Haemolysins of Salmonella, their role in pathogenesis and subtyping of Salmonella serovars

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Haemolysin patterns of 175 strains of different Salmonella enterica subspecies enterica serovars isolated from different animal sources and places were determined using 11 different blood agar media made with either non-washed horse/sheep erythrocytes or with washed erythrocytes of cattle, sheep, horse, goat, rabbit, guinea pig, and human A, O and B blood groups. Study on 47 strains belonging to 10 serovars of Salmonella from buffalo meat (buffen), 42 strains of 11 serovars from goat meat (chevon); 16 strains of Salmonella enterica serovar Paratyphi B and 25 of S. enterica serovar Paratyphi B var Java from fish, meat, meat products and clinical cases; 45 isolates of S. Abortusequi from aborted mares (18); fetal contents (21), aborted donkey mares (2) and 4 reference strains, revealed that all host restricted Salmonella namely, S. enterica serovar Gallinarum, S. enterica serovar Anatam, S. enterica serovar Abortusequi and S. enterica serovar Paratyphi B could be divided into different haemolysin types based on their inability to produce haemolysis on one or more types of blood agar, while strains of all zoonotic Salmonella serovars induced haemolysis on all the 9 types of blood agar made of washed erythrocytes. None of 175 Salmonella could produce hemolytic colonies on blood agar made of non-washed horse/sheep erythrocytes. Haemolysin type I (lysing all types of washed erythrocytes) was the commonest one among all serovars except S. Abortusequi, none of which lysed horse erythrocytes. Salmonella enterica serovar Abortusequi having hemolytic activity against sheep erythrocytes were more invasive but had lesser ability to survive in sheep mononuclear cells than non-hemolytic strains. Multiplicity of haemolysins appeared significant epidemiological tool.

Keywords: Enterolysins, Haemolysins, Pathogenicity, Salmonella

Salmonella organism, as pathogen of man and animals, is of ubiquitous nature with multiple modes of transmission and exists as an ever persisting problem for public health professionals. Most of the Salmonella infecting warm blooded animals are Salmonella enterica subspecies enterica serovars, generally abbreviated as S. followed by serovar name written with capital letter as S. Paratyphi B, S. Typhimurium etc. There are more than 2400 Salmonella serovars, a few have adapted to various hosts and few have established themselves as zoonotic pathogen, due to their rapid adaptability to different hosts. Multiplicity of serovars causing variable syndromes, have created lot of ambiguity and confusion among clinicians dealing with typhoid and paratyphoid fever. Though it appears to be impossible to eradicate salmonellosis, its risks can be minimized through systematic approach to discriminate between prevalent and rare strains and understanding their virulence factors employed in host system during infection. Commonly investigated and some well established virulence factors of Salmonella include adhesins on bacterial surface, lipopolysaccharides, hydrophobic moieties of bacterial surface, Congo red dye binding and iron chelating properties, bacteriocins and colicins, colonization factors, invasins and many other plasmid mediated virulence factors. Besides, drug resistance coexisting on some plasmids with some virulence markers is also significant determinants of pathogenicity. Many other factors viz. cytotoxins, enterotoxins and invasins etc. have been shown to play variable role in pathogenesis. However, haemolysins, important virulence markers of many pathogens, and also produced by several members of Enterobacteriaceae seem to be a very rare trait among Salmonella. Libby et al. reported a salmolysin of 16 kDa molecular weight to be hemolytic and cytolytic and encoded by SlyA gene in S. Typhimurium. Though precise role of this gene is still unclear, it has been shown to be implicated in survival of Salmonella within mouse peritoneal macrophages and also in regulation of other genes and may also act as a haemolysin activator for other bacteria, without having any role in entero-pathogenesis. Another...
haemolysin reported in *Salmonella* strains is cytolitic phospholipase C\(^{27,29}\). It induced lysis of erythrocytes, cytolysis of Vero and MDBK (Maiden Derby Bovine Kidney) cells, and is dermonecrotic on intra-dermal inoculation in guinea pigs and rabbits but its role in pathogenesis is yet not clear\(^{28}\). The present study has been undertaken to find out the prevalence of haemolysins in strains of different serovars of host adapted (S. Abortusequi, S. Paratyphi B and S. Gallinarum) and zoonotic (of goat meat, chevon and buffalo meat, buffalo origin) *Salmonella*. Attempts have been made to determine the role of haemolysin in adhesion, invasion and intracellular survival (S. Paratyphi B and S. Abortusequi) in sheep blood mononuclear cells (MNCs). Utility of different hemolytic patterns generated through haemolysis of washed erythrocytes has also been examined for its potential for epidemiological subtyping of the strains under study.

