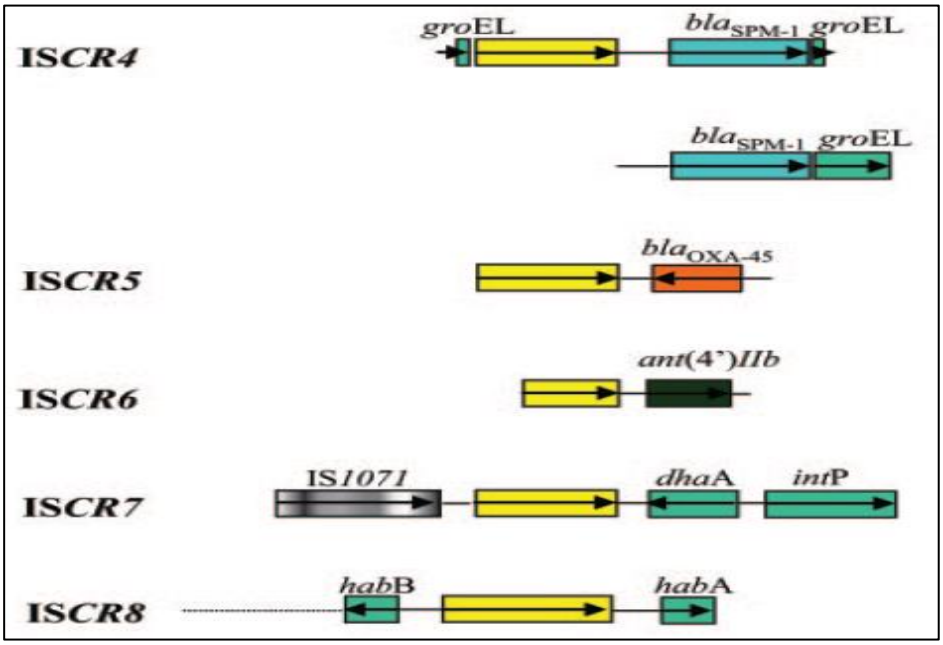

Figure 16: Genetic context of different group of ISCR elements*. Transcription process indicated with arrows.* ***Source: Toleman, 2006.***

ISCR5 is associated with the class D oxacillinase gene bla_{OXA-45} found on a small plasmid (Tolemn, 2003), ISCR6 was found adjacent to an aminoglycoside-modifying gene, ant(4´)–Iib which confers resistance to amikacin but not netilmicin (Sabtcheva, 2003). Other group of ISCR elements are possess several putative transposase.

2.4 Gene captures mechanism:

2.4.1 Evolution of Integron: The evolution of a wide range antibiotic resistance mechanism in pathogenic gram bacteria has contributed the presence of Lateral gene transfer and its association with transposons and plasmids

(Chowdhury et al., 2013). For the rapid proliferation of antibiotic resistance bacterial population showing various adaptation and integron system is one of them adaptation process (Stalder et al., 2012). Integron was introduced as a discrete genetic element that was capable of capture antibiotic resistance genes via site specific recombination (Hall, 2006). The word “Integron” first came to importance as magnitudes of their infiltrating among pathogenic Gram negative bacteria and possess multiple cassettes with the associated gene conferring (Chowdhury et al., 2013). The distribution of integron in chromosomes indicates that they are equally prevalent in non–clinical environmental microorganisms (Stalder et al., 2012). Two major types of integron have been described: “Multidrug resistant integrons” (MRIs) and “Chromosomal integrons” (CIs) or “Super integron” (SIs).

2.4.2 **Multidrug Resistant Integron (MRI):** Based on the amino acid sequence of the intI protein MRI have been characterized into different classes which are mostly clinically important and associated with antibiotic resistance genes i,e; class I, class II, class III (Chowdhury et al., 2013). Class I MRI has been most prominent in clinical settings. They have been mainly found to be associated with functional and non-functional transposons which derived from Tn402 (Stalder et al., 2012). The non-functional type of integron are grouped under class I integron, commonly known as “Clinical integrons” (Gillings et al., 2008) and frequently embedded in plasmids or larger transposons, such as Tn3 family. Class I integron possesses the highly conserved intI1 gene and found in a 5´Conserved Sequence (5´CS). The 5´CS of class I integron contains the core site for site specific recombination, result of which catalyses the insertion and excision of genes present in gene cassette.

