EFFECT OF NOREPINEPHRINE ON CONJUGATION OF *ESCHERICHIA COLI* STRAINS

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Abstract

Antibiotics are substances produced by bacteria or fungi that are inhibitory to other bacteria and fungi. Antimicrobial compounds include substances that are naturally produced, chemically modified or completely synthetic (chemically designed or synthesized). The chemical modification of naturally produced antibiotic generally results in increase stability, solubility, increased spectrum of activity, or efficacy. Antimicrobial compounds are used in animals to treat and control infectious diseases, and also for growth promotion. Bacteria may gain resistance to antibacterial agents via a variety of mechanisms. There is growing evidence that antimicrobial resistance has significant public health consequences. Rationale use of antimicrobial drugs using appropriate medication at the proper dosage and for duration is one of the important means to reduce selective pressure that helps reduce life of resistant organism. It is also vital to reduce the spread of multi drug resistant organisms in the environment especially in health care facilities. Bacteria evolve rapidly not only by mutation, but also by horizontal gene transfer through the transformation, transduction, and conjugation. Conjugation involves a close contact between two bacteria and transfer of the plasmid that carry many genetic elements. The pathogenic bacteria have the ability to sense as well as respond to the stress in the recipient. The epinephrine and norepinephrine play a key role in stress situations in animals. A previous study showed that norepinephrine (NE), a catecholamine at physiological concentrations promoted the conjugation efficiencies of a conjugative plasmid from a clinical strain of *Salmonella typhimurium* to an *E. coli* recipient in vitro. The objective of this study was to determine the effect of norepinephrine on conjugation of two *E. coli* strains. Both filter mating and liquid mating assays were used. The results revealed that there was no significance difference between the presence and the absence of norepinephrine on conjugative transfer of RP4 plasmid between *E. coli* strains (FS1290 and C600N) either in filter mating or liquid mating. Further studies are needed to determine whether higher concentration of (more than 20 mM) has any effects on conjugation in *E. coli.*

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Dedication

I would like to dedicate this report to my parents. My late father's advices are always with me, and my mother for her constant support in all my endeavors. I would also like to dedicate this report to my dear wife Saleha. Thank you for your love and support throughout this process. And my children: Fatema, Mohamed and Khadeeja for being with me during this period.

Chapter 1 - Literature Review

Antibiotics and Antimicrobial agents

Antibiotics are substances produced by bacteria or fungi that are inhibitory to prokaryotes and eukaryotes (Kohanski *et al.,* 2010). Inhibition of bacteria and fungi results in killing or prevention of growth by targeting cellular processes, and the compounds generally cause little or no damage to the recipient (Prescott, 2000; Walsh, 2003; Giguere, 2006; Guardabassi and Courvalin, 2006). Because of the limitation of the nutrient in the environment, there is usually a competition between microorganisms, which is one of the reasons why some organisms produce the antibiotics to have a competitive advantage to survive. Antibiotics can be naturally produced by bacteria, fungi, or chemically modified compound that is produced naturally or completely synthetic (chemically designed or synthesized). The chemical modification of naturally produced antibiotic generally results in increase stability, solubility, increased spectrum of activity, or efficacy (Hemaiswarya *et al.,* 2008). A number of semisynthetic penicillins and cephalosporins have been developed which have increased the spectrum of activity, long acting, and less likely to become resistant (Miller, 2008). The term antimicrobial agent is used to describe any compound, naturally produced or chemically synthesized, which are inhibitory to microorganisms. Antimicrobial agents are important tools in the fight against and elimination of infectious diseases (Hancock *et al.,* 2005).

Antimicrobial compounds are classified based on the following criteria:

a. Chemistry and structure
b. Spectrum of activity
c. Mode of action of inhibition

a. Chemistry and Structure

Structurally, the antimicrobial compounds have simple to complex chemistry of purified and chemically modified compounds increase stability, solubility, or efficacy of naturally produced antibiotics compounds are synthetic, which are chemically synthesized (Table 1.1)

Table 1.1 Classification of Antimicrobial Compounds based on the Chemistry and Structure

Class of Compounds	Chemistry and Structure	Examples
1. Aminocyclitols	Amino polyhydroxy cycloalkanes.	Spectinomycin
2. Aminoglycosides	Hexose nucleus to which amino sugar is linked by glycosidic bonds.	Amikacin, Apramycin, Gentamicin, Kanamycin, Dihydrostreptomycin
3. Bacitracin	Polypeptide, consist of phenyl alanine, histidine, lysine and cystine	
4. Beta-lactams	β-Lactam ring	Amoxicillin, Ampicillin, Carbapenams, Ceftiofur, Cephalosporins, Cephalothin, Penicillins
5. Diaminopyrimidines	Two amine groups on a pyrimidine ring	Trimethoprim
6. Fluoroquinolones	Addition of fluoride in the place of nitrogen in the position 6 to the quinolone molecule	Enrofloxacin, Ciprofloxacin, Danafloxacin, Difloxacin, Marbofloxacin, Orbifloxacin

7. Lincosamides	Proline residue, attached by a peptide bond to a galactoside ring	Clindamycin, Lincomycin,
8. Macrolides	12 – 16-member macro cyclic lactone ring connected to two or more sugar molecules	Azithromycin, Clarithromycin, Erythromycin, Tilmicosin, Tildipirosin, Tulathromycin, Tylosin
9. Nitroimidazoles	Heterocyclic compounds with a five-membered nucleus	Dimetronidazole, Ipronidazole, Metronidazole, Ronidazole, Tinidazole
10. Phenicols	Derivatives of dichloroacetic acid and contain a nitrobenzene moiety	Chloramphenicol, Florfenicol
11. Pleuromutilins	Derivatives of naturally occurring diterpene antibiotic pleuromutilin	Tiamulin
12. Polymyxin	Polypeptide	Polymyxins B
13. Rifamycins	Structurally related to Macrolides	Rifampin, Novobiocin
14. Sulfonamides	Sulfonamide group, structurally analogous to *p*-aminobenzoic acid	Sulfamethoxazole, Sulfadiazine Sulfachlorpyridazin, Sulfamethazine, Sulfaquinoxaline

15. Tetracyclines	Naphthacene ring with hydrochloride	Chlortetracycline, Doxycycline, Minocycline, Oxytetracycline, Tetracycline
16. Vancomycin	Glycopeptide	Vancomycin

b. Spectrum Activity of Antimicrobial Compounds

Broad-spectrum antimicrobial compounds are effective against gram-negative and gram-positive bacteria, and tend to have higher toxicity to the recipient, such as tetracyclines. Narrow-

Table 1.2. Spectrum of Activity of Common Antimicrobial Compounds

Antibiotics	**Gram-positive bacteria**	**Gram-negative bacteria**	***Mycoplasma***	***Rickettsia***	***Chlamydia***
Aminocyclitols	-	+	-	-	-
Aminoglycosides	-	+	-	-	-
Beta-Lactams	+	-	-	-	-
Fluoroquinolones	+	+	+	+	+
Lincosamides	+	-	+	-	-
Macrolides	+	-	+	-	+
Phenicols	+	+	+	+	+
Pleuromutilins	+	-	+	-	+
Tetracyclines	+	+	+	+	+
Sulfonamides	-	+	+	-	+
Diaminopyrimidines	+	-	-	-	-

spectrum drugs are effective against a limited group of microbes and exhibit lower toxicity to the recipient, such as penicillin (Table 1.2)

c. Mode of Action of Antimicrobial Compounds

Antimicrobial agents work in two ways, by either killing the bacteria, which is called bactericidal, or by the preventing of the growth, which is called bacteriostatic. The important targets for antimicrobial agents in the cell are the cell wall, cytoplasmic membrane, nucleic acids (DNA to RNA or DNA to RNA), and ribosomes (protein synthesis). Antimicrobial compounds have four major modes of action:

1. Inhibition of cell wall synthesis

According to this mechanism of action, the synthesis of peptidoglycan, present in the cell wall of all bacteria, except *Mycoplasma* (Holtje *et al.,* 1998), is inhibited. The peptidoglycan structure is basically the same in both Gram-positive and Gram-negative organisms. However, there are important differences. Gram-negative bacteria have a thin peptidoglycan layer, which in loosely cross-linked. Gram-positive bacteria, on the other hand, possess a very thick peptidoglycan layer. Penicillins, cephalosporins, carbapenems, bacitracin and vencomycin are the antimicrobial compounds that prevent the synthesis of peptidoglycan (Park and Uehara, 2008; Figure 1.1).

2. Inhibition of protein synthesis

Some antibiotics inhibit the process of protein synthesis by interfering with the ribosome. Bacteria have 70S ribosome, which consist of 50S and 30S subunits, whereas eukaryotic cells (animal or human cells) have 80S ribosomes, which make bacterial ribosome as a suitable target for selective inhibition. Drugs that inhibit the protein synthesis are among the broadest classes of antibiotics, and can be divided into two subclasses; the 50S ribosome inhibitors and 30S ribosome inhibitors (Nissan *et al.,* 2000). The classes of antibiotics that inhibit protein synthesis are tetracyclines, macrolides, aminoglycosides, lincosamides, and chloramphenicol (Table1.3).

3. Disruption of the Cytoplasmic Membrane

These antimicrobials have an effect on the external membrane of Gram-negative bacteria. The compounds interact with phospholipids of the cell membranes, and disrupt the structure and function (Gilleland *et al.,* 1984). The end result is leakage of cytoplasmic contents and death of the cell. Polymyxin, produced from *Bacillus polymyxa*, is an example for this mode of action. They are cyclic decapeptides, and they act like detergents, hence react with phospholipids of cell membranes and disrupt their integrity (Figure 1.1).

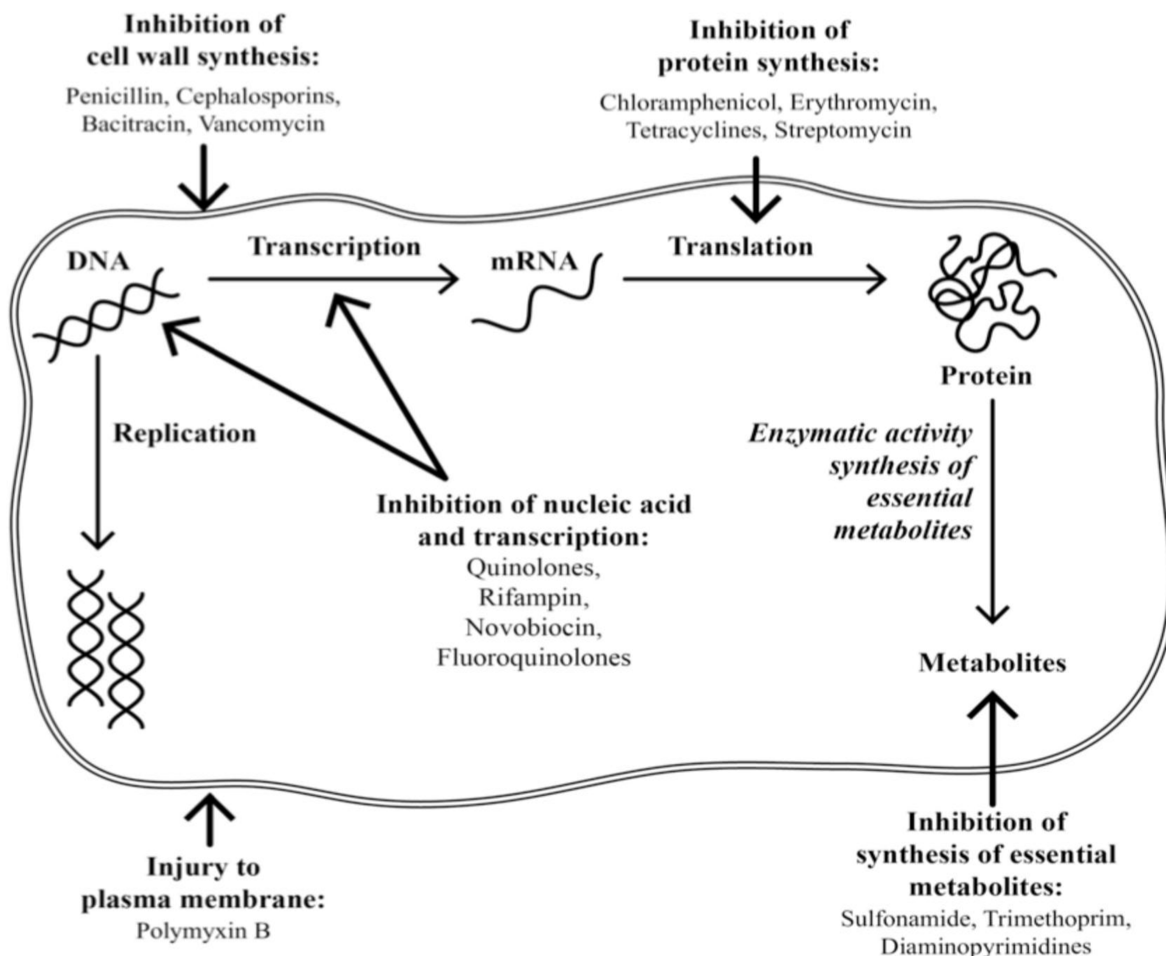
Table 1.3. Classification of antimicrobial compounds based on mode of action

