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A Comprehensive Review on the Development of Salmonella Biofilm on Gallbladder Surface

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Abstract:- Salmonella is a group of rod-shaped bacteria belonging to the family Enterobacteriaceae. They are gram-negative and facultatively anaerobic. These bacteria reside mainly in the intestinal tract of humans and other animals. The pathogen, Salmonella enterica serovar Typhi primarily causes typhoid fever, a disease specific to humans. Salmonella can exist in the human gallbladder in an asymptomatic carrier state. Salmonella species have the capability to form biofilms. The production of biofilms serves as an advantage because bacteria in the biofilm are resistant to chemical, physical, and mechanical stresses as well as disinfectants. Biofilm formation also assists in Salmonella virulence. It is because the bacterial biofilm serves as a resistant barrier to antibiotics and immune attacks by the host. This results in a chronic infection accompanied by the development of Salmonella carrier state. In this review, we present a comprehensive overview of Salmonella biofilm formation, factors affecting biofilm formation, complications arising from biofilm formation, and available treatments for biofilm-mediated infections.

Keywords:- *Salmonella; Biofilm Formation; Gall Bladder; Typhoid; Antibiotic Resistance; Factors; Complication; Treatment.*

I. INTRODUCTION

Salmonella enterica serovar Typhimurium is a primary enteric pathogen that infects both humans and animals [1]. *Salmonella* Typhimurium and Typhi are the best-characterized serovars. It is seen that *S.* Typhimurium is involved in localized gastroenteritis in many hosts while *S.* Typhi causes a systemic human-specific disease [2]. These are a diverse group of pathogens that have evolved themselves to survive in a wide range of environments and across multiple hosts [3]. Although non-typhoidal *Salmonella* mainly causes gastroenteritis, typhoidal serovars (*S.* Typhi and *S.* Paratyphi A) are known to cause typhoid fever, the treatment of which is threatened due to increasing drug resistance of the pathogen [4]. People suffering from typhoid develop diarrhea, fever, and abdominal cramps within 8 to 72 hours.

S. Typhi and *S.* Typhimurium are capable of forming bacterial biofilms in mammalian and/or environmental niches. Biofilms are mainly aggregated mixtures of sessile bacteria that get implicated in many chronic infections and are known to ease the process of bacterial persistence by increasing resistance against the microbes and interfering with the host immune response [5,6,8,9]. Biofilms are encased within a mixture of secreted and cell wall-associated poly-saccharides, glycoproteins, and glycolipids, as well as extracellular DNA, known collectively as extracellular polymeric substances (EPS) [5,7]. Biofilms are frequently associated with implanted devices, such as catheters, prosthetics, and contact lenses [8,9]. For patients with gallstones, antibiotic treatment against *Salmonella* generally becomes ineffective and elimination of gallbladder infection in these individuals usually requires surgery and gallstone removal [10]. In addition to this, biofilm production by *S.* Typhi may be regarded as a key factor for the promotion of persistent infection in the gallbladder, thus enduring a chronic local inflammatory response and exposing the epithelium to repeated damage caused by carcinogenic toxins.

Salmonella biofilm formation depends on certain genes, environmental factors, the presence of flagella, fimbriae, bile, and quorum sensing. Quorum sensing is being used in many different bacteria as a mechanism for cell signaling based on cell density which is thought to regulate a variety of processes, such as conjugation, virulence, motility, and biofilm formation [11,12,13,14,15,16].

According to the Centre for Disease Control and Prevention (CDC) *Salmonella* bacteria causes about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year. Even with the use of adequate antibiotic therapy typhoid records a mortality rate of (2-3) %. Controlled human infection models (CHIMs) have played an important role in accelerating the development of conjugate vaccines against *Salmonella* Typhi [17].

The recent evolution of *S.* Typhi, which is a multidrug-resistant (MDR) strain has evolved as a notable problem for patients as low-priced and readily available antibiotics like streptomycin, chloramphenical, ampicillin and trimethoprin-sulfamethoxazole are often unproductive against them [18,19]. Although hostility to ciprofloxacin, which is a second-generation fluoroquinolone is increasing, medical practitioners still recommend it as first-line therapy for children and adults [20,21,22].

