Research article

Baseline titres of O, H and AH agglutinins to *Salmonella* Typhi and Paratyphi A in blood donors in Sri Lanka.

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Abstract

Background: Sri Lanka is considered as an endemic country for enteric fever. Due to difficulties in performing blood cultures, the Widal test is still commonly carried out for the diagnosis of enteric fever. However, there are no published data on current baseline Widal titres in the country. This study was carried out to determine the baseline titres of O, H and AH agglutinins among the Sri Lankan population.

Method: Five hundred and one (501) serum samples of blood donors from 31 blood banks in Sri Lanka were collected during 2012 and 2013 and were screened for *Salmonella* O, H and AH agglutinins using the Widal tube test. A titre of 20 and above was considered positive. Age and gender of the study participants were recorded.

Results: Of the 501 sera tested, 58% were positive for at least one of the O, H and AH agglutinins. *Salmonella* O, H and AH agglutinins were positive in 46.1%, 26.5% and 8.4% of the study population respectively. Of the study population, 97.5% had O, H and AH agglutinin titres less than or equal to 80, 160 and 80 respectively. A significantly higher percentage of females (H-36.7%, p=0.019; AH-15.3%, p=0.15) were positive for H and AH agglutinins than males (H-24.9%; AH-7.4%). The baseline titre of AH agglutinins was higher in females (160) than males or the total population (80). Highest test positivity (40%) was seen among the 31-40 year age group. A significant number of donors below 20 were negative for *Salmonella* O agglutinins (p=0.024).

Conclusion: We recommend baseline titres of 80, 160 and 80 for *Salmonella* O, H and AH agglutinins respectively to be used in Sri Lankan settings. As there is a variation in baseline titre with age and gender it is necessary to consider both when interpreting Widal test results.

***Keywords**: Widal test, Baseline values, Enteric fever, Typhoid, Paratyphoid, Sri Lanka*

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Background

Enteric fever includes both typhoid and paratyphoid fevers and is a global health problem. Typhoid fever is caused by *Salmonella* Typhi whilst the causative organism of paratyphoid fever is *Salmonella* Paratyphi A. The global estimate for typhoid fever in year 2000 was 21,650,974 with 216,510 deaths. In the same year, the estimated number of paratyphoid fever cases was 5,412,744.[1] By 2010, there was an increase in the incidence of typhoid fever with the estimated number being about 26.9 million with around 1% of global deaths attributed to this disease.[2]

Enteric fever is typically a disease associated with poverty and poor sanitation, hence commonest in low and middle income countries (LMIC). Disease transmission is via the faecal-oral route and contamination of food and water with infected faeces facilitates transmission of these diseases. With increasing global incidence and the development of multi-drug resistance to commonly used antibiotics, enteric fever is among the top public health problems in many LMICs, including Sri Lanka. However, basic information on enteric fever from some endemic countries is missing due to lack of recent research.

In order to arrive at a definitive diagnosis, isolation of either *S.* Typhi or *S.* Paratyphi A by culture of blood, bone marrow, stool, urine, rose spots or intestinal secretions is necessary.[3] Of these, bone marrow culture is considered to be the most reliable[4], but its invasive nature has limited its use.[5] Blood culture is considered the next best test in diagnosing enteric fever. However, its use is compromised in LMICs due to empirical antibiotic usage, cost, lack of laboratory facilities and time taken to obtain results.[5,6] Thus, the Widal test, based on the demonstration of antibodies against the H (flagellar) and O (somatic) antigens of *S.* Typhi and *S.* Paratyphi A in the serum of patients, is still commonly used for diagnosis of enteric fever in many countries, including Sri Lanka. However, demonstrating a four-fold rise in titre in paired acute and convalescent sera, as recommended, is not commonly practiced and the antibody titre in a single acute sample of serum is often used to decide on the clinical management of patients.