Mode of action	Mechanism	Examples
Interference with cell wall synthesis	Prevent the synthesis of peptidoglycan, and the cell wall became weakened and the cell undergoes lysis	**β-Lactams** Carbapenems, Cephalosporins Monobactams, Penicillins **Glycopeptides** Bacitracin, Isoniazid, Teicoplanin Vancomycin
Interference with protein synthesis inhibition	Bind to 50S ribosomal subunit	Chloramphenicol, Clindamycin, Linezolid, Macrolides, Quinopristin- Dalfopristin
	Bind to 30Sribosomal subunit	Aminoglycosides, Tetracyclines
Disruption of bacterial membrane structure	React with phospholipids of the cell membranes and disrupt their integrity	Daptomycin, Polymyxins

Interference with nucleic acids	Inhibit DNA synthesis:	Fluoroquinolones, Novobiocin
	Inhibit RNA synthesis:	Rifamycins
Interference in metabolic pathway	Block folic acid synthesis	Sulfonamides, Diaminopyrimidines

Adapted from Tenover, 2006.

Figure 1.1 The mechanism of action of antimicrobial on bacteria



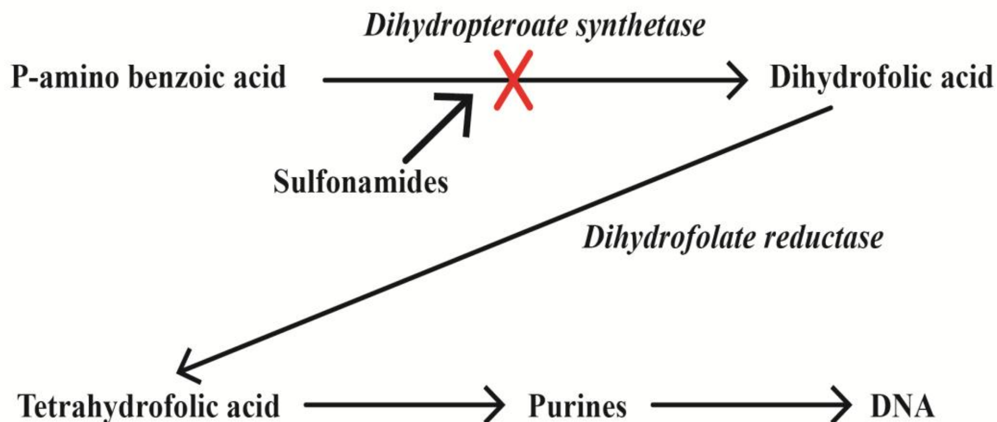
4. Interference with Nucleic Acids

DNA is the main storage form of genetic information in living organisms. It is composed of two strands of nucleotides connected through a backbone of sugar and phosphates. When the cells divide, DNA is replicated to produce new identical DNA copies that are incorporated into the new cells. DNA is also transcribed to nucleic acid, messenger RNA, which directs the synthesis of proteins. There are many enzymes that participate in the synthesis of nucleic acids that are potential therapeutic targets including DNA gyrase, and topoisomerase IV. The differences between the enzymes used to synthesize nucleic acids in prokaryotes and eukaryotes, allow for selective action of antibiotics against prokaryotes by inhibiting nucleic acid synthesis. The rifamycin family prevents RNA synthesis and the reason is they bind to RNA polymerase, which is responsible for transcribing bacterial DNA to RNA. Quinolone group works by inhibiting DNA gyrase. DNA gyrase is responsible for DNA supercoiling, an important step in the process of DNA replication (Kohanski *et al.,* 2010). These groups of antibiotics have the ability to enter the tissues and reach appropriate level to have an effect. The process involves numerous enzymes and other proteins because enzymes are selectively targeted from specific organisms. Therefore, in human medicine it is used to treat tuberculosis, and even leprosy. However, resistance develops rapidly and hence not often used alone (Kohanski *et al.,* 2010). In animals, it is used in combination with a macrolide, such as erythromycin, clarithromycin, to treat *Rhodococcus equi* infection that cause pneumonia in horses and Potomac Horse Fever caused by *Neorickettsia risticii*. Some antimicrobial drugs can interfere with the termination of RNA transcription. The antibiotics that inhibit nucleic acid include rifamycins, novobiocin, quinolones and fluoroquinolones, and nitroimidazoles (Gilleland *et al.,* 1984).

5. Interference in metabolic pathways

Para Amino Benzoic Acid (PABA) is key to mechanism of sulfonamide action. The bacteria that are sensitive to sulfonamides need para amino benzoic acid for the growth. Sulfonamides or sulfa drugs are structurally analogous to p-amino benzoic acid, which is a part of the vitamin folic acid, a precursor in the synthesis of nucleic acids. Sulfa drugs mainly inhibit the process of folic acid synthesis in bacteria by competing with p-amino benzoic acid for the enzyme dihydropteroate synthetase. The mammalian cells do not synthesize folic acid, but the bacteria synthesis their folic acid when growing in the host, and sulfa drugs are effective only against bacterial cells. Sulfa drugs have broad-spectrum activity including effects on protozoa such as coccidia and are bacteriostatic. The sulfonamides and trimethoprim are known to inhibit folate biosynthesis in bacteria, and they are widely clinically used drugs (Holtje *et al.,* 1998); Figure 1.2)

Figure 1.2 Inhibition of folic acid synthesis



Use of Antimicrobial compounds in animals

Antimicrobial compounds are used in animals to treat and control infectious diseases, and for growth promotion. If we protect the health of animals, then we protect human health as well. This is because 60% of diseases that affect humans come from animals, and the relationship between the animal health and human health is very strong (Karesh *et al.,* 2012). In the United States, the yearly overall production of antibiotics in 1979 was 17.5 million Kg. Of those, 12.5 million Kg are used for non-therapeutic purposes in livestock production, and only 1.5 million Kg are used for human medical treatments (Mellon *et al.,* 1979). Food and Drug Administration (FDA) has approved the use of some antimicrobials for the promotion of growth in certain livestock, and poultry (Table 1.4). The most important uses of antibiotics in animals are to treat bacterial infections and to promote animal growth. In the absence of preventive treatments, the infections would occur more frequently and would require more therapeutic interventions (Schwarz *et al*., 2001). In the USA and Canada, agricultural use of antibiotics is regulated, and there are three ways of use: as feed antibiotics, as over the-counter drugs, and as veterinary prescription drugs. Feed antibiotics include antibiotics used for growth promotion and those used for sub therapeutic (including prophylactic, and some growth-promotion use), and therapeutic purposes (Prescott, 1993). Antimicrobial agents are commonly used in animals for the following purposes:

a) **Therapy:** Treatment of bacterial infections
b) **Prophylaxis:** Treatment of the healthy animals to prevent the onset of diseases
c) **Metaphylaxis**: Treatment of all animals in a herd after one or two show clinical signs.

d) **Growth promotion**: Inclusion antimicrobial agents in the feed to improve the growth.

Table 1.4 Antimicrobial Agents approved for use in livestock in the US

Antibiotic class	Example	Species	Used in feed
Beta-lactams	Amoxicillin	Beef, Dairy cattle, Poultry, Swine	No
	Ampicillin	Beef, Dairy cattle, Poultry, Swine	No
	Penicillin	Beef, Dairy cattle, Poultry, Swine	No
Aminocyclitol	Spectinomycin	Beef, Dairy cattle, Poultry, Swine	No
Aminoglycoside	Apramycin	Swine	Yes
	Gentamicin	Beef, Dairy cattle, Poultry, Swine	No
	Hygromycin	Poultry, Swine	Yes
	Neomycin	Beef, Dairy cattle, Poultry, Swine	Yes
Bacitracin	Bacitracin	Beef, Dairy cattle, Poultry, Swine	Yes
Bambermycin	Bambermycin	Beef, Dairy cattle, Poultry, Swine	Yes
Cephalosporin	Ceftiofur	Beef, Dairy cattle, Poultry, Swine	No
Diterpene	Tiamulin	Swine	Yes
Fluoroquinolones	Enrofloxacin	Beef	No
	Danofloxacin	Dairy cattle	No
Lincosamines	Lincomycin	Beef, Dairy cattle, Poultry, Swine	Yes
	Pirlimycin	Beef, Dairy cattle	No
Macrolides	Erythromycin	Beef, Dairy cattle, Poultry, Swine	No
	Oleandomycin	Beef, Dairy cattle	No
	Tilmicosin	Poultry, Swine	Yes
	Tylosin	Beef, Dairy cattle, Poultry, Swine	Yes

Novobiocin	Novobiocin	Beef, Dairy cattle, Poultry	Yes
Orthosomycin	Avilamycin	Swine	Yes
Phenicols	Florfenicol	Beef, Dairy cattle, Poultry	No
Polypeptides	Colistin	Poultry	Yes
	Polymyxin	Beef, Dairy cattle	Yes
Quinoxaline	Carbadox	Poultry, Swine	Yes
Streptogramins	Virginiamycin	Poultry, Swine	Yes
Sulfonamides	Sulfachlorpyridizine	Beef, Dairy cattle, Swine	No
	Sulfadimethoxine	Beef, Dairy cattle, Poultry, Swine	No
	Sulfamethazine	Beef, Dairy cattle, Poultry, Swine	Yes
	Sulfaethoxypyridazin	Beef, Dairy cattle, Poultry, Swine	No
	Sulfathiazole	Beef, Dairy cattle, Swine	Yes
Tetracyclines	Chlortetracycline	Beef, Dairy cattle, Poultry, Swine	Yes
	Oxytetracycline	Beef, Dairy cattle, Poultry, Swine	Yes
	Tetracycline	Beef, Dairy cattle, Poultry, Swine	No

(Adapted from Mathew *et al.,* 2007)

Mechanisms of Antimicrobial Resistance

Bacteria may gain resistance to antibacterial agents via a variety of mechanisms. Some species of bacteria are innately resistant to more than one class of antimicrobial agents. In such cases, all strains of that bacterial species are resistant to all the members of that antibacterial class. For example, *Mycoplasma* is resistant to penicillins because they do not have cell wall. There are many cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antimicrobial agent, and proliferate and spread under the selective pressure of use of those antimicrobial agents. There are a variety of mechanisms of acquired antimicrobial drug resistance and they are:

a) Change in the target inside the cell so that it will not bind to the antibiotic.