**Materials and Methods**

**Bacterial strains**—All 175 *Salmonella* strains belonging to different serovars of *Salmonella enterica* subspecies *enterica* were revived from the glycerol stocks of field isolates submitted from different parts of India during recent years to the centre [National Salmonella Centre (Vet.), IVRI, Izatnagar]. The freeze-dried stocks of reference cultures used in the cultures were maintained on nutrient agar slants at 4°C.

The strains included a total of 47 isolates of *Salmonella* isolated from buffen samples belonging to 10 serotypes namely S. Anatum (14), S. Lagos (2), S. Dublin (1), S. Gallinarum (9), S. Orion (2), S. Rostock (3), S. Stockholm (1), S. Typhimurium (5), S. Weltevreden (7) and rough strains (3); 42 *Salmonella* isolates from chevon samples belonging to 11 different serovars e.g. S. Altona (1), S. Rovaniemi (1), S. Chicago (2), S. Czernyting (2), S. Weltevreden (1), 6,7:z126 (1), 3,10,15:z6- (2), 52:r:1,5 (1), 1,3,19:r:1,5 (1), 1,3,19:z26 (1) and rough (29); 16 isolates of classical *Salmonella Paratyphi* B (CSPB) and 25 S. Paratyphi B var Java (SPBJ) isolated from fish, meat and meat products and clinical cases; 45 strains of S. Abortusequi isolated from clinical cases of salmonellosis in equines viz. vaginal swabs of aborted mares (18), aborted fetae contents (21), vaginal swabs of aborted donkey mares (2) and 4 reference strains. Besides, one non-hemolytic *Escherichia coli* (E288) and a beta hemolytic *Streptococcus pyogenes* (St-124) were used as negative and positive control, respectively.

**Haemolysin assay**—To perform the test\(^{29,30}\) blood samples from sheep, goat, cattle, horse, rabbit, guinea pig, humans blood group A, B and O were collected in Alsever’s solution. Erythrocytes were pelleted after centrifugation of blood at 1800x\(g\) for 10 min and washed twice with sterilized normal saline solution (NSS, 0.85% NaCl). Thereafter, the original volume was made up with sterile NSS and 5 ml of it was added aseptically to 95 ml of molten blood agar base (Difco India Ltd., Mumbai) maintained at 50°C and mixed well. Blood agar plates were made and incubated for 24 hr at 37°C to test the sterility. Blood agar plates were also made with defibrinated whole sheep and horse blood i.e. without washing the RBC’s.

To determine the production of haemolysins all the test and reference strains were spot inoculated on blood agar plates containing RBC’s of different species. After overnight incubation at 37°C, zone of clearance was measured around the colonies in case of haemolysin positive isolates.

**Pathogenicity assay**—All the 41 *Salmonella* Paratyphi B (16 classical and 25 var java) and 45 S. Abortusequi isolates were tested for pathogenicity in inbred Balb/c mouse model\(^31\) through intraperitoneal inoculation of 0.1 ml of 6 hr LB (Luria bertani Medium) grown culture [\(\sim 10^8\) colony forming units (cfu) per dose]. Mice, 45-50 days old and weighing 25-30 gm each, were procured from *Salmonella* free colony maintained in Laboratory Animal Research Section of the Institute. Three mice were sacrificed from the procured mice and blood from heart was taken to obtain serum, for detecting *Salmonella* antibodies\(^32\) while spleen and intestinal contents were collected for isolation of *Salmonella*\(^32\). Once the negative results were obtained for *Salmonella* antibodies and isolation then only mice were used in the study. Inoculum was prepared by diluting the LB grown culture to 0.6 OD at 540 nm, and *Salmonella* count was made in each inoculum by pour plate method\(^31\). A group of 3 mice, kept in one cage was used for each isolate. Mice were observed for 21 days for clinical symptoms, moribund mice or terminally ill mice (starry coat, loose faeces, prostration and inability to move, eat and drink) were sacrificed humanely. NSS was inoculated similarly in mice of control group.

