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Dihydrofolate Reductase (*dfr*) and Dihydropteroate Synthase (*sul*) Gene Mutations in *Escherichia coli* Trimethoprim-Sulfamethoxazole Resistance

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ABSTRACT

Escherichia coli is a rod-shaped bacterium with Gram-negative properties that colonizes the human digestive tract. Generally, *E. coli* is harmless, but can become pathogenic if it obtains additional virulence genes from other microorganisms through the mechanism of gene transfer (transformation), transfer by pili or pilus (conjugation), or gene transfer through bacteriophages (transduction). This pathogenic *E.coli bacteria* can cause enteric diseases such as diarrhea, urinary tract infections, and sepsis or meningitis.One of the empirical therapies in dealing with *E. coli* infection is the antibiotic trimethoprim-sulfamethoxazole. This antibiotics work by inhibiting the enzymatic reaction of bacterial folate synthesis at two successive stages in bacteria, so that this drug combination can provide a synergistic effect. Both combinations of antibiotics are reported to inhibit the bacterial synthesis of tetrahydrofolic acid, which is an important cofactor for the synthesis of thymidine and purines, the basic bases of DNA and RNA. Sulfonamides (sulfamethoxazole) are analogs of para-aminobenzoic acid (PABA) and compete with PABA to bind dihydropteroate synthetase (DHPS), thereby inhibiting dihydrofolate acid synthesis. Trimethoprim binds to dihydrofolate reductase (DHFR), thereby blocking the conversion of dihydrofolic acid to tetrahydrofolic acid. The mechanism of acquired resistance has often been identified, mainly due to mutational modifications in the genes that code for target enzymes. This can occur if there is resistance to the acquisition of the *sul* gene that codes for *dihydropteroate synthetase* so that it is not sensitive to sulfonamides, or it can occur in the *dfr* gene that codes for dihydrofolate reductase that is not sensitive to trimethoprim.

Keywords: *Escherichia coli*, resistensi, trimethoprim-sulfamethoxazole, *dfr* and *sul* gene

INTRODUCTION

Escherichia coli is a Gram-negative coliform bacterium that is cylindrical (rod), has flagella and is classified as facultative anaerobic bacteria [1]. *E. coli* bacteria belong to the family Enterobacteriaceae that can live colonizing the human digestive tract [2]. *Escherichia coli is generally* harmless and commensal, as it can contribute to both *the innate* and adaptive immune systems [3]. However, *E. coli* can be classified as a pathogenic bacterium if it acquires virulence genes from other microorganisms through displacement mechanisms by pili or pilus (conjugation), gene transfer through bacteriophages (transduction), or gene transfer (transformation) [3]. Due to some genetic changes in *E. coli* bacteria, they can cause varying impacts on human health [4]. And some examples of enteric diseases caused by bacterial infections *E. coli* are diarrhea, urinary tract infections (UTIs), as well as perception, and meningitis [3].

Morphology *of Escherichia coli*

Escherichia coli is a bacterium in the form of a short rod (rod-shape) or commonly called coccobacilli [7]. E. *coli* bacteria are about 1–3 μm × 0.4–0.7 μm (micrometers) in size and are flagella. *E. coli* cannot form spores, and is non-motile, but some are motile due to the arrangement of peritrichous flagella [7]. *E. coli* has polysaccharide capsules that can be easily demonstrated upon identification with a microscope using Indian ink. So that the results

obtained will appear as a clear halo on a dark background [8].

E. coli is a Gram-negative bacterium that can be characterized in its cell envelope, which consists of a cell membrane, inner cytoplasm, peptidoglycan cell wall, and outer membrane [9]. The outer membrane of *E. coli* bacteria is made of *lipid bilayer*, membrane proteins, and lipopolysaccharides (LPS), which will cause a toxin reaction if it undergoes lysis [8]. In addition, there are also primary antigens that can be found in *E. coli* bacteria, namely H or flagellular antigen, O or somatic antigen, K or capsular antigen, and F antigen or fimbria (Figure 1) [7,8].