Example: Change in the penicillin binding protein in pneumococci, which can confer resistance to penicillin.

b) The organisms may destroy the antibacterial agent before it can have an effect by acquiring genes that encode for the enzymes.

Example: β-lactamases, erythromycin ribosomal methylase in staphylococci.

c) Bacteria may acquire efflux pumps that extrude the antimicrobial agent from the cell before it can reach its target site and exert its effect. These pumps are present in the cytoplasmic membrane of Gram-negative and Gram-positive bacteria. Some are single component pumps and the other multicomponent pumps.

Example: efflux of fluoroquinolones in *Staphylococcus aureus* (McManus, 1997).

d) Biochemical pathway some bacteria may become resistant by the changing of their metabolism by altering biochemical pathway.

Example: Sulfonamides inhibit the production of folic acid synthesis (Tenover, 2006).

Normally, susceptible populations of bacteria may become resistant to antimicrobial agents via mutation as well as selection, or via gaining the genetic information that encodes resistance from other microbes, called horizontal gene transfer. The gene transfer may occur via one of three genetic mechanisms, transformation, conjugation, or transduction. Because of genetic exchange, many bacteria can become resistant to multiple classes of antibacterial agents, and these multidrug resistance bacteria are of serious concern (McManus, 1997).

Horizontal Gene Transfer

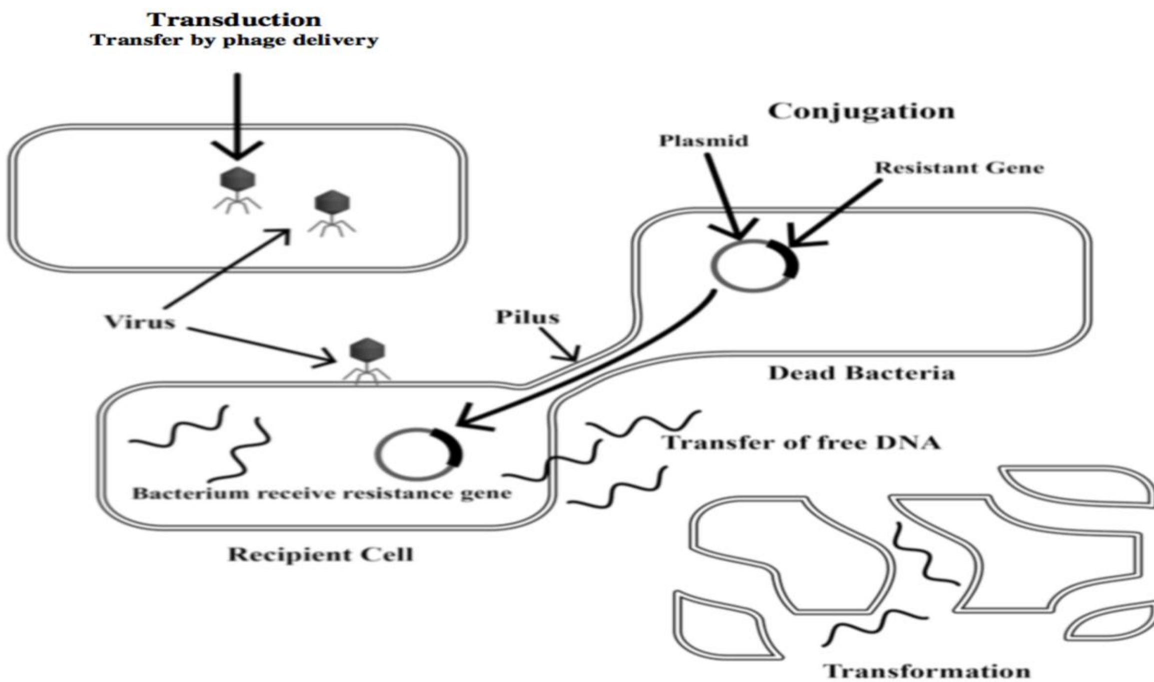
The spread of antimicrobial resistance within species or different species of bacteria is basically the outcome of horizontal gene transfer, a phenomenon of mobile genetic elements that carry the resistance genes from one organism to the other. The transmission of antimicrobial

resistance genes from mother cell to daughter cells is called vertical gene transfer. It is difficult to account for modification, inactivation, or differential regulation of the genes by mutations alone (Narra and Ochman, 2006). The bacteria reproduce by binary fission, which result in the genetic replication of one bacterial cell into two daughter cells. The result of this type of reproduction is producing identical bacterial cells genetically and both have same susceptibilities to environmental pressures like antimicrobial compounds. These are three main classes of horizontal gene transfer, and they are: transformation, transduction and conjugation (Figure 1.3)

1. **Transformation**

The genetic variation will be increased via transformation method between the competent bacteria that take the DNA from the surrounding environment and using it into the genetic material of the recipient to complete its cellular functions. It was first noticed in 1928 by

Figure 1.3 Horizontal gene transfer



Fredrick Griffith, who reported that a strain of *Streptococcus pneumoniae* had two different phenotypes and had different effects on mice (Griffith, 1928). The initial conditions for the natural transformation include the release of extracellular DNA in the environment, and capturing of the free DNA by the donor cells. The release of DNA from dead bacteria occurs after self-induced lysis, which results in broken cell walls and membranes and the release of the cell contents, including DNA, into the environment (Palmen and Hellingwerf, 1995; Schwarz *et al.,* 2001). The free DNA comes from the lysed bacterial cells, which are degraded under most environmental conditions. However, few bacteria, such as *S. pneumoniae* or *Bacillus* spp, have the natural ability to take up DNA from the surrounding environment. A restriction of transformation is that not all bacteria in a population will uptake DNA, be able to take in sufficient amounts of gene needed for survival, or the required gene may not be in the immediate environment of bacteria (Lorenz and Wackernagel, 1994).

2. Transduction

Transduction process is mediated by bacteriophages. Bacteriophages are bacterial viruses, and are capable of transferring the DNA into new recipient cells. Transduction has been shown to be responsible for the mobility of Shiga toxin genes (*stx*1 and *stx*2) from *Shigella* species to the virulent *E. coli* O157:H7 (Plunkett *et al.,* 1999). The restriction of transduction is similar to transformation, as it is relies on the required gene to be present and taken up by the proper phage at the perfect time, and the donor and the recipient must be sensitive to the same bacteriophage (Lorenz and Wackernagel, 1994; Muniesa and Jofre, 2004).

3. Conjugation

Conjugation is transfer of an antimicrobial resistance gene via conjugative plasmid or transposon from a donor cell to a recipient cell. This is the most important method of horizontal transfer of antimicrobial resistance gene in bacteria. It has been shown to be important for the promotion of the survival and evolution of many bacterial species. Close contact between donor cells and recipient cells is one of the major requirements for the conjugation process; it is performed by horizontally transferring genetic elements that was packaged in plasmids or transposons into the cytoplasm for the passage into the recipient cell (Narra and Ochman, 2006). These transposons and plasmids increase the high efficiency transfer of antimicrobial and virulence genes from single resistant donor bacterium to many recipient bacterial cells, and the outcome is the donation of the resistance genes to several recipient bacterial cells (Thomas and Nielsen, 2005). Horizontal transfer by conjugation method is not exclusive to the bacteria of the same species, but it can be performed between different species. Also, the conjugative horizontal transfer is not exclusive to bacterial species, because there are studies that have shown transfer of genetic materials between bacteria and yeasts, and between bacteria and plant cells, and also between the bacteria and the mammalian cells (Rosenberg *et al.,* 1998; Vicky, 1987). The transfer by conjugation between the bacteria that carry antimicrobial resistance genes has very high impact on human and animal health (van den Eede *et al.,* 2004).

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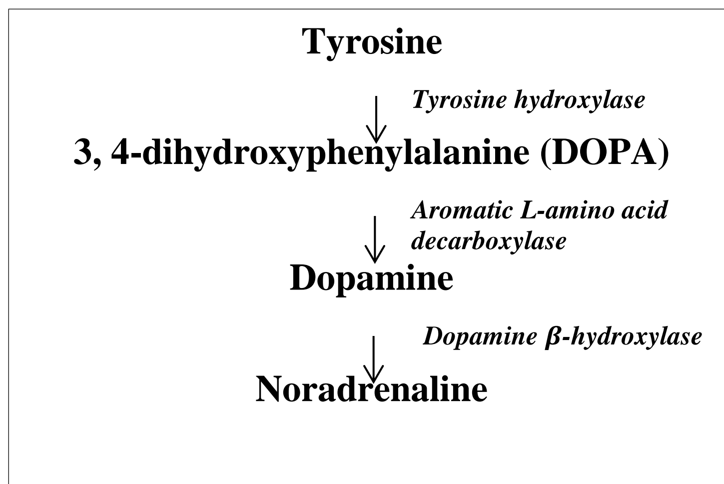
Chapter 2 - Norepinephrine on Conjugation of *Escherichia Coli*

Norepinephrine and Bactria

Catecholamines are a group of hormones that include epinephrine (adrenaline), norepinephrine (NE; noradrenaline) and dopamine. Norepinephrine is produced in the body

Figure 2.1 Noradrenaline Synthesis



from tyrosine (Figure 2.1), released from the adrenal medulla into the blood, and it is also a neurotransmitter in the central and sympathetic nervous systems, where it is released from noradrenergic neurons. The epinephrine and NE hormones are the sympathetic neuroendocrine mediators of fight or flight (acute stress) response of the host. The elevated catecholamine levels make the blood more prone to clotting, thus reducing the risk of heavy bleeding in case of tissue damage (Karasek *et al.,* 1982; Krantz and Manuck, 1984). However, high catecholamine level increases the risk of arterial obstruction and myocardial infarction (Rozanski *et al.,* 1988; Yusuf *et al.,* 2004). More than half of the NE hormone is synthesized and utilized in the enteric nervous system (Furness, 2000). It is estimated that the physiological concentration of NE in the gastrointestinal tract is as high as 50 µM (Thomas, and Nielsen. 2005). The human and animals gastrointestinal tracts particularly the rumen in ruminants and hindgut in monogastrics and ruminants, are inhabited by a dense population of bacteria (10^{11}/g - 10^{12}/g of contents), whose composition is influenced by the health of the host. The gut flora has evolved specific system to detect or sense neuroendocrine secretions, and use

such mediators as environmental cues to alter their growth and virulence. A serum-based iron-depleted medium showed that catecholamines increased the growth of bacteria (Lyte, and Ernst. 1992), which was initially assumed to be due to enhanced iron acquisition through the use of catecholate-specific iron transport system (Bearson *et al.,* 2008; Freestone *et al.,* 2000). Additional studies have shown that catecholamines can influence the production of virulence factors, such as toxins, adhesins, biofilm formation, and quorum sensing molecules, even under conditions where iron was not a limiting factor (Lyte *et al.,* 1996; Reading and Sperandio, 2006). For example, *E. coli* O157 when exposed to catecholamines will respond by increasing the expression of Shiga toxins (Dowd, 2007), increasing adherence to eukaryotic cells (Chen *et al.,* 2003), promoting attachment and effacement (A/E) lesions (Reading and Sperandio, 2006), and increasing flagella expression and motility (Clarke *et al.,* 2005). Catecholamines have also been shown to stimulate motility and promote colonization of *Salmonella* in the gut of swine. This observation provides a non-immunological interpretation for increased incidence and severity of infectious diseases during the period of stress. Peterson *et al.,* (2011) have reported that norepinephrine at physiological concentrations increase the horizontal gene transfer efficiency of a conjugative plasmid from a donor, *Salmonella typhimurium*, to a recipient, *E. coli in vitro.* The mechanism of the effects of catecholamines on horizontal gene transfer in bacteria is presently not known (Peterson *et al,* 2011).

