Synopsis submitted and approved by the Research Degree Committee in Microbiology, Himachal Pradesh University, Shimla
PHENOTYPIC AND GENOTYPIC CHARACTERIZATION
OF SALMONELLA ENTERICA SEROVAR TYPHI
ISOLATES IN INDIA

SYNOPSIS

Of

Research Work

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1. INTRODUCTION:

Typhoid fever is a systemic infection caused by Salmonella enterica serovar Typhi. The disease remains an important public health problem in developing countries being a cause of high morbidity and mortality. In 2000, it was estimated that over 2.16 million episodes of typhoid occur worldwide, resulting in 21,600 deaths, and that more than 90% of this morbidity and mortality occurred in Asia (Crump et al., 2004). Most of the developing countries are facing the burden of typhoid fever due to poor sanitary conditions (Parry et al., 2002).

Although, mortality from typhoid fever has been greatly reduced by effective therapies and the availability of vaccines, morbidity is still high due to the global emergence of multidrug resistance and subsequent treatment failures (Chandel and Chaudhry, 2001). Furthermore the condition gets worse by several complications (Arora et al., 2008; Naithani et al., 2008).

The incidence of typhoid fever varied substantially between different developing countries in Asia, being high in India and Pakistan, intermediate in Indonesia and low in China and Vietnam (Ochiai et al., 2008). These findings highlight the considerable, but geographically heterogeneous, typhoid in endemic area of Asia, and underscore the importance of evidence of disease burden in making policy decisions about interventions to control this disease. Typhoid fever is endemic in India with an annual incidence of 214.2/100,000 per year with majority of patients (493.5) within an age group of 5 to 15 years (Ochiai et al., 2008).

Moreover, the alarming ability of the microbe to acquire persistent, high level resistance to the clinically most relevant antibiotics posed a serious problem in developing
countries like India (Anand et al., 1990; Koul et al., 1991; Jesudasan et al., 1990; Gupta et al., 1990; Gaind et al., 2006). More recently, highly flooroquinolone resistant *Salmonella* Typhi strains have been reported to circulate in the country (Dutta et al., 2008).

One of the main approaches for combating typhoid fever today is the control of dissemination of the causative agent. Therefore, epidemiological investigation of the *Salmonella* Typhi is of major importance using different typing methods. Typing of bacteria can be used to determine whether isolates recovered from different patients or different area are related and in so doing, provide evidence of common source of transmission of the agent (Kang and Dunne, 2003).

Many phenotypic and genotypic typing methods have been applied to epidemiologically trace *Salmonella* Typhi infections. Traditional epidemiological methods include biotyping, serotyping and phage typing of isolates as well as antimicrobial susceptibility testing. Although, these methods do not always give enough information for epidemiological purposes, these methods lay down the basis of the phenotypic characterization of the isolates. Moreover, the antimicrobial susceptibility testing elaborates the present scenario of the antimicrobial resistance. The antibiotic resistance pattern is helpful in the delineation of the appropriate control measures required for the prevention of enteric fever (Mohanty et al., 2006). Characterization studies can further be expanded to molecular techniques for epidemiological patterns of *Salmonella* Typhi.

Various genotypic methods have been applied for the characterization of salmonella Typhi. Restriction Fragment Length Polymorphism (RFLP) analysis of chromosomal DNA (Maher et al., 1986; Nair et al., 1994; Reeves et al., 1989; Selander et al., 1990) and
Ribotyping (Grimont and Grimont, 1986; Stull et al., 1988) have led to a significant increase in discriminatory power.

By using these techniques, various distinct fingerprint types can be distinguished, even within phage types (Altwegg et al., 1989; Nasatasi et al., 1991). The mobile genetic element IS200, initially described by Lam and Roth (1993), has also been described for DNA fingerprinting. The number of IS copies in Salmonella Typhi varies from 10 to 15 (Gilbert et al., 1990), suggesting its usefulness in epidemiological investigations. The discriminatory power of IS200 fingerprinting was found to be poor among strains isolated in areas such as India and Pakistan, where a restricted number of phage types occur (Threlfall et al., 1993; Threlfall et al., 1994).

Other genotypic methods include analysis of plasmid content and plasmid restriction pattern (Liu et al., 1995), Random Amplified Polymorphic DNA (RAPD) analysis (Hermans et al., 1996) and Pulse Field Gel Electrophoresis (PFGE) (Hudson et al., 2001; Lindstedt et al., 2000; Thong et al., 1995; Hermans et al., 1996; Navarro et al., 1996). Lipopolysaccharide profile (Jimenez-Lucho and Foulds, 1990) and envelope protein profile (Franco et al., 1992) have also been used as discriminative tools.

