

Analysis of Attributing Characteristics of *Salmonella enterica* serovar Paratyphi A, B and C across India during 6 years (2010 to 2015)

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Abstract

Paratyphoid fever has been emerged as a global public health problem, especially in developing countries. There should be a consistent monitoring of the isolation throughout the countries to analyze the spectrum of the *Salmonella enteric* serovar Paratyphi. This study examined current isolation pattern of *Salmonella Paratyphi* A, B and C over a period of 6 years at National Salmonella and Escherichia Centre (NSEC), Central Research Institute (C.R.I), Kasauli. Miscellaneous suspected cultures of *Salmonella* had been received from various regions of India during six years span of January 2010-December 2015. These samples were characterized by biotyping as well as serotyping at NSEC situated at Central Research Institute, Kasauli. Isolates were serotyped on the basis of somatic O and phase 1 and phase 2 flagellar antigens by agglutination tests with antisera according to the Kauffmann White scheme. Out of 71 isolates of *Salmonella enterica*, 51 (71.830%) were *Salmonella Paratyphi* A, 16 (22.532%) were *Salmonella Paratyphi* B and 4 (5.633%) *Salmonella Paratyphi* C. Among total 71 samples of *Salmonella Paratyphi*, 11.267% were obtained from North India while 88.732% cases were from South India. 81.96% samples were isolated from blood while rest of samples were from feces, urine, pus etc. Increasing rates of antibiotic resistance among *S. enterica*, particularly in *Salmonella Paratyphi* A strains, is of concern, as *Salmonella Paratyphi* A infection is becoming increasingly common and is not prevented by current vaccinations. This study caters the *Salmonella Paratyphi* A, B, C characterization by biotyping and serotyping status in various regions of India irrespective of their co-relation to the region of isolation, source of sample isolation, types of isolates including their age, gender and season during period of 2010 to 2015 across India.

Keywords: *Salmonella Paratyphi*: A-B-C; Biotyping; Serotyping; India

Introduction

Salmonella Paratyphi A has begun to replace *Salmonella enterica* serovar Typhi as the main causative agent of enteric fever in many Asian countries in recent years. Globally, three serotypes of *Salmonella Paratyphi* are described. These are *Salmonella Paratyphi* type A, type B and type C. Among these three, only *Salmonella Paratyphi* A is most prevalent while very few cases of illness are reported due to *Salmonella Paratyphi* B and C serotypes. Fewer studies have been done for the surveillance of *Salmonella Paratyphi* 'A' infections and because of similarities of clinical picture to *Salmonella Typhi*, clinicians have been unable to assess the incidence rates *Salmonella Paratyphi* 'A' infection.

The disease caused by *Salmonella Paratyphi* A is well known as paratyphoid fever. Although there is no major outbreak of paratyphoid fever in recent years, *Salmonella Paratyphi* A infection still remains a public health problem in many tropical countries. Therefore, surveillance studies play an important role in monitoring infections and the emergence of multidrug resistance, especially in endemic country such as India. However, the incidence of paratyphoid fever is increasing gradually worldwide, especially in certain endemic regions, such as certain provinces in China and Pakistan where *Salmonella Paratyphi* A infection has become a major health problem [1,2].

Generally, *Salmonella Paratyphi* A is a Gram-negative bacterium that belongs to the Enterobacteriaceae family. Based on the serotyping scheme developed by Kauffman-White, *Salmonella Paratyphi* A is classified as serogroup A with an antigenic formula as [1,3,4]. *Salmonella Paratyphi* A produces a weakly positive reaction for H₂S test and subsequently fails to show evidence of H₂S production during the first of 14 days of incubation. Gas produced by *Salmonella Paratyphi* A is also relatively little and *Salmonella Paratyphi* A is not lysine decarboxylase-positive, xylose fermenting organism [1].

When *Salmonella Paratyphi* A is sub-cultured on blood agar and MacConkey agar, after overnight incubation at 37°C, blood agar showed non pigmented (grey-white) colonies of size 1-2 mm, opaque, non-hemolytic, moist, circular with a smooth convex surface and the entire edge. The growth on MacConkey agar consisted of non-lactose fermenting colonies of similar morphology. Colonies are catalase positive and oxidase negative. Gram stained smear from the growth revealed Gram-negative bacilli, 2-4 µm × 0.6 µm in size, non-capsulate and non-sporing. The bacilli are motile [3].

