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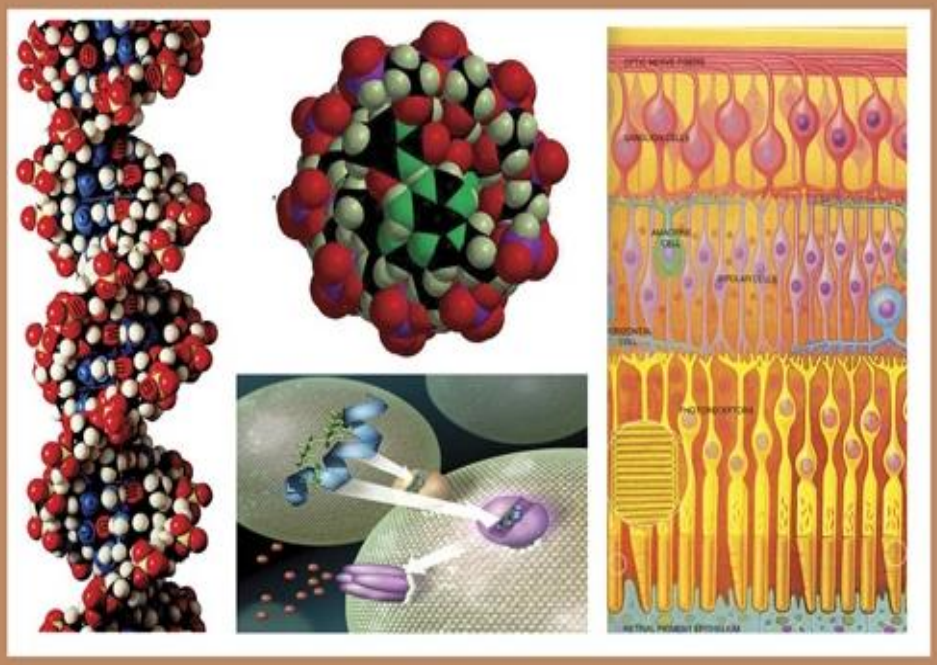

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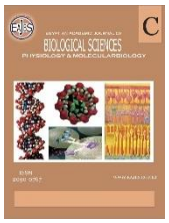

Prevalence of Biofilm Formation in *Salmonella typhi* Isolated from Enteritis Patients in Al-Najaf-Iraq

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ABSTRACT

Several types of bacteria enhance their survival by attaching to non-living surfaces or tissues, presenting them as multicellular communities covered by a protective extracellular matrix called biofilm. There has been a clear interest in assessing the relationship between antibiotic resistance phenotype and biofilm production. The aim of this paper was to present additional experimental results on this topic and to test the ability of *Salmonella typhi* isolates to biofilm formation using *in vitro* methods in the context of their antibiotic resistance. In the result of the study, 28 isolates of *S. typhi* were included. All 28 clinical specimens 100% showed strong biofilm formation and all clinical specimens showed the presence of *pml* gene by clear bands in gel electrophoresis. The results of the antibiotic sensitivity test of *S. typhi* isolates by disk diffusion method against 18 types of commonly used antibiotics showed a large variation in their rates of antibiotic resistance, as the highest percentage of resistance to the antibiotic to *S. typhi* resistance to the most common antibiotics used in treatment. The highest rate of resistance was seen with Ciprofloxacin and Gentamycin, Ceftrazidime, Cefotaxime, Amoxicillin, Ampicillin 28/28 (100%) followed by Azithromycin and Cephalothin 26/28 (92.9%), Levofloxacin and Erythromycin 22/28 (78.6%), Chloramphenicol 20/28 (71.4%), Clindamycin 8/28 (28.6%), Imipenem 4/28 (14.3%), Tobramycin, Netilmicin and Tetracycline 3/28 (10.7%) as all isolates were sensitive. The study found a positive correlation between interesting study genes and biofilm formation and antibiotics in *S. typhi*. Strains that appeared multidrug-resistant (MDR) were given a high mean of biofilm. It has been demonstrated that some correlations exist between antibiotic resistance and the biofilm-forming ability of *S. typhi* isolates. There is an association between biofilm production with persistent infection and antibiotic failure.

