Plant Archives Vol. 18 No. 1, 2018 pp. 1081-1087 *ISSN 0972-5210*

PHENOTYPIC DETECTION OF SOME VIRULENCE FACTORS IN *SALMONELLA TYPHI* CARRIER ASSOCIATED WITH GALL BLADDER CHRONIC INFECTION

Mahdi H. AL-Ammar[1] and Zahraa Hameed Oda AL-Quraishi[2]

[1]College of Health and Medical techniques, Kufa, Kufa University, 31003 Al-Kufa, Iraq.
[2]Department of Biology, College of Science, Al-Furat Al-Awsat Technical University, Iraq.
E-mail: Dr.mahdi65@ymail.com, zahraa.alquraishi1989@gmail.com

Abstract

A total of (50) clinical specimens were collected from patients suffering from gall bladder attending to AL-Sadder Medical City and AL-Furat General Hospital include (gall bladder tissue, stool and blood) during the period from December 2016 to September 2017 from different age and sex. The identification of *Salmonella typhi* isolates were depended on colonial morphology and biochemical tests as a primary identification. The final identification was performed with the automated VITEK-2 compact system and PCR technique. According to the results obtained by the VITEK tests and PCR technique, thirty five clinical isolates of *Salmonella typhi* were obtained. This study revealed that totally 35 (70%) positive result include that 23 (65.7%) isolates was positive from gall bladder tissue , 11 (31.4%) from stool and 1 (2.8%) from blood. The study investigated the virulence factors of *Salmonella typhi*, which play a major role in enterococcus pathogenicity. *Salmonella typhi* had the ability to produce colonization factor antigen, biofilm L. form and flagellar swarming variation while did not produce hemolysin. The study showed that the virulence factors of *Salmonella typhi* , which play a major role in their pathogenicity among gall bladder chronic infection.

Key words : *Salmonella typhi*, VITEK-2 compact system, PCR and virulence factors.

Introduction

The gallbladder is a small organ located on the right side of the abdomen, just below the liver. The gallbladder's main function is to store bile made by the liver and secrete it into the small intestine to help digestion. Bile is made of water, cholesterol, fats, bile salts (natural detergents that break up fat) and a pigment called bilirubin (Schirmer *et al*., 2005).

Propagation of *S. typhi* infection is due to its ability to enter a dormant state through the formation of a biofilm in the human gallbladder (typhoid carriers), enabling it to evade the immune system and do not show any symptoms. The only reservoir for *S. typhi* which is transmitted via contaminated water or food (WHO, 2006).

Because *S. typhi* is a human-specific pathogen, these carriers serve as a critical reservoir for further spread of the disease through bacterial shedding in feces, which is a sporadic and intermittent event (Bhan *et al*., 2005)

Particularly in areas of high endemicity, the carrier state is linked to the presence of gallstones, as approximately 80 to 90% of chronically infected carriers have this gallbladder abnormality (Parry *et al*., 2002).

Salmonella typhi can form biofilms on the surfaces of cholesterol gallstones in the gallbladders of mouse and human carriers and on the gallbladder epithelium of mouse carriers (Gonzalez-Escobedo and Gunn, 2013). This biofilm formation has been demonstrated to be a mechanism of persistence and chronic colonization in the gallbladder (Crawford *et al*., 2010).

Flagella are thin, rigid appendages of bacteria, and are bacterial locomotive structures. A flagellum has three basic parts: the outmost and longest part is a filament which consists of around 20,000 protein subunits of a single protein called flagellin (Flic) with a molecular weight of 50 to 60 KDa (Chang *et al*., 2007).

Flagella provide the *S. typhi* with motility and may play a role in cell entry are down regulated inside cells,

and it has been suggested that they are used for escape from an intracellular site (Ibarra & Steele-Mortimer, 2009).

Methodology

Identification of bacteria

A total of 50 clinical specimens were collected from patients suffering from gall bladder infection attending to AL-Sadder Medical City and AL-Furat General Hospital include (gall bladder tissue, stool and blood) during the period from December 2016 to September 2017 from different age and sex. A single colonies were isolated from primary positive cultures and identified according to the criteria of Collee *et al*. (2011) using specific media such as MacConkey agar, XLD and S-S agar, which identified by morphological, biochemical tests, the automated VITEK-2 compact system (VITEK-2 GN-ID kit) was used in bacterial diagnosis (BioMérieux – France) and finally by PCR technique.