Escherichia coli

Escherichia coli are Gram-negative bacteria and the species is a member of the *Enterobacteriaceae* family. *Escherichia coli* occurs widely in nature, including the intestinal tracts of humans, animals and other vertebrates, such as birds and reptiles. Most *E. coli* does not cause illnesses. However, there are few types that can cause infections in animals and people.

These types of *E. coli* that cause diseases are classified based on the patterns of attachment on recipient, the types toxins produced, and invasiveness.

1. Enterotoxigenic (ETEC)

Enterotoxigenic *E. coli,* known as Cholera-like, is the most common cause of *E. coli* diarrhea in farm animals. This type of *E. coli* is characterized by the production of enterotoxins. The enterotoxin that is produced may be a heat stable (100 °C for 15 min), or heat labile (60 °C for 15 min). Also, it does not cause histological changes or invade the enterocytes. The attachment is mediated by pili.

2. Attaching and Effacing (AEEC), Enterohemorrhagic (EHEC), Shiga toxigenic *E. coli* (STEC), or Verotoxigenic (VTEC)

Biologically and structurally, the toxin produced by this type resembles the cytotoxin of *Shigella dysenteriae,* which is why they are called Shiga toxin. The type also invades the tissues. The toxins are heat labile and lethal for cultured Vero cells, which is why they are called Verotoxins. They are chemically proteins and have A and B subunits. The subunit A has cytotoxic activity, and B subunit has the binding function. There are two types of shiga toxins. Shiga toxin 1 is identical to shiga toxin of *Shigella dysenteriae,* and shiga toxin 2 has 56% homology to shiga toxin 1.

3. Enteroinvasive (EIEC)

This type is referred to as *Salmonella*-like. It invades the tissues and destroys the cells, and may be able to multiply inside living cells (facultative intracellular pathogen). It causes bacteremia or septicemia, mostly seen in poultry. The strains are rarely found in mammals.

4. Enteropathogenic (EPEC)

This type causes diarrhea in many animals, most often in pigs, rabbits, and dogs. The attachment is mediated by pili and it causes loss of microvilli (effacement). It dose not produce Shiga toxins (verotoxins) or enterotoxins. The main virulence factors are pili and cytotoxin.

5. Enteroaggregative (EAggEC)

This group tends to clump in small aggregates, both *in vitro* and *in vivo*. The clumping is because of thin fibrillar structures, pili. They do not invade the small intestinal cells, but they bind to them. They cause a persistent form of diarrhea in children. This type produces ST-like toxin called EAST (heat stable enterotoxin for Enteroaggregative) and hemolysin-like toxin.

Norepinephrine and *E. coli*

There are several highly adapted *E. coli* strains that have acquired specific virulence attributes, which confer an increased ability to adapt to new niches and allow them to cause a variety of diseases (Stins *et al.,* 1999). The prevalence of bacteria resistant to antimicrobial compounds within animal and human population complicates infection control (Stins *et al.,* 1999). The growth-stimulating effect of catecholamines was assumed to be the result of increased iron acquisition and consumption through catecholate-specific iron carrying system with participation of enterobactin and enterochelin ways (Bearson *et al.,* 2008). The growth and virulence of many Gram-negative and Gram-positive bacteria has been stimulated by catecholamines. Bacteria respond to the increase of concentrations of stress hormones by increasing the growth and promoting the possibility to cause disease, which leads to an increased transmission to a new healthy host (Freestone *et al.*, 2008). Studies have been confirmed that

catecholamines can have an effects on the production of virulence factors like adhesins, toxins, biofilm development, and quorum sensing, even in iron-replete circumstances (Lyte and Ernst, 1992; Sperandio *et al.,* 2002). Dowd *et al.,* (2007) have reported that *Escherichia coli* O157 responds to catecholamines by increasing the expression of shiga toxins. On the small intestinal epithelium, norepinephrine increases the growth of enterotoxigenic *E. coli*, and its expression of F5 fimbrial adhesin, which mediates the attachment of the bacterium to epithelial receptors (Lyte et al., 1997; Nagy and Fekete, 2005). Peterson *et al.,* (2011) have reported that norepinephrine at normal concentrations increased the horizontal gene transfer efficiency of a conjugative plasmid from *Salmonella* Typhimurium to *Escherichia coli in vitro.* Also, they observed an upregulation of the expressions of plasmid encoded transfer genes, which are necessary for conjugation in the presence of NE.

Effects of Norepinephrine on Conjugation in E. coli

The pathogenic bacteria have the ability to sense as well as respond to the stress in the host. The epinephrine and norepinephrine play a key role in stress situations in animals. The horizontal gene transfer is an important mechanism that contributes to bacterial diversity. Transformation is one mechanism that involves the uptake of the free DNA from the environment and has the potential to transmit DNA between far related organisms (Chen and Dubnau, 2004). The genetic material also can be introduced into a bacterium by a bacteriophage that has replicated into the donor organism and packaged random DNA fragments, or the DNA adjacent to the phage attachment site (Ochman *et al.,* 2000). Conjugation is mechanism that involves physical contact between donor and recipient cells and can mediate the transfer of genetic material between microorganisms (Ochman *et al.,* 2000). Peterson *et al.,* (2011) have

shown that NE at physiological concentrations enhanced horizontal gene transfer efficiencies of a conjugative plasmid from a strain of *Salmonella* Typhimurium to an *E. coli in vitro.*

Objective of the Experiment

The primary objective was to determine the effect of norepinephrine on conjugation between two *E. coli* strains. The effects of norepinephrine were tested at two concentrations, 5 mM and 20 mM. *Escherichia coli* FS1290 was as the donor and strain C600N was the recipient in the conjugation assay. Both filter mating and liquid mating assays were performed.

Materials and Methods

Bacterial strains and culture medium: The bacterial strain used in this experiment was *E. coli* FS1290 with a conjugative self-transmissible plasmid. This plasmid is 64-kb in size and carries genes that confer resistance to ampicillin, tetracycline, streptomycin and kanamycin (Table 2.1). The recipient *E. coli* C600N has a chromosomal mutation that confers resistance to nalidixic acid (Table 2.2). Strains were grown at 37° C in Luria Bertani broth (LB; Franklin Lakes, NJ. USA).

Norepinephrine medium. Stock solution (50 mg/ml in 0.5 M HCl) of NE (Sigma-Aldrich, St Louis, MO) was prepared. Further dilutions to achieve 5 mM and 20 mM of NE were performed in LB broth and mixed with media for plating. All procedures (preparation of the solution, dilution, and plating) were done in the dark.

Table 2.1 ***Escherichia coli*** **FS1290 genotype**

Mutation	Location	Certainty	Display
Lac-3350	7.77	1	*lac-3350*
galK2(Oc)	16.99	1	*galK2(Oc)*
galT22	17.01	1	*galT22*
LAM-	17.40	1	λ^-
IN(rrnD-rrnE)1	73.74	1	*IN(rnnD-rnnE)1*
rpsL179(strR)	74.84	1	*rpsL179(strR)*
DeoA21	99.50	1	*deoA21*

The resource of these data is Coil Genetic Stock Center, available on http://cgsc.biology.yale.edu/Strain.php?ID=122766

Table 2.2 ***Escherichia coli*** **C600N genotype**

Mutation	Location	Certainty	Display
Thr-1	0.01	1	*thr-1*
leuB6(Am)	1.74	1	*leuB6*(Am)
fhuA21	3.61	1	*fhuA21*
Cyn-101	7.72	0	*cyn-101*
LacY1	7.79	1	*lacY1*
glnV44(AS)	14.99	1	*glnV44*(AS)
LAM-	17.40	1	λ^-
rfbC1	45.43	1	*rfbC1*
GlpR200(glp^c)	76.68	1	*glpR200(glp^c)*
thiE1	90.34	1	*thiE1*

The resource of these data is Coil Genetic Stock Center, available on http://cgsc.biology.yale.edu/Strain.php?ID=11195

Conjugation by filter mating

Strains FS1290 and C600N were streaked on Luria Bertani (LB) agar plates containing appropriate antibiotics and individual colonies were picked to check for purity. Strain FS1290 was grown on LB agar containing ampicillin at 50 µg/mL and strain C600N was grown on LB

agar containing 12.5 μg/mL of nalidixic acid. Strains were inoculated separately in to tubes of LB broth with 50 μg/mL ampicillin for FS1290 or 12.5 μg/mL nalidixic acid for C600N and incubated at 37° C in shaking incubator overnight (16 hours). Five mL LB broth in a 15 mL-tube was inoculated with FS1290 (donor) by using a sterile loop. In another 15-mL tube, 5 mL of LB broth was inoculated with *E. coli* C600N (recipient). Broths were incubated at 37° C in a shaking incubator overnight (16 hours). Overnight cultures were diluted in pre-warmed LB broth (1 mL overnight culture + 5 mL pre-warmed LB broth) and incubated for 1.5 hours until they reached a turbidity of 0.3 at 600 nm (Spectronic $20D^+$ Milton Roy, Warminster, PA). One mL of donor culture and 5 mL of recipient culture were run through a filter-mating unit (Millipore Filter Corp) and the filter was placed on LB agar plate containing 20 mM NE. Another filter containing mixture was placed on LB agar plate without NE (control) and incubated for 16 hours (Table 2.3)

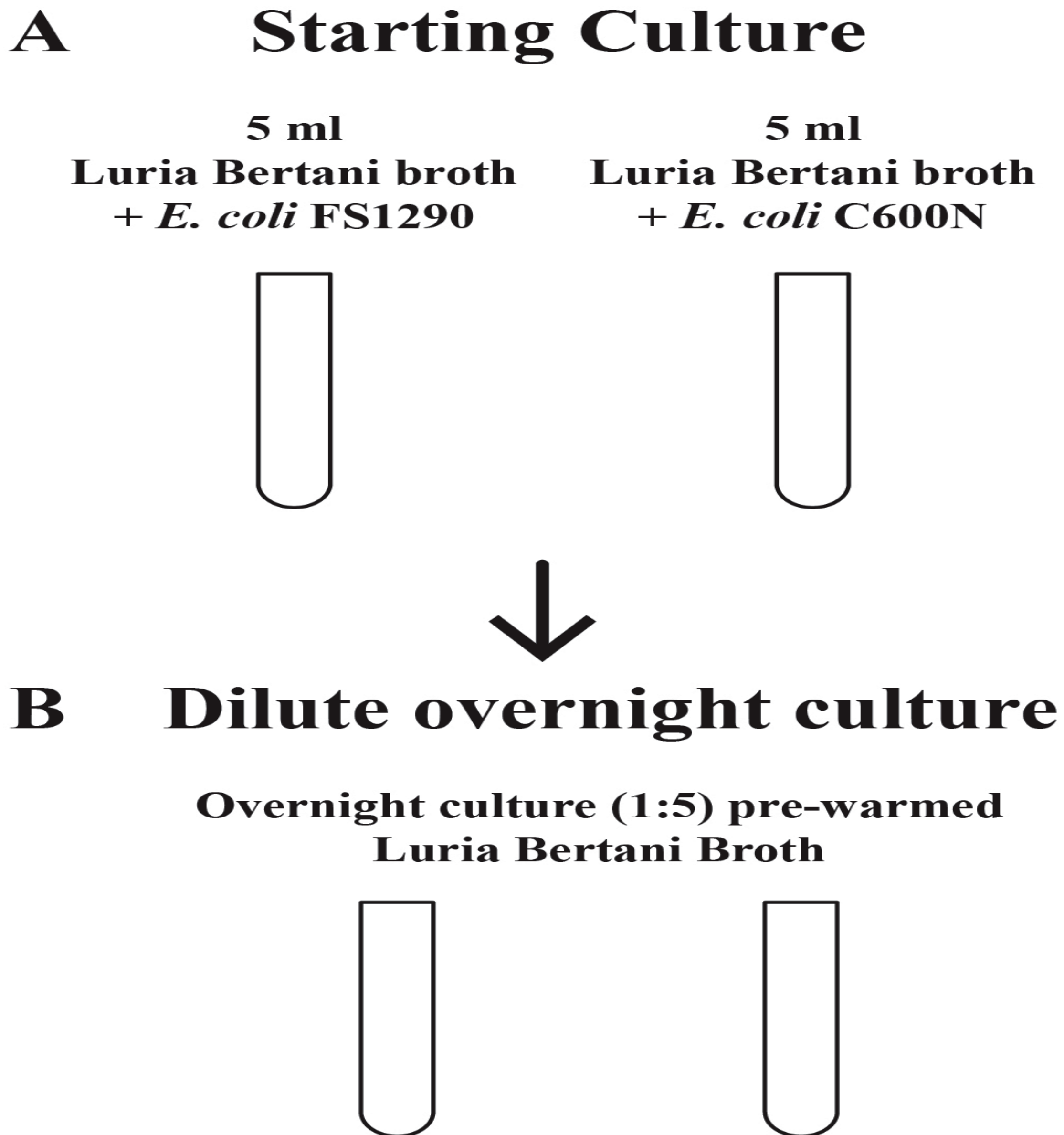
Serial dilutions. Each membrane was taken from the plates and placed in 50 ml tube containing 5 mL LB broth and vortexed. Ten-fold dilutions were carried out in LB broth by mixing 100 μL from the culture + 900 μL of plain LB. Fifty μL of each dilution (10^{-1} – 10^{-10}) were plated on LB agar with selected antibiotics; LB agar + tetracycline (10 μg/mL), or LB agar + streptomycin (50 μg/mL) for FS1290, LB agar + nalidixic acid (12.5 μg/mL) for C600N, and LB agar containing tetracycline (10 μg/mL) + nalidixic acid (12.5 μg/mL) for transconjugant and incubated overnight (figure 2.2).