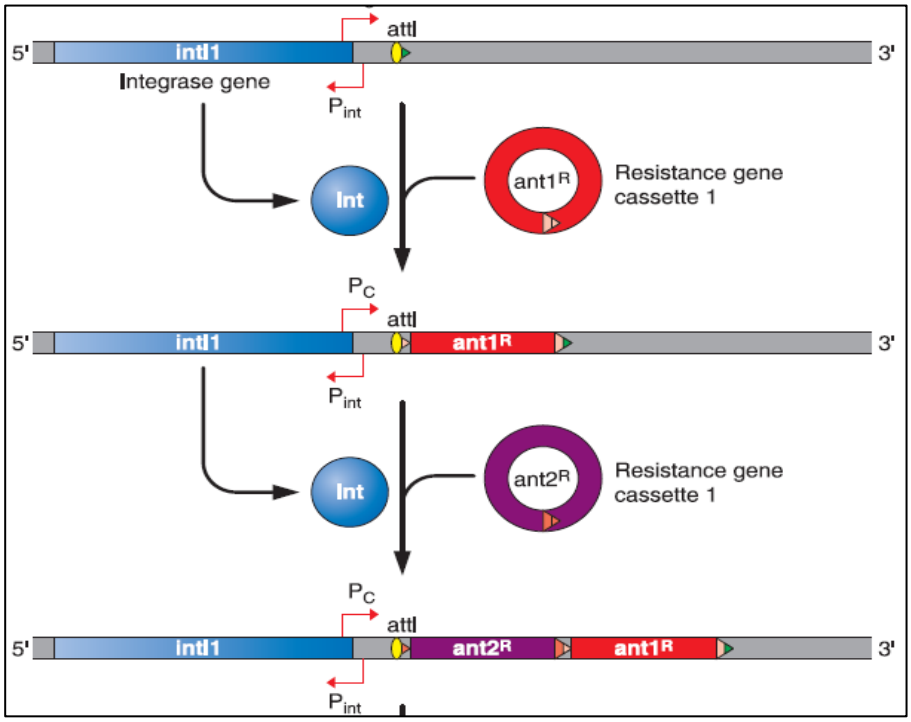

Figure 17: Integron mediated gene capture mechanism.

Source: Larouche and Roy, 2011.

The 3´Conserved Sequence (3´CS) region is composed of qacEΔ1 gene, a functional region for deletion of the qacE gene and conferring resistance to quaternary ammonium compounds (Paulsen et al., 1993). The qacEΔ1 gene is followed by sul1 gene conferring resistance to sulphonamides and orf5 encoding a protein of unknown function (Stalder et al., 2012).

Class II integrons are the second most important group of integron and consists of intI2 protein in 5´CS. The intI2 gene is interrupted by a stop codon and resulting in a truncated and non-functional protein (Stalder et al., 2012). Due to the presence of a stop codon at amino acid 179 it produce short and probably inactive classes of integron(Barlow and Gobius, 2006). Class II integron mostly

associated with Tn7 transposons and carries gene cassette dfrA1 (conferring the resistance aginst trimethoprim), sat2 (involved in the resistance to streptothricin), aadA1 (involve in the resistance to streptomycin and spectinomycin) and orfX (for unknown function) (Hansson et al., 2002). Variability of class II integron have identified a number of novel arrangements and found to be inserted sat2, catB2 and aadB gene within the upstream region (Barlow and Gobius, 2006).

Class III integrons are very rare group of integron in clinical setting as well as in non-clinical environment. This was first isolated from a carbapenem-resistant Serratia marcescens strains (Arakawa et al., 1995). According to the (Collis et al., 2002) this structure consists of intI3 protein, attI3 site and Pc promoter region.