To re-isolate the injected *Salmonella*, the whole spleen of sacrificed (after 21 days or when the animals were found moribund) or dead mouse was homogenized in 10 ml of buffered peptone water and
incubated at 37°C for 18 hr. Aliquots of 0.1 and 1 ml were transferred to modified Rappaport Vassiliadis and tetra thionate broth medium (Hi Media, Mumbai), respectively and incubated at 37°C for 24 hr. After enrichment, streaking was done on hektoen enteric agar (HEA)/brilliant green agar (BGA) plates (Hi Media, Mumbai). After 24 hr of incubation, suspected colonies were confirmed serologically using specific ‘O’ and ‘H’ factor serum.

Assays in sheep mononuclear cells (MNCs). Adhesion assay—MNCs were harvested from freshly collected heparinized (10 IU/ml) blood sheep blood on Histopaque (Sigma Chemicals Co. USA). After washing the cells with sterile phosphate buffer saline (PBS, 0.15 M, pH 7.2), viable cells were counted by trypan blue dye (0.1% solution in 1:10 ratio) by exclusion method. Plates (12 well tissue culture) were seeded at the concentration of 5 x 10^5 cells/well with 1 ml RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) and 100 µg/ml gentamicin. Plates were incubated overnight at 37°C in a humidified 5% CO_2 incubator. For infection, two classical Salmonella Paratyphi, two Salmonella Paratyphi B var Java and two S. Abortusequi strains (one of which lysing and other not lysing sheep RBCs) were chosen. Pellet from 8 hr broth cultures grown at 37°C in trypticase soy broth (TSB, HiMedia, Mumbai) were suspended in RPMI-1640 medium to contain about 5 x 10^6 Salmonella organisms/ml. Before infection to MNCs non-adherent cells were washed off with PBS, then culture medium was replaced with RPMI-1640 (1 ml/well) with 5% FCS without any antibiotic in control wells and RPMI containing Salmonella in test wells designated to be infected with specified Salmonella strains in triplicate. After 30 min of incubation of cell culture plates at 37°C, wells were washed aseptically with sterile PBS. Thereafter, MNCs were lysed by adding 1 ml of sterile 0.1% sodium deoxycholate solution, then incubating for 10 minutes and thereafter rupturing them with vigorous pipetting. A 10 µl of 10-fold serially diluted lysate from each of the 3 Salmonella infected and control wells was plated onto MacConkey lactose agar (MLA, HiMedia, Mumbai) plates in triplicate and incubated overnight at 37°C to count colony-forming-units (cfu) of Salmonella.

Intracellular invasion assay—The test was performed using gentamicin protection assay. It was done similar to adhesion assay up to infection and incubation for 30 min. Then after washing twice with PBS, each well was filled with 1 ml RPMI-1640 supplemented with gentamicin (100 µg/ml). Cell culture plates were incubated at 37°C for next one hour. Thereafter, cells were washed twice with PBS and lysed with 0.1% sodium deoxycholate to count Salmonella cfu in the lysates as above.

Intracellular survival assay—The assay was performed similar to invasion assay up to the step of last washing with PBS. Then 1 ml of RPMI-1640 with 5% FCS and gentamicin (100 µg/ml) was poured into each well. Plates were incubated for next 18 hr at 37°C. Thereafter, each well was washed twice with PBS then cells were lysed to count Salmonella cfu in lysate as described earlier.