Salmonella Typhi infection is endemic in India (Ochiai et al., 2008). Emergence and circulation of antibiotic resistant strains (Dutta et al., 2008; Joshi and Amarnath, 2007; Chitnis et al., 1999) and the increased incidence of typhoid fever in human immunodeficiency virus infected individuals are further cause of concern. In relation to effective surveillance and the development of rational control strategies for this important human disease, the detailed accurate data, related to epidemiology of Salmonella Typhi, should be generated time to time.
Although several localized studies have been conducted at different areas of the country, for infection due to multidrug resistant *Salmonella* Typhi (Joshi and Amarnath, 2007; Mandal et al., 2002; Dutta et al., 2008), trends of *Salmonella* Typhi infection (Saha et al., 2002) and characteristics of *Salmonella* Typhi strains circulating in these area (Saha et al., 2003; Mohanty et al., 2006), these studies are not sufficient to depict the distribution trends of *Salmonella* Typhi in all areas of the country.

National Salmonella and Escherichia Centre (NSEC) was established at Central Research Institute, Kasauli, India in the year 1958 in collaboration with World Health Organization and assistance from Central public Health Laboratory, Collindale (UK) and Pasteur Institute (Paris). The centre is involved in the serological identification of *Salmonella* and *Escherichia*. Centre receives Salmonella isolates from different parts of the country for serological identification.

*Salmonella* Typhi isolates received from different parts of the country can be characterized phenotypically as well as genotypically for assessing the distribution trends of the bacterium in the country. Therefore, the purpose of the present study is to assess the level of phenotypic and genotypic diversity and possible relationships among *Salmonella* Typhi strains isolated from different parts of the country at different time.
2. AIM AND OBJECTIVES

AIM: Phenotypic and Genotypic characterization of *Salmonella enterica* serovar Typhi isolates in India

The above aim will be achieved by the following objectives:

1) To study the prevalence of *Salmonella enterica* serovar Typhi in India

2) Phenotypic characterization of isolates by
   a) Biochemical characterization
   b) Serological characterization
   c) Phage typing
   d) Antimicrobial susceptibility testing
   e) Determination of MIC levels
   f) Characterization of antimicrobial resistance
   g) Outer membrane protein profile analysis

3) Genotypic characterization of isolates by
   a) PFGE (Pulse Field Gel Electrophoresis) analysis
   b) RAPD (Random Amplified Polymorphic DNA) analysis
   c) Plasmid profile analysis

4) Comparison of the studied characteristics with those of standard strain (Ty2)
3. MATERIALS AND METHODS

3.1 Materials:

3.1.1 Media:

1) Nutrient broth
2) Nutrient agar
3) Mac Conkey agar
4) Luria-Bertani broth
5) Muller Hinton agar
6) SOC medium
7) SOB medium

3.1.2 Biochemicals:

1) Glucose
2) Lactose
3) Dulcitol
4) Mannitol
5) Salicine
6) Peptone water
7) Glucose phosphate media
8) Ortho-nitrophenyl-β-galactoside
9) Triple sugar iron
10) Lysine, Arginine and Ornithine
11) Cragie’s tube
12) Malonate
13) Urea

3.1.3 Antisera:

1) Salmonella ‘O’ and ‘H’ antisera

3.1.4 Bacterial cultures:

1) E. coli ATCC 25922
2) E. coli DH5α
3) E. coli HMS174
4) S. Typhi Ty2
3.1.5  **Restriction enzymes:**

1) *EcoRI*
2) *HindIII*
3) *XbaI*
4) *Spe*
5) *NotI*

3.1.6  **Primers:**

1) *ERIC1*
2) *ERIC2*
3) *RAPD1*

3.1.7  **Chemicals and reagents:**

1) Crystal violet
2) Gram’s iodine
3) Ethanol
4) Carbol fuschin
5) Catalase
6) Oxidase
7) Antibiotic discs
8) Antibiotic (powered)
9) Glucose
10) Tris-HCl
11) Ethylene diamine tetraacetic acid
12) Sodium hydroxide
13) Sodium dodecyl sulphate
14) Phenol
15) Chloroform
16) Potassium acetate
17) Glacial acetic acid
18) Ethidium bromide
19) Agrose
20) Boric acid
21) Potassium chloride
22) Magnesium chloride
23) Cetyl trimethyl ammonium bromide
24) Gelatin
25) Triton X
26) Deoxynucleotide triphosphates
27) Taq polymerase
28) Glycerol
29) Bromophenol blue
30) Calcium chloride
31) Sucrose
32) Sodium lauryl sarcosinate
33) Acrylamide
34) Bis-acrylamide
35) Glycine
36) Ammonium persulphate
37) Tetramethylethlenediamine
38) 2-Mercaptoethanol
39) Sodium chloride
40) Sodium deoxycholate
41) Lysozyme
42) Proteinase K
43) Dithiothreitol
44) Bovine serum albumin
45) DNA marker
46) Protein molecular weight marker