2.4.3 **Super Integron:** Super integron are found in chromosome 2 of V. cholera and identified as a repetitive DNA sequences (VCR) (Rowe-Magnus and Mazel, 2001) (Figure 19). The large numbers of resistance genes with high homology are gathered between the attC sites of these cassette and the key features named as “Super Integron” (SI) (Rowe-Magnus and Mazel, 2001). SI possesses a specific and related integrases that are responsible for the insertion of ORFs into unique chromosomal attachment sites and forming a tandem array (Rowe-Magnus and Mazel, 2001). They share the same generalised structure but the mechanism by which cassettes are inserted remains unknown. The preliminary data indicates that SI gene cassettes encode adaptive functions beyond pathogenicity and antimicrobial resistance and in V. Cholera carries three pathogenicity genes stog genes (to carries the heat stable toxin), mrhA (for mannose-fucose-resistant haemagglutinin gene) and mrhB (presence in the same

operon as mrhA and as well as lipoprotein encoded gene) (Rowe-Magnus and Mazel, 2001).

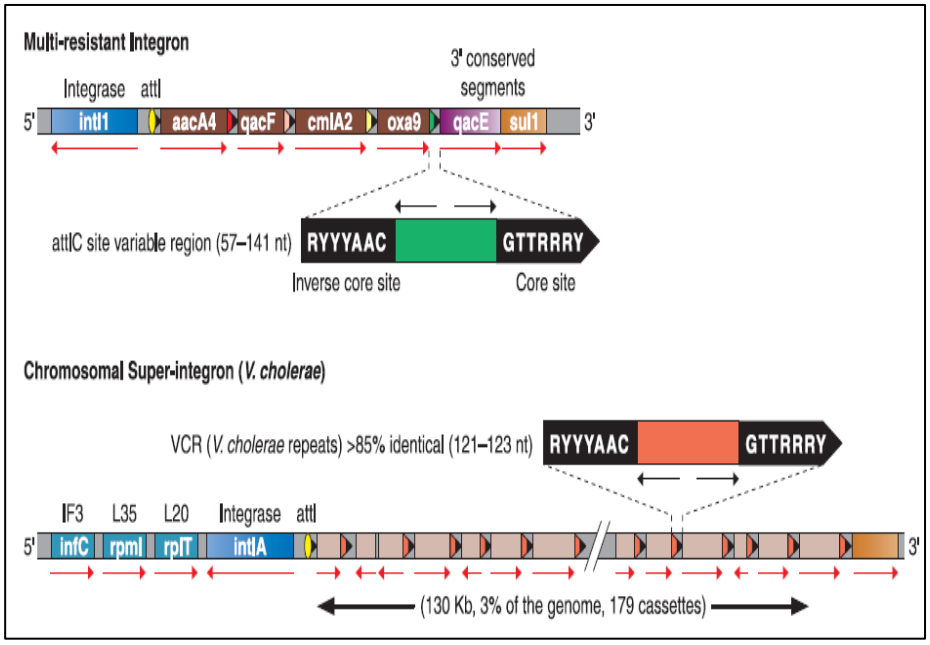

Figure 18: Structural comparison of super integron.

Source: Rowe-Magnus, Guérout, Mazel, 1999.

2.5 Gene cassette arrangement: Gene cassettes are small mobile elements that generally include a single gene or open reading frame and downstream recombination site, known as 59-base element. The cassette are incorporated into integron by site-specific recombination between the attI site of integron and the 59-be in a circularized cassette (Hall et al., 1991) and both intregration and excision of cassettes by intI. Gene cassette basically does not include a promoter and transcription of cassette-associated genes is from a common promoter Pc (Stokes 1997). The cassette associated recombination sites are not highly conserved, length vary from 57-141bp and can be identified by their location and relationship at their outer ends to consensus sequences that are

imperfect inverted repeats of one another. The recombination occurs close to one end of the 59-be, within a conserved core site with a consensus sequence GTTAGGC or GTTRRRY and it occurs at the unique position between the adjacent G and T residues (Stokes et al., 1997).The bases that are not matched by a base in the other half of an attC site bulge out from the double standard part of a hairpin folded single strand (Hall et al., 1999). Variants of gene cassettes that have lost most of the central part of the attC site, effectively fusion the cassette to the next cassette or to the DNA segment next to it.

