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Factors influencing production of *Salmonella* enterotoxin (Stn)

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Abstract

Salmonella enterotoxin (Stn) is an important food borne agent affecting public health. The study evaluated the influence of different physico-chemical factors on *in-vitro* production of Stn. The Stn production was tested by Staphylococcal Co-agglutination test. The highest secretion of Stn was found in cell free culture supernatant (CFCS) of tryptic soya broth culture followed by brain heart infusion broth and biken broth, while the least production was observed in nutrient broth. Growth with constant shaking (200 rpm) had positive influence on Stn production. Maximum Stn production was also recorded during an incubation temperature of 32 °C to 37 °C than 22 °C and 27 °C while minimum was at 42 °C. Peak production was at 18 to 24 hours incubation which sustained beyond 48 hours. The pH (6-8) influenced Stn production and decreased at pH beyond 9. Bacterial cells sonicates yielded more Stn than CFCS. Results showed that the physico-chemical factors influence the Stn production.

Keywords: *Salmonella*, enterotoxin, Stn, CFCS, cell sonicates

Introduction

Diarrhoeal diseases are the most common illnesses resulting from unsafe food, 550 million people falling ill each year, including 220 million children under the age of 5 years. *Salmonella* is 1 of the 4 key global causes of diarrhoeal diseases. While all serotypes can cause disease in humans, a few are host-specific and can reside in only one or a few animal species (WHO, 2018) [33]. Salmonellosis can be acquired through consumption of infected raw or undercooked eggs (Martelli and Davies, 2012) [20]. Despite significant advances in sanitation, provision of potable water and highly controlled food chain surveillance, transmission of *Salmonella* spp. continues to affect communities, preferentially children, worldwide (Flor *et al.* 2011) [7]. *Salmonella enterica* subsp. *enterica* remains a main cause of infection and disease in human and animals worldwide. Much of the public health and economic problem originated from diseases or infected Animals carriage (Demirbilek, 2018) [6]. The pathogenicity of *Salmonella* in induction of diarrhea is a complex phenomenon involving several pathogenic mechanisms including the production of enterotoxin (Ashkenazi *et al.* 1988; Khurana *et al.*, 1992; Rahman *et al.*, 1994; and Malik *et al.*, 1995) [1, 15, 22, 19]. Various *Salmonella* serotypes including *S. Typhimurium* (Koupal and Deibel, 1975 [16]; Baloda *et al.*, 1985; Rahman *et al.*, 1991b) [4, 23] and *S. Virchow* (Rahman *et al.*, 1991c) [25] have been shown to produce enterotoxins.

Although most of the *Salmonella* serovars were shown to produce enterotoxins (Stn), enterotoxigenicity of *Salmonella* has been found to be difficult to detect by different assays since salmonellae produce a very low level of enterotoxin when cultured by conventional methods (Baloda *et al.*, 1983b) [23]. Moreover, a major portion of Stn is retained as cell bound content, which is not released in the extracellular milieu (Kaura and Sharma, 1980 [12] and 1988) [13]. The *stn* gene is widely distributed among *Salmonella* irrespective of the serovars and sources of isolation. Since the *stn* gene is found in all the isolates it can be a viable target gene to explore the possibility and to direct detection of *Salmonella* from biological sources. (Murugkar *et al.*, 2003) [21]. The *stn* gene could be amplified in all 102 strains of 87 serovars of *Salmonella* tested and no products were detected in 57 of non-*Salmonella* strains. (Srisawat and Panbangred, 2015) [30] and All of 37 *Salmonella* from two serovars, namely Enteritidis and Typhimurium produced amplicon for enterotoxin (*stn*) gene (Chaudhary *et al.* 2015) [5].

There are some *Salmonella* strains, which produce enterotoxins but do not release extracellularly and thus may escape tests and be declared to be non-enterotoxigenic if only extracellular occurrence of Stn is tested Rahman *et al.*, 1991b) [23]. The present study was undertaken to study the effect of various factors on production of Stn *in-vitro*.

Materials and methods

Bacterial strains

Three enterotoxigenic strains of *Salmonella enterica* subsp. *Enterica* serovars *S. Typhimurium* DT096 (Stn⁺), *S. Typhimurium* DT 003 (Stn⁺), and *S. Virchow* PT 33 (Stn⁺) and a non-enterotoxigenic strain of *S. bongori* (Stn⁻) as negative control were used for the study. A strain of *Staphylococcus aureus* Cowan1 was used for coagglutination (CoA) test used for the assay of Stn. All the bacterial strains were maintained on nutrient agar slants at 4°C before use.