Molecular study

Extraction of Genomic DNA

Genomic DNA was extracted by using a commercial extraction system (Genomic DNA promega Kit).

PCR assay

The PCR assay was performed to detect the flagellar *FliC-d* gene for confirmation the identification of *Salmonella typhi*.

Primers selection

The primer in this study was synthesized by Bioneer company (Korea) as shown in table 1.

PCR Program

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in table 2.

Detection of DNA content by agarose gel electrophoresis

Gel electrophoresis was used for detection of DNA by UV transilluminator (Sambrook and Russell, 2001). Each well is loaded with 7ml of DNA specimen and standard molecular weight of DNA ladder (marker) is loaded in a first well. Electrophoreses run at 80 volt/cm for 1hr. Gel was visualized with UV transilluminator and photographed by using digital Camera (Mishera *et al*., 2009).

Detection of virulence factors

Hemolysin production was detection according to the method of Baron *et al*. (1994)). Colonization factor antigen (CFA)was detection according to the method of Ofek *et al*. (1977). Detection of biofilm formation : tissue culture plate method (TCP)was carried out according to Christensen *et al*. (1985). L. form detection of *S. typhi* was detection according to the method of Casadesus (2012). Flagellar swarming variation production was detection according to the method of Darnton *et al*. (2010).

Results and Discussion

***Salmonella typhi* identification :** Microscopically *S. typhi* appeared gram negative bacilli, peritrichous flagellated, motile, non sporulating forming bacteria , encapsulated with small polysaccharide capsule. Biochemically all *Salmonella enterica serovar typhi* isolates gave negative result for haemolytic on blood agar and showed pink colour colonies with black centre on XLD agar as in fig. 1(A).

All *S. typhi* isolates were able to ferment-arabinose but not xylose, the isolates were aerobic and facultative anaerobic, it was grown on simple laboratory media in temperature optimally at 37C° and required enrichment medium such as amino acids or vitamins. *Salmonella typhi* were isolated from different clinical specimens using specific media such as MacConkey agar, XLD and S-S agar.

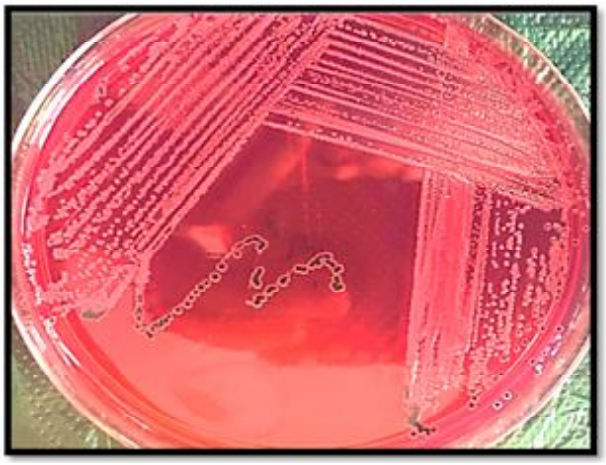
The result obtained showed that the *S. typhi* appeared on S-S agar is highly selective medium formulated to inhibit the growth of most *E. coli* form organisms and

Table 1 : Sequences and Product size of the primer.

Primer type	Primer sequence (5'-3')		Product size(bp)	References
FliC-d	F	ACTCAGGCTTCCCGTAACGC	763	(10)
	R	GGCTAGTATTGTCCTTATCGG		

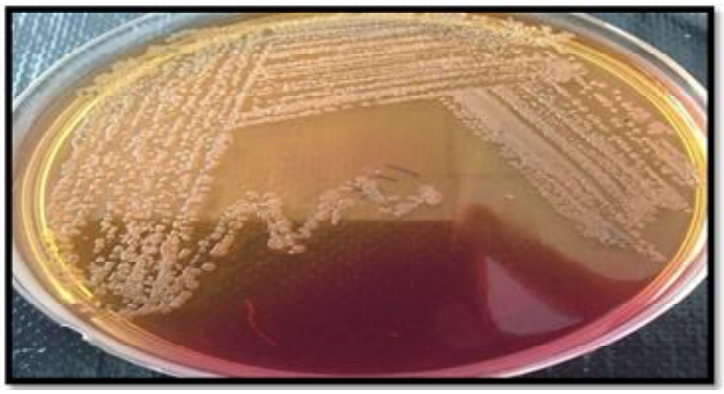
Table 2 : PCR program that apply in the thermocycler.