2.6 Resistance markers of Integron: Inappropriate use of antibiotics is a now become rise a global problem with an antibiotic resistance. As results, multidrug resistance rates increases among clinical environment as well as in commensal bacteria. Sulphonamide group of drugs first came in use during 1932 and Trimethoprim introduced in 1962 (Huovinen, 1985). The combination of these two drugs showed synergistic in vitro and later on this use for the prophylaxis of urinary tract infection as co-trimoxazole drug (Huovinen, 1995). Because of the wide range of clinical indications co-trimoxazole used extensively everywhere and microorganism became resistance against those drugs (Huovinen, 1995). The rapid development of antibiotic resistance suggests that the selection pressure of the corresponding resistance genes may be responsible for such acquired resistance (Grape, 2005). To encounter the mechanism behind the antibacterial resistance, molecular approaches are used and, being surprisingly found that integrons are associated with such resistance mechanism. The structure of class I integron promotes most of the antimicrobial resistance within bacteria due the presence of sul1 and qacEΔ1 genes in the downstream (Grape, 2005).

2.6.1 Trimethoprim resistance in Integrons: Trimethoprim (TMP) is a broad spectrum antibiotic agent and mostly active against enteric pathogen (Adrian et al., 2000) (Figure 20). The common mechanism of trimethoprim is the production of plasmid mediated Dihydrofolate reductase (DHFR) and replaces the chromosomal enzyme to make them less sensitive by preventing the dihydrofolate to convert into tetrahydrofolate (Adrian et al., 2000).The high incidence of trimethoprim resistance gene cassettes inserted into class1 integrons which normally harbour sul1 at the 3´CS which most likely to be occurs by the strong selection pressure of trimethoprim-sulphonamide combination (Adrian et al., 2000). These resistance genes mainly encoded two distinct groups of enzymes families: type A DHFR and type B DHFR family.

Type A DHFR family includes most of the known trimethoprim resistance genes and they are having 64-80% identical amino acids (Lee et al., 2001). dfrA1, dfrA5, dfrA7 and dfrA14 were found to be associated with trimethoprim gene cassette, although dfrA6 was not found in an identical cassette (Lee et al., 2001). dfrA12 and dfrA13 are closely related to one another found in gene cassette but not a member of this family (Lee et al., 2001). The type A family encoded the DHFR enzyme in between 57-187 residues and after alignments of 17 these enzyme reveal high conservation within termini of these proteins and 12 residues strictly conserved across all enzymes (White and Rawlinson, 2001).

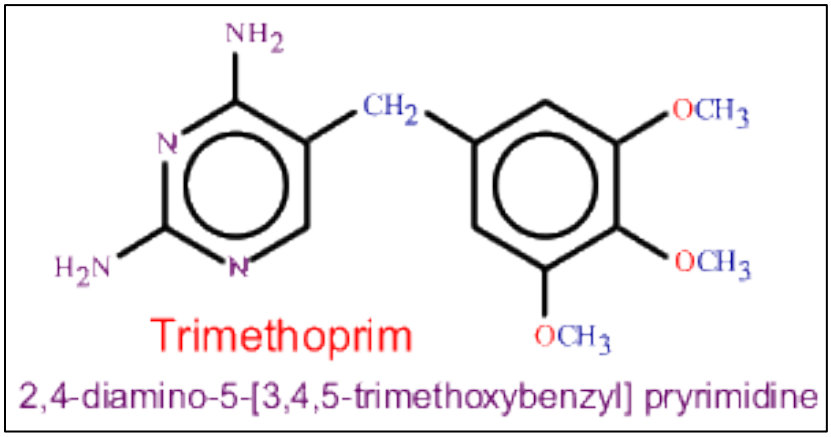

Figure 19; Chemical structure of Trimethoprim

Source:http://www.greenerindustry.org.uk/pages/vanillin/images/vanilla_Diag05.gif.

Type B DHFR family have shorter residue against type A and encode the (dfrB1, dfrB2, dfrB2 and dfrB3) in their gene cassette and encoded the enzyme DHFRIIa, DHFRIIb, DHRFIIc respectively (White and Rawlinson, 2001).