There are many limitations in culture-based methods. The accuracy of diagnosis depends on the adequate amount of sample taken from patients, the appropriate media to be used, the stage of disease, and other variables during the isolation procedure. Unlike typhoid fever, the number of bacteria throughout the duration of paratyphoid fever has not been reported previously. Therefore, this makes the diagnosis of paratyphoid fever more challenging. However, for both typhoid and

paratyphoid fever, culture from the bone marrow gives the most accurate results.

Salmonella Paratyphi A, B, C are the causal organisms of paratyphoid fever. However no statistically significant differences in individual symptoms between patients with infection due to *Salmonella* Typhi or *Salmonella* Paratyphi A are found. The most common presenting complaints were fever and headache for both cases respectively. Frequent other symptoms included anorexia, abdominal pain, chills, malaise, anorexia, nausea and cough. Physical findings included coated tongue, splenomegaly, and abdominal tenderness. Overall, the cytokine profile of *Salmonella* Paratyphi A infection is similar to *Salmonella* Typhi but distinct from other non-typhoidal *Salmonellae* [5]. Also, the disease phenotype for the two infections is identical-with no difference in disease presentation or complication rate between infections due to *Salmonella* Paratyphi A and *Salmonella* Typhi. The studies demonstrate that both *Salmonella* Typhi and *Salmonella* Paratyphi A infections can be highly prevalent at the same time in the same population.

The paratyphoid fevers largely follow an extra-household transmission, mostly by consumption of contaminated food prepared outside home; as children tend to consume street food or eat in restaurants, they are likely to be infected with *Salmonella* Paratyphi A [6]. Transmission of the pathogen is via the fecal-oral route, with humans as the sole reservoir of infection. Transmission is via consumption of contaminated food/water or contact with chronic asymptomatic carriers [7]. Family contact could be a factor of transmission as well, but a recent report on the risk factors for the disease showed that paratyphoid infections occur mostly outside the household [8]. The important vehicles of transmission in many countries include shellfish harvested from sewage-contaminated beds, raw fruits, vegetables fertilized by night soil and eaten raw, milk and milk products. Preparation of food by hands of carriers or infected food handlers may also contribute to the disease transmission [1,9]. Humans are the major reservoir for *Salmonella* Paratyphi B, with spread occurring by contamination of food products or water by excreta [7].

Enteric fever caused by *Salmonella* Typhi and *Salmonella* Paratyphi A still continues to be a major public health problem in developing countries like India. Typhoid fever was estimated to have caused 21.6 million illnesses and 216,500 deaths globally, and the less severe paratyphoid fever caused an estimated 5.4 million illnesses in 2000. Although *Salmonella* Typhi was observed to be the predominant serovar worldwide, a shift in the most prevalent *Salmonella* serotype from *Salmonella* Typhi to *Salmonella* Paratyphi A has been reported recently by many researchers *Salmonella* Paratyphi A is the second most common cause of enteric fever after *Salmonella* Typhi with infection rate between *Salmonella* Typhi to Paratyphi being approximately 4:1. Population based studies have estimated the global burden of enteric fever, to be >21 million cases of typhoid fever in the year 2000 and >5 million cases of paratyphoid fever with regions of high incidence of typhoid fever (>100/100 000cases/yr) at South Central Asia and South East Asia [1,8].

Salmonella Paratyphi A was the less frequent cause of enteric fever in earlier days, with isolation rates being 3-17% from India. The incidence of enteric fever due to *Salmonella* Paratyphi A is increasing since 1996 [6].

Among the enteric fever cases that occurred in an urban slum in New Delhi from October 1995 to October 1996, 25% was caused by

Salmonella Paratyphi A. Moreover, in 1996, 36 cases of paratyphoid fever were reported in a residential area of New Delhi, India within 1 month period (September-October). Subsequently, infections due to *Salmonella* Paratyphi A are increasing in India. A few retrospective cohort studies have been conducted in India to monitor the trends of *Salmonella* Paratyphi A infection. Based on the data collected from the All India Institute of Medical Sciences in New Delhi, the isolation rate of *Salmonella* Paratyphi A has increased from 6.5% in 1994 to 44.9% in 1998 [1]. Data from 1999 to 2004 were collected again from the same institution by Mohanty et al. [10] and the isolation rate was dropped to 23.8%. In another study conducted by Capoor et al. [11] a significant increase in *Salmonella* Paratyphi A isolation from 2001 to 2006 has been observed in New Delhi.