For the correct interpretation of a Widal test on a single sample of serum, it is important to know the baseline antibody titres in the normal population in a particular geographical area.[6,7] This is necessary as a significant number of healthy persons may also carry antibodies to *S.* Typhi and *S.* Paratyphi A.[6,8,9] In an endemic area, there is often a high background titre in the general population due to previous clinical or subclinical infection with *S.* Typhi or *S.* Paratyphi or previous vaccination for typhoid. Previous infection caused by non typhoidal *Salmonella* with O antigen type 9 or 12[10], *Salmonella* Enteriditis[4], other Enterobacteriaceae and even *Burkholderia pseudomallei*[11] and malaria[10] may lead to the development of Widal agglutinins. Lack of data on baseline *Salmonella* agglutinin titres in endemic populations may therefore lead to incorrect interpretation of the Widal test. At present, no literature is available on current baseline agglutinin titres of *S.* Typhi and *S.* Paratyphi in Sri Lanka. The purpose of this study was to determine the baseline agglutinin titres of *S.* Typhi and *S.* Paratyphi A in the healthy population in Sri Lanka.

Methods

The study population was healthy blood donors from Sri Lanka. The study sample included 501 serum samples obtained from donors from 31 of the 96 blood banks in Sri Lanka during 2012/2013 (Figure 1).

Fig. 1: Map of Sri Lanka showing locations of blood banks enrolled in the study and number of blood samples tested for each site.

Serum was separated and stored at -20 °C until further testing. Haemolysed as well as samples with inadequate sera were excluded from the study. Anonymized data on age and sex of blood donors was collected, and therefore the requirement for informed consent was waived by the Ethics Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka.

Standard *Salmonella* Typhi O, *Salmonella* Typhi H and *Salmonella* Paratyphi AH antigens (Biotech Ltd, Suffolk, UK) were used to perform the tube Widal test. The antigens were validated using relevant antisera with known titres (Murex Biotech Ltd, Dartford, UK).

Tube agglutination tests were carried out according to the manufacturer's instructions. Two fold dilutions, from 1:20 to 1:1280, of each of the 501 serum samples was prepared as follows. Three sets of 8 Kahn tubes were used for each serum sample in order to test for agglutinins to each of the antigens *Salmonella* O, *Salmonella* H and *Salmonella* AH. To tube one 1.9ml of 0.85% saline was dispensed whilst1.0ml of 0.85% saline was dispensed into the other seven tubes. Thereafter, 0.1ml of undiluted patient's serum was pipetted into tube 1 and mixed thoroughly by vortexing.

Next, 1.0ml from tube 1 was pipetted into the second tube and mixed thoroughly. Similarly, doubling dilutions were continued through to the sixth tube. Serum was not added to the seventh tube which acted as the control tube. One drop of the appropriate undiluted antigen suspension was added to each tube. The tubes were vortexed and incubated in a water bath at 50 °C. The tubes containing *Salmonella* O antigen were incubated for 4 hours and the tubes with *Salmonella* H and AH antigens were incubated for 2 hours. The tubes were covered with aluminum foil, left overnight in a refrigerator, and examined for agglutination after 24 hours.

Large flakes of agglutination, visible to the naked eye, in the tubes with *Salmonella* H and AH antigens and small granules of agglutination, visible with a magnifying glass, in the tubes with *Salmonella* O antigen were considered as giving positive results after ensuing that the control tubes did not show auto-agglutination. The reciprocal value of the highest dilution at which agglutination was visible was taken as the end point titre. Any titre above 20 was taken as a positive test according to the manufacturer's instructions.

The percentage of positive sera in each blood bank was mapped. The percentage of the study population positive for each agglutinin was determined. The baseline titres of O, H and AH agglutinins were determined by finding the titre of each agglutinin present in 97.5 percent of the study population. Distribution of positivity for any agglutinin at any titre and test positivity for O, H and AH agglutinin by sex and age was determined and baseline titres for males and females was determined. A p value of < 0.05 in the Chi square test was considered significant.

Results

Of the 501 participants in the study, data on age were available from 459 individuals. Age range of the study population was 18 to 72 years, with the majority being in the 21-30 year age group (43.3%). Only 0.4% of blood donors were above 60 years of age. A majority in the study population were males (78%).

Distribution of positive serum samples

Positivity of any agglutinin, at any titre, is shown in Table 1. Of the 501 samples tested, 57.9% (290/501) showed titres of ≥ 20 for one or more of the three agglutinins O, H or AH (Table 1). A titre ≥ 40 for one or more agglutinins was shown by 187 of the 501 sera (37.3%).