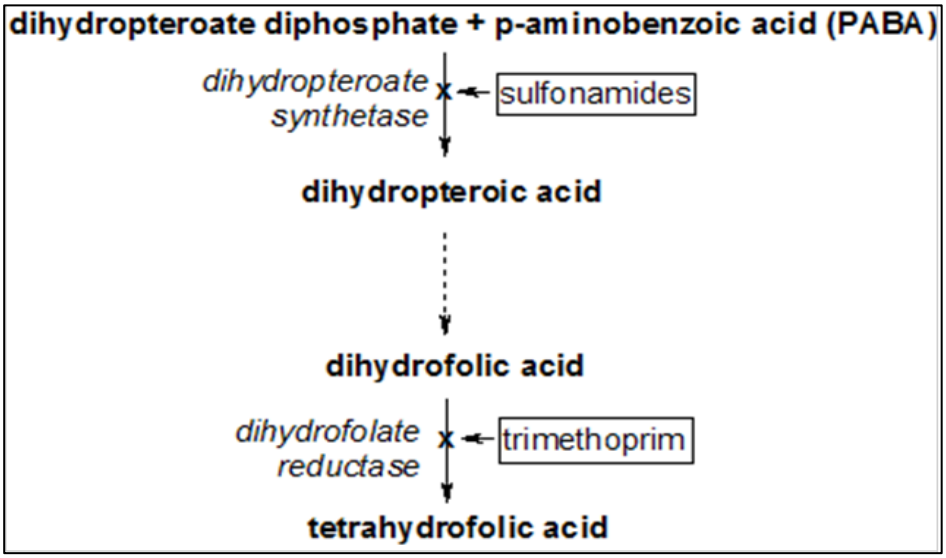

Fig 20: Tetra-hydrofolate synthesis pathway.

Source:http://upload.wikimedia.org/wikipedia/en/e/e9/THFsynthesispathway.png

2.6.2 Sulfonamide resistance in Integron: The enzyme dihydropteroate synthase (DHPS) catalyses the formation of dihydropteroic acid in bacteria, Sulphonamide (SUL) drugs acts as a inhibitors of DHPS and

blocking folate biosynthesis (Huovinen et al., 1995) (Figure 22). Suls are the structural analogs of normal p-aminobenzoic acid and act as a substrate to sulfa containing pteroateanalog (Huovinen et al., 1995). Despite the massive reduction in the use of sulphonamide drugs resistance has persists at high rates among clinical isolates (Bean et al., 2009).

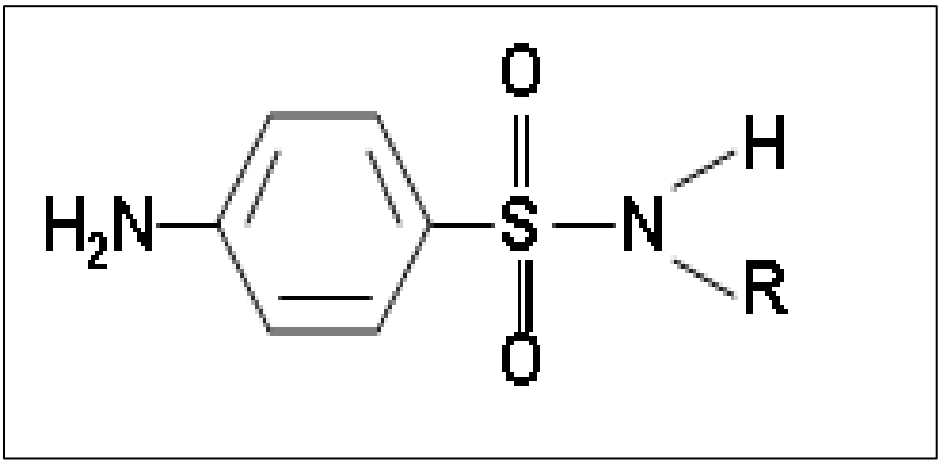

Fig 21: Chemical structure of Sulphonamide antibiotic.

Source: http://classroom.sdmesa.edu/eschmid/Lectur83.gif.

There are three acquired genes imparting resistant to sulphonamide among clinical isolates such as: sul1, sul2 and sul3 (Grape et al., 2004).While sul1 gene typically associated with 3´CS of class I integron and sul2 gene has remained more prominent and found both alone and in combination of sul1 gene (Bean et al., 2009). Sul2 gene is not considered as a part of genetic element but frequently found adjacent to the streptomycin resistance gene pair strAB (Bean et al., 2009). A newly plasmid -borne sulphonamide resistance Sul3 gene was first discovered in 2003 among E.coli isolates and samples collected from pig in Switzerland (Grape et al., 2005).