Results

Mouse pathogenicity assay—Of the 45 S. Abortusequi, 16 S. Paratyphi B and 25 S. Paratyphi B var Java tested for mouse pathogenicity, all except one S. Paratyphi B induced morbidity and mortality in mice within 24 hr to 7 days of inoculation. Symptoms of salmonellosis (starry coat, diarrhoea, prostration and sleepy demeanour) were evident within first 3-5 days of inoculation and no death was observed after 7 days of inoculation. Inoculated Salmonella strain could be re-isolated from spleen of all the moribund and dead mice during experiment. The mortality pattern varied from 53 to 100% in S. Abortusequi isolates. All Salmonella Paratyphi B var Java isolates killed mice (33 to 100%) on ip inoculation. All classical S. Paratyphi B were also proved to be lethal to mice but one isolate from Hyderabad from pig, neither induced any observable sickness nor proved lethal to mouse on ip inoculation. Non-pathogenic strain of Salmonella could not be isolated from any of the organs even after sacrificing the mice.

Haemolysis on blood agar plates made with unwashed erythrocytes of horse and sheep—None of the 175 Salmonella isolate under study produced hemolytic colonies on sheep or horse blood agar made with unwashed erythrocytes (defibrinated blood). However, reference hemolytic strain of Streptococcus pyogenes (St-124) formed colonies surrounded by big zone of beta haemolysis on both sheep or horse blood containing media.

Haemolysis of washed erythrocytes by Salmonella isolates from buffalo meat—Most of the 47 Salmonella isolates from buffalo formed hemolytic colonies on blood agar made with washed erythrocytes of horse, rabbit, cattle, sheep, goat, guinea pig and humans. The best haemolysis was on blood agar made from horse RBC followed by those
from rabbit and guinea pig erythrocytes. Colonies of almost every strain irrespective of serovar had haemolytic zone of about 6 mm in diameter, around them after 24-36 hours of incubation, on blood agar plates made of guinea pig erythrocytes. Haemolysis was seen either just under the colonies of Salmonella (on human A group RBCs) or colonies grew with narrow zone of haemolysis on media containing sheep, goat, calf and human O and B group RBCs. Human blood group A erythrocytes were the least affected ones. Erythrocytes of human O and B groups were more sensitive than that of cattle and sheep but less than that of goat, guinea pig and horse RBCs to haemolysins of Salmonella of buffalo origin.

There was no variation in hemolytic pattern of isolates of S. Lagos (2), S. Dublin (1), S. Orion (2), S. Stockholm (1), S. Rostock, (3), S. Typhimurium (5), S. Weltevreden (7) and 3 rough strains and all of them lysed all the 9 types of RBCs. However, strains of S. Gallinarum and S. Anatum varied in their hemolytic profile.

Among 14 isolates of S. Anatum from buffalo, 3 haemolysin types were evident. Twelve of the isolates haemolysed all 9 types of RBCs (Hly I) similar to other non-host-restricted Salmonella strains. One of them yielded colonies with the biggest (diameter, 22 mm, after 36 hr of incubation) zone of haemolysis on human blood group A erythrocyte containing medium. Of the remaining two isolates of S. Anatum, one failed to lyse human blood group A (Hly II) while other failed to lyse rabbit and horse RBCs besides human blood group A erythrocytes (Hly III). Seven of the S. Anatum isolates from buffalo samples from Bareilly slaughter house belonged to 3 different Hly types while isolates from other parts of India were all alike in lysing the different erythrocytes.

Haemolysis of washed erythrocytes by Salmonella Gallinarum—Sensitivity of different erythrocytes to S. Gallinarum strains of buffalo origin was no different than the strains of other serovars. All strains haemolysed human O, rabbit and goat RBCs but variation was evident on blood agar containing RBCs of other 6 types. Nine isolates of S. Gallinarum from buffalo could be divided into 6 haemolysin types (Table 1) depending on haemolysis of different types of RBCs. Four isolates belonged to Hly type I while rest 5 belonged to 5 different Hly types. Isolates belonging to different haemolysin types were isolated from samples collected from the same slaughterhouse but the animals were brought for slaughter from different places, reared under different conditions. Strains from 3 distant located places belonged to Hly I.