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Epidemiological studies in regions where *S.* Typhi is endemic revealed that the greater part of chronically infected carriers also harbor gallstones, which in turn, have been indicated as a primary predisposing factor for the onset of gallbladder cancer. But now, it is well recognized that *S.* Typhi strain produces a typhoid toxin with a carcinogenic potential that induces DNA damage and cell cycle alterations in intoxicated cells.

II. BIOFILM FORMATION ON THE GALL BLADDER SURFACE

In humans, *Salmonella enterica* serovar Typhi can cause an asymptomatic and persistent infection of the gallbladder, indicating that this strain uses novel mechanisms to increase colonization in bile-rich environments. Gallstones are among the most dangerous threats to growing carriage, and formerly in some of the studies, it has been confirmed in vitro that there are *Salmonellae*-shaped biofilms on gallstones. As a result, we can infer that gallstone surfaces formed by bile-induced biofilms are colonized by gallbladder bacteria [23]. Gallstones, in particular, are commonly associated with chronic typhoid carriage when they develop in the gallbladder [24].

The organism infects the intestinal epithelium, invades macrophages, and spreads throughout the body [25]. The organism may colonize the liver and then shed into the gallbladder, where it chronically colonizes the gallbladder wall [26,10]. The persistent colonization of *S.* Typhi with gallstones and gallbladder cancer is associated with persistent colonization of these organisms, but it is not known whether this organism is a causal factor in gallstone formation or whether gallstones promote persistent colonization. Several recent studies indicate that *S.* Typhi forms bacterial biofilms on cholesterol gallstones. It is possible that this biofilm formation could allow persistent colonization and protect the organism as the organism is treated with antibiotics. The formation of biofilms requires the presence of bile, as organisms not cultured with bile would not form biofilms [26].

In *Salmonella* carriers, the growth of a biofilm may protect microorganisms from antimicrobial agents and excessive levels of bile for an extended period of time. Bile acids are amphipathic molecules, surface active, and are a kind of detergent. As a detergent, bile acids have strong antimicrobial activity [27]. The absence of bacterial biofilm on *S.* Typhi gallstones suggests that *S.* Typhi followed a similar strategy, by forming a biofilm on the gallstone surface, enabling it to persist inside the gallbladder for many years and assist in shedding and reattachment, followed by diffusion through urine and feces [28]. A number of studies have shown that people with gallstones who are infected with *S. enterica* serovar Typhi are much more likely to develop the infection and become carriers than people without gallstones, who do not have obvious gallbladder abnormalities [28,7]. This indicates that gallstones are involved in chronic disease development. Gallstones are strong and stable surfaces and can act as a firmly attached organism that can avoid being washed away into the surrounding tissues due to the continuous emptying of the gallbladder.

Formation of *Salmonella* biofilm depends on the action of certain genes. Moreover, it has been seen that the presence of flagella, fimbriae, and bile can affect biofilm formation. Quorum sensing is another mechanism that favors microbial interactions and regulation of population-level virulence of bacteria.

- *Genes involved in biofilm regulation*

In stressful conditions, *S.* Typhi can use the biofilm phenotype in order to increase the production of the membrane matrix, protect and adhere to the surface, and enter an energy-conserving state [29].

The galE gene is crucial for the formation of wild-type biofilms. galE encodes for a uridine diphosphogalactose-4-epimerase, which is a structural gene needed for the synthesis of galactose, that is added to both the outer core and the O-antigen. This gene mutation results in a lipopolysaccharide (LPS) that lacks all sugars beyond the heptose region of its internal core (thus producing a tough or incomplete LPS) [25].

Gene STY0893, a bssR gene involved in biofilm regulation, is significantly downregulated within the biofilm cells. In a study, researchers compared the bssR gene in *E*. coli and *Salmonella*. The bssR gene, also called yliH in E. coli cells, becomes upregulated during biofilm formation [30]. The transcriptome study on *S*. Typhi biofilm cells, however, confirmed that the BssR gene (STY0893) was markedly downregulated compared to planktonic cells. The BLAST analysis of *E*. coli K-12 and *S.* Typhi CT18 showed that the gene bssR is found in both species, although it has only 75% nucleotide similarity. Additionally, since the base pair length of both species differs, it is likely that both proteins are unique [30].

The gene which codes for biofilm stress and motility protein A is Yjfo gene. According to a study conducted on *E*. coli, yjfO was related to cellular survival based on the formation of biofilms in response to peroxide pressure [31]. Comparison of the outcomes of the study using statistics shows that *S.* Typhi has an exclusive feature for the gene yjfO because the gene becomes downregulated in *S*. Typhi during biofilm formation. The yjfO gene is also responsible for microcolony formation. It is also possible that the gene transcription rate can become suppressed in mature biofilm tissues.