Counting the colonies. After the incubation period hours, plates that had colonies between 20 and 200 were counted to determine conjugation ratio (transconjugant/donor, and transconjugant/recipient).

Conjugation by liquid mating

Overnight cultures and media preparations were same as described before. The liquid mating protocol was the donor + recipient were diluted at 1:5 ratio and incubated in liquid medium for 4, 6, 8, or 16 hours (Table 2.4)

Figure 2.2 Illustration of the experiment sequence



C

Incubation with and without norepinephrine (NE) for conjugation

Liquid mating with 5 mM or 20 mM concentration of NE for 4, 6, 8, and 16 hours

Plain Luria Bertani broth (Control) | **NE**

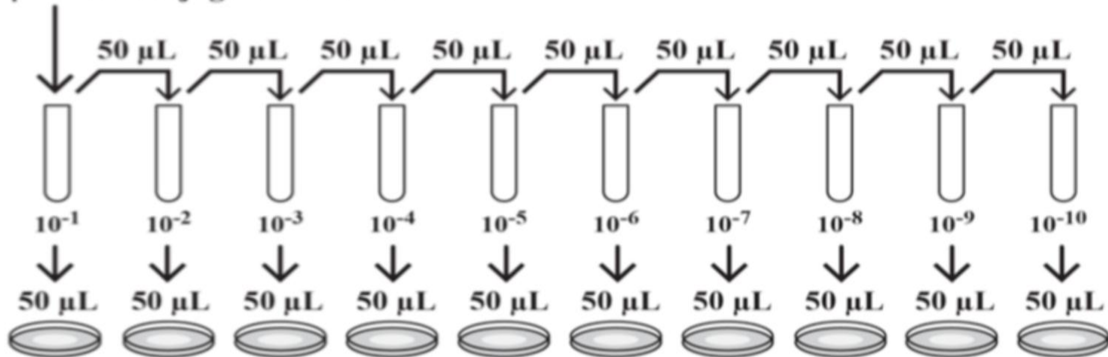
Filter mating with 20 mM concentration of NE for 4 hours

Plain Luria Bertani agar (Control) | **NE**

D

Serial Dilution

50 µL from conjugation culture



E

Incubation Overnight

Tetracycline (10 µg/mL)

Donor *E. coli* FS1290

Streptomycin (50 µg/mL)

Donor *E. coli* FS1290

Nalidixic Acid (12.5 µg/mL)

Recipient *E. coli* C600N

Tetracycline (10 µg/mL) + Nalidixic Acid (12.5 µg/mL)

Trans conjugant

Polymerase chain reaction (PCR): A PCR assay was carried out to determine if the plasmid was transferred from FS1290 (donor) to the transconjugant. Ten randomly picked transconjugant from LB agar plates containing tetracycline and nalidixic acid were tested. The primers that were used in the PCR assay were: MA-ApRF and the sequence was 5-TTG CCG GGA AGC TAG AGT AA -3, the other primer was MA-ApRR and the sequence was 5-GCT ATG AGG CGC GGT ATT AT -3. The primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA).

Statistics. Graph Pad prism 5.03 was used to analyze the data. One-way ANOVA with posttest using Dunns multiple comparison tests were, used for all analysis.

Results

Filter mating assay

Effect of 5 mM NE on conjugation. This experiment was done one time, and in the absence of NE, the count of the donor was 1.5 x 10^8 CFU/mL, and the count of the recipient was 3.4 x 10^8 CFU/mL, and count of the transconjugant was 1.9 x 10^8 CFU/mL. In the presence of NE, the donor count was 6.4 x 10^8 CFU/mL with count of recipient 7.8 x 10^8 CFU/mL, and transconjugant was 7.0 x 10^8 CFU/mL (Figure 2.3).

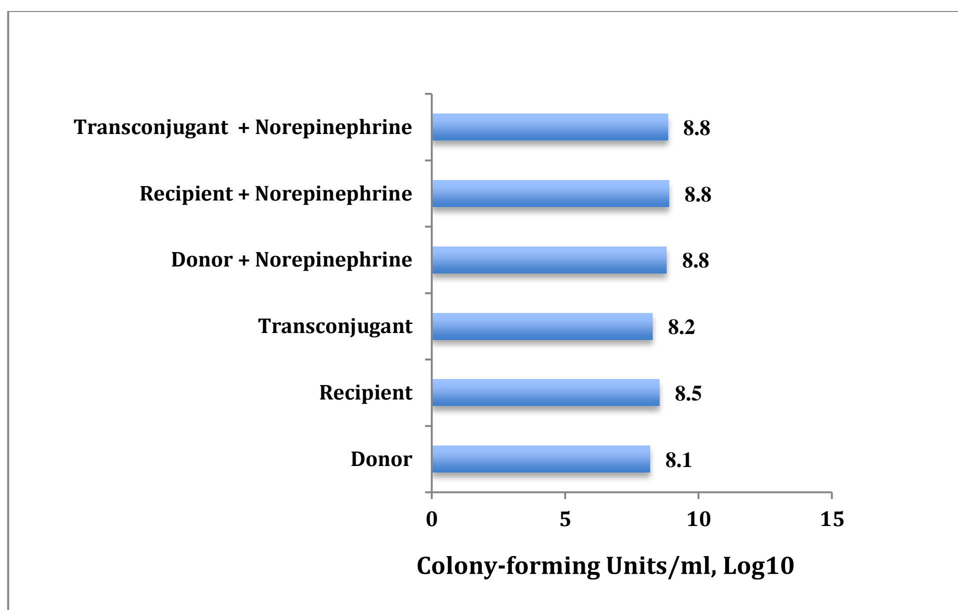


Figure 2.3 Effect of 5 mM norepinephrine on conjugation in filter mating assay with 4 hours of incubation

Effect of 20 mM NE on conjugation. This experiment was done three times. Results from individual experiments and the mean are presented (Figures 2.4, 2.5, 2.6 and 2.7). In the absence of NE, the mean count of the donor was 2.7 x 10^8 CFU/mL (Log 8.4 CFU/mL), and the mean count of the recipient was 4.0 x 10^8 CFU/mL (Log 8.6 CFU/mL), and mean count of the transconjugant was 2.6 x 10^7 CFU/mL (Log 7.4 CFU/mL). In the presence of NE, the average of donor was 4.0 x 10^9 CFU/mL (Log 9.6 CFU/mL), with average of recipient 5.2 x 10^9 CFU/mL (Log 9.7 CFU/mL),, and transconjugant was 5 x 10^7 CFU/mL (Log 7.6 CFU/mL). The *P*-value for donor vs donor +NE was 0.73. The *P*-value for recipient vs recipient + NE was 0.82 The *P*-value of transconjugant vs transconjugant + NE was 0.87.

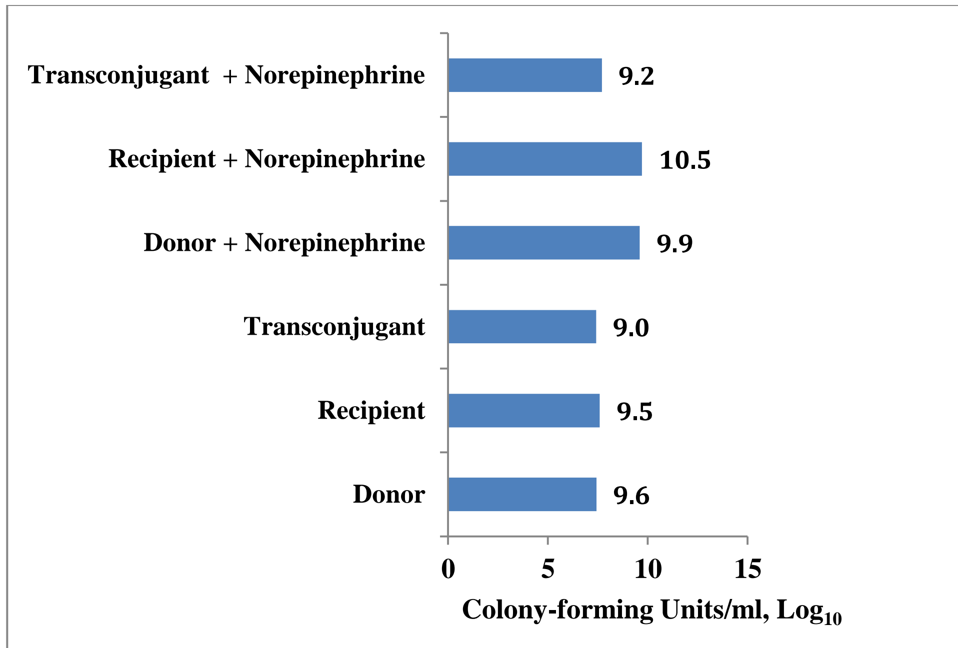


Figure 2.4 Effect of 20 mM norepinephrine on conjugation in filter mating assay with 4 hours of incubation (Expt.1)

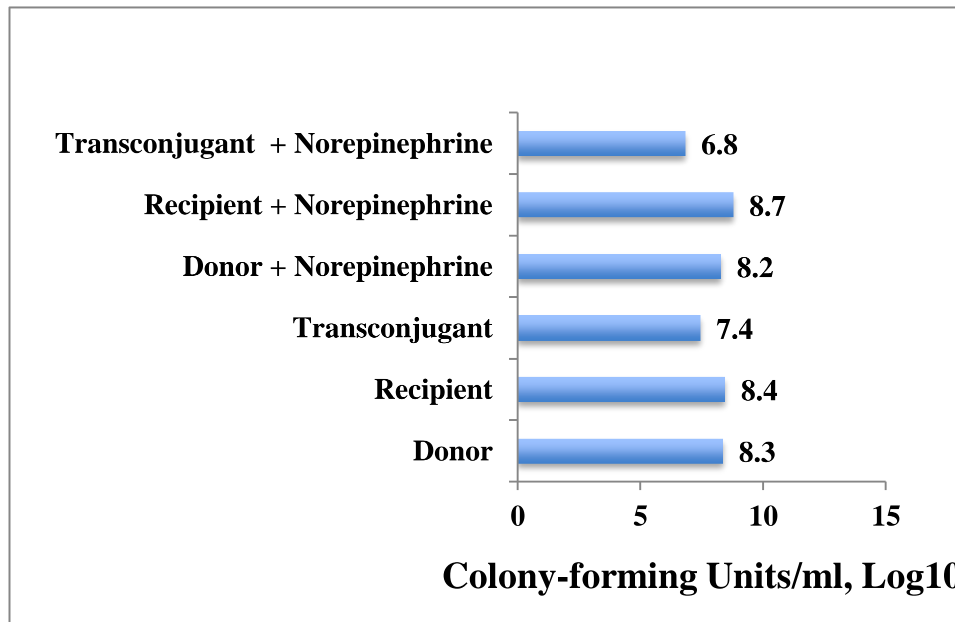


Figure 2.5 Effect of 20 mM norepinephrine on conjugation in filter mating assay with 4 hours of incubation (Expt.2)