Gene Name	Temperature (°C) / Time					CyclesNumber
	Initial Denaturation	Cycling Conditions			Final Extension	
		Denaturation	Annealing	Extension		
Flic-d	95/5 min	94/35 s	55/30 sec	72/40 sec	72/5 min	35



A: Growth of *S. typhi* on XLD agar.

B: Growth of *S. typhi* on S-S agar.



C: Growth of *S. typhi* on macConkey agar

Fig. 1 : *S. typhi* colonies on different culture media.

permit the growth of species of *Salmonella* and *Shigella* from environmental and clinical specimens as in fig. 1(B).

The present study revealed that *S. typhi* colonies of most strains were moderately large 2-3 mm in diameter after 24 hr. at 37C°. The colonies of *Salmonella typhi* on MacConkey agar appeared after 18-24 hour at 37^0C were pale yellow, 1-3 mm in diameter and easily distinguished from the pink red colonies of lactose fermenting commensally coliform bacilli e.g. *Escherichia coli* colonies (none lactose fermentation), slightly mucoid less than *klebsiella* with regular edges as in fig. 1(C).

IMVC tests was used to differentiated genus *S. typhi* from *Shigella* and *Citrobacter*. The results showed that *S. typhi* isolates were gave negative result to simmons citrate, voges proskauer, oxidase tests and positive to H_2S production and catalase test, all isolates of *S. typhi* were positive to methyle red test. In triple sugar iron slants, both the butt and slant turned into yellow and red colour respectively indicating the fermentation of glucose alone and no production of acid in the butt.

The *S. typhi* isolates showed production of hydrogen sulphide and no gas production in TSI. Isolates were negative for oxidase test, indole production, urease production and citrate utilization. All the result (morphology and cultural) were identical with Nalbantsoy (2012).

The results show that almost isolates of *S. typhi* carrying *FliC-d* gene that is characteristic of *S. typhi*, 35 (70%) positive result include that 23 (65.7%) isolates was positive from gall bladder tissue, 11 (31.4%) from stool and 1 (2.8%) from blood.

This result is associated with Khan *et al*. (2012), who found out of 80 suspected typhoid fever cases, flagellin gene (*fliC-d*) was detected by PCR in 56 (70%) cases which corresponds with the result of similar study done in Bangladesh, where PCR was positive in 88.7% of suspected typhoid fever cases. The reported positivity rate of PCR is (65%-71.9%) in different studies (Khan *et al*., 2012).

The results obtained by VITEK system is the same which obtained by PCR technique, This results similar with the results of (20) who found the positive result was

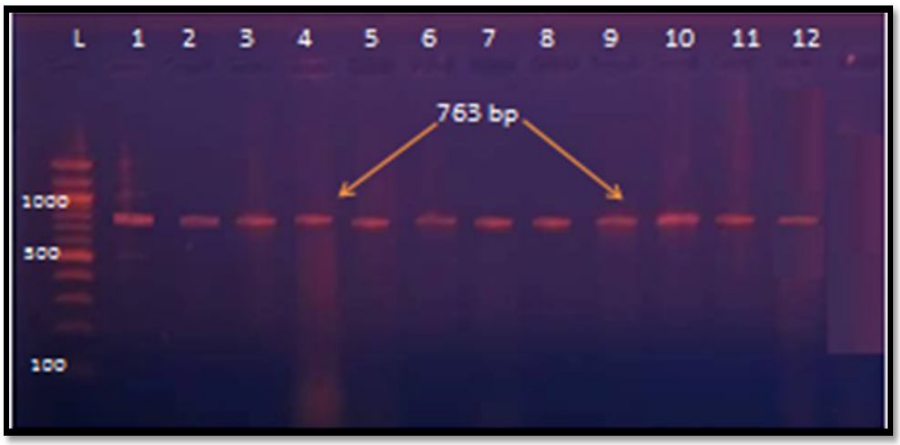


Fig. 2 : Ethidium brmide-stained agarose gel electrophoresis of PCR amplification products of *S.typhi* isolates that amplified with *FliC-d* gene primers with product 763 bp for 1 hr. at 80volt/cm.