- *Flagella*

The presence of flagella has been proven to be very crucial to biofilm formation, especially during the early stages when microcolonies are being formed. In order to propel microorganisms throughout the surface, flagella are required to move them to the surface, for attachment, and to propel them to find different microorganisms. Some research has been conducted to determine if flagella play a part in *Salmonella* biofilm formation on gallstones. It was found that a mutant showed a severe defect in the ability to form flagella (and therefore non-motile) which were analyzed using a scanning electron microscope (SEM). After 14 days of growth, a weak biofilm was observed. However, the

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phenotype differed significantly from that of the *S. enterica* serovar Typhimurium wild kind [25].

Researchers have demonstrated that motility is crucial to biofilm development on glass using the motA and fliA mutants of *S.* Typhimurium [25,32]. However, it has been defined that *Salmonella* mutants with defective flagella (flhC or flgE) cannot form complete biofilms in the presence of bile [33,34]. Earlier, works involving QseBC two-component system (TCS) functioning in *S.* Typhimurium found out that QseBC plays a role in flagella formation, biofilm formation, and virulence mechanism [35,36,37]. Recent research has proven that the cyclic di-guanylate monophosphate (c-di-GMP) receptor YcgR and the phosphodiesterase YhjH can distinctively inhibit flagellar motility in *S.* Typhimurium [38]. Therefore, flagellar motility plays a very important role in the development of Salmonella biofilm.

Salmonella flagella can also additionally play a significant role in EPS secretion or production. In addition, it also helps in establishing primary adhesion and microcolony formation (aided by using bacterial movement throughout the surface) on gallstones [25].

- *Fimbriae*

Fimbriae play a role in the adhesion of the microorganism to different microorganisms and to the surface of the gallbladder. A fimbria-mediated attachment on the surface indicates the initiation of a microcolony. The fimbriae of different organisms participate in the formation of biofilms as well. Various *S. enterica* serovar Typhimurium fimbrial mutants have been tested in association with gallstones and strains with individual mutations in four fimbrial operons (*fim, agf, lpf, and pef*). In addition, a strain wherein all these four mutations were combined and tested for gallstone biofilm formation [39]. The *fim* operon encodes a type I fimbria that is peritrichous and *agf* operon encodes a thin aggregative fimbria which is also peritrichous in nature. A *lpf* operon encodes polar fimbriae, which are essential for virulence, while a *pef* operon is on a plasmid and is needed for adhesion to organ surfaces [25].

The DT104 strain of *S. enterica* serovar Typhimurium exhibits a rugose phenotype on certain media, which gives prominence to its biofilm-forming ability. SR11 strain shows a rugose phenotype, but it overproduces an EPS that allows rapid biofilm formation.

A biofilm matrix of *Salmonella* is mainly composed of curli fimbriae, the O-antigen capsule, cellulose, colanic acid, vi-antigen, and biofilm-related proteins [40,41]. The essential protein element is curli fimbria, which is crucial for biofilm formation and it plays an important role in virulence and alterations to the immune system [42,43]. Curli fibers are much like eukaryotic amyloid fibers and are involved in molecular aggregation and adhesion. Curli genes are organized in divergent curli-particular gene (csg) operons with independent promoters: one consists of the structural components CsgA and CsgB (csgBAC), and the second consists of the CsgD, which acts as regulator and different structural proteins (csgDEFG). CsgD is a transcriptional regulator which is very crucial for biofilm production and it also regulates the csgBAC operon, cellulose, and the BapA protein [44,45].

- *Quorum sensing*

Cell-cell communication is achieved by a process known as quorum sensing, which depends on gene expression in response to population cell density. A quorum-sensing signal is activated in *Salmonella* biofilms, and this signal triggers the maturation and disassembly of the biofilm in an organised manner. As part of quorum sensing, acylated homoserine lactone signaling molecules are produced, released, and detected in the environment. These molecules are known as autoinducers [46]. Several studies have confirmed that *S. enterica* serovar Typhimurium has a homologue for luxS, an autoinducer-2 gene [15,16]. The autoinducer has been implicated in supporting the transformation of pathogenic bacteria to free-living bacteria within the surrounding environment [15].