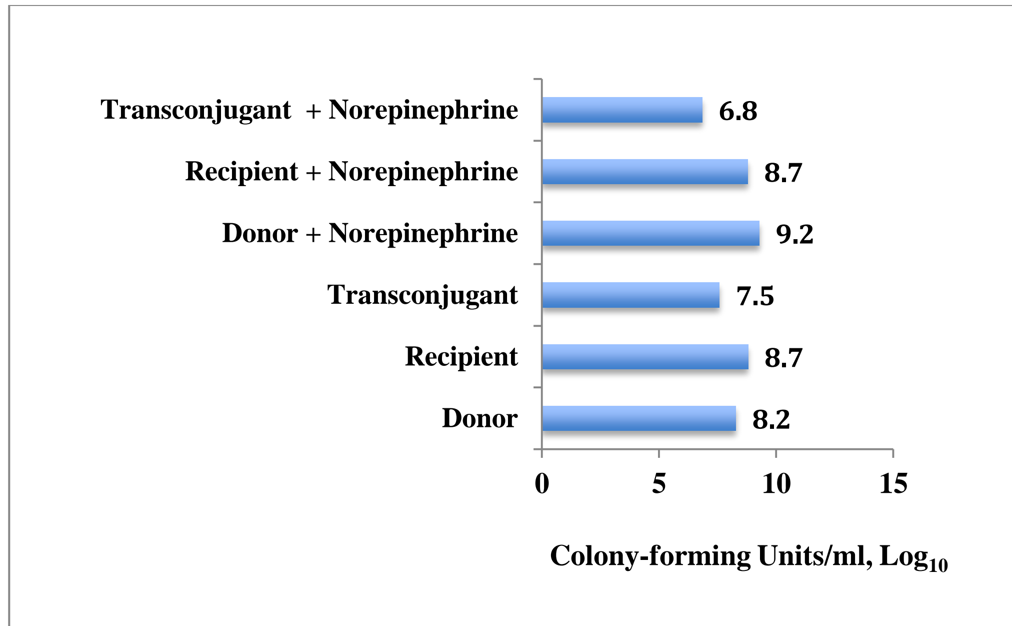


Figure 2.6 Effect of 20 mM norepinephrine on conjugation in filter mating assay with 4 hours of incubation (Expt. 3)

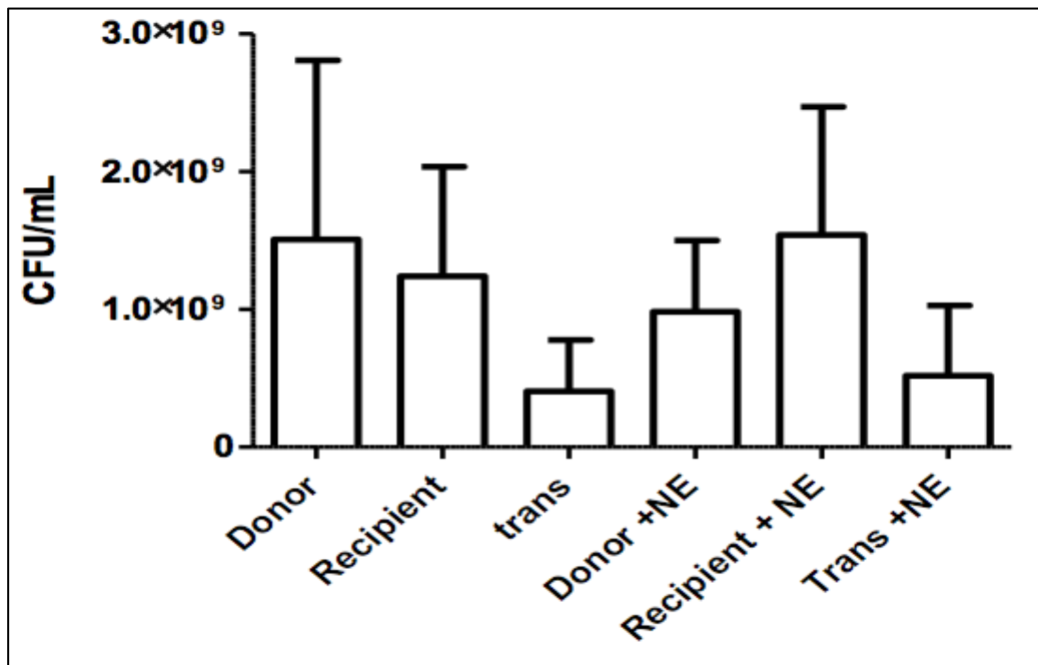


Figure 2.7 Effect of 20 mM norepinephrine on conjugation in filter mating assay with 4 hours of incubation (mean of three experiment)

Liquid mating assay

Effect of 5 mM NE on conjugation with 4 hours incubation. In the absence of NE, the mean count of donor was 3.6 x 10^8 CFU/mL (Log 8.7 CFU/mL), and the mean count of the recipient 4.4 x10^8 CFU/mL (Log 8.0 CFU/mL), but no growth for transconjugant. In the presence of NE, the mean count of donor was 4.2 x 10^7 CFU/mL (Log 8.6 CFU/mL), and the mean count of recipient was 2.6 x 10^8 CFU/mL (Log 8.1 CFU/mL), and the mean count of transconjugant was 5.2 x 10^4 CFU/mL (Log 4.7 CFU/mL). The *P*-value for donor vs donor +NE was $P = 0.9$ which is more than $P = 0.05$ so there is no significant difference. *P*-value for recipient vs recipient +NE was $P = 1.0$ which was more than $P = 0.05$, so there was no significant difference. The *P*-value of transconjugant vs transconjugant +NE was 0.51 which is also more the $P = 0.05$ and there was no significant difference (Figures 2.8, 2.9, 2.10 and 2.11).

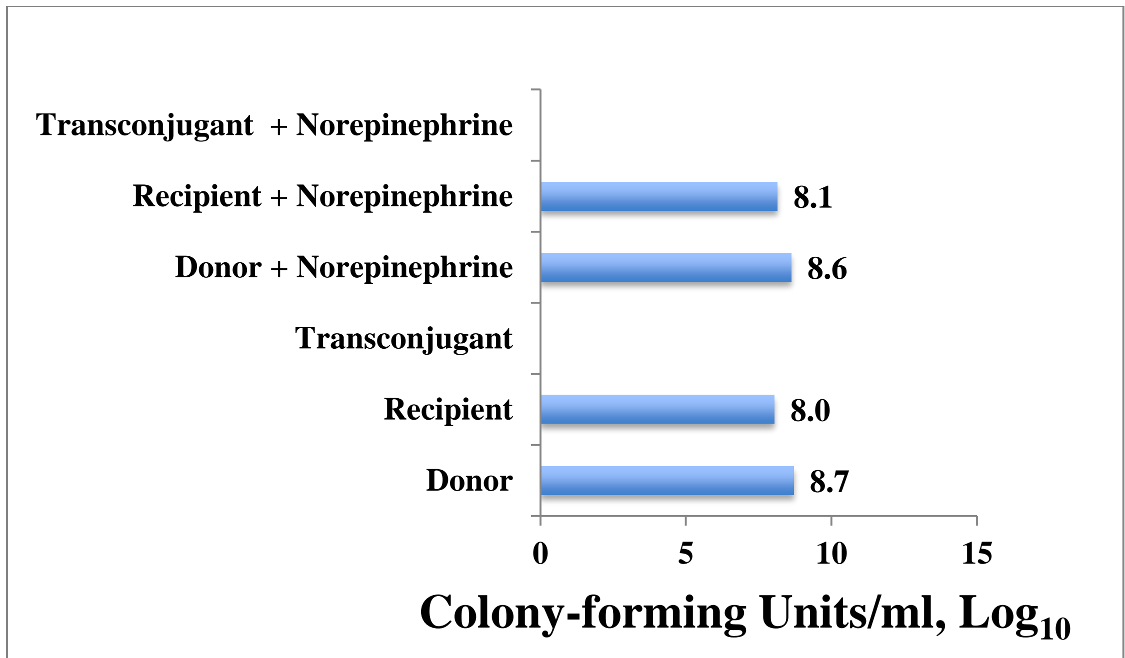


Figure 2.8 Effect of 5 mM norepinephrine on conjugation in liquid mating assay with 4 hours of incubation

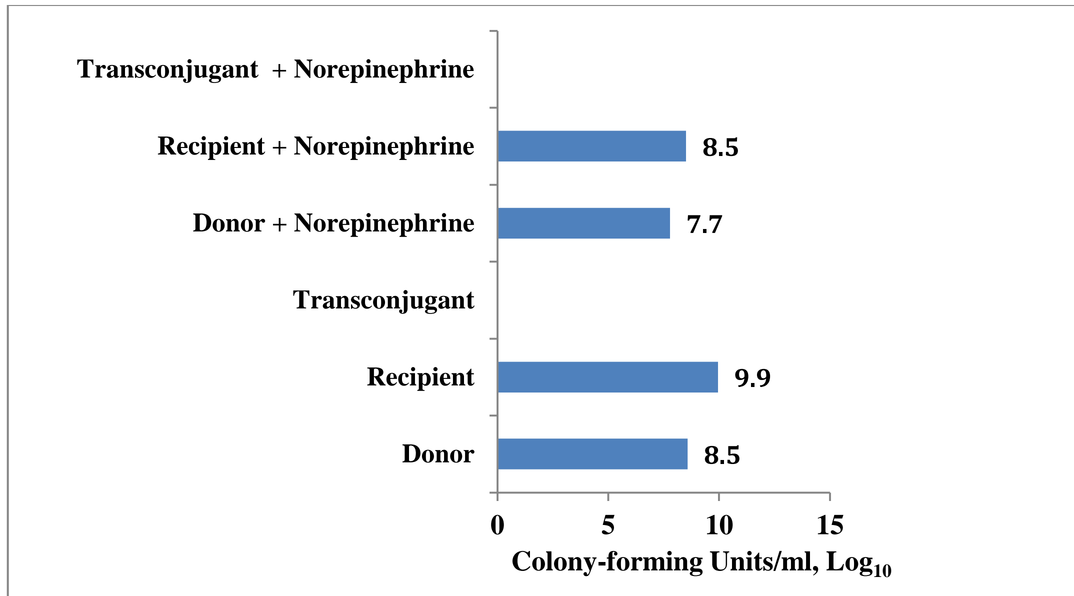


Figure 2.9 Effect of 5 mM norepinephrine on conjugation in liquid mating assay with 4 hours of incubation