Table 3 : Virulence factors of *S. typhi* isolates.

Virulence factors		*S. typhi* No.= 35			
		Positive isolates		Negative isolates	
		No.	(%)	No.	(%)
Hemolysin		0	0	35	100
CFA	CFA / I	32	91.4	3	8.5
	CFA / II	6	17	29	28.8
	CFA/III	11	31	24	68.5
Biofilm Formation	Strong biofilm	11	31	24	68.5
	Moderate biofilm	21	60	14	40
	Weak biofilm	3	8.5	32	91.4
L. form detection		25	71.4	10	28.5

Table 4 : Classification of bacterial biofilm formation by TCP method.

Mean of OD value at 630nm	Biofilm formation
<0.120	Non
0.121.240	Moderately
>0.240	High

65 (32.5 %) from both Vitek system and PCR technique (fig. 2).

Virulence factors determination (Virulence Factors of *S. typhi* associated with gall bladder carrier)

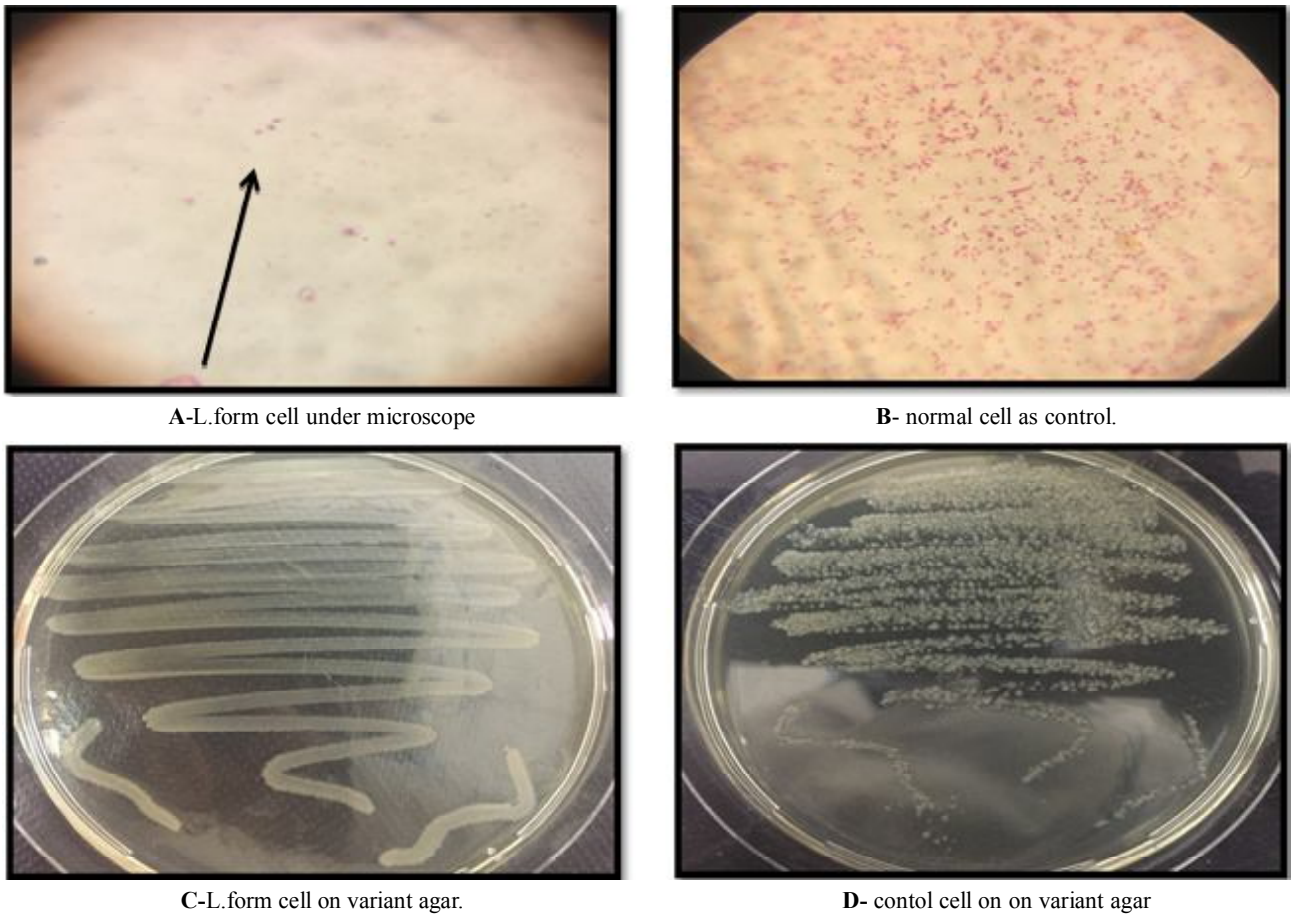
1. Hemolysin Production : The results of virulence factors of *S. typhi* isolates, showed that all *S. typhi* did not produce hemolysin as in the table 3. This results was agree with the findings of Israa (2017).