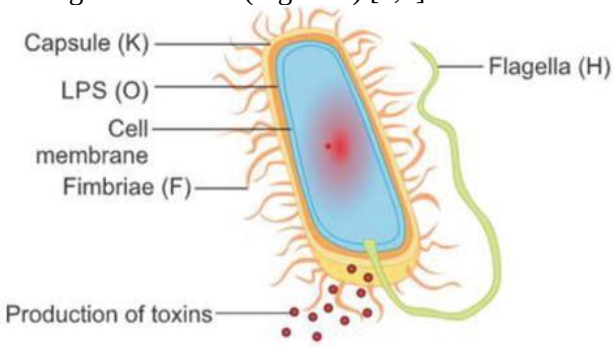

Figure 1. Antigenic structures in *E. coli* show that there are four primary antigens found on the outer membrane of bacteria[7]

Growth of *Escherichia coli*

E. coli bacteria generally live and develop in the digestive tract of humans and animals. The growth and survival of *E. coli* in the natural environment can be influenced by biotic and abiotic factors [10]. Abiotic factors include temperature, availability of water and nutrients, pH, and solar radiation. Similarly, biotic factors, where *E. coli* has the ability to obtain nutrients from other micro-organisms, compete with other micro-organisms and form biofilms in the natural environment [11,12]. Infectious diseases caused by *E. coli* bacteria are generally caused by the ability of these bacteria to adapt and survive in different environments. *E.* coli can survive at pH 4.5–9.5, but maximum growth for this bacterium is at pH 7.0 (neutral pH). However, the pH levels required vary depending on each strain of *Escherichia coli* [12].

Escherichia coli can replicate quickly under optimal growth conditions, which is in approximately 20 minutes. E. *coli* bacteria can survive temperatures of 10–40°C, but the optimum temperature for growth in most strains is 37ºC [13]. *E.* coli is also known to grow relatively faster when cultured on growth media with an estimated generation time of about 20-30 minutes, so the bacteria can be useful in a study with a short time and can also be used as a conventional diagnostic method of *E. coli* [14].

Genome

E. coli bacteria *have approximately* "4.5 to 5.5 million base pairs (Mbp) on chromosomes and plasmids that code for about 4,500 to 5,500 genes [15]. The length of *E. coli's* chromosomes is up to a thousand times longer than its cells [16]. The genome of *E. coli*, both pathogenic and nonpathogenic bacteria, exhibits complex segmentation, and both are known to share linearly primary sequences except for a few replication points [17]. The genome sequence *of E. coli* has a lot of diversity in each species, it is based on the interaction of bacteria with hosts such as pathogenicity factors and serotypes. All genetic information of *E. coli* is obtained from the chromosomes and plasmids of bacteria. Similarly, pathogenicity in bacteria is found on chromosomes and on plasmids that contain many genes encoding virulence traits or pathogenicity *island* (PAI) [12].

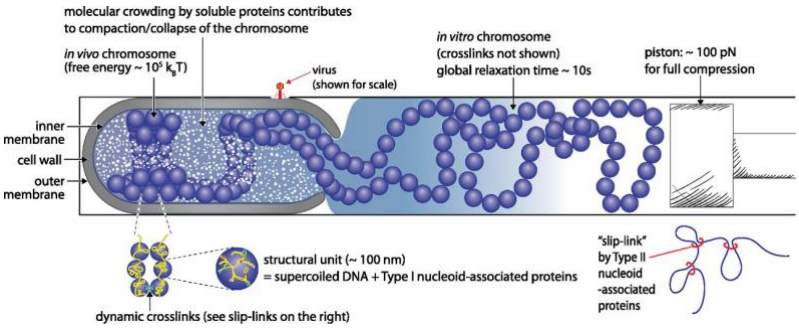

Figure 2. The chromosomes of *E. coli* bacteria have a manic-like structure with a size of up to a thousand times longer than the cell.[12]