2.7 Molecular approach detection of Integron mediated resistance:

PCR amplification for the detection of whole class I integron cassettes was performed with the primers 5´CS and 3´CS (Levesque et al., 1995) to determine the size of any inserted cassette in integron system (Figure 23). For PCR detection of the int1 and int2 integrase genes (integrase gene PCR) oligonuceutide primer based on the int1 and int2 genes were designed by Koeleman et al., 2001. Primers intI1F and intI2R, and intI2F and intI2R were used to amplify with fragment size 160bp and 288bp.

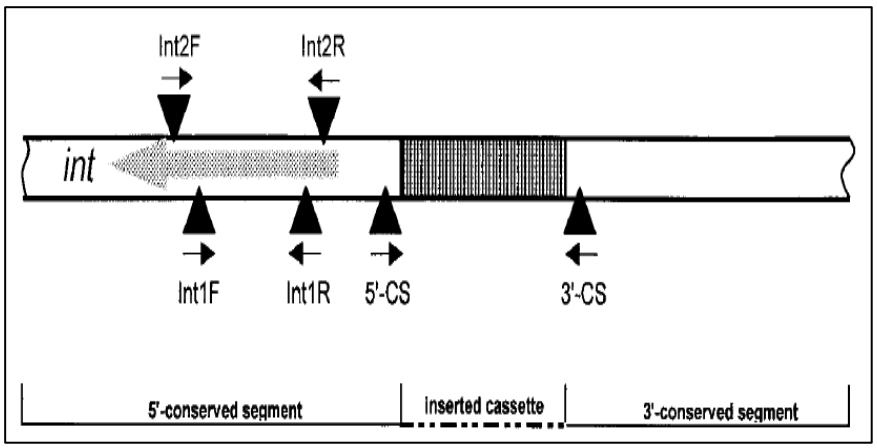

Figure 22: Scheme for PCR detection of class I and class II integron structure.

Source: Koeleman et al 2001.

The features of the integron-gene cassette system suggested that intact novel genes could be recovered directly from environmental DNA which are flanked by conserved sequences (59-be sites) that are potential targets for PCR primers. HS 286-HS287 is degenerated primer which designed to amplify flanking region of 59-be element (Stokes et al., 2001) (Figure 24).

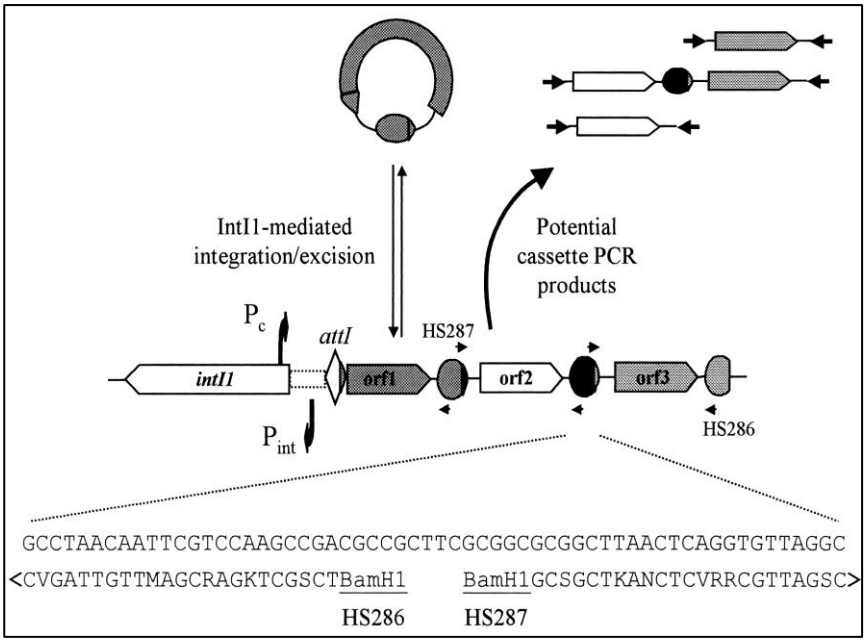

Figure 23: Exploitation of the integron-gene cassette system for recovery of intact gene by PCR.