Table 1. Distribution of positive sera for one or more of the three agglutinins O, H or AH

Widal status	N	%
Positive	290	57.9
Negative	211	42.1
Total	501	100

Distribution of agglutinin titres

Of the study population, 231/501 (46.1%) had O agglutinin titres of ≥ 20. Among them, 230 (99.6%) had titres between 20 and 80. Only one had a titre of 160 (Table 2.). Of the study population 133/501 (26.5%) had H agglutinin titres of ≥ 20. Among them, 126 (94.7%) had titres between 20 and160, 6 (4.5%) had a titre of 320 and only one had a titre of 640 (Table 3). Of the study population, 42/501 (8.4%) had AH agglutinin titres ≥ 20. Among them, 40 (95.2%) had titres between 20 and 160. Only two had a titre of 320 (Table 4).

Table 2. Distribution of O agglutinin titres among 501 blood donors from Sri Lanka

Highest Titre	N	%	Cumulative %
0	270	53.9	53.9
20	105	21	74.9
40	79	15.8	90.6
80	46	9.2	99.8
160	1	0.2	100
Total	501	100	

Table 3. Distribution of H agglutinin titres among 501 blood donors from Sri Lanka

Highest titre	n	%	Cumulative %
0	368	73.5	73.5
20	46	9.2	82.6
40	47	9.4	92
80	19	3.8	95.8
160	14	2.8	98.6
320	6	1.2	99.8
640	1	0.2	100
Total	501	100	

Table 4. Distribution of AH agglutinin titres among 501 blood donors from Sri Lanka.

Highest titre	n	%	Cumulative %
0	459	91.6	91.6
20	16	3.2	94.8
40	10	2	96.8
80	7	1.4	98.2
160	7	1.4	99.6
320	2	0.4	100
Total	501	100	

Table 5. Summary of antibody titres with percentiles.

Percentiles	O highest titre	H highest titre	AH highest titre
50	0	0	0
90	40	40	0
95	80	80	40
97.5	80	160	80

Percentile distribution of baseline antibody titres in sera of blood donors.

For all three antigens, the agglutinin titre at the 50th centile was zero. At the 97.5 percentile, the titre of O, H and AH agglutinins in the study population was less than or equal to 80, 160 and 80 respectively (Table 5).

Table 6: Distribution of titres of *Salmonella* O, H and AH agglutinins by sex

Agglutinin		Male		Female		Pearson Chi-Square Tests	
		n	%	n	%	Chi-square	P
O	Negative	207	56.60	47	48.00	2.307	0.129
	Positive	159	43.40	51	52.00		
H	Negative	275	75.10	62	63.30	5.48	.019*
	Positive	91	24.90	36	36.70		
AH	Negative	339	92.60	83	84.70	5.903	.015*
	Positive	27	7.40	15	15.30		

Details on the gender of blood donor were available in 464 samples. Test positivity (titre ≥ 20) of these sera for O, H, and AH agglutinins at any titre, distributed by gender, is shown in Table 6.

The rate of H and AH agglutinin positivity was significantly higher in females. This gender difference was not seen for O agglutinins (Table 6).

Percentile distribution of baseline titres by sex

The O and H agglutinin titres at the 97.5 percentile were equal in males and females and similar to the baseline titre of the study population. However, the titre of AH agglutinins in females was 160, which is greater than that in males and in the study population (80) (Table 7).

Table 7. Baseline titres of O, H and AH agglutinins

Percentiles	O highest titre		H highest titre		AH highest titre	
	Male	Female	Male	Female	Male	Female
50	40	20	0	0	0	0
90	40	80	40	80	0	20
95	80	80	80	160	40	80
97.5	80	80	160	160	80	160

Distribution of titres of *Salmonella* O, H and AH agglutinins by age

Age distribution of the 459 sera samples is shown in Table 8. Percentage distribution of positives by age for all agglutinins was lower in the population below 20 years. The highest positivity rate was seen in the 31-40 year age group (40.2%).

