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In Vitro Relationship Between Differing Levels of Virulence and Antibiotic Resistance in *Salmonella typhi*

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ABSTRACT

Typhoid is a major health problem. Its causative agent, *Salmonella typhi*, can cause severe disease in very low concentration. This high invasiveness has been a subject of great interest. Unfortunately, animal models cannot be used for this strict human pathogen. In vitro production of a lipopolysaccharide material, called biofilm, has been related to virulence in *Salmonella enteritidis*. In the present study, different clinical isolates of *Salmonella typhi* were evaluated for biofilm production and its relationship to drug resistance. Thirty strains, including 16 drug resistant strains, were studied. Maximum levels of biofilm production, ++++ and +++, were produced by 81.82 and 75% drug resistant strains respectively. It is clearly indicted that there is a relationship between virulence (represented by level of biofilm production) and drug resistance.

Key Words: Typhoid; Virulence; Antibiotic resistance; *Salmonella typhi*

INTRODUCTION

Salmonella typhi is the etiologic agent of human typhoid. Typhoid fever continues to be a major public health problem in developing countries. There are many interrelated factors, including increased urbanization, inadequate supplies of clean water, antibiotic resistance, the variable efficacies of vaccine preparations, and the increased regional movements of large number of migrant workers.

Until the mid 1970s chloramphenicol was the undisputed drug for the treatment of typhoid fever and it reduced the mortality from 10% to < 2%, but it can no longer be regarded as the first line drug for typhoid fever (Bernard *et al.*, 1999). Since 1989, outbreaks caused by strains of *Salmonella typhi*, resistant to chloramphenicol, ampicillin, and trimethoprim and with additional resistance to streptomycin, sulfonamides, and tetracyclines (ACSSuTTm) have been reported in many developing countries, especially in Pakistan (Karamat, 1990) and India (Parkash & Pillai, 1992) and in several countries in South Asia (Tinya-Superable *et al.*, 1995) and both North and South Africa (Mourad *et al.*, 1993).

During its passage through the body, *Salmonellae* must be able to tolerate several environments with hostile conditions such as low gastric pH and the antimicrobial actions of peptides secreted by the enterocytes. In this journey, an intimate interaction takes place between the bacteria and the host cells through a series of biochemical signals (Finlay *et al.*, 1995). It is generally assumed that virulence in *Salmonella spp*. and many other micro-organisms is an induced property. Accordingly, osmolarity, oxygen tension, pH, the concentration of free iron and magnesium and many other factors significantly influence the phenotype and the expression of invasion genes (Lee & Falkow, 1995; Garcia *et al.*, 1996; Galan, 1996).

Virulent isolates of *Salmonella enteritidis* produce a *Salmonella typhi* like lipopolysaccharide and the amount of this antigen polysaccharide was found to be twice in amount in virulent as compared to the avirulent strain (Rehman *et al.*, 1997). Difference in virulence and invasiveness of different strains of *Salmonellae* has already been found with a variety of animal models (Hinton *et al.*, 1990; Gast & Benson, 1995; Humphrey *et al.*, 1996; Mckee *et al.*, 1996). However as Humphrey *et al.* (1996) pointed out, there is a need to be able to differentiate between virulent and avirulent strains of *Salmonellae* without necessarily resorting to the use of animal model. Solano *et al.* (1998) have reported an *in vitro* method, based on biofilm production, for discriminating strains of *Salmonella enteritidis* according to level of virulence, and showed its reliability by comparison with a chick model.

We thought that establishment of an in vitro method would be extremely useful in case of *Salmonella typhi* because these bacteria do not cause disease in laboratory animals. Therefore, we based our study on similar lines for individualizing different *Salmonella typhi* strains according to the virulence, and to establish a relationship, if any, with drug resistance.

MATERIALS AND METHODS

Bacterial strains: A total number of 30 strains of *Salmonella typhi* were studied. These were isolated from blood samples of patients from different parts of the country. Strains were preserved in 10% dimethyl sulfoxide and were kept at –20°C till further use.