2. Colonization Factor Antigen (CFA) : Table 3 shows 11(31%) of isolates were had ability to produce CFA / III, while the ability of isolates to produce CFA / II were less 6 (17 %) . The high production of CFA, appeared to produce CFA / I, 32 (91.4%) of *S. typhi* isolates were producing this factor. CFA/ III and CFA/II also were found in isolates of *S .typhi* but in percentage less than that in CFA/I. This results agree with the findings of (20) that shows 31% of isolates were had ability to produce CFA / III, 15% had ability to produce CFA / II and I, 92% of *S. typhi* isolates were producing CFA I.

Fimbriae are assumed to play critical roles in attachment to epithelial cell surfaces. Binding to specific host receptors, fimbriae mediates the bacterial colonization, host cell signaling. Fimbrial adhesins determine the fate of the bacterial pathogen in the host as well as the progress of the corresponding disease process. Type-1 fimbriae also play an important role in deciding the virulence of the organism. Experiments conducted by Jaroni indicated that a mannose-resistant haemagglutinin was required for the attachment of *Salmonella* to target cells (Jaroni, 2014). The present result assumed important role of fimbriae 1 in attachment to epithelial cell surface (mediates the bacterial colonization) and deciding the virulence of the *S. typhi*.

The relationship between mannose – sensitive hemagglutinin (MSHA) or type 1 fimbriae and pathogenisity of bacteria was established from adherence of bacteria in mucous surfaces or epithelial cells of gastric tract. CFA/II was appeared that the lower presence among isolates. This factor causes agglutination of chicken blood, and act to adhere of bacteria with specific and complex carbohydrate receptors of epithelial cells of small intestine (Nowsheen and Jain, 2008).

3. Detection of Biofilm Formation : Over the past two decades, bacterial biofilms have been increasingly implicated as burdens to food and public safety worldwide, and are broadly defined as structured communities of



A-L.form cell under microscope

B- normal cell as control.

C-L.form cell on variant agar.

D- contol cell on on variant agar

Fig. 3 : L. form of *S. typhi* isolates.