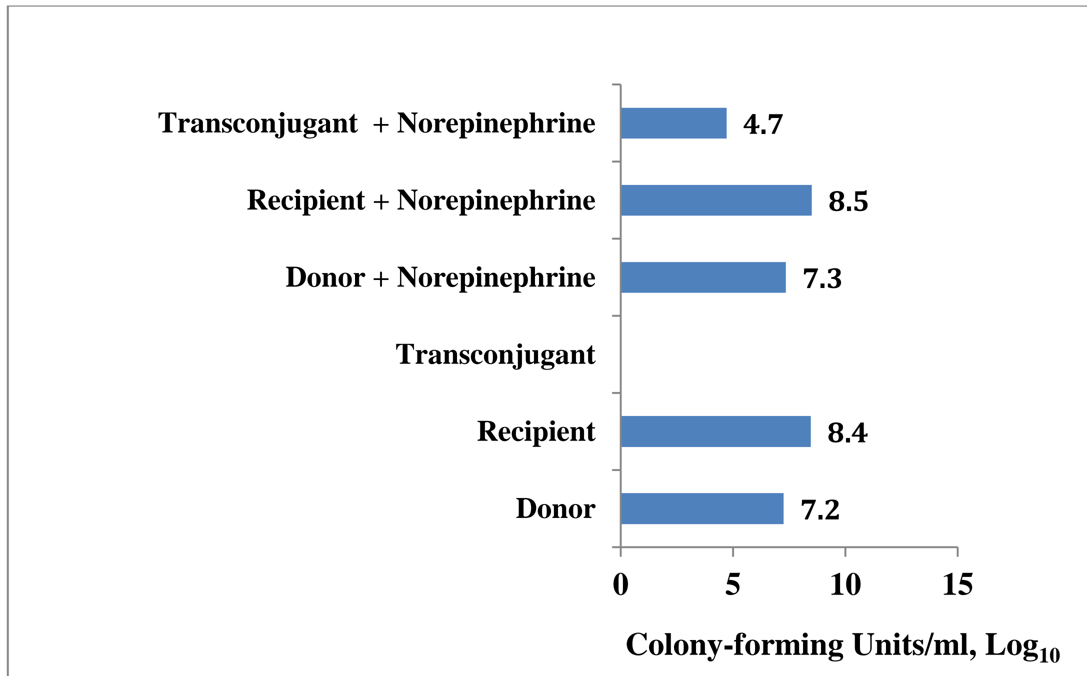


Figure 2.10 Effect of 5 mM norepinephrine on conjugation in liquid mating assay with 4 hours of incubation

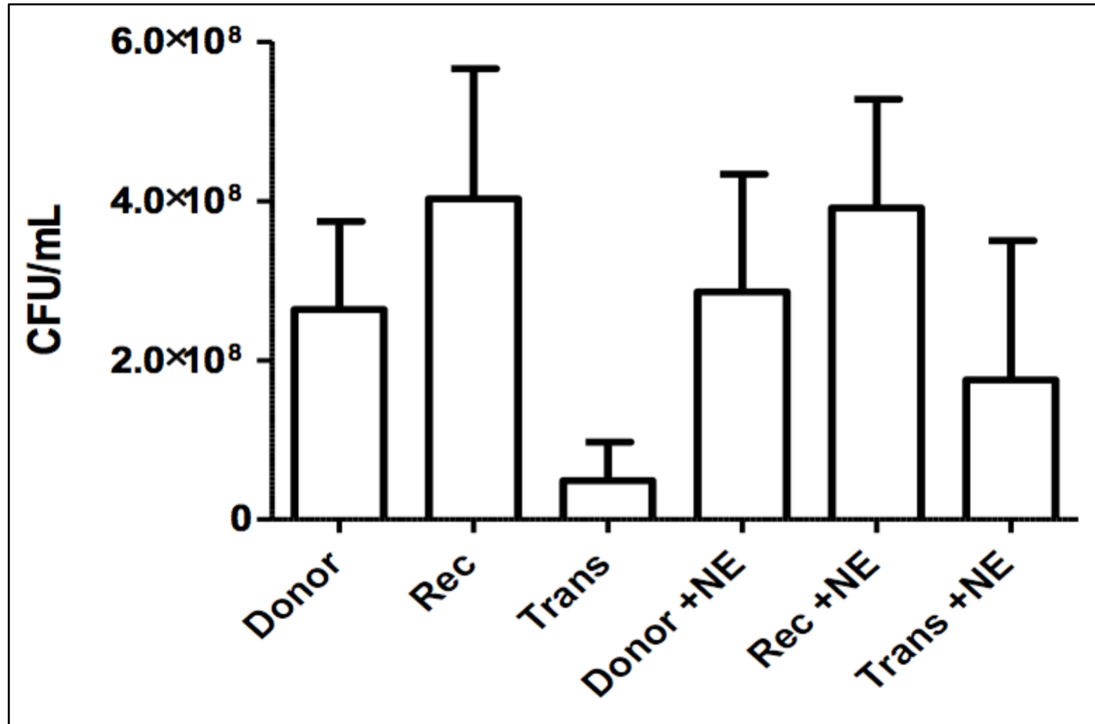


Figure 2.11 Effect of 5 mM norepinephrine on conjugation by liquid mating assay with 4 hours of incubation (Mean of three experiments)

Effects of 5 mM NE in 6, 8, and 16 hours of incubation on conjugation. In the absence of NE, the mean count of the donor was 5.5 x 10^7 CFU/mL, and the mean count of recipient 2.9 x10^7 CFU/mL, and mean count of transconjugant 2.8 x 10^5 CFU/mL. In the presence of NE, the mean count of donor was 5.6 x 10^7CFU/mL, and mean count of recipient 5.1 x 10^7 CFU/mL, and mean count of transconjugant 5.3 x 10^4 CFU/mL. The *P*-value for donor vs donor +NE was P = 0.50 which is more than P = 0.05 there is no significant difference. The *P*-value for recipient vs recipient +NE was P = 0.50, also it was more than P = 0.05, so there was no significant difference. The *P*-value of transconjugant vs transconjugant +NE *P*-value was 0.67 which is also was more the P = 0.05 and there was no significant difference (Figures 2.12, 2.13 and 2.14).

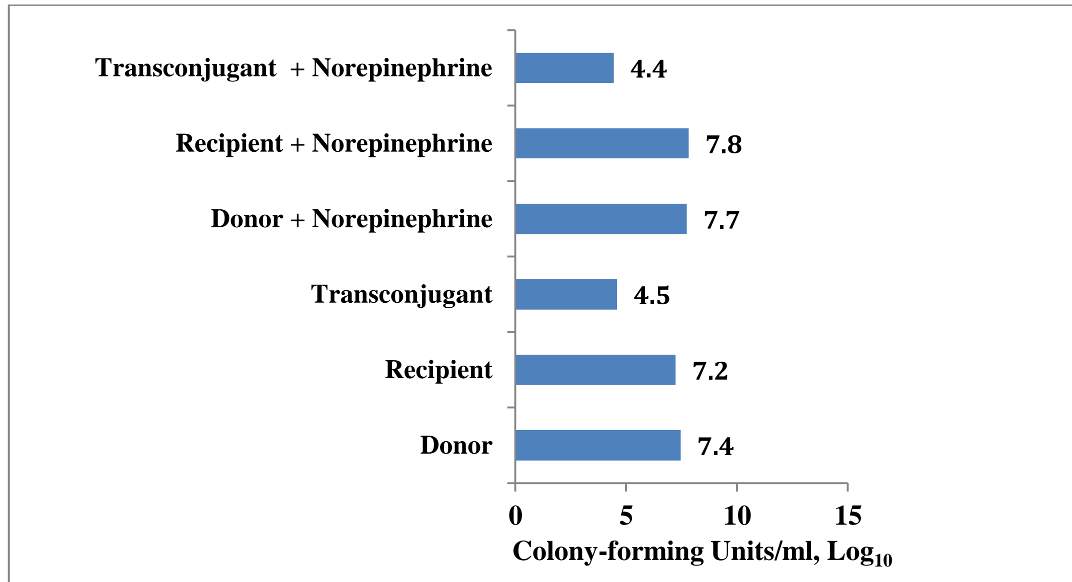


Figure 2.12 Effect of 5 mM norepinephrine on conjugation in liquid mating assay with 6 hours of incubation

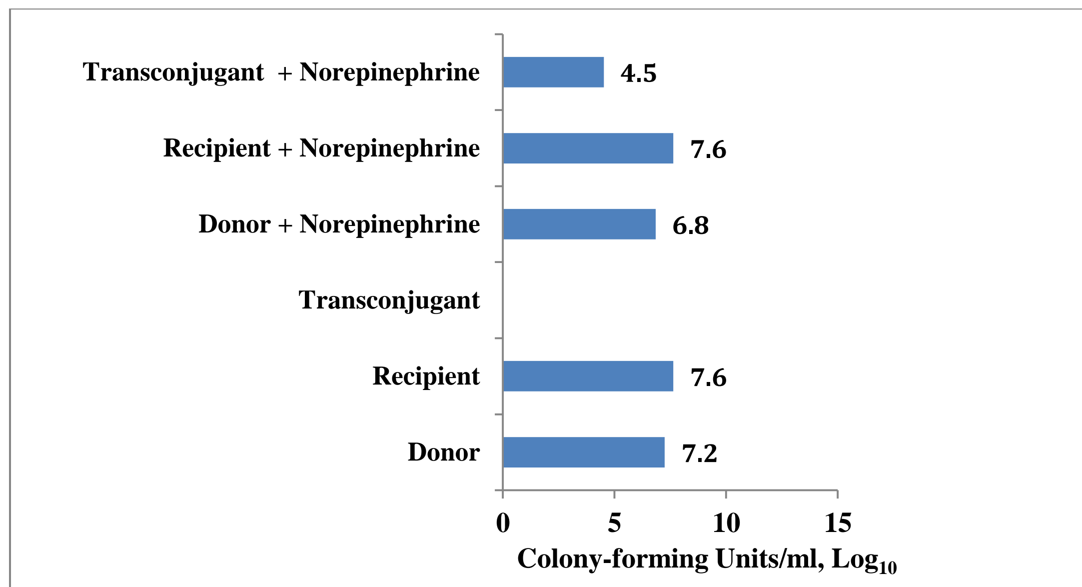


Figure 2.13 Effect of 5 mM norepinephrine on conjugation in liquid mating assay with 8 hours of incubation

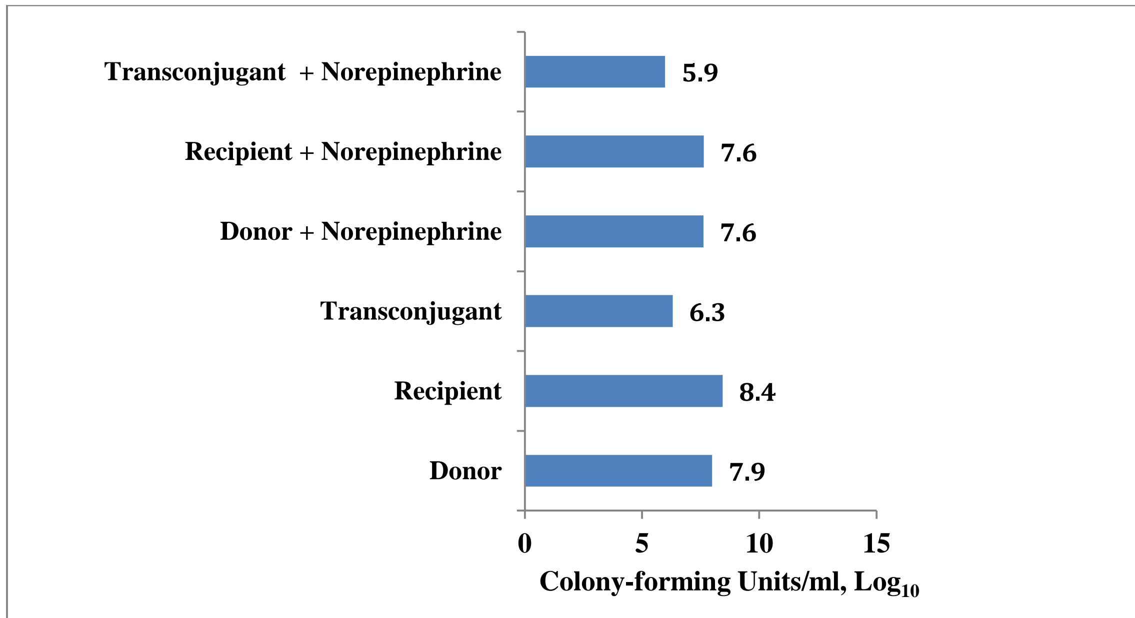


Figure 2.14 Effect of 5 mM norepinephrine on conjugation in liquid mating assay with 6 hours of incubation

Effect of 20 mM NE on conjugation incubation in liquid mating assay with 4 or 6 hours of incubation. In the absence of NE and the mean count of donor 6.8 x 10^{6} CFU/ml, and the mean count of recipient 7.1 x 10^{7} CFU/ml, and mean count of transconjugant 4.5 x 10^{4}CFU/ml. In the present of NE the mean count of donor 3.2 x 10^{7}CFU/ml, and the mean count of recipient 2.2 x 10^{7} CFU/ml, and mean count of transconjugant 5.6 x 10^{4} CFU/ml. The *P*-value for donor vs donor +NE was $P = 0.81$ which is more than $P = 0.05$ there is no significant difference. *P*-value for recipient vs recipient +NE was $P = 0.05$ which was less than $P = 0.05$, so there was a significant difference. The *P*-value of transconjugant vs transconjugant +NE *P*-value was 0.7719 which was more the $P = 0.05$ and there was no significant difference (Figures 2.15 and 2.16).