Identification and confirmation of strains: For the identification of *Salmonella typhi* isolates different biochemical tests were performed (Ewing, 1986). Confirmation was made

by polymerase chain reaction. For PCR, DNA from bacterial cells was extracted by conventional phenol/chloroform method, followed by RNase treatment for the removal of contaminating RNA. The conditions were the same as described by Haque *et al.* (1999). Two sets of primers, which target the flagellin gene of *S. typhi*, were used for performing regular and nested PCR. For regular PCR the primers were: ST1 (5'-TATGCCGCTACATATGATGAG -3') ST2 (5'-TTAACGCAGTAAAGAGAG -3'). For nested PCR the primers were: ST3 (5'- ACTGCTAAAACCACTACT -3'), ST4 (5'- TGGAGACTTCGGTCGCGTAG -3'). In each case, 100 µl DNA amplification mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 150 pmol of each primer, 95 nmol of each dNTP, 1U *Taq* polymerase, 20 µl of DNA mixture (1: 50 dilution of extracted DNA) and distilled water to make the volume. The amplification mixture was subjected to 30 cycles of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C each. It was followed by 7 minutes at 72°C (Perkin-Elmer Gene Amp 2400). The amplification products were fractionated electrophoretically on 2% agarose gels at 100V for one hour.

Sensitivity Testing: Antibiotic sensitivity was checked by using the disc diffusion method according to the recommendation of National Committee for Clinical Laboratory Standards (1990).

In vitro Aggregation and Adherence: This *in vitro* study was conducted using the methods reported by Solano *et al.* (1998) for discriminating strains of *Salmonella enteritidis*. Some modifications were made in the medium preparation and experimental conditions. Adherence test media (ATM) contained 60 mM NaCl; 20 mM KCl; 111mM Glucose; 30 mM $NaHCO_3$. Supplemented ATM contained 20 mM NH_4Cl, 40 mM Na_2HPO_4, 50 mM $(NH_4\}H_2PO_4$, 50 :M $CaCl_2$, 999 :M $MgCl_2$, 86 :M $FeCl_3$ and 40 mM Na_2SO_4. Strains were retrieved from stock cultures into trypticase soy broth (TSB) from where they were transferred on MacConkey agar plates and incubated overnight at 37°C. Several colonies were transferred to 20 mL TSB and were incubated at 37°C till the optical density ($O.D_{590}$) reached 0.4. After washing, pellet was suspended in ATM till the ($O.D_{590}$) reached 0.125. This suspension was transferred to test tubes. The test tubes were placed in a shaker at 160 rpm and 37°C temperature. The result was noted after 24 hours. The level of biofilm production was recorded as -. +. ++, +++, and ++++ on visual differences (Fig. 1).

RESULTS

Drug sensitivity: The drugs used were chloramphenicol, trimethoprim, and ciprofloxacin. Out of 30 tested strains, 16(53.33%) were resistant to one or more drugs, whereas 14 (14.67%) were drug sensitive. Among the drug resistant strains, 4(25%) and 11(68.75%) strains were resistant to chloramphenicol and trimethoprim respectively. None of the strains showed resistant to ciprofloxacin.

In vitro biofilm production: No biofilm production was seen in ATM, at low temperature and without shaking, but when 37°C temperature and 160 rpm shaking was provided, there was some biofilm production. However, maximal biofilm production was seen when ATM was supplemented with salts.

Fig. 1. Biofilm production by *Salmonella typhi.* From left + + + +, + + +, + +, +, and – levels of biofilm respectively