INTRODUCTION

Salmonella is a serious food-borne illness that affects people all over the world and causes serious morbidity, mortality, and financial loss (Siddiky *et al*., 2021). A leading cause of foodborne illness around the globe, *Salmonella* is estimated to have a 3.7-billion-dollar annual economic cost burden. One of the most prevalent foodborne organisms, *Salmonella*, is responsible for outbreaks of foodborne illness around the world (Shamma *et al*., 2016). The most important genus in the Enterobacteriaceae family is *Salmonella*. The *Salmonella* genus is a group of Gram-negative, facultative anaerobes that may live on a wide range of hosts, including humans, animals, poultry, pigs, and the environment (Appling *et al*., 2018).

Salmonella species may be pathogenic to domestic or wild humans and animals not producing spores, mostly motile, rod-shaped and flagellated (peritrichous flagella-all around the cell body) with cell lengths ranging from 2.0 to 5.0 m and diameters between 0.7 and 1.5 m (Abdul-Hassan *et al*., 2020). The major ways that these bacteria are disseminated are through tainted food and water People are more likely to get typhoid fever in heavily populated places When someone has typhoid fever, these bacteria live in their blood and intestinal tract. They enter the body through contaminated food and water, reproduce, and spread throughout the bloodstream (Rana *et al*., 2022). According to O and H antigens, Kauffman-White proposed a classification based on the one serotype-one species rule. Through this classification, the *Salmonella* genus is divided into more than 2500 species. These days, there are two species of *Salmonella: enterica and bongori. S. enterica* has six subspecies*: enterica, salamae, arizonae, diarizonae, houtenae,* and *indica,* based on an examination of the genome and biochemical traits. Based on antigenic traits, the subspecies are split into serogroups (O antigen) and serovars (H antigen) (Popa and Popa, 2021).

The two most prevalent serotypes of *Salmonella*, Typhimurium and *S. enterica* serovar Enteritidis, can infect a variety of hosts, including people and birds. The primary cause of acute gastroenteritis in humans is *S. Enteritidis*, while *S. Typhimurium* produces a systemic sickness similar to typhoid (Wang *et al*., 2020). Thus, the main aim of this study was to present results that show the association between the ability of *S. typhi* to produce biofilms and antibiotic resistance phenotypically and genotypically using *in vitro* experiments.

MATERIALS AND METHODS

The study was conducted at Bacteriology and Molecular Laboratories in Biology Department/Sciences Faculty/Kufa University/Iraq.

1. Specimen Collection and Bacterial Isolation:

Blood samples were collected from patients who attended consultation clinics in Al-Najaf hospitals who suffer from enteric fever. Each blood specimen (2-3 ml) was added to 10-15 ml of brain heart infusion (BHI) broth. The bottle of BHI broth was incubated at 37°C for 1-2 days. Each BHI was subcultured on SS agar and then incubated at 37°C for 24 hr to give the microorganism more chance to grow.

2. Identification of *S. typhi*:

The identification of *S. typhi* from blood samples was done with the Gram stain, IMViC tests and then was confirmed by using Vitek-2 compact system (Al Naiemi *et al*., 2008; Christner *et al*., 2010).

2.1 Antibiotic Profile of *S. typhi* Isolates:

The disk diffusion method, recommended by the Clinical and Laboratory Standards was used to determine the susceptibility of all *S. typhi* isolates to different antibiotics (Bioanalyse Company, Turkey).