The cell free culture supernatant (CFCS) of *S. Typhimurium* strain was prepared according to the procedure described by Singh *et al.* (1983) [27]. The CFCS was tested for the presence of Stn by rabbit ligated ileal loop (RLIL) test and CoA test. The CFCS was subjected to partial purification of Stn by (NH₄)₂SO₄ salt precipitation and concentrated against polyethylene glycol (PEG) 6000 (Hudson and Hay, 1991) [10]. The estimation of protein was done by Lowry's method (Lowry *et al.* 1951) [18]. The antiserum used for CoA test was in rabbit against partially purified Stn of *S. Typhimurium* as per the procedure described by Honda *et al.*, (1981a) [9]. To determine the specificity and cross reactivity of the antiserum, serum agglutination test (SNT) was performed in rabbit according to the procedure as described by Jiwa (1981) [11].

Preparation of co-agglutination reagent

The CoA reagent was prepared following the method described by Kronvall (1973) [17] for detection of polysaccharide of pneumococci and as modified by Rahman *et al.* (1991a) [24] for detection of Stn. Briefly, the bacterial strain *Staphylococcus aureus* Cowan type 1 was grown on brain heart infusion broth (BHIB) at 37°C for 24 hours, centrifuged at 3000 x g for 20 minutes, washed with PBS (0.05 M phosphate buffer in 0.15 M saline solution, pH 7.2) and suspended in 0.5 per cent formalin (BDH) prepared in phosphate buffered saline. The formalized suspension was incubated at room temperature for 3 hours. After washing thrice in PBS (pH 7.2) the suspension was heated at 80°C in a water bath for 30 minutes, cooled at room temperature and centrifuged at 3000 x g for 20 minutes. A 10 per cent suspension of the sediment was made in PBS and stored at 4°C.

To 1 ml of cell suspension, 0.1 ml of antiserum against Stn was added and incubated for 1 hour at room temperature and centrifuged at 2000 x g for 30 minutes at 4°C. Finally, a 5 per cent suspension of the sediment was made in PBS (pH 7.2) and stored at 4°C.

Effect of media

The four different media viz. brain heart infusion broth (BHIB), tryptic soya broth (TSB), Biken broth (BB, Honda *et al.* 1981b) [8] and nutrient broth (NB) were used for production of CFCS.

Effect of shaking

All the strains were grown in TSB in two sets; one under shaking condition and another set in non-shaking condition.

Effect of incubation temperature

The incubation temperature under which the Stn production was studied were 22°C, 27°C, 32°C, 37°C and 42°C in shaking condition.

Effect of period of incubation

Different sets of cultures of *Salmonella* were incubated for 3, 6, 9, 12, 15, 18, 21, 24, 36 and 48 hours and Stn production was measured by CoA test.

Effect of pH

The effect of different pH levels at 5, 6, 7, 8, 9, 10 and 11, on Stn production were studied. The required pH was adjusted with 1 N HCL or 1 N NaOH, as the case may be (Sobeh and Vadehra 1983) [29].

Detection of cell-bound and cell-free content of enterotoxin

For detection of cell-free enterotoxin, CFCS from 10 ml of the 20 ml culture of each of the *Salmonella* strains were prepared. For detection of cell-bound enterotoxin, another CFCS from 10 ml culture of the *Salmonella* strains were sonicated at 4°C for 30 minutes in a vibrionic sonicator at its maximum output. The sonicated cultures were centrifuged at 15,000 rpm for 1.5 hours at 4°C. Thus, the supernatant obtained was designated as cell sonicates (CS).

Results and discussion

The dilution indices (DI) with CFCS of the TSB cultures were 0.85, 0.82 and 0.75 and BHIB were 0.75, 0.73 and 0.71 and for the *Salmonella* strains DT096, DT003 and PT33, respectively. The DI was higher in the CFCS of TSB cultures than that of the BHIB cultures. The CFCS of *S. bongori* cultures in all the media, the uninoculated TSB medium and PBS used as control inocula caused no fluid accumulation in the ligated segments in RLIL test.