- *Bile*

The gall bladder is responsible for storing bile in the human body. It has been hypothesized that bile can also be used as a signal to induce biofilm formation. In a mouse model study, gallstones had been incubated within the presence of LB broth alone and LB broth containing 3% bile. The presence of bile facilitates complete biofilm formation on gallstones after 14 days in *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi cultures, while the absence of bile prevents the formation of biofilm [25]. Thus, *Salmonella* biofilm formation on gallstones is dependent on bile within the culture medium. Bile is necessary for biofilm formation on gallstones in 14 days, so it is possible that bile gives the signal to the microorganism to form biofilm and makes the surfaces of the gallstones smooth, making adhesion easier [10].

III. ENVIRONMENTAL FACTORS AFFECTING *SALMONELLA* BIOFILM FORMATION

Biofilm formation is a complicated process that begins with the initial adherence of bacterial cells to the substratum, leading to physiological changes within the microbe and multiplication of attached cells to form microcolonies, and finally leads to maturation of the biofilm [47]. Bacteria that have the ability to form biofilms present distinct features as compared to their planktonic counterparts which are free-living, such as different physiology and high resistance to the immune system and antibiotics. Hence, biofilms serve as a key factor in chronic and persistent infections [48]. It has been seen that the change of phenotype from planktonic to the sessile form occurs when there are changes in environmental conditions [49]. Nutrient level, temperature, pH, oxygen concentration and osmolarity are the key environmental factors that can affect biofilm formation through several pathways [50]. In order to obtain the best nutrients to survive and reproduce, mature biofilms change with environmental conditions [51].

➢ *pH*

The environmental pH can highly influence the formation of biofilms [50]. The first step in biofilm formation is microbial adhesion to surfaces. It has been seen that this step is influenced by environmental pH [52,53]. pH is considered as an important factor in the initial adhesion to surfaces in bacteria, such as *Staphylococcus epidermidis* [54]. One study showed that the optimal pH for *Salmonella* biofilm formation is pH 7.0. However, biofilm formation was significantly reduced at pH 10.0. *Salmonella* biofilm formation is inhibited at alkaline pH however most cells can survive the alkaline stress [55].

➢ *Temperature*

The formation of biofilm is also affected by temperature. Optimum temperature can enhance bacterial growth and assist in the rapid formation of biofilm. However, when optimum temperature is not present, bacterial growth can be decreased, due to a reduction in reaction rates, and as a result, the biofilm development might be affected [56]. The biofilm formation of *V.*parahaemolyticus is temperature-dependent. It has been seen that a higher temperature range (15°C to 37°C) gives rise to stronger biofilm formation, but a lower temperature (4°C or 10°C) induces the monolayer adherance of bacterial cells [57]. In one paper, among the 243 analyzed *Salmonella* strains, 224 (92.2 %) were able to produce biofilm in atleast one of the tested temperature conditions, but to various extents. It was seen that there is a strong influence of temperature on biofilm production as strains showed different behaviors at different incubation temperatures [58].

➢ *Oxygen Concentration*

Oxygen availability can also affect biofilm formation as it determines bacterial energy production. If there is a production of microenvironments within biofilms, such as reduced oxygen zones or restricted nutrient diffusion through the biofilm, it leads to slow growth of the bacteria. Deficiency in oxygen and nutrient within biofilms often results in a decrease in bacterial metabolic activity and termination of bacterial growth [59]. Hence, this state does not provide sufficient energy to maintain cell attachment. As a result, detachment occurs. For example, the presence of oxygen is required for the formation of biofilm in some microbes, such as *E.* coli. The lack of oxygen can act as a signal for detachment for the cells [60]. Another study demonstrated that the biofilm formation of *Salmonella* on stainless steel is highly influenced by oxygen levels [61].

➢ *Nutrient Level*

It has been seen that bacterial attachment to surfaces is also influenced by the availability of nutrients in the surrounding medium; thus, an increase in nutrient concentration increases the microbial attachment rate. Characteristics like biofilm morphology, biomass, thickness, composition and activity and are determined by nutrient availability in the environment. It was shown in one experiment that microbes produced less dense biofilms with lower biomass content in batch mode compared to biofilms formed in continuous mode with constant supply of nutrients [62]. In some microbes such as *E. c*oli, biofilm formation is enhanced when glucose is added as a carbon source to the medium [63]. However, the presence of glucose has been reported to slow down the biofilm formation in *Salmonella.* It is suspected that glucose inhibits a component required in the early phase of bacterial adhesion [64].