An increase in isolation of *Salmonella* Paratyphi A was reported with 11.1% (2001) to 59% (2003) from Calicut (now known as Kozhikode) [12] Between 2001 to 2003 an unusually high rate of isolation was reported from 20.3% in Mumbai, Nagpur (46.15%), Sevagram (53.33%) and Rourkela (11,12). 23.5% in Calcutta from September 2003 to August 2004. 38.4% in Shimla from 2000 to 2006, 23.3% in Chennai between 2007-2009. A study from Chandigarh also showed an 40.6% increase of isolation of *Salmonella* Paratyphi from January 2003 to April 2007 [14,15]. More recently, a multi-center surveillance study has been carried out by the Indian Network for Surveillance of Antimicrobial Resistance Group [16]. A total of 764 *Salmonella* Paratyphi A strains have been isolated between January 2008 to December 2010 in the 15 participating centers throughout India [17-19]. The incidence of enteric fever caused by *Salmonella* Paratyphi A is increasing in many parts of the world. *Salmonella* Paratyphi A appears to be responsible for a growing proportion of enteric fever in a number of Asian countries, sometimes accounting for 50% of *Salmonella* bloodstream isolates among enteric fever patients [2,20-23].

The disproportionate increase in the numbers of *Salmonella* Paratyphi A cases may be due to the vaccine effect (Ty21a and Vi vaccines), which only protects individuals from *Salmonella* Typhi infection and also the inappropriate preventive strategies against *Salmonella* Paratyphi A. The reduction strategies that were effective against *Salmonella* Typhi might not be useful against *Salmonella* Paratyphi A [1].

Studies have been conducted to define the antibiogram pattern of *Salmonella* Paratyphi 'A' and these shows that the multiple antibiotic resistance for *Salmonella* Paratyphi 'A' is emerging in India [21,24-27]. Multidrug resistant typhoid and paratyphoid infections are more severe with higher rates of toxicity, complication and mortality than infections with sensitive strains. This may be related to the increased virulence of multidrug resistant *Salmonella* as well as a higher number of circulating bacteria [28].

The rise in incidence and prevalence of *Salmonella* Paratyphi 'A' may be due to the fact that current vaccines only offer protection against typhoid fever. Due to this *Salmonella* Typhi infections are getting under control and isolation of *Salmonella* Paratyphi is becoming more common.

All of these studies highlight *Salmonella* Paratyphi A as the emerging pathogen of enteric fever. This study is designed at NSEC for the analysis of indications of the overall trends followed by *Salmonella* Paratyphi and its subtypes. The NSEC is a national reference centre for *Salmonella* and *Escherichia* for India. It receives samples from research

laboratories, hospitals and institutions for biochemical and serological identification.

The retrospective analysis was done during this time period of 2010 to 2015 for parameters like the region, subtype, source of collection of sample, season of isolation, age and gender were studied to explore the ongoing pattern of *Salmonella* Paratyphi in India during these years.

Materials and Methods

The National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India, has provided a service to the nation for the past five decades, and is a national reference laboratory formerly under the control of the World Health Organization. Isolates of *Salmonella* Paratyphi were obtained at NSEC from all over India. During the year 2010-2015, 71 isolates of *Salmonella* Paratyphi were received by the NSEC from various regions of North and South India. These constituted the material for this study. The records of all the isolates were retrieved from laboratory records of the department.

For the biotyping, all media and biochemicals were obtained from Hi Media Lab. Pvt. Ltd., Mumbai, India. All isolates were identified as *Salmonella* Paratyphi by conventional biochemical tests [29,30] and confirmed by serotyping on the basis of somatic O and phase 1 and phase 2 flagellar antigens by agglutination test using standard *Salmonella* agglutinating factor sera (Seiken Laboratories, Tokyo, Japan) according to the White-Kauffmann-scheme [31,32].