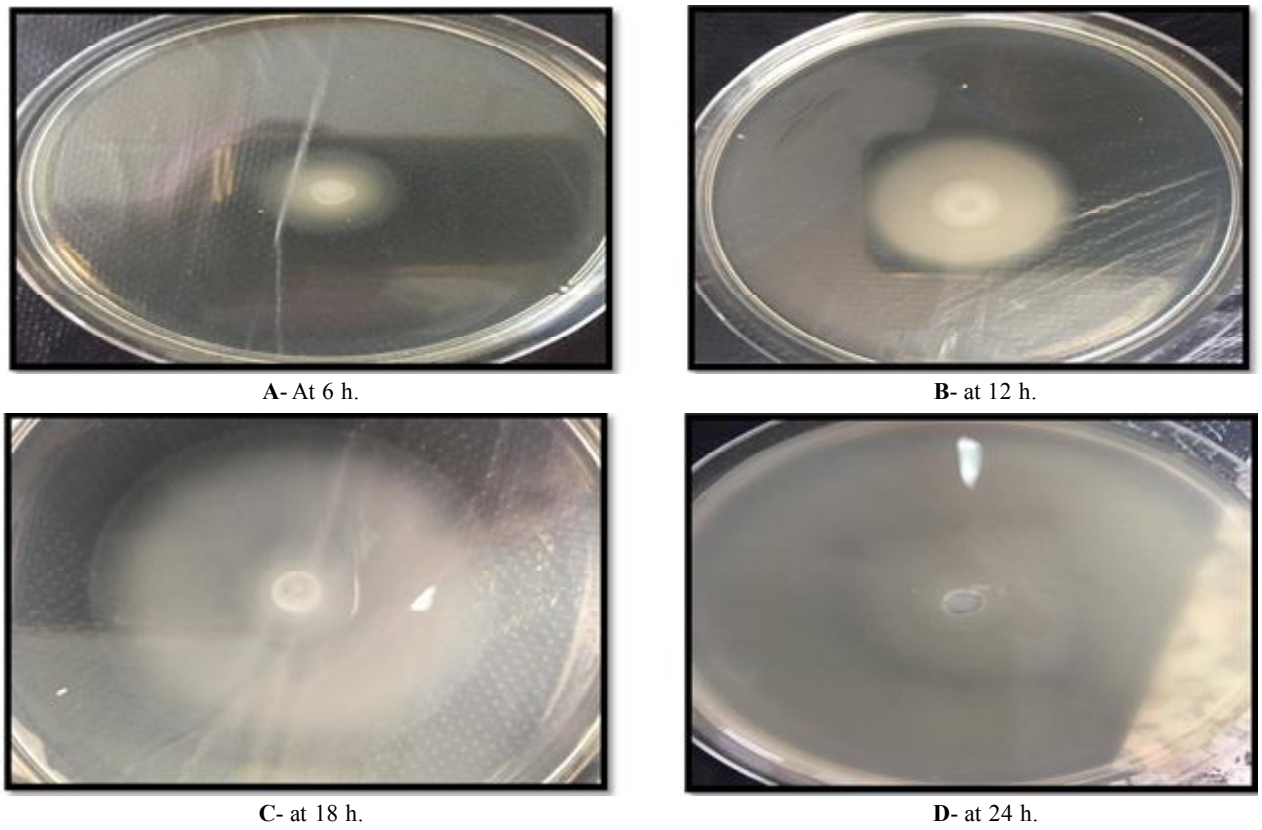
microorganisms that adhere to each other and to inert or live substrates by a self-produced polymeric matrix (Monds and O'Toole, 2009). They demonstrate heightened resistance to immune host responses, antibiotics, nutrient stress and disinfectants which enhances their spread and persistence inside and outside the host, thus being very difficult to eradicate (Tabak *et al*., 2009; Hoiby *et al*., 2010).

Biofilm formation occurs in sequential, highly regulated stages that begin with adherence of free-swimming, planktonic bacteria to a surface. Subsequent biofilm maturation is characterized by the production of a self-initiated extracellular matrix composed of exopolysaccharides, proteins and nucleic acids; that encase the community of microorganisms and provide structure and protection (Monds and O'Toole, 2009). Planktonic cells from this sessile, matrix-bound population are continuously shed, which can result in reattachment and fortification of the biofilm or release of the organism into the environment (Pasmore and Costerton, 2003). Typically considered as a response to stress, biofilms have been implicated in many chronic and acute infections. Approximately 80% of all bacterial infections are related to biofilms (Hall-Stoodley and Stoodley, 2009).

The estimation of biofilm production of *S.typhi* isolates was done according to the value in the table 4. A total isolate *Salmonella typhi* were 35 for their ability to produce biofilm. According to the table 3, which distribute *S. typhi* of biofilm formation to 11 (31%) of strong biofilm formation,21(60%) moderate biofilm formation and 3 (8.5%) weak biofilm formation. This results was agree with the findings of (21) that found 9 (30%) of *S.typhi* form strong biofilm, 19(63.3%) form moderate biofilm formation and 2 (6.6%) form weak biofilm.

4. L. Form Detection : Multiple studies have shown that when one of the Beta-lactam antibiotics (a class of antibiotics that includes penicillin) are applied to wild-type bacteria in a petri dish, small colonies of L-form bacteria form on the edges of the plate. Treatment with penicillin does not merely select for L-forms (which are penicillin resistant) but actually induces L-form growth (Casadesus, 2007).

Table 3 shows 25(71.4%) of isolates were had ability to produce L. form. This results was agree with the findings of Shaimaa (2003) that found 82.3% of isolates



A- At 6 h. **B-** at 12 h.