3.1.8 Equipments:

1) Weighing balance
2) pH meter
3) Water bath
4) Incubator
5) Refrigerator
6) PAGE assembly
7) Horizontal electrophoresis assembly
8) UV transilluminator
9) Gel documentation system
10) PFGE CHEF apparatus
11) Centrifuge
12) Thermocycler
3.2 Methods:

3.2.1 Phenotypic characterization of isolates:

a) **Receipt of samples:**

National Salmonella and Escherichia centre is a national reference laboratory and receives isolates from different parts of the country. Isolates submitted to National Salmonella and Escherichia Centre will be checked for any leakage or breakage of the container. Details of the isolates including source, place and name of the sender will be recorded. This data will be used for estimating prevalence of Salmonella Typhi in the country.

b) **Isolation:**

Individual isolates will be inoculated on Mac Conkey’s agar plates and incubated at 37°C to obtain discrete colonies. Five smooth, isolated non-lactose fermenting colonies (with characteristic colony morphology of *Salmonella*) will be picked up and inoculated on to the nutrient agar slopes for biochemical characterization. Simultaneously, nutrient broth will also be inoculated for further use in serotyping. Both nutrient agar slants and broth tubes will be incubated at 37°C for overnight.

c) **Morphology and Gram’s reaction:**

All isolates will be observed for Gram’s reaction and morphological characteristics. Motility will be observed by hanging drop preparation. Catalase and oxidase tests will also be performed.
d) **Biotyping:**

Individual isolates will be biotyped using a battery of biochemicals (William, R.H., 1994; Cowan, S.T., 1983). Cragle’s tube test will also be performed to confirm motility. Isolates giving characteristic reactions of genus *Salmonella* will further be processed for serotyping.

e) **Serotyping:**

Isolates suggestive of being *Salmonella* on the basis of morphological, cultural and biochemical characteristics will be identified on the basis of their serological reactions (Popoff and Minor, 1992). Isolates identified as *Salmonella* Typhi will be selected for further study.

f) **Phage typing:**

All *Salmonella* Typhi strains will be analyzed by phage typing using the method described by Guinee and Leeuwen (1978).

g) **Antimicrobial susceptibility test:**

Antimicrobial susceptibility testing (AST), of the isolates confirmed as *Salmonella* Typhi, will be done by Kirby Bauer method according to the guidelines of CLSI (Clinical Laboratory Standard Institution) (NCCLS, 2002).
h) **Determination of MIC levels:**

Minimum inhibitory concentration of different antibiotics for *Salmonella* Typhi isolates will be determined by using agar dilution method (NCCLS, 1997).

i) **Characterization of antimicrobial resistance:**

To ascertain that whether antibiotic resistance will be plasmid encoded we will investigate the ability to transfer resistance phenotypes via conjugation and transformation to *E. coli* (Robertson, F.M. *et al.*, 2002). Plasmid isolation and transformation of *E. coli* DH5α will be performed according to standard procedures (Sambrook *et al.*, 1989).

j) **Outer membrane protein profile analysis:**

Outer membrane protein of *Salmonella* Typhi isolates will be extracted by the method of Koga and Kawata (1983). These proteins will be run on SDS-PAGE for their analysis.

3.2.2 **Genotypic characterization of isolates:**

a) **Plasmid profile analysis:**

Isolation of the plasmid DNA will be done as described by Sambrook *et al.* (1989). Samples of plasmid DNA will be electrophoresed in horizontal 0.8%
agarose. For further discrimination, plasmid DNA will be digested with EcoRI and HindIII. DNA ladder will be used as molecular marker.

b) Random Amplification of Polymorphic DNA:

Random amplification of polymorphic DNA (RAPD) will be done according to the method described by Hermans et al. (1996). Genomic DNA will be extracted by treatment with hexadecyl-trimethylammonium bromide according to current protocols in molecular biology (Elbing, K. et al., 2002).

c) Pulsed-Field Gel Electrophoresis:

Salmonella Typhi isolates will be analyzed by pulsed-field gel electrophoresis (PFGE) by the method described by Tsen et al. (1999).

All animals used in the research study will be cleared by IAEC.

The findings of the research study will be subjected to statistical analysis for validity.
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