The sera raising for serotyping was performed in rabbits by following an immunization schedule with formalin killed *Salmonella* suspension of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml dose by subcutaneous route on day 1,2,7 and 11. The animals were test bled after one week of the last injection and tested for homologous titres by agglutination test. The animals were bled by cardiac bleeding method. Blood was collected in dry sterile test tubes and then stored in a refrigerator (+4°C) overnight. The blood clot was removed by centrifugation and all the sera from different bleedings were pooled together and filtered by 0.45 µm micro-filter.

For serotyping testing an 18-24 hour broth culture of the test strain was taken. It was then formalinized and kept at room temperature for 30 minutes. The culture was centrifuged at 2,000 rpm for 15-20 minutes. The supernatant was discarded and the deposit was used for serotyping work. The deposit was resuspended in sterile PBS.

4-5 drops of the centrifuged deposit were placed on a clean glass slide. To the first drop was applied a drop of O polyvalent Group A-E antisera. If agglutination was positive, the *Salmonella* serotype was suspected. 80-90% strains exhibit agglutination with this antisera. If there was no agglutination, additional group antisera were tested.

In case, agglutination with group polyvalent A-E was negative, agglutination with individual factor sera was required. The next step was to check for phase 1 antigens. For this purpose the help of polyvalent Rapid *Salmonella* Diagnostic sera (RSD 1,2,3) was taken for serotyping.

Results

Salmonella Paratyphi A was sub-cultured on blood agar and MacConkey agar, after overnight incubation at 37°C, blood agar showed non pigmented (grey-white) colonies of size 1-2 mm, opaque, non-hemolytic, moist, circular with a smooth convex surface and the entire edge. The growth on MacConkey agar consisted of non lactose

fermenting colonies of similar morphology. Colonies were catalase positive and oxidase negative. Gram stained smear from the growth revealed Gram-negative bacilli, 2-4 µm × 0.6 µm in size, non-capsulate and non-sporing. The bacilli were motile.

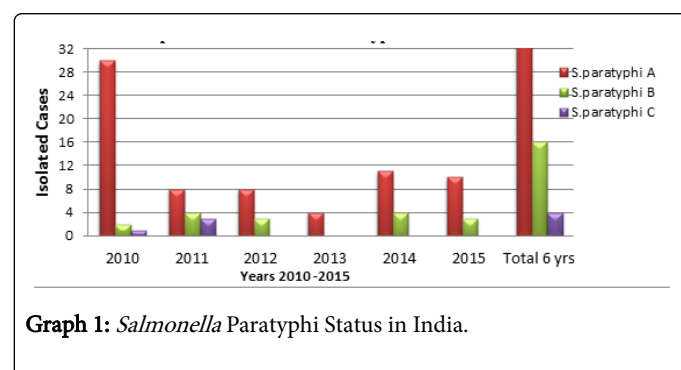
Biotyping was performed on *Salmonella* Paratyphi A cultures which were found Lactose negative, Catalase positive, Oxidase negative, Indole negative, Methyl Red positive, Vogus Proskaur negative, Citrate negative, Urease negative, Triple Sugar Iron showed K/A glucose acid with gas, Nitrate reductase positive and Lysine negative. The isolates were cultured on nutrient agar, Mac-conkey agar and blood agar plates. In serotyping tests the positive agglutination of *Salmonella* Paratyphi A antisera with *Salmonella* Paratyphi A bacteria was observed.

A retrospective analysis of laboratory records at NSEC, from January 2010 to December 2015 showed an increasing rate of isolation *Salmonella* Paratyphi from cases of enteric fever.

Highest number of 27 isolates of *Salmonella* Paratyphi A were isolated in 2010 and lowest number of 1 case in 2011 (Table 1 and Graph 1). For *Salmonella* Paratyphi B maximum isolation of 4 numbers was done in 2011 and nil case in 2013. However *Salmonella* Paratyphi C, 1 isolate was obtained in 2010 and 3 cases were isolated in 2011. From 2012 to 2015, no new isolates of *Salmonella* Paratyphi C were obtained.

S. No.	Year	Total Isolation	Salmonella Paratyphi A	Salmonella Paratyphi B	Salmonella Paratyphi C
1	2010	30	27	2	1
2	2011	8	1	4	3
3	2012	8	5	3	Nil
4	2013	4	4	Nil	Nil
5	2014	11	7	4	Nil
6	2015	10	7	3	Nil
	2010-15	71	51	16	4

Table 1: Results of *Salmonella* Paratyphi isolation during six years 2010-2015.