C- at 18 h. **D-** at 24 h.

Fig. 4 : Swarming of S. typhi isolates.

had ability to lose their cell wall and produce L. form after culturing on special media was prepared for this target. After staining with gram stain and examining with light microscope the bacterial colonies appear as spherical or ovoid shape and agglutinated with each other (Kalaivani *et al*., 2014) as in fig. 3.

5. Flagellar Swarming variation : Swarmer differentiation can also be coupled to increase expression of important virulence determinants in some species. Besides that, swarming is also linked to biofilm forming ability in bacteria, which serve as another important virulent factor of human pathogens (Murray *et al*., 2010). In fact, bacteria move in various modes, including swimming and surface swarming. Swimming occurs when bacterial cells move in the aqueous environment (low agar concentration) while swarming motility is a collective behaviour of bacterial cells associated with migration on semi-solid surfaces.

Unlike the classical swimming motility in aqueous environment, vegetative cells must first differentiate into elongated and hyperflagellated swarmer cells to migrate on the surface (Kalaivani *et al*., 2014).

In this study, all of the tested strains for swarm motility assay were measured more than (2 cm-8 cm) after 24 hrs of incubation. Strain showing migration of cells (increase in colony diameter) were considered as positive swarming. Initially when this work was done, the rates of the migration of bacteria from the point of inoculation were measured at 0, 6, 12, 18 and 24 hr.

However in this study, at 0 hr, *S. typhi* isolates showed no migration. At 6 hr and 12 hr, *S. typhi* isolates showed similar rate of migration. After 18-24 hrs, most of the *S. typhi* isolates colonised the entire surface of the petri plate and reached the maximum size.

This results was agree with the findings of Kalaivani *et al.* (2014) that showing swarm motility assay were measured more than 1.5 cm–7.7 cm after 24 hrs of incubation (fig. 4).

References

Angham, J. M. A. (2015). Identification of *Salmonella typhi* Isolated from Patient's with Typhoid Fever Immunomolecular Study. A Thesis Submitted to the Council of faculty of Science University of Kufa In Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy of Science in Biology / Microbiology.

Baron, E. J., I. R. Peterson and S. M. Finegold (1994). Baily and Scott's. Diagnostic Microbiology. **9**th ed. Company. USA.

Bhan, M. K. R. Bahl and S. Bhatnagar (2005). Typhoid and paratyphoid fever. *Lancet*, **366** : 749–762.

Christensen, G. P., W. A. Simpson, J. J. Younger, L. M. Baddour, Barrett, D. M. Melton and E. H. Beachey (1985). Adherence of coagulase –negative *Staphylococci* to plastic tissue culture plates aquantitative model for the adherence of *Staphylococci* to medical derices. *J. Clin. Microbiol.*, **22** :996-1006.

Casadesús, J. (2007). Bacterial L-forms require peptidoglycan synthesis for cell division. *BioEssays : news and reviews in molecular, cellular and developmental biology*, **29(12)** :1189-91.

Chang, G-N., C-S. Chen and K. Chieh Ho (2007). Molecular cloning and expression of a phase I flagellin gene from *Salmonella enterica serovar Cholerasuis*. Taiwania. **53(3)** :201-209.

Collee, J. G., R. S. Miles and B. Watt (2011). Tests for the identification of bacteria. In: Collee JG., Fraser AG., Marimion BP., Simmons A., editors. Mackie and McCartney practical medical microbiology, 14th ed. London: Churchill Livingstone . 131-49.

Crawford, R. W., R. Rosales-Reyes, L. Ramirez-Aguilar Mde, O. Chapa-Azuela, C Alpuche-Aranda and J. S. Gunn (2010). Gallstones play a significant role in *Salmonella spp*. gallbladder colonization and carriage. *Proc Natl Acad Sci USA*, **107** : 4353-4358.

Darnton, N. C., L. Turner, S. Rojevsky and H. C. Berg (2010). Dynamics of bacterial swarming. *Biophys J*., **98(10)** : 2082–2090.

Gonzalez-Escobedo, G. and J. S. Gunn (2013). Gallbladder epithelium as a niche for chronic *Salmonella* carriage. *Infect. Immun*., **81** : 2920–2930.

Hall-Stoodley, L. and P. Stoodley (2009). Evolving concepts in biofilm infections. *Cellular Microbiology*, 11 : 1034-1043.