The number of genes present in *E. coli* is known to reach more than 20,000 genes. *E. coli* DNA in certain strains can contribute to the virulence of bacteria. E. *coli* chromosomes are circular and have a double strand. Generally, the number of nucleotide bases on the pathogenic *E. coli* chromosome is greater than that of non-pathogenic *E. coli* strains, this is due to the substitution of several sequences that encode virulence genes [18] Similarly to plasmids in E. coli bacteria, *plasmids are found in double-stranded DNA that is*

mostly arranged into supercoils [16]. Generally plasmids are used to encode genes needed by bacteria to survive in unfavorable environments, or even as one of the factors causing virulence in pathogenic bacteria [19].

Pathogenicity and Virulence

Pathogenicity is the ability of an organism to be able to cause infection with diseases. *E. coli* bacteria can cause disease infection if the bacteria can enter the host's body, then adapt and survive in the host's body, which will then disrupt the immune system causing disease infection [12]. The pathogenicity is caused by the presence of chromosomes or plasmids indigenus in *E. coli* bacteria. The combination of virulence genes found in bacterial chromosomes or plasmids will determine the trait or pathotype of *E. coli*, where each bacterial pathotype will cause different clinical symptoms [20]. *E. coli* is generally non-pathogenic, but due to the substitution of virulence genes from other microorganisms, the bacteria will undergo a change in nature to become a pathogen. The process of substitution or addition of virulence genes can be through the mechanism of gene transfer (transformation), plasmid transfer (conjugation), or it can also be by the process of gene transfer through bacteriophage (transduction). So that diseases caused by *E. coli* can vary due to the virulence factor and the mechanism of pathogenesis [20,21].

Based on its pathogenic nature, *E. coli* is grouped into several types. This is based on the mechanism of bacterial pathogenicity, virulence properties, and clinical syndromes caused [22]. Based on its pathogenicity, *E. coli is divided into six types:* Enteropathogenic E. coli (EPEC), Inherent diffusion *E. coli (DAEC), Enterotoxigenic* E. coli (ETEC), Enteroaggregative E. *coli (EAEC), Enterohemorrhagic E. coli (*EHEC), and *Enteroinvasive* E. *Coli (EIEC)* [22]. The pathogenesis mechanism of *E. coli* bacteria can go through several stages, namely the occurrence of colonization processes in certain locations on the surface of intestinal mucosal cells, then cell changes, destruction of mucosal cells, then bacteria will enter the intestine, and also enter the bloodstream, which will then lead to tethering to the target organ and eventually cause organ damage [12].

E. coli bacteria can attach to the surface of the intestinal mucosa due to the presence of pili (fimbriae antigen) found on the outer membrane of the bacteria. Each *E. coli* has a unique structure of fimbriae and varies in both shape, size, and function of virulence genes. Therefore, there can be varying mechanisms in each group of *pathogenic E. coli* to cause damage to host cells [23] Most *pathogenic E. coli* can damage intestinal mucosal surface cells, but in the group of enteroinvasive bacteria *E. coli* (EIEC) can replicate in intestinal mucosal cells and macrophages, so it is called intracellular pathogens [24].

Antibiotic Resistance

Several antimicrobials have been found that can be used in the treatment of Urinary Tract Infections (UTIs) caused by *E. coli bacteria*. E. coli *bacteria* are intrinsically sensitive to almost all clinically relevant antimicrobial agents [32]. However, it turns out that these bacteria also have a large capacity to be able to accumulate resistance genes. Resistance is the ability of bacteria to adapt so that they can protect themselves by eliminating or weakening the action of antibiotics [33]. Most *E. coli* bacteria perform antimicrobial resistance by horizontal gene transfer [33]. In addition, one way *E. coli* bacteria do self-defense is by producing *extended spectrum beta-lactamases* (ESBL) enzymes [34]. ESBL is an enzyme that causes bacteria to be resistant to antibiotics by splitting the beta-lactam ring of β-lactam antibiotics, cephalosporins and monobactams resulting in antimicrobial inactivation [27].