Haemolysis of washed erythrocytes by Salmonella isolated from chevon samples—All 42 Salmonella isolates of goat meat origin belonging to S. Altona (1), S. Rovaniemi (1), S. Chicago (2), S. Czernyering (2), S. Weltevreden (1), 6,7:r:z6 (1), 3,10,15:z6:- (2), 52:r:1,5 (1), 1,3,19:r:1,5 (1), 1,3,19:z6 (1) and rough (29) groups, irrespective of serovar, haemolysed all types of washed RBCs to almost an equal extent. The best haemolysis was observed on horse erythrocytes followed by that of rabbit, guinea pig, goat, human O, A, B blood group, sheep and cattle RBCs. The pattern was similar as given by the Salmonella of buffalo origin except that RBCs of human A blood group stood in between human O and B for sensitivity to Salmonella haemolysins of chevon origin isolates instead of being least sensitive as for Salmonella of buffalo origin. All 42 Salmonella strains of goat meat origin though belonging to different serovars can be classed into single haemolysin type (Hlyf).

Haemolysis of washed erythrocytes by Salmonella Abortusequi—All the 41 field strains of S. Abortusequi were of equine origin and were isolated from north India only and were the most homogenous group as far as the source and place of isolation is concerned. Surprisingly, none of the S. Abortusequi isolates (including the 4 reference strains) could haemolysse horse erythrocytes and varied extensively in their hemolytic activity. All haemolysed human A, B and O blood group as well as guinea pig RBCs but...
none haemolysed horse RBCs. Forty-five isolates could be classified into 9 haemolysis (Hly) types (Table 2) viz. Hly-I to IX, of these type V and IX were equally prevalent in different parts of north India. All strains belonging to type IV and VIII were exclusively isolated from Meerut and those of type II from Babugarh. Best haemolysis was seen on cattle erythrocytes, rest of the sensitivity series remained same as for strains of other serovars i.e. rabbit (however only 8 isolates were able to haemolysed rabbit RBCs), guinea pig, goat, human O, A, B groups and sheep (in decreasing order of sensitivity).

**Haemolysis of washed erythrocytes by Salmonella Paratyphi B** — All the 41 isolates (16 CSPB and 25 SPBJ) haemolysed guinea pig, horse and human B group erythrocytes. *Salmonella* Paratyphi B isolates could be classified into 11 Hly types (Table 3) on the basis of inability to haemolysse RBCs of human blood group A and O, cattle, sheep, goat and rabbit erythrocytes. Of the 11 haemolysin types, type I and II were the commonest and most prevalent types among all and has the same haemolysis pattern as the strains of *S. Gallinarum* Hly I and II had. Sixteen isolates of CSPB belonged to 6 Hly types while 25 strains of SPBJ could be classified into 10 Hly types.

**Studies on sheep mononuclear cells** — There was significant difference in strains of different serovars as well as in two biovars (SPBJ and CSPB) of *Salmonella* with respect to their adhesion, invasion and intracellular survival (Fig. 1). Adhesion studies revealed that *S. Paratyphi* B strains adhered much better than *S. Abortusequi* strains. Non-hemolytic strains adhered better than hemolytic ones but for invasion, picture was quite reverse as more percentage of adhered hemolytic bacteria got internalized than the non-hemolytic ones (Fig. 1). *Salmonella* Paratyphi B strains adhered significantly (*P* < 0.01) better than *S. Abortusequi* strains but latter has better ability to invade and survive in MNCs. More than 97% of the adhered *S. Abortusequi* invaded the MNCs while about 70% of classical *S. Paratyphi* B and about 60% of adhered *S. Paratyphi* B var Java failed to invade. After 18 hr of intracellular exposure only about 25, 13, 21 and 19% of invaded non-hemolytic SPVJ, hemolytic SPVJ, non-hemolytic CSPB and hemolytic CSPB, respectively could survive therein. On the other hand, there was an increase of 20 and 26% in count of intracellular sheep erythrolytic and non-erythrolytic *S. Abortusequi* strains, respectively.