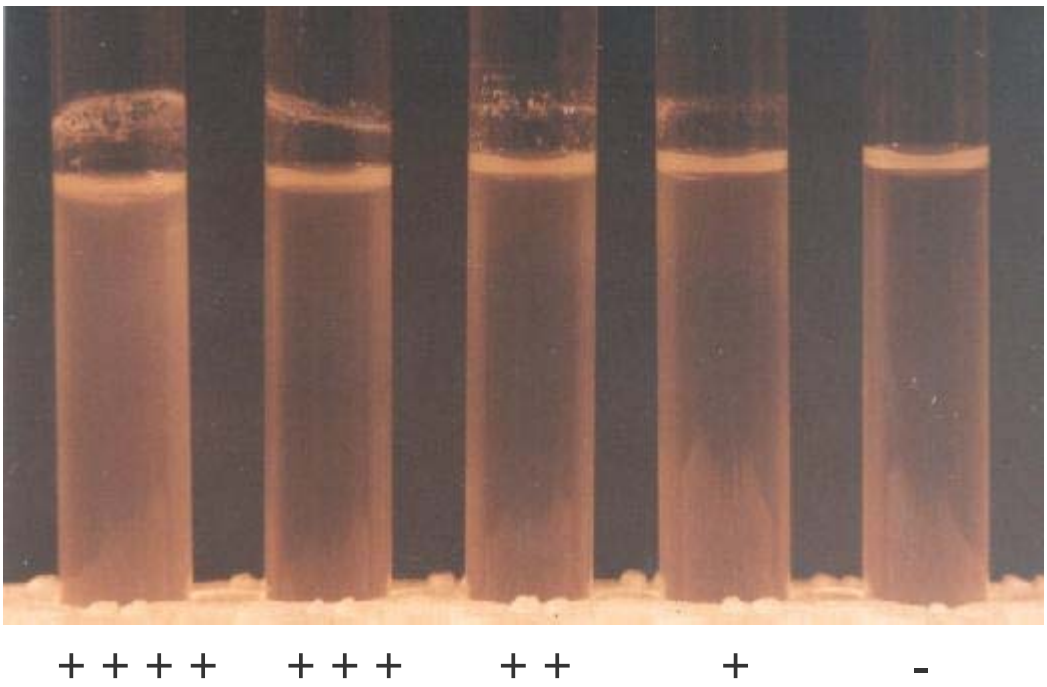


Table I. MIC of supplementing salts in the formation of biofilm

Supplementing Salts	Minimum inhibitory concentration in mg/mL
NH_4Cl	6.6
Na_2HPO_4	6.6
$(NH_4)H_2PO_4$	3.33
$CaCl_2$	2.5
$MgCl_2$	0.02
$FeCl_3$	0.2
Na_2SO_4	6.6

When salt concentrations were increased, the biofilm production diminished and finally stopped. The MICs of supplementing salts are given in Table I.

Levels of biofilm production: Differing levels of biofilm production were observed. Only one strain failed to produce biofilm; +, + +, + + +, and + + + + levels of biofilm production were shown by 23.33, 23.33, 13.33, and 36.6% strains respectively (Fig. 2).

Relationship between virulence and antibiotic resistance: The results are summarised in Table II. It is evident that antibiotic resistant strains produced greater amount of biofilm. Among the strains that produced maximum amount (+ + + +) of biofilm, 81.82% were drug resistant. Similarly 75% of strains that showed + + + level of biofilm production were drug resistant.

DISCUSSION

Typhoid fever is very common in developing countries, where it remains a major health problem despite the great advances in diagnosis and treatment. Reduction in mortality and morbidity due to the infection with *Salmonella typhi* requires effective antimicrobial chemotherapy. This task is made more difficult by the fact that *Salmonella typhi* is unique in being exceptionally virulent. Even 8-10 bacteria/mL can cause severe disease. It can be hypothesised that the present disease causing strains of *Salmonella typhi* have emerged through natural selection and are not only more difficult to treat but also more virulent. So an effort to find a relationship between drug resistance and virulence, if any, is very much a need of the hour for devising future strategies against this lethal disease.

Chloramphenicol, trimethoprim, streptomycin, and ampicillin have been the commonly used drugs against typhoid but the preferences have changed over the years and this organism has become resistant to many drugs. There is evidence that its genomic DNA has undergone rearrangements (Liu & Sanderson, 1995), and this may be the reason for development of drug resistance. In present study we found 16 out of 30 (53.33%) isolates as multiple drug resistant (Table II).

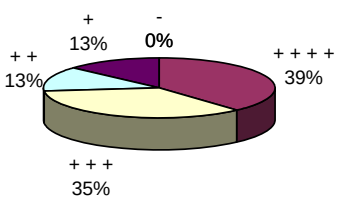
We found only 25% resistance (4/16) to chloramphenicol, which is much lower in comparison with other studies. This may be due to demographic and geographic differences. Unlike chloramphenicol, we found maximum resistance (68.75%) against trimethoprim. Mirza *et al.* (1995) reported resistance to flouroquinolones among some strains of *Salmonella typhi* in Pakistan. But elsewhere, researchers found total susceptibility of this organism towards flouroquinolones (Panigrahi *et al.*, 1996; Bhat *et al.*, 1998). We also found complete susceptibility to one of the leading flouroquinolones, ciprofloxacin. The resistance observed by Mirza *et al.* (1995) may be because of use of flouroquinolones of lesser potency in comparison with ciprofloxacin. Present results are also supported by the findings of Bernard *et al.* (1999). We think that ciprofloxacin can be a drug of great importance against typhoid.