E. coli bacteria have also been found to be resistant to other mostly first-line antimicrobial agents, such as tetracycline, phenyhol, sulfonamides, trimethoprim, and phosphomycin [33]. In particular, the antimicrobial co-trimoxazole (trimethoprim-sulfamethoxazole) is currently considered in some regions as first-line therapy for urinary tract infections (UTIs) [35]. According to the study of Lescure *et al* (2001), short-term treatment using co-trimoxazole is the recommended empirical treatment for uncomplicated acute cystitis [36]. But the ability of *E. coli* to cause urinary tract infections is increasing, where the treatment process of co-trimoxazole in UTIs is increasingly difficult to understand, due to multidrug antibiotic resistance that occurs in these first-line antibiotics [37].

Trimetrophrim antibiotics

Trimetophrim antibiotics have been widely used as antibacterial treatment, one of which is in urinary tract infections (UTIs) [35]. Trimetoprhim works by

interfering with the process of folate synthesis. More specifically, Trimetrophrim works to bind to dihydrofolate reductase (DHFR), thereby blocking the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF) [6,7]. The process of inhibition of tetrahydrofolic acid (THF) reduction is very appropriate in inhibiting the replication of *E. coli* bacteria. This is because THF plays a central role in thymidine synthesis pathways, and thymidine synthesis plays an important role in the process of bacterial DNA synthesis. Therefore, it can be said that the binding process of *dihydrofolate reductase* (DHFR) to the antibiotic Trimetrophrim causes reduced folate synthesis in *E. coli* bacteria [38].

Cotrimoxazole (trimethoprim-sulfamethoxazole)

The pathogenicity of *Escherichia coli* is supported by its ability to survive in acidic environmental conditions (low pH), various temperature changes, and osmotic pressure. One of the empirical therapies in dealing with *Escherichia coli infection* is the antibiotic trimethoprim-sulfamethoxazole [4]. Trimethoprim-sulfamethoxazole is a combination of two antimicrobial agents. The antibiotic trimethoprim-sulfamethoxazole works in a bacteriostatic way49, namely by inhibiting the enzymatic reaction of bacterial folate synthesis at two consecutive stages, so that this drug combination can provide a synergistic effect [5]. Wüthrich, D *et al* 2019 stated that these two antibiotic combinations are reported to inhibit the synthesis of tetrahydrofolic acid bacteria, which are important cofactors for the synthesis of thymidine and purines, the basic bases of DNA and RNA [41]. The combination of these two agents is intended to create a synergistic anti-folate effect, especially in tetrahydrofolate which plays an important role in synthesizing purines so as to disrupt the production process of DNA and proteins in bacteria (Figure 3) [31,41].

Cotrimoxazole consists of a combination of the antibiotic trimethophrim with sulfametoxazole [11]. The mechanism of action of the two antibiotics works by inhibiting the synthesis of tetrahydrofolic acid. Where tetrahydrofolic acid is an essential metabolic cofactor in the process of synthesis of purines, thymidine, glycine and methionine in bacteria. So that if there is an obstacle in the synthesis of these components, then bacteria cannot carry out metabolism and growth [41]. When co-trimoxazole enters the body, trimethoprim will be absorbed faster than sulfamethoxazole. Similarly, the duration of the trimethoprim absorption process is 2 hours, while in sulfametoxazole for 4 hours [42]. Both trimethoprim and sulfamethoxazole are lipophilic. Trimetoprim can be distributed quickly and concentrated within tissues. As much as 44% of trimethoprim and 70% of sulfamethoxazole bind to plasma [40,42]. Protein binding to sulfamethoxazole significantly reduces protein binding to trimethoprim. Co-trimoxazole can be metabolized in the liver, where the antibiotic trimethoprim is metabolized to oxide metabolites and hydroxylation; While sulfametoxazole will conjugate with glucoronic acid [40,43].