Overnight-grown cultures of each isolate in brain heart infusion (BHI) broth were prepared. The turbidity of the broth was checked to 0.5 McFarland standard tube. Each isolate was spread on the surface of Mueller-Hinton (MH) agar with a sterile cotton swab and after 15 minutes, antibiotic discs were placed on plates (the distance between discs was 15 mm at least), then incubated the plates at 37°C for 18-20 hrs. The zone diameter around each disc was measured and compared with CLSI guidelines to interpret the results.

2.2 DNA Extraction:

Using the boiling technique, genomic DNA was successfully recovered from *S. typhi* isolates. The RNA/DNA spectrophotometer (Biodrop) instrument directly evaluated the concentration and purity of extracted DNA; extracted DNA purity ranged between (1.8-2). Gel electrophoresis was used to confirm and analyze the extracted DNA.

2.2.3 Detection of biofilm formation:

Semi-quantitative measurements of

biofilm formation were determined using tissue culture-treated, 96-well polystyrene plates (microtiter plates MTP), based on the methods of Lizcano *et al*., (2010).

2.2.4 Primer type of *pml*:

Primer *pml* Sequence was 5′-3′ F: ACTCAGGCTTCCCGTAACGC, R: GGCTAGTATTGTCCTTATCGG in the Product size 563 bp (Abbas *et al*., 2015) The condition of the primer was included: Initial Denaturation 94\2m, followed 35 cycles of (Denaturation 95\30 sec., Annealing 52\30 sec and Extension 72 \30 sec), finally the Final Extension was 72\7 min. The reaction mixture was held at 4°C until use while the final extension step took place at 72°C for about 10 minutes (Abbas *et al*., 2015). Each and every PCR amplification was performed using a Verity Thermal Cycler (Agilent, UK). Then, 1% agarose gel electrophoresis was used to analyze all of the PCR products, and they were all stained with red ethidium bromide dye. Finally, the gel documentation system was used to identify the electrophoresis results.

Statistical Analysis

Experimental data were presented in terms of observed numbers and percentage frequencies; SPSS (Statistical Package for Social Science) program version was used. Regarding other data were analyzed Correlation.

RESULTS AND DISCUSSION

1. Isolation and Identification of *S. typhi*:

During the collection period, a sum of 100 samples was gathered from the patients with suspected enteric fever from the main hospitals in Al-Najaf Al-Ashraf. After observing the culture and morphological characteristics of bacterial isolates and performing the classical IMViC tests, then confirmed by the Vitek-2 compact system. A total of 28 (28%) isolates had been identified as *S. typhi*.

The Gram-negative bacilli bacteria *S. typhi* can be found in food and water. Despite the fact that the disease has been linked to a wide range of food sources, poultry, in particular, has been identified as the sole major cause of salmonellosis in humans. In addition to harming the poultry business, avian salmonellosis can also infect people and is brought on by eating tainted chicken products like meat and eggs **(**Oscar, 2021). One of the most dangerous zoonotic and foodborne infections that endanger people's overall health and well-being is *Salmonella*. Despite this, *Salmonella* continues to be a significant human disease and a significant global public health concern Salmonellosis, often known as food poisoning, is an illness brought on by *Salmonella* and typically presents as moderate diarrhea (Balasubramanian *et al*., 2019)**.**

2. Antimicrobial Susceptibility of *S. typhi*:

All of the 28 *S. typhi* isolates were evaluated for susceptibility to 18 different antibiotics A summary of susceptibility rates (according to (CLSI, 2021) guidelines as resistant, intermediate resistant, and susceptible) for all antibiotics against *S. typhi* is given in the following Table (1).

Table (1): Antibiotic susceptibility test for 28 *S . typhi* isolates.