Table 8. Percentage distribution of positives by age for any one or more of the three agglutinins O, H or AH

	Negative		Positive	
Age	**N**	**%**	**N**	**%**
<20	7	3.6	6	2.3
21-30	95	49.2	88	33.1
31-40	50	25.9	107	40.2
41-50	31	16.1	43	16.2
51-60	9	4.7	20	7.5
>60	1	0.5	2	0.7
Total	193	100	266	100

Test positivity of 459 serum samples for O, H, and AH agglutinins distributed by age is shown in Tables 8 and 9. Positivity for O agglutinins is significantly low below the age of 20 years (Chi square = 5.062 p=0.024). It doubles between 21-30 years of age and then remains static. Positivity for H agglutinins is low below the age of 20 and rises with age. However, the difference between test positivity for H agglutinins among the age group below and above 20 years was not statistically significant (Chi square = 3.569 p=0.058). Widal positivity for AH agglutinins was low (8.38%). Positivity for AH agglutinins is low below the age of 40 and then doubles between 41-50 years of age. However, the difference between positivity for AH agglutinins in the age group below 20 years and above 21 years was not statistically significant (p=0.786 with Yates correction) (Table 9).

Table 9. Distribution of test positivity of O, H and AH agglutinins by age

Age group	O agglutinin				H agglutinin				AH agglutinin			
	Negative		Positive		Negative		Positive		Negative		Positive	
	n	%	n	%	n	%	n	%	n	%	n	%
<20	17	77.30	5	22.70	20	90.90	2	9.10	21	95.50	1	4.50
21-30	108	54.30	91	45.70	159	79.90	40	20.10	187	94.00	12	6.00
31-40	74	50.30	73	49.70	97	66.00	50	34.00	135	91.80	12	8.20
41-50	38	58.50	27	41.50	42	64.60	23	35.40	52	80.00	13	20.00
>50	15	57.70	11	42.30	16	61.50	10	38.50	24	92.30	2	7.70

Discussion

In this study, blood bank donors were chosen to represent the healthy Sri Lankan population. Blood donors are often used as representatives when reference ranges are calculated or assay methods validated because they are considered as healthy individuals in a population.[12] Many workers have used blood bank donors as representing the general population in studies on baseline titres of *Salmonella* agglutinins.[12,18] The blood banks in this study covered all Districts and Provinces of Sri Lanka, except for the Mullaitivu District which does not have a blood bank (Figure 1).

In the present study, the seroprevalence of one or more of *Salmonella* O, H, and AH agglutinins among blood donors was 57.8%, which shows that a large number of individuals in Sri Lanka are positive for antibodies to *Salmonella* antigens. This seropositivity rate is higher than that found in Kenya (21.25%)[12] and North Kerala (25.2%)[16], but much lower than that found in studies done in Maharashtra and Hubli-Dharwad[17,18], probably reflecting exposure rates in each population. The high seropositivity seen in Maharashtra[17] was also hypothesized to be due to exposure to cross reacting bacteria such as *Citrobacter freundii,* a harmless gut bacterium which share antigenic properties with *Salmonella* serotypes.

The seropositivity rate of 37.3% at a titre ≥ 40 found in the present island-wide study is higher than the 12.7% reported in a previous study done in Sri Lanka in 1965 which was limited to the Western Province where the starting dilution was 1:50.[8] The seroprevalence at a titre ≥ 40, of O, H and AH agglutinins found in the present study (O=24.75%, H=17.37%, AH=5.1%) is also considerably higher than the corresponding percentages in a previous study conducted in the Western Province.[8] This higher rate is maintained, even if we compare only the results for Colombo (O=25%, H=25%, AH=12.5%) with that found in 1965 in the Western Province (1.8%, 10.6% and 2.4%).[8] Further, the titres for Jaffna in the present study (O= 37.5%, H=25%, AH=6%) are higher than those found in Jaffna in 2012/2013 (7%, 9% and 0%).[19] This may be due to differences in methodologies, antigen kits and starting dilutions used in the different studies (1/20, 1/50 and 1/30 respectively). The higher positivity rate in Colombo in this study, compared to 1965 may also be explained as being caused by an increase in cross-reacting antibodies in the population of the Western Province. These cross reacting antibodies may have arisen due to increased prevalence of infections such as dengue[20], leptospirosis[21] or food poisoning due to *Salmonella* Enteritidis[22], as these organisms share antigens with typhoidal