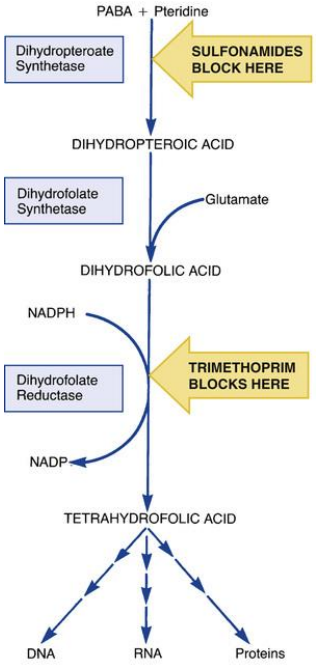

Figure 3. Mechanism of Action of Trimetroprim-Sulfamethoxazole[39]

Gene Mutations

E. coli is also a major cause of resistance genes that cause treatment failure in humans. An increasing number of resistance genes have been identified in *E. coli* isolates, many of which are acquired through horizontal gene transfer [33]. In enterobacterial gene pools, *E. coli* can act as a donor as well as a recipient of resistance genes. Therefore, *E. coli* bacteria can obtain resistance genes from other bacteria but can also pass their resistance genes to other bacteria [33,37]. Multiresistant plasmids in bacteria are easily mutated genetic elements, such as transposons and gene tapes in class 1 and class 2 integrons, apparently playing a major role in the spread of resistance genes [33].

Based on data obtained from the Microbiology Laboratory (LMK FKUI, 2020), it was reported that as many as 38-40% of *Escherichia coli* isolates isolated from clinical samples of urinary tract infection (UTI) patients showed phenotypes resistant to trimethoprim-sulfamethoxazole. The mechanism of acquired resistance has often been identified, mainly due to mutational modifications in the genes that code for target enzymes, namely in *dihydropteroate synthase* or *dihydrofolate reductase respectively* [38,39] This can occur if there is resistance to the acquisition of the *sul* gene that codes for *dihydropteorate synthetase* so that it is not sensitive to sulfonamides, or it can also occur in the *dfr* gene that codes for dihydrofolate reductase that is not sensitive to trimethoprim [33,44].

The incidence of resistance of *Escherichia coli* to the antibiotic trimethoprim-sulfamethoxazole has also been reported from various studies, with the contribution of mobile genetic elements *dfrA1, dfrA5, dfrA7, dfrA8, dfrA12, dfrA14, dfrA17* and integrons correlated with resistance to trimethoprim and *sul1, sul2* genes with sulfamethoxazole [9,10]. The study of Majeed Issa, O *et al* (2022) involved (490) patients with UTIs, and urine samples cultured on media. 116 *Escherichia coli* isolates were isolated from urine specimens, 35 of which were resistant to trimethoprim/sulfamethoxazole, and 81 isolates sensitive to trimethoprim/sulfamethoxazole*; Escherichia coli* isolates were subjected to PCR examination to detect several *sul* resistance genes. Resistant isolates with the prevalence of the Sul1 gene were 11 (31%), while isolates sensitive to the Sul1 gene were 1 (6%). While isolates that are resistant to the *Sul2* gene prevalence is 8 (23%), while isolates that are sensitive to the Sul1 gene are 0 (0%). The number of resistant isolates is (11) and (8) carrying the *Sul1* gene and the *Sul2* gene respectively, while the number of sensitive isolates is (1) and (0) respectively [9]. The increasing ability of *E. coli* to cause urinary tract infections and the difficulties encountered in treating these infections due to multidrug antibiotic resistance require updating knowledge about their drug resistance in certain environments [37].