Type of antibiotic	Antibiotic disk	No. (%)		
		Resistance	Intermediate	Sensitive
Quinolones	Levofloxacin	22 (78.6)	0	6 (21.4)
	Ciprofloxacin	28 (100)	0	0
Carbapenemase	Imipenem	4 (14.3)	0	24 (85.7)
Aminoglycosides	Gentamycin	28 (100)	0	0
	Tobramycin	3 (10.7)	0	25 (89.3)
	Netilmicin	3 (10.7)	0	25 (89.3)
Sulfonamides	Trimethoprim	0	0	28 (100)
Macrolides	Erythromycin	22 (78.6)	0	6 (21.4)
Tetracycline	Tetracycline	3 (10.7)	0	25 (89.3)
Phenicol	Chloramphenicol	20 (71.4)	0	8 (28.6)
β-lactams cephalosporines	Azithromycin	26 (92.9)	0	2 (7.1)
	Ceftrazidime	28 (100)	0	0
	Cephalothin	26 (92.9)	0	2 (7.1)
	Cefotaxime	28 (100)	0	0
Lincomycin	Clindamycin	8 (28.6)	4 (14.3)	16 (57.1)
Polymyxim	Colistin Sulphate	0	2(7.1)	26 (96.9)
Penicillin	Ampicillin	28 (100)	0	0
	Amoxicillin	28 (100)	0	0

Patients with shock and enteritis should be treated with comprehensive antibiotic coverage for gram-negative, gram-positive, and gas-producing bacteria at first, and then deescalate when cultures and sensitivities improve. Patients with positive results of cultures for *S. typhi* were studied by von Graevenitz *et al*., (2010) who discovered that both species were sensitive to tetracycline, colistin, piperacillin/ tazobactam, higher generation cephalosporins, aminoglycosides, carbapenems, and polymyxin. However, research has revealed that multidrug-resistant carbapenem species exist (Kim *et al*., 2012). Aggressive source control, in addition to antibiotics, was discovered to be important for successful therapy in these individuals (Tram-Anh Duong *et al*., 2019)**.**

3. Detection of Biofilm Formation:

The ability of all isolates of *S. typhi* 28 to form biofilm was detected by using microtiter plates (MTP). Biofilms were measured by quantifying the absorbance of stained biofilms at 630 nm with a microtiter plate reader. The results in this study were indicated according to Salwa *et al*., (2011) in which 28 (100%) of *S. typhi* e isolates appeared high biofilm formation (strong positive adherence) (Fig. 1).

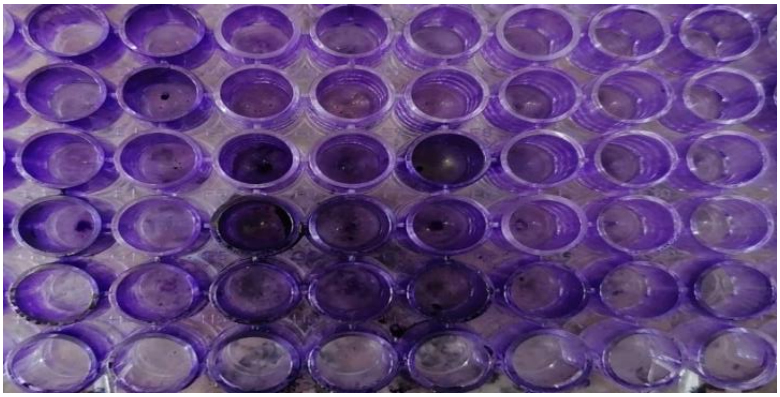

Fig.1: Biofilm formation of *S. typhi* by microtiter plates (MTP) method.

The MTP method is a precise and reproducible method used to screen and determine biofilm production Therefore, we used this method in our study. Bacteria utilize an assortment of methods to continue in their specific niche in the host (Munŏz-Elías *et al*., 2002). Microorganisms form biofilms that are present in the environment, whether on living animals or inanimate objects, the bacteria attach to these surfaces, such as aquatic systems and soil, and have been recorded in the human literature developing on medical equipment, within surgical implants, middle ear, lungs, external ear, heart valves, and tooth enamel (Singh *et al*., 2013)**.** Biofilm production in G-ve bacteria happens when bacterial cells first swim along a surface, utilizing flagellar-mediated motility until their attachment is initially reversible at a specific site (O'Toole, 2000).