Graph 1: *Salmonella* Paratyphi Status in India.

Analyzing the retrospective data, among total 71 samples of *Salmonella* Paratyphi, 11.267% were obtained from North India while 88.732% cases were from South India with maximum isolation from Mumbai, Pune and Chennai. The number of isolation of *Salmonella*

Paratyphi was highest in 2010 (42.253%) followed by 15.492% in 2014 and 14.084% in 2015.

In preview of type of isolates it had been observed that among total *Salmonella* Paratyphi isolates, 71.830% were of *Salmonella* Paratyphi A, 22.535% were of *Salmonella* Paratyphi B and 5.633% were of *Salmonella* Paratyphi C.

In contrast to the source of isolation from the blood samples were dominating with total of 81.690%, feces with 12.676% and other miscellaneous samples 5.634% were from urine, liver, pus and environment (Table 2).

S. No.	Parameter	Observation	Total Cases	Percent
1	Region	North India	8	11.267
		South India	63	88.732
2	Isolate type	<i>Salmonella</i> Paratyphi A	51	71.830
		<i>Salmonella</i> Paratyphi B	16	22.535
		<i>Salmonella</i> Paratyphi C	4	5.633
3	Source of collection	Blood Sample	58	81.690
		Feces	9	12.676
		Urine, Liver, Pus, Tree	4	5.634

Table 2: Interpretation of the results of 5 years 2010 to 2015.

Of the total cases studied, both children and adults were included. The incidence of Paratyphi was higher in children as compared to adults, while gender-wise distribution was not much different. It indicated that both males and female were equally susceptible to the *Salmonella* Paratyphi infection. Every year, most of the isolation of *Salmonella* Paratyphi occurred throughout the year showing that paratyphoid had been prevalent during all seasons in the country.

Discussion

Recent insights into the evolution of *Salmonella* Paratyphi A from genomics confirm that the organisms are genetically monomorphic and show other features of host-adapted pathogens. These features remind us of the organism vulnerabilities and the potential for major gains in disease control. Added to the increasing complexity of managing enteric fever due to antimicrobial resistance, there is a strong case for much greater effort in disease control through improvements in sanitation, greater access to safe water and food, identification and treatment of *Salmonella* Paratyphi carriers, and the more widespread use of currently available vaccines in high-risk populations.

However, the obvious application of biotyping of salmonellae is in epidemiological investigations. The findings should assist not only in interpreting the epidemiology but also in following the emergence and disappearance of particular epidemic strains.

In the longer term, though, biotyping is likely to make an even greater contribution when applied to the selection of strains for detailed analysis. Such selection is required when choosing cultures for an investigation into phylogenetic relationships between strains within a serotype and between serotypes within the genus *Salmonella* factors relating to virulence.

On the otherhand, serotyping is based on the long standing observation that the microorganisms from the same species can differ in the antigenic determinants expressed on the cell surface. Serotyping

is method used to differentiate isolates of *Salmonella* beyond the subspecies level. *Salmonella* serotypes are designated based on the immunoreactivity of two cell surface structures, the O and H antigens. A substantial amount of diversity exists in these two antigens, resulting in the designation of more than 2,500 serotypes and the regular recognition of new serotypes.

The biotyping and serotyping is an efficient tool for studying the characteristic role of *Salmonella* Paratyphi strains circulating in the country.

Conclusion

Current study emphasize the mutual analysis by biotyping as well as by serotyping, the isolation of the *Salmonella* Paratyphi serovars throughout India. Paratyphoid had been the next most common disease caused by *Salmonellae* after typhoid. So there should be a continuous monitoring of the epidemiological behavior of these isolates at diagnostic level. This can on long term, increase the impending analysis of the potential antibiogram pattern as well as the chances of detection of neglected strain detection can be enhanced.

The vastly resistant Paratyphi strains had been isolated from various regions of the world which lay down the need of antibiotic treatment as well as the requirement of new candidate vaccines against *Salmonella* Paratyphi to have a control over the escalating incident rates of paratyphoid cases. The studies should be started at the national level for development of new paratyphoid vaccines so that the infectivity, severity and morbidity due to these prevalent *Salmonella* Paratyphi strains should come to a check rate.

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