The titre of Stn was highest in CFCS obtained from TSB (1:128) for both the strains of *S. Typhimurium* and 1:64 for the strains of *S. Virchow*. All of the three enterotoxigenic strains gave a titre of 1:32 in the CFCS of BB, while in NB cultures, the titre of 1:16 was found with DT096 and 1:8 with DT003 and PT33.

The study revealed that some of the growth factors were essential for the production of enterotoxin since a considerably higher amount of Stn could be detected in the CFCS from the cultures grown in enriched media like TSB and BHIB followed by BB than the CFCS from NB cultures which is a simple medium. Rahman *et al.* (1991b) [23] reported similar findings of observing more amount of toxin production in enriched media like BHI, Casamino acid yeast extract (CYE) medium than that in NB. The findings were also in agreement with that of Sedlock *et al.* (1978) [26] and Sobeh and Vadehra (1984) [28].

A two fold increase of Stn production in shaking cultures was observed in CFCS of serovars of *S. Typhimurium* and four fold increase of CFCS of serovar *Virchow* than that of the cultures incubated at steady condition. These results were in conformity with that of the other workers (Sobeh and Vadehra 1983 [29]; Rahman *et al.* 1991b) [23]. Increased production of Stn under shaking condition might be the result of increased growth of the organism facilitated by maximal utilization of the nutrients from the media due to uniform suspension of the organisms and enhanced release of enterotoxin under such condition.

The maximum enterotoxin production was observed at a temperature between 32°C and 37°C in all the cultures. However, the titre of Stn could be obtained at 27°C in case of *S. Typhimurium* strain DT096. Comparatively, a lesser amount of enterotoxin was produced at 22°C and 42°C, when the environment became hostile for the bacterial strains. A titre of only 1:8 was found at 42°C as compared to the maximum titre of 1:128 detected at 32°C and 37°C for all the strains of *Salmonella*. Similar findings were reported by Rahman *et al.* (1991b) ^[23] with *S. Typhimurium* (P/536 and P/603) strains.

Enterotoxin of *S. Typhimurium* strains could be detected as early as 3 hours of incubation, while in case of *S. Virchow*, it could be detected only after 6 hours of incubation. Although *Salmonella* enterotoxin could be detected within 3 to 6 hours of incubation, a considerable amount of toxin was produced only after 15 hours of incubation and the optimum production was observed within 18 to 24 hours of incubation, as detected by CoA test. The findings corroborated the findings of Rahman *et al.* (1991b) ^[23] who reported that the detection of *Salmonella* enterotoxin was possible as early as 1.5 to 3 hours of incubation. In the present study, the peak titre of 1:128 was maintained on incubation beyond 24 hours up to 48 hours. Similar findings were earlier reported by Rahman *et al.* (1991b) ^[23]. The results showed that toxin production was related to the number of cells in the culture (Sobeh and Vadehra 1983) ^[29]. There was maximum toxin production during the log phase of growth. During the stationary and decline phases of bacterial growth, no increase in the level of toxin production was observed, as the bacteria ceased to grow and multiply during these phases.

It was observed that Stn production occurred at pH 6 to 8, although a considerable amount of toxin was produced at pH 5 and 9 also. At pH beyond 9, production of enterotoxin dropped abruptly and the least production was recorded at pH 11. Similar results were also reported by Sobeh and Vadehra (1983) ^[29] from their study with *S. Enteritidis*. However, they suggested that the optimum pH for production of *Salmonella* enterotoxin was 7.3. The effect of hydrogen ion concentration on growth and metabolism of the organism and the stability of proteinaceous material is a biological phenomenon, which is species and strain specific. Although no generalization could be made, it appeared that slight alkaline condition induced better production of enterotoxin by most of the organisms (Sobeh and Vadehra 1983) ^[29].

For studying the cell-free and cell-bound nature of *Salmonella* enterotoxin, cell sonicates (CS) were prepared by ultrasonic lysis of the bacterial cells. The titre of Stn was found to be increased by 2 to 4 folds in CS compared to CFCS titres as detected by CoA test. The results indicated that besides the extracellular enterotoxin that appeared in the CFCS, cell-bound enterotoxin was also present, which were not released in culture media. The results were in conformity with that of Rahman *et al.* (1991b) ^[23] who found an increased titre of enterotoxins in CS than that in CFCS preparations of the same strains. The cell-bound nature of the enterotoxin was also reported by Baloda *et al.* (1983a) ^[2], Kaura and Sharma (1983) ^[14], and Kaura and Sharma (1988) ^[13].

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