Correlation between Biofilm and Antibiotic Resistance in *S. typhi*:

Figure (2) revealed a positive significant moderate correlation (0.569**) in *S. typhi* isolates between the number of antibiotic resistance and the mean of biofilm formation.

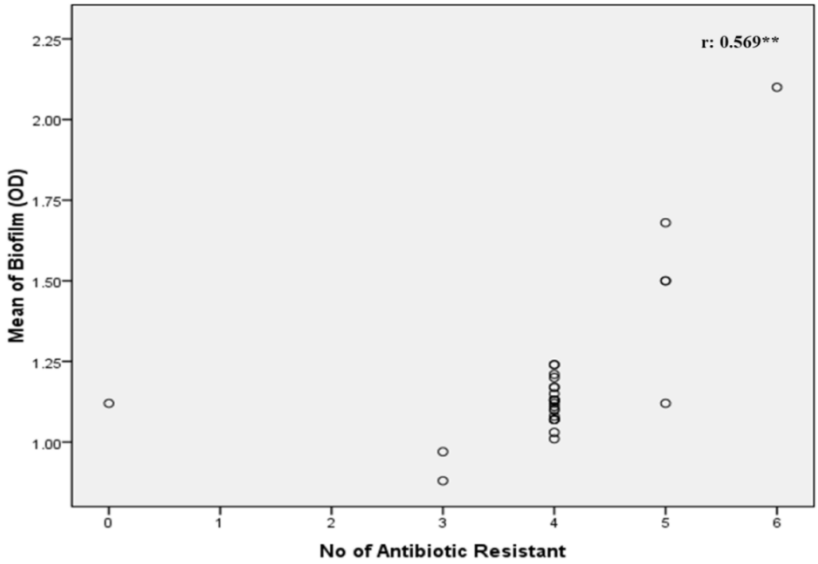

Fig.2: Correlation between the number of antibiotic resistance (No.) and the mean of biofilm formation (OD) in 28 *S. typhi* isolates.
(**. Correlation is significant at the 0.01 level (2-tailed).

To date, it has been demonstrated that some correlations exist between antibiotic resistance and the biofilm-forming ability of *S. typhi* isolates. There is an association between biofilm production with persistent infection and antibiotic failure. Gilbert *et al*., (2002) reported that biofilm producers were to be 10-1000 times less susceptible towards antibiotics than are the equivalent cells growing planktonically. Curtin *et al*., (2003) revealed that biofilm hampered the penetration of antimicrobials and the concentrations required to eradicate biofilm-producing bacteria are higher than those required to eradicate strains that did not produce biofilm. Also, Keren *et al*., (2004) explained this issue as bacterial populations produce persister cells that neither grow nor die in the presence of antibiotics and that persisters are largely responsible for high levels of biofilm tolerance to antimicrobials.

So, the process of biofilm formation is particularly relevant for clinicians because biofilm-associated microorganisms exhibit dramatically decreased susceptibility to antimicrobial agents (de-Silva *et al*., 2002). There are many virulence factors in *Enterobacteriacae* strains associated with its pathogenicity. Among these factors is the

capacity of *Enterobacteriacae* strains to form a biofilm that protects it from the host immune response as well as from antibiotics (Bandeira *et al*., 2014; Chung and Rimal, 2016).

Molecular Study of Biofilm Formation by the Detection of *pml* Gene:

The molecular detection of *pm1* gene by using a specific primer for *S. typhi* isolates revealed positive amplification for 100% as shown in Figure (3).