Current results for the most part, are consistent with those of other researchers. Only major discrepancy was seen in case of chloramphenicol, the first drug used for treatment of typhoid. The level of resistance in our strains was much less when compared with some recent reports. As already

Table II. Biofilm production by susceptible and MDR isolates of *Salmonella typhi*

S. No	Strain	Susceptible/MDR	Level of biofilm
1	ST1	Susceptible	-
2	ST2	Susceptible	+ +
3	ST3	Susceptible	+
4	ST8	Susceptible	+ +
5	ST9	Ctx	+ + + +
6	ST10	Susceptible	+
7	ST13	Susceptible	+ + + +
8	ST15	Susceptible	+
9	ST17	Susceptible	+ +
10	ST20	Susceptible	+ +
11	ST23	Susceptible	+ +
12	ST24	AtmZoxSmCfm	+ + + +
13	ST28	AtmCfpCmSmTm	+
14	ST32	Susceptible	+ + +
15	ST35	CfpVTm	+ + +
16	ST36	Susceptible	+
17	ST37	Cfp	+ + +
18	ST39	AtmCmTm	+ +
19	ST42	Susceptible	+ + + +
20	ST43	Susceptible	+
21	S1	SmClrTm	+ + + +
22	S2	ClrTm	+ + + +
23	S3	AtmZoxCtxCfm	+ + + +
24	S4	SmClrTm	+ + + +
25	S5	Tm	+ + + +
26	S6	SmClrTm	+ + +
27	S7	SmClrTm	+ + + +
28	S10	Clr	+ +
29	S11	CmSmClrTm	+
30	S12	AtmCmSmTmCfm	+ + + +

Fig. 2. Relationship between level of virulence and antibiotic resistance



discussed, this may be attributed to demographic variations. In our experiments, we exploited the properties of adherence and virulence present in *Salmonella typhi.* Although we do not exactly know the importance of aggregation and adherence to pathogenicity; the ability to adhere to epithelial cells does play a significant role in mucosal colonization. This is because *Salmonella typhi* are enteroinvasive pathogens that require attachment to the luminal surface to counter the peristaltic cleansing motion of the intestine and to initiate penetration through the mucus. Several reports have suggested that contact with eukaryotic cells or even with glass surfaces could be a signal that triggers the transcription of virulence genes. These virulence genes are necessary for efficient adherence and entry of these organisms into cultured epithelial cells. These genes are also induced when grown under the conditions of high osmolarity (0.3 M NaCl). Expression is optimal at pH 6.5 and strongly reduced at low pH (5.0). Moreover, the transcription of genes is initiated under anaerobic conditions (Galan & Curtis, 1991; Altmeyer *et al.*, 1993). Our study encompassed these considerations as well.

Our experiments clearly showed that at low temperature and without shaking, there was no adherence to glass wall and no biofilm production took place. The phenomenon was temperature dependent and was enhanced at 37°C. It was less pronounced at 20°C, and was not visible at 4°C. A variety of attractive forces are probably involved between the biofilm and the glass and include electrostatic and hydrophobic interactions. Significant biofilm production was not observed in static cultures or when incubation was performed at low turbulence under normal shaking conditions. The observation that the turbulence was important could imply that the phenomenon was related to aeration and contact. In fact the biofilm was produced in the area of the surface where the shear forces are maximum, that is, on the border of the cone created by shaking.

In vitro incubation of strains in ATM revealed the ability of some of them to adhere to glass wall at the interphase between the medium and the air and in some strains, the biofilm was seen. Although in many strains there was no biofilm production, on examination by ordinary microscope, cell aggregation was observed even when they were not attached to the glass. This phenomenon became visible after 17 h. This biofilm was stable and it remained attached to the glass even after vigorous shaking.

We observed maximum biofilm production when we added supplementary salts. Our MIC of salts (Table I) was different from that determined by Solano *et al.* (1998). This discrepancy can be explained by keeping in view the fact that *Salmonella typhi* is a strictly human pathogen where as *Salmonella enteridis* can survive in animal models. Although both produce similar lipopolysaccharide, receptors for binding to epithelial cell lining of intestine are different, so it is logical to assume that there must also be a difference in conditions for induction of virulence genes. The quantitative influence of lipopolysaccharides can be explained by the findings of Rehman *et al.* (1997) who concluded that the virulent strains had twice the amount of O antigen polysaccharides in comparison with avirulent strains.

From the present study, it is clear that multiple drug resistance is not an isolated problem. The strains which are naturally endowed with greater facility of virulence survive better against the stress of natural selection and the main instrument of their survival is the ability to develop drug resistance. We are confident that our findings will provide new dimensions to the understanding of the pathogenicity of *Salmonella typhi* which in turn will make the control of typhoid more practical.

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