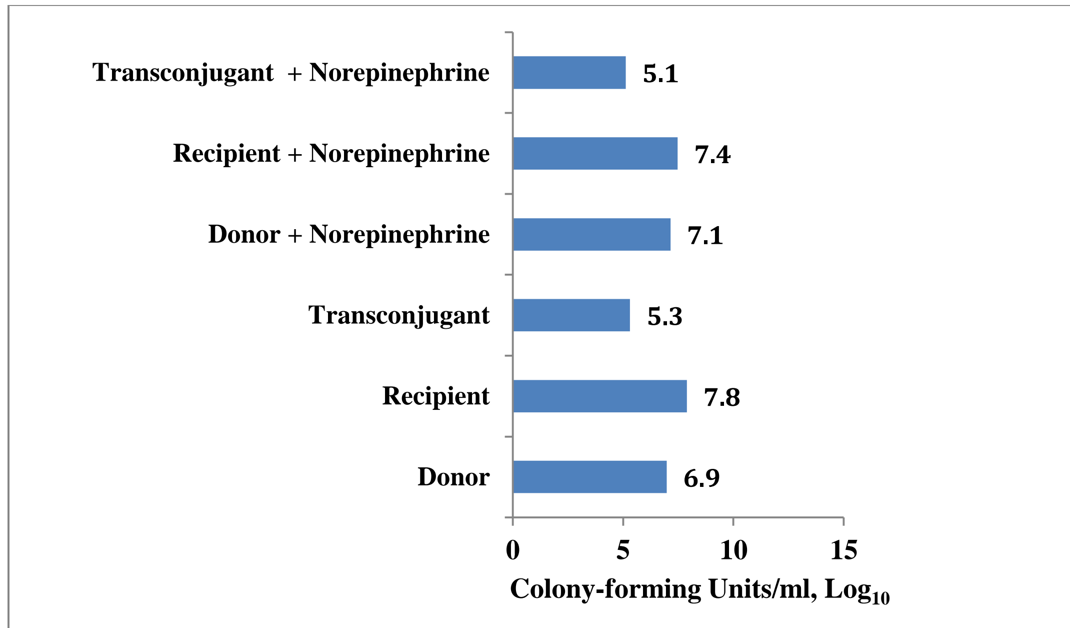


Figure 2.15 Effect of 20 mM norepinephrine on conjugation in liquid mating assay with 4 hours of incubation

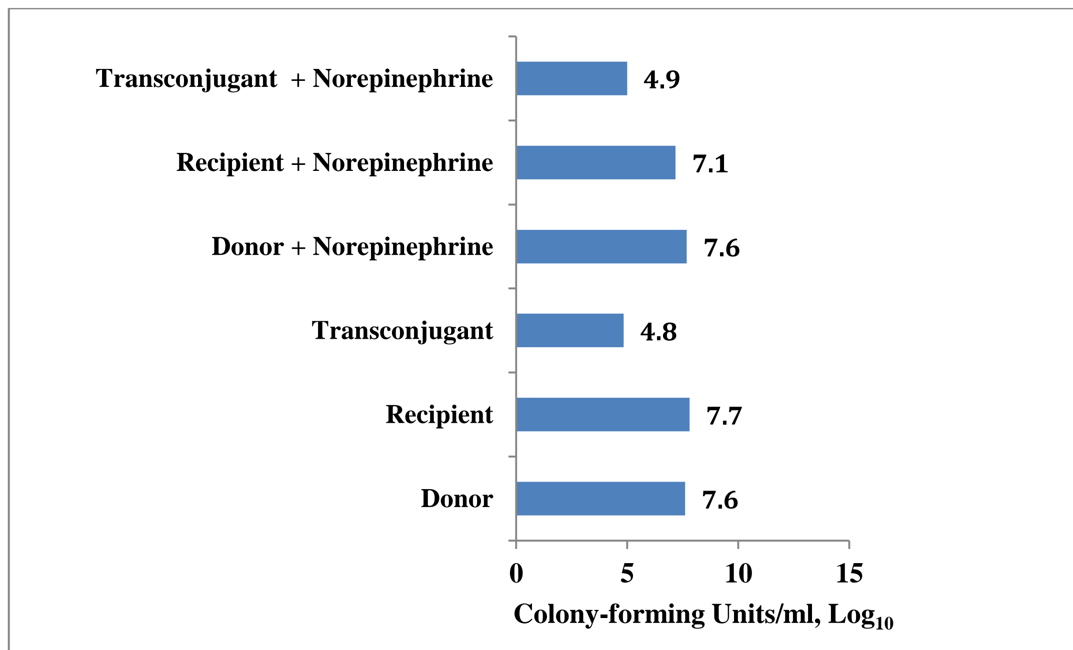


Figure 2.16 Effect of 20 mM norepinephrine on conjugation in liquid mating assay with 6 hours of incubation

Polymerase Chain Reaction

The PCR assay showed that donor and transconjugant had the band, but there was no band with the recipient and the control (Figure 2.17)

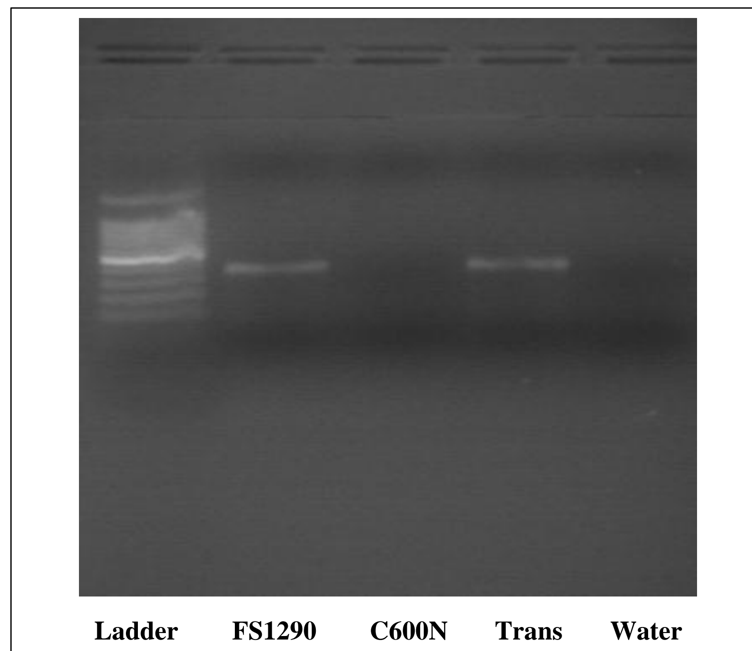


Figure 2.17 Gel image of the ampicillin resistant gene present in *Escherichia coli* strain FS1290 and the transconjugant and absent in *E. coli* strainC600N

Conclusion

In conclusion, norepinephrine did not have any significant effect on conjugation efficiency of plasmid between *E. coli* FS1290 and *E. coli* C600N. Although, we did not use NE at many concentrations, we observed that NE at 5 mM and 20 mM concentration did not have any significant effect either in filter mating or liquid mating on conjugation. The transconjugant ratio is very high in our experiment making it difficult to evaluate augmentation of conjugation by

NE. Therefore, a plasmid system with lower efficiency of transfer may be employed in further studies. The various incubation periods 4, 6, 8, 16 did not have any effect on efficiency of conjugation. Further research is needed to elucidate more on the effects of norepinephrine on conjugation. Increasing the NE concentration more than 5 mM and 20 mM may improve the efficiency of conjugation.

Table 2.3 Effects of norepinephrine on *Escherichia coli* conjugation determined by filter mating assay.

		No Norepinephrine			With Norepinephrine		
Experiment	**Incubation period**	**Donor**	**Recipient**	**Transconjugant**	**Donor**	**Recipient**	**Transconjugant**
Norepinephrine, 5 mM							
Experiment 1	4 hours	1.5×10^8	3.4×10^8	1.9×10^8	6.4×10^8	7.8×10^8	7.0×10^8
Norepinephrine, 20 mM							
Experiment 1	4 hours	4.0×10^9	2.9×10^9	1.2×10^9	8.1×10^9	3.4×10^{10}	1.5×10^9
Experiment 2	4 hours	2.3×10^8	2.8×10^8	2.8×10^7	1.9×10^8	6.1×10^8	6.7×10^6
Experiment 3	4 hours	1.9×10^8	6.3×10^8	3.8×10^7	1.9×10^9	6.1×10^8	7.0×10^6
Mean	4 hours	2.7×10^8	4.0×10^8	2.6×10^7	4.0×10^9	$5.2 \times 1\ 0^9$	5.0×10^7

Table 2.4 Effects of norepinephrine on *Escherichia coli* conjugation determined by liquid mating assay

		No Norepinephrine			With Norepinephrine		
Experiment	**Incubation period**	**Donor**	**Recipient**	**Transconjugant**	**Donor**	**Recipient**	**Transconjugant**
Norepinephrine, 5 mM							
Experiment 1	4 hours	5.1×10^8	1.1×10^8	N/A	4.2×10^8	1.4×10^8	N/A
Experiment 2	4 hours	3.8×10^8	8.7×10^8	N/A	6.0×10^7	3.2×10^8	N/A
Experiment 3	4 hours	1.8×10^8	2.9×10^8	N/A	2.2×10^7	3.2×10^8	5.2×10^4
Mean	4 hours	3.6×10^8	4.4×10^8	N/A	4.2×10^7	2.6×10^8	5.2×10^4
Norepinephrine, 5 mM							
Experiment 1	6 hours	2.9×10^7	1.7×10^7	3.6×10^4	5.5×10^7	6.7×10^7	2.8×10^4
Experiment 2	8 hours	3.6×10^7	4.4×10^7	N/A	6.9×10^6	4.3×10^7	3.4×10^4
Experiment 3	16 hours	9.9×10^7	2.7×10^8	2.0×10^6	4.1×10^7	4.3×10^7	9.7×10^5
Norepinephrine, 20 mM							
Experiment 1	4 hours	9.6×10^6	7.8×10^7	2.1×10^5	1.4×10^7	2.9×10^7	1.3×10^5
Experiment 2	6 hours	4.0×10^7	6.3×10^7	6.9×10^4	4.8×10^7	1.5×10^7	9.8×10^4

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