➢ *Osmolarity*

Bacteria are exposed to osmolarity which is one of the abiotic stresses. In *Salmonella* species, biofilm formation is inhibited when there is an increase in NaCl concentrations [65]. In a study, it was found that when the growth medium was supplemented with a low concentration of sodium chloride (0.5 to 2%), biofilm formation was slightly enhanced for the strain S. enterica serovar Enteritidis 110. However, biofilm formation by the strain S. enterica serovar Newport 193 was sharply reduced or abolished [66].

IV. COMPLICATIONS ARISING FROM BIOFILM FORMATION ON GALL BLADDER SURFACE

Salmonella typhi forms biofilm on cholesterol gallstones which is surface-specific and bile dependent, but this stage results in a carrier state. Gallstones are one of the most critical risk elements for growing carriage states. Once the carrier state is formed, biofilm is tough to treat [23,67].

Most biofilm infections are persistent, as biofilm-residing microorganisms may be resistant to the immune system, antibiotics, and various treatments. During their dormant phase, bacteria can infect nearby tissues, which can later cause acute infection. The microorganisms within the biofilm adapt to environmental anoxia and nutrient deficiency by changing their metabolism, gene expression, and protein production, resulting in a reduced metabolic rate and slowing down the molecular division process [68].

Biofilms might additionally have a major role to play in the colonization and chronic persistence of *S.* Typhi. This notion is supported using numerous reviews and researches that bile, a lipid-rich, detergent-like digestive secretion with antimicrobial characteristics contained within the gallbladder, induces an exopolysaccharide matrix O-antigen production that allows *S.* Typhi biofilm formation on human gallstones [23,69]. Gallstones can block the tubes (ducts) via which bile flows out of the gallbladder or liver into the gut. As a result, severe pain, jaundice, and bile duct contamination can also occur.

Biofilm embedded gallstones might also additionally constitute favorable surroundings for bacterial persistence within the gallbladder and might result in reseeding of the gut bacteria and fecal shedding, followed by transmission to a new host and exposing the gallbladder epithelium to bacterial factors potentially carcinogenic for prolonged periods of time [70]. Different products like bacterial glucuronidase and nitroso compounds are released by *S.* Typhi, which have the capacity to promote carcinogenesis [71].

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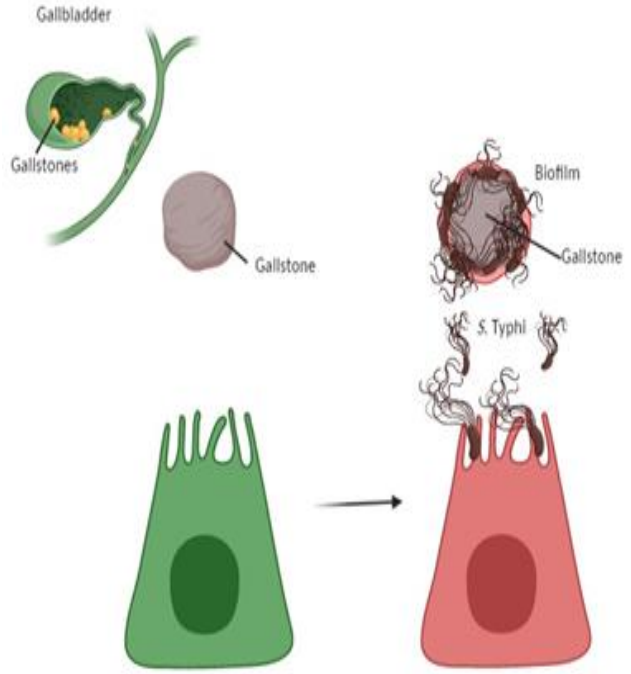

Fig 1: The development of gallbladder cancer may be related to biofilm-producing S.Typhi. S. Typhi infection is strongly related to the development of gallstones and gallbladder cancer. Gallstones provide S. Typhi strains with an ideal substrate to form biofilms. Biofilm formation may cause bacteria to release carcinogens, which trigger genomic instability and chronic inflammation, which are both necessary for GC to form.