Salmonella serotypes[23]. Such cross reacting antibodies are usually found at low titres[24] as seen in this study. Similar to Gunjal *et al*. (2013)[17] and Bijapur *et al.* (2014)[16], the seropositivity rate for O agglutinins was higher than that for H and AH agglutinins. This may be because of constant exposure to bacteria in the environment which share O antigens with enteric fever causing *Salmonellae.*

This high background level of test positivity makes it even more important to determine the baseline cut off titres for the Sri Lankan population before performing the Widal test to diagnose acute enteric fever. In this study, 97.5% had O, H, and AH agglutinin titres of less than or equal to 80, 160 and 80 respectively. The highest titre found in 97.5% of the population is considered as the background titre for that particular geographical area. Therefore, these titres can be considered as baseline titres of O, H and AH agglutinins in Sri Lanka and titres above these can be considered as indicators of acute infection.

The baseline Widal titres obtained in the present study are consistent with those found in other studies conducted in endemic areas such as India, Kenya and Nigeria.[6,16,17,18,12,25] These baseline titres are also consistent with a study done in Sri Lanka by Thevanesam (1992)[26] where the diagnostic cut off titres for typhoid fever were ≥ 120 for O agglutinin and ≥ 120 for H agglutinin respectively. However, they contrast with a study by Gnanakarunyan *et al* (2012)[19] in Jaffna, where the cut off titres were 480 and 60. This may be due to differences in study populations and source of SAT antigens, test methodology and starting serum dilutions.

The percentage of females with O agglutinins was similar to that in males but H and AH agglutinin positivity was significantly higher in females. This contrasts with a study done in Nigeria[25] where a higher positivity in males was seen, with males showing 39%, 41% and 51% positivity to O, H and AH agglutinins versus 10.7%, 29.5% and 17% in females. This shows that males and females in Sri Lanka are equally exposed to bacteria with antigens that cross react with *Salmonella.*

Exposure appears to be maximum in the 31-40 year age group as the highest positivity rates were seen in this group. O agglutinin positivity rates remain almost the same with increasing age after 20 years while H positivity rates gradually increase. O agglutinins are short lived and reflect current exposure to cross reacting bacteria. They also do not show an anamnestic reaction.[10,13] Therefore all age groups should have similar O agglutinin positivity. The findings of the present study agree with this. In contrast, H and AH agglutinins persist for longer after exposure and show an anamnestic reaction to re-exposure[13] and positivity increases with age. The findings of the present study are in accordance with this as well.

Our results are similar to the study conducted in 1965 by Velaudapillai and Singho[8] where an increase in H agglutinin positivity was seen with age in the non-vaccinated normal population. This increasing trend of seropositivity with age is important when interpreting a Widal test result. Widal positivity for any one or more of the three agglutinins O, H or AH is low (2.3%) under 20 years of age. However, the difference between those under 20 years and those over 20 years was significant only for O agglutinin titres. The low positivity rate in this age group reflects less exposure to bacteria with cross reacting antigens. A consequence of this result is that baseline titres in the paediatric population are different (lower) than for adults and that cut off titres should

be adjusted to reflect this difference. Further studies are needed to elucidate the cut off titres in the paediatric age group.

Limitations

The baseline agglutinin titres that are reported in this study may not truly reflect that of the general population due to the smaller percentage of females (21.1%) in the study population and the lack of samples from persons aged <20 years (4.8%) and >50 years (5.6%). The employment of different antigen kits, some commercial and some in-house, and different methodologies in various laboratories in Sri Lanka make comparisons between baseline titres derived from different studies difficult.

Conclusions

Although the background prevalence of *Salmonella* O, H and AH agglutinins in the healthy population in Sri Lanka is high, the titres were seen to be low, with baseline titres being 80, 160 and 80 for *S.* Typhi O, *S.* Typhi H and *S.* Paratyphi AH agglutinins respectively. Therefore, titres above these values can be used to diagnose enteric fever. It is important to consider sex and age when interpreting a positive test. It would be advisable to regularly update the baseline titre values for different geographical areas.

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