Hoiby, N., T. Bjarnsholt, M. Givskov, S. Molin and O. Ciofu (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*, **35** : 322-332.

Israa Abdul Ameer Flayyih (2017). Molecular characterization of MDR *Salmonella* sp. Isolate from typhoid patients in Al.Najaf Al.Ashraf province-IRAQ.A Thesis Submitted to the Council of faculty of Science University of Kufa In Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy of Science in Biology.

Ibarra, J. A. and O. Steele-Mortimer (2009). *Salmonella* –the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cell Microbiol.*, **11** : 1579 – 1586 .

Jaroni (2014). *Salmonella* / *Salmonella typhi* Encyclopedia of Food Microbiology (Second Edition), 349–352.

Kalaivani Kalai Chelvam1, Lay Ching Chai1 and Kwai Lin, Thong1 (2014). Variations in motility and biofilm formation of *Salmonella enterica serovar typhi*. *Kalai Chelvam. Gut Pathogens* **6** : 2.

Khan, B. N., G. A. Harish, N. S. Menezes, Acharya and S. C. Parija (2012). Early diagnosis of typhoid fever by nested P.C.R. for flagellin gene of *Salmonella enterica serotype typhi*. *Indian J Med Res*., **136** : 850-854.

Levy, H., S. Diallo, S. M. Tennant, S. Livio, S. O. Sow, M. Tapia, P. I. Fields, M. Mikoleit, B. Tamboura, K. L. Kotloff, R. Lagos, J. P. Nataro, J. E. Galen and M. M. Levine (2008). PCR method to identify *Salmonella enterica serovars typhi*, *Paratyphi A* and *Paratyphi B* among *Salmonella* Isolates from the blood of patients with clinical enteric fever. *J Clin Microbiol.*, **46(5)**:1861-1866.

Monds, R. D. and G. A. O'Toole (2009). The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol*., **17** : 73– 87.

Mishera, V., V. L. Nag, R. Tandon and S. Awsthi (2009). Response surface methodology-based optimization of agarose gel electrophoresis for screening and electropherotyping of rotavirus. *Appl. Biochemi ND Biotech*.

Murray, T. S., M. Ledizet and B. I. Kazmierczak (2010). Swarming motility, secretion of type 3 effectors and biofilm formation phenotypes exhibited within a large cohort of *Pseudomonas aeruginosa* clinical isolates. *J. Med. Microbiol*., **59(5)** : 511–520.

Nalbantsoy, A. (2012). Prevention and identification of *Salmonella*, **4** : 1614-1622.

Nowsheen, H. and S. K. Jain (2008). Characterization of an outer membrane protein of *Salmonella enterica serovar typhi* that confers protection against typhoid clinical and vaccine immunology, 1461–1471.

Ofek, I., D. Mrelman and N. Sharon (1977). Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptor. *Nature* (London), **265** : 623 – 625

Parry, C. M., T. T. Hien, G. Dougan, N. J. White and J. J. Farrar (2002). Typhoid fever. *New England Journal of Medicine*, **347(22)** : 1770-1782.

Pasmore, M. and J. W. Costerton (2003). Biofilms, bacterial signaling, and their ties to marine biology. *J Ind Microbiol Biotechnol*., **30** : 407-413.

Schirmer, B. D., K. L. Winters and R. F. Edlich (2005). Cholelithiasis and cholecystitis. *J Long Term Eff Med Implants*, **15** : 329-338.

Sambrook, J. and R. W. Russell (2001). Molecular cloning : A laboratory manual, **3**rd ed. Cold spring harbor laboratory press, cold spring harbor, N.Y.

Shaimaa, J. M. Al- Sultany (2003). Biology of cell wall defective bacteria associated with cryptic enteric fever in human. A thesis Submitted to the college of science. University of Babylon In A partial fulfillment of the requirements for the degree of Master In Biology/ Microbiology.

Tabak, M., K. Scher, M. L. Chikindas and S. Yaron (2009). The synergistic activity of Triclosan and Ciprofloxacin on biofilms of *Salmonella typhimurium*. *FEMS Microbiology Letters*, **301** : 69-76.

World Health Organization (2006). 6th International Conference on Typhoid Fever and other salmonelloses). Geneva, WHO. Ref Type: Pamphlet.