Cytolethal distending toxins (CDTs) are toxic bacterial molecules produced by S. Typhi, which are capable of causing irreversible DNA damage and causing the cell cycle to stop and apoptosis to occur [72]. Gallbladder infections damage deoxyribonucleic acid (DNA), leading to repeated tissue growth attempts to reverse the damage, released cytokines and growth factors, and thus predisposing cells to oncogenic transformation [73]. The characteristics of gallstones further influence the development of gallbladder cancer. As the stone length increases, the chance of gallbladder cancer increases. Stones that are longer than 3 cm convey a tenfold elevated risk as compared to smaller stones [74].

S. Typhi appears to utilize biofilm formation as an adaptive strategy in order to facilitate microbial persistence. Therefore, it ensures the spread of bacteria both within the host and community as well as immunity against antibiotics. The persistence of bacterial toxins on target cells may contribute to their mutagenic effects, which could additionally cause cumulative damage and transformation [71].

V. TREATMENT

- *Using small molecules of JG-1 and M4*

JG-1 and M4 are two compounds that are found to show effective anti-biofilm activity against *Salmonella. S.* Typhi resides in humans and does not colonize mice and *S.* Typhimurium, which is a related species is used as a model for typhoid infection in mice. In a study, the abilities of JG-1 and M4 was observed using both *S.* Typhi and *S.* Typhimurium grown in a particular media. In *S.* Typhimurium both compounds showed similar inhibiting capacity however in *S.* Typhi, biofilms were inhibited more efficiently by M4 than JG-1. In order to detect the anti-biofilm effects of M4 and JG-1 and whether they could be accredited as bacteriostatic or bactericidal in nature, the liquid cultures of S. Typhimurium were incubated. This was done in the presence of a vehicle or a low/high concentration of either compound for a period of 24 hours. However, no significant differences were observed with respect to growth progression at any of the time points assessed [75].

- *JG-1 and M4 disrupt pre-formed Salmonella biofilms*

Destruction of already existing biofilm is of great importance to therapeutic relevance. Studies were conducted by exposing *S.* Typhimurium after 1-2 hours of inoculation to observe the anti-biofilm effects of JG-1 and M4 at various phases during biofilm formation. When a bare minimum amount of JG-1 or M4 was added to *S.* Typhimurium nearly 8 hours after inoculation, both compounds were seen to inhibit biofilm growth and maturation. Then it was tested on 24-hour biofilm with the compounds at various concentrations and the results proved that both JG-1 and M4 disrupted the biofilm to a certain degree but M4 was more efficient in disrupting *S.* Typhimurium biofilm than JG-1. In case of *S.*Typhi, both compounds were equally competent in disrupting the biofilm [75].

- *Administration of Ciprofloxacin enhances the biofilm disrupting capabilities of JG-1 and M4*

Ciprofloxacin is the first-line therapy for chronic and acute typhoidal infections in majority of countries. Using this agent, scientists have tried to evaluate the therapeutic potential of JG-1 and M4 against *Salmonella* biofilms. In congruence with the prior data, scientists treated 24-hour *S.* Typhimurium biofilm with some amount of ciprofloxacin but it had no significant effect but treatment with JG-1 or M4 caused a major reduction in biofilm formation. Viewing these results, researchers further combined M4 or JG-1 with ciprofloxacin and found that this combination of drugs amplifies the destructive effects of both compounds despite ciprofloxacin displaying no anti-biofilm effects on its own [75].

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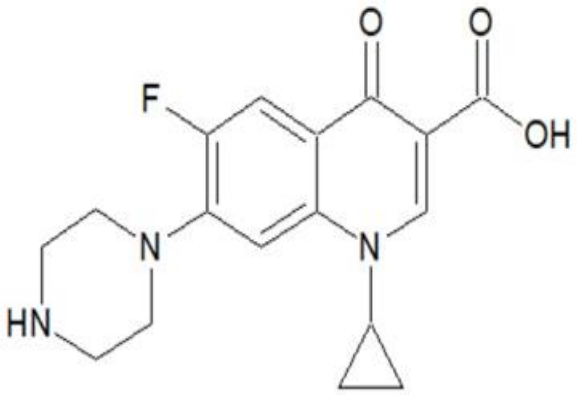

Fig 2: Structure of Ciprafloxacin.