Source:http://femsre.oxfordjournals.org/content/femsre/35/5/820/F1.large.jpg

The method of amplifying class I cassette arrays by targeting adjacent conserved regions was applied with the primers HS 458-HS 459 was carried out with intI1 positive isolates (Figure 25).

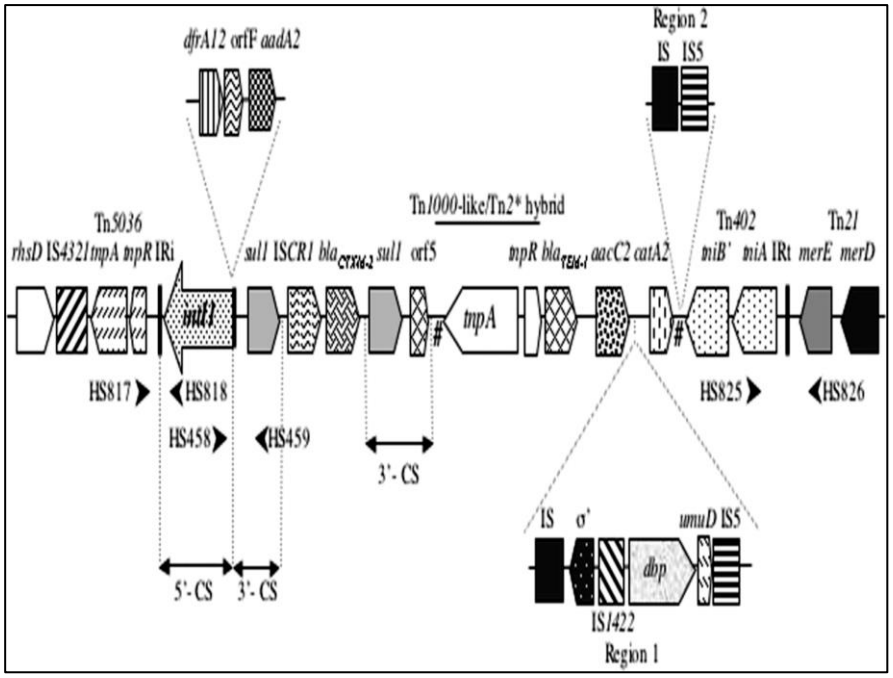

Figure 24: Physical mapping of the class Iintegron.

Source: Marquez C et al., 2008.

For the detection of class II integrons by PCR with degenerated primers hep74 and hep 51, which bind with the attI2 and orfX sites of class II integron respectively within the downstream of the cassette region of Tn7 (White, 2001) (Figure 26).

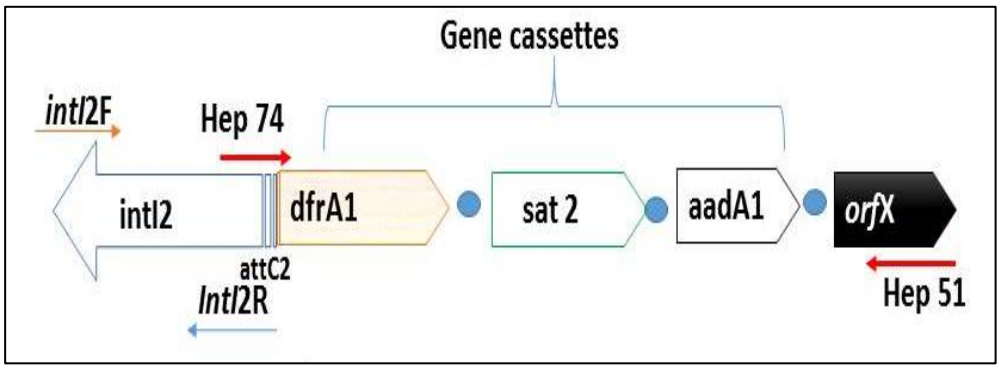

Figure 25: Schematic diagram of class II integron.

Increasing trend of multidrug resistance now becomes serious problem among bacterial population; there was an enormously successful period in medical history but during the last half century the situation is become threatened by the increasing incidence of antibiotics resistance. Directed by a strong antibiotic selection pressure, microbes gradually develop many different processes to establish resistance.