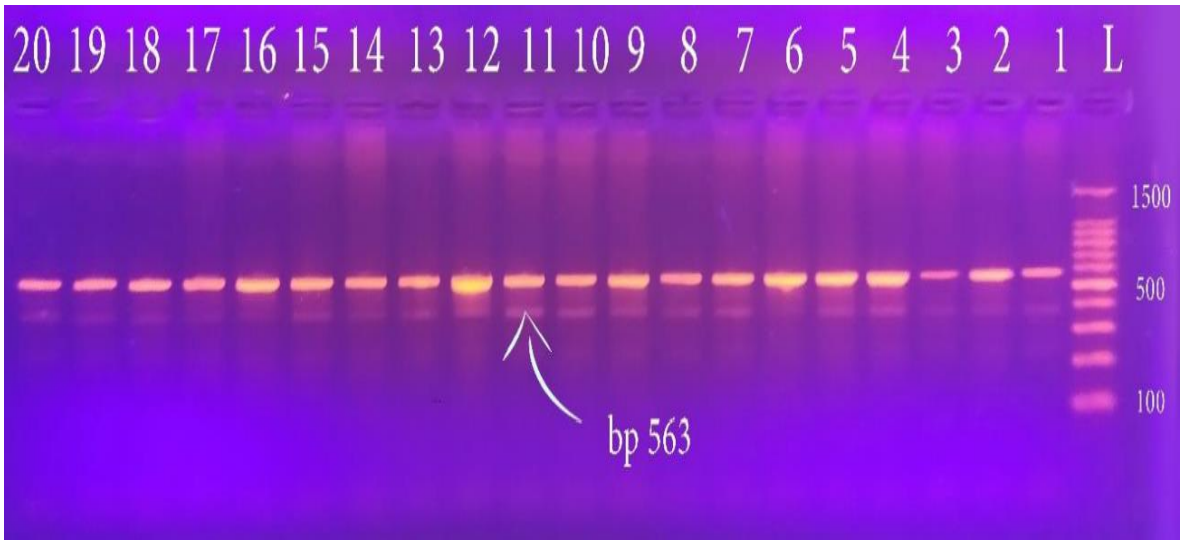

Fig.3: Electrophoresis on agarose gel of *pm1* gene amplification products (563 bp) in isolates of *S. typhi* (1% agarose, 70 V for 120 minutes). Column L: DNA size guide -100 base pairs. Columns 1-20: isolates carrying the *pm1* gene.

The results of the study showed that 100% of the isolates belong to the PM1 bacteria strain, which is characterized by its distinctive phenotypic characteristics and its possession of all virulence factors that enable it to cause infection (Abbas *et al*., 2015).

Al-Dahhan, (2017) confirmed that the PM1 strain is characterized by the homogeneity of its colonies on the solid media and the lack of clumping growth in the liquid culture media (Brooks *et al*., 2007), and they also indicated Fusco *et al*., (2017) the high ability of the PM1 isolate to adhesion in the epithelial cells. And the formation of the biofilm on the biological surfaces after 2 hours of incubation, up to the full phenotypic characteristics of the biofilm after 6 hours of incubation through its high ability to swarm and thus using this strain to create treatments targeting specific pathways of virulence factors.

According to the study's findings, all of the isolates are members of the PM1 bacteria strain, which is distinguished by its unique phenotypic traits and by its possession of all the virulence factors necessary to spread infection. The study's findings revealed that 100% of isolates carried the *mrpA* and pm1 genes, indicating the prevalence of the PM1 *S. typhi* strain enteritis. According to Abbas *et al*., (2015), the gene was present in 47% of all *S. typhi* bacterial isolates, the PM1 isolate has a high capacity for adhesion in epithelial cells and is distinguished by the homogeneity of its colonies on solid media and the absence of clumping growth in liquid culture media (Brooks *et al*., 2007).

Conclusion:

This study demonstrated that *Salmonella typhi* was one of the important pathogens causing enteric fever, enterocolitis, and other types of invading *Salmonella* infections, such as septicemia, it accounted for 19.5% of bacterial isolates. It was highly resistant to many common antibiotics, particularly ß-lactam. All bacterial isolates have the ability to produce strong biofilm. The study found a positive correlation between study interesting genes,

biofilm formation and antibiotics in *S. typhi*.

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