- *Combining with ciprofloxacin helps to enhance the efficacy of JG-1 and M4 treatment in mice with chronic Salmonella gallbladder carriage*

After experimenting on in-vitro models, scientists have further addressed *Salmonella* activity in an in-vivo model. A specific mouse strain was fed with a lithogenic diet to help in the formation of cholesterol gallstones. Infection with *S.* Typhimurium shows the same result as that of S. Typhi in humans. Later, in the research, the mouse model was treated with different regimens: JG-1 or M4 alone; ciprofloxacin alone; a combination of ciprofloxacin with either JG-1 or M4, and a control was setup. For this purpose, mice were used and their livers, spleens, and gallbladders were collected for further assessment of *Salmonella* burden.

The results showed that ciprofloxacin alone did not inhibit biofilm growth gallbladder of infected mice but slightly reduced biofilm growth in the liver and spleen. In contrast, M4 or JG-1 alone could reduce the bacterial burden in the gallbladder but increased the number of bacteria in the liver and spleen. This also resulted in an increased mortality rate in mice. The main cause of this was found to be the reduction in gall-bladder contents, but there was a decline in bacteria associated with gall-bladder tissue. The fecal matter was also tested and it was found to have increased fecal bacteria (*Salmonella*) when treated with the JG-1 or M4 alone than with ciprofloxacin. In in-vitro biofilms, co-administration of ciprofloxacin with M4 or JG-1 decreases *Salmonella* burden in the gallbladder as well as in the liver and spleen with no associated mortality. Thus, scientists could say that the compounds M4 and JG-1 effectively and remarkably reduced chronic carriage in the mouse model [75].

Another research was conducted using Compound 7955004 [3-(2-furylmethyl)-2-[[(5-hydroxy-1*H*-pyrazole-3-yl) methyl]thio]-3,5,6,7-tetrahydro-4*H*cyclopenta[4,5]thieno [2,3- *d*]pyrimidin-4-on] which inhibits *S.* Typhimurium biofilm formation in a dose-dependent manner. It was seen that biofilm formation was dependent on the amount of the compound applied. Compound 7955004 was further tested in *S.* Typhi and *S.* Typhimurium for 60 hours. It was observed that biofilm inhibition by *S.* Typhi was more modest than S. Typhimurium. Compound 7955004 was again tested to disperse pre-existing 24-hour biofilms but no significant decrease in biofilm presence was observed [76].

VI. CONCLUSION

Salmonella biofilm is formed through the action of a few genes. The quorum-sensing mechanism contributes to microbial interactions and regulates virulence at a population level. Gallstone surfaces formed with a biofilm could protect the microorganism from antimicrobial agents as well as excessive concentrations of bile for a long time. Flagella of *Salmonella* can also be useful for secreting or producing EPS. It also acts as an aid in primary adhesion and microcolony formation on gallstones. The fimbriae are important in the attachment of microorganisms to microorganisms and to the surface of the gallbladder. Gallbladder colonization seems to be associated with chronic persistence of *S.* Typhi caused by biofilm formation on gallstone surfaces. The main cause of gallbladder cancer in individuals with particular predisposing factors is the presence of chronic *S.* Typhi infection, despite the presence of gallstones. Gallstones are not the only sites where *S.* Typhi infection can persist; the gallbladder epithelium and other cavities could also serve as alternative niches. Environmental factors affect the formation of Salmonella biofilms to a great extent. *Salmonella* prefers a neutral pH for biofilm formation. Biofilm formation also shows a variation at different temperature ranges. Generally, at higher oxygen and nutrient levels, *Salmonella* biofilm formation is better whereas a higher salt concentration adversely affects biofilm formation. Glucose in culture media inhibits biofilm formation in *Salmonella*. Studies show that the treatment of chronic carriage with anti-biofilm compounds alone may be able to reduce the burden of bacteria able to produce biofilms in the gallbladder. However, in few cases it may lead to increased acute disease severity, sepsis, or even death. Further, it is seen that the adverse outcomes can be avoided by using a dual-therapy approach in which traditional antibiotics like ciprafloxacin are administered in combination with anti-biofilm compounds.

Further research has to be done to observe the combined effect of dual-drug therapy and environmental factors, both in-vivo and in-vitro models. It has already been seen that the dual-drug therapy approach yields good results. Hence, further studies should be done considering proper environmental conditions so that better results may be obtained and the disease can be managed more efficiently. Research should also be carried out to produce a single drug as a substitute to dual or multiple drugs in combination that have been proven to be effective against the disease.

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