Gen Dihydrofolate Reductase (*dfr*)

Antibiotic resistance to trimethoprim has been detected in Enterobacteriaceae and other Gram-negative bacteria [33]. Resistance to trimethoprim antibiotics occurs mostly in the part of the *dfr* gene located in the plasmid section of *E. coli bacteria*. The *dfr gene* is a gene that codes for the DHFR enzyme as the target of the antibiotic trimethoprim. Based on their size and structure, *dfr* genes are classified into two, namely *the dfrA and dfrB genes* [45]. In both families, *dfr* genes can encode evolutionarily unrelated proteins of very different sizes. Based on phylogeny analysis, the dfrA gene is homologous to the chromosomally encoded *folA* gene, while the *dfrB* gene is a functional analog of unknown origin. According to the study of Estrada, A *et al* (2016), DHFR enzymes in *Escherichia coli* resistant to trimethoprim are known to have plasmids *dfrA1, dfrA12, and dfrA17* with DHFR mutated in F98Y. The *dfrA gene codes for proteins from 152 to 189 amino acids, while the dfrB-encoded protein measures only 78 amino acids.* Most of *the dfrA and dfrB genes found in E. coli* of animal origin are located on gene cassettes inserted into class 1 or class 2 integrons [33]. In the study by Seputiené, *et al* (2010), the *dfrA8 gene is located neither in class 1 nor class 2 integrons*. In addition, only seven of the 13 *dfrA14 genes in E. coli isolates* came from animals associated with the integron [46].

Gen Dihydropteroate Synthase (*sul*)

In most gram-negative enteric bacteria, sulfonamide resistance is mostly transmitted via plasmids and is associated with drug-resistant DHPS variants with substantial sequence divergence [47]. Chromosomal mutations at the DHPS locus such as point mutations, duplicate amino acid insertions, or larger sequence changes as a result of recombination can also cause resistance [48]. In other organisms, such as *E. coli* and *Plasmodium falciparum*, nonsynonymous point mutations that result in amino acid substitutions in DHPS can lead to sulfamethaxazole (*sul*) resistance [47]. The *sul* gene is a gene encoding the enzyme DHPS that is targeted by sulfamethoxazole antibiotics and this gene is commonly found in transposons. In *E. coli* sulfonamide resistance is mediated by one of the following three sul genes: *sul*1, *sul*2, or *sul*3 [33]. The sul1 gene is very widespread because it is part of the conserved 3′ segment of the class 1 integron. The sul1 gene is located on plasmids, including multiresistance plasmids carrying the ESBL gene [49]. Thus, the sul1 gene is often found along with other antimicrobial resistance genes located on gene tapes in the variable section of class 1 integrons [33,50]. Genetic analysis showed that the *sul* 1 and *sul* 2 genes encoding only DHPS were 57% identical

in amino acid levels to each other, and their origin was unknown [51]. A study from Yun et al (2012), showed that DHPS amino acid mutations at the F28L/I and P64S positions in Escherichia coli are in loop 1 and loop 2 close to the active site of DHPS and affect the binding site of para-aminobenzoic acid (PABA) so that the bacteria provide phenotypic resistance [51,52].

CONCLUSION

E. coli is also a major cause of resistance genes that cause treatment failure in humans. An increasing number of resistance genes have been identified in *E. coli* isolates, many of which are obtained through horizontal gene transfer. In enterobacterial gene pools, *E. coli* can act as a donor as well as a recipient of resistance genes. Therefore, *E. coli* bacteria can obtain resistance genes from other bacteria but can also pass their resistance genes to other bacteria. The mechanism of acquired resistance has often been identified. This can occur if there is resistance to the acquisition of the *sul* gene that codes for *dihydropteroate synthetase* so that it is not sensitive to sulfonamides, or it can occur in the *dfr* gene that codes for dihydrofolate reductase that is not sensitive to trimethoprim.

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