**Discussion**

The study revealed that almost every *Salmonella* isolates of *S. Paratyphi* B and *S. Abortusequi* was pathogenic to mouse, however, to a variable extent proving that the difference in lethality potential exist not only between strains of different serotypes but also exist between strains within a serotype. Mouse lethality due to *S. Paratyphi* B strain ranged from 0 to 100% while it ranged between 33-100% on inoculation of *S. Abortusequi* isolates. Similar variability has been reported in *S. Typhimurium* and *S. Enteritidis* (20-100%) strains. Mouse model is valued since ages to evaluate pathogenicity potential of *Salmonella*, but it is often disputed that mouse-lethal-*Salmonella* are equally pathogenic to different hosts or to their specific hosts probably due to role of different genes in pathogenicity in different hosts.

Virulence genes are present either on plasmids or on chromosome or on both acting in concert may be encoding for adhesion, colonization, invasion, *in vivo* spread and toxicity. The haemolysins have been shown to be very important virulence determinants of a disease outcome as enterohaemolysins of *Escherichia coli* (VTEC) are associated with hemolytic uremic syndrome, pathogenicity of *Streptococcus* and *Staphylococcus aureus* (toxic shock syndrome and exfoliative disease) is all dependent of their haemolysins. Although haemolysin production by *Salmonella* is said to be a rare trait, it has been shown to be a determinant of intracellular survival.

All 175 strains of *Salmonella* used in this study produced haemolysins and it appeared to be universal trait; this contradiction to earlier observations appears to be mainly due to use of washed erythrocytes in the present study. Similar to earlier observations, none of the strain induced haemolysis of unwashed RBCs as none of the 175 strains under study produced hemolytic colonies on horse or sheep blood agar made of defibrinated blood using unwashed erythrocytes. The results indicated that most of the *Salmonella* may be producing haemolysin(s) similar to enterohaemolysin of VTEC acting only on washed RBC's and haemolysins acting on un-washed RBCs appears to be produced very rarely as reported earlier.

Observations on different strains of *Salmonella* with absence or presence of potential to produce haemolysin active on sheep washed erythrocytes revealed that, in *Salmonella*, haemolysins have no major role in adhesion. Hemolytic strains have
Table 2 — Different types of haemolysins and pathogenicity of S. Abortusequi

<table>
<thead>
<tr>
<th>Hly type</th>
<th>RBCs (animal species) lysed</th>
<th>Types of RBCs lysed (No. of isolates)</th>
<th>Strain</th>
<th>Place and time of isolation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7</td>
<td>All but goat and horse (1)</td>
<td>E157</td>
<td>Reference-1976</td>
<td>Mukteshwar India</td>
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<tr>
<td></td>
<td>6</td>
<td>All but sheep, rabbit and horse (4)</td>
<td>E843, E846, E833, E836</td>
<td>Babugarh, Aug-1978</td>
<td>Fetal contents</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>All but cattle, rabbit and horse (3)</td>
<td>E156, E113, E137</td>
<td>Reference -1976</td>
<td>Mukteshwar India</td>
</tr>
</tbody>
</table>

All haemolysed human A, B and O blood group as well as guinea pig RBCs but none haemolysed horse RBCs.
Table 3—Different haemolysin types among S. Paratyphi B strains

<table>
<thead>
<tr>
<th>Hly type</th>
<th>RBCs (animal species) lysed</th>
<th>Hemolytic activity on erythrocytes of</th>
<th>S. Paratyphi B var Java</th>
<th>Classical S. Paratyphi B</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>All types</td>
<td>1991-Kolkata fish</td>
<td>896-Hyderabad pig</td>
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<tr>
<td></td>
<td>Same as haemolysin type I of</td>
<td>2604-Mumbai meat</td>
<td>1294-Chennai khoa</td>
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<td></td>
<td>S. Gallinarum</td>
<td>2605-Mumbai poultry</td>
<td>3258-Ranchi fish</td>
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<td></td>
<td></td>
<td>2600-Mumbai poultry</td>
<td>3260-Ranchi fish</td>
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<td>3264-Ranchi fish</td>
<td>3267-Ranchi fish</td>
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<td>3275-Ranchi fish</td>
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<td>3391-Bareilly goat</td>
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<tr>
<td>II</td>
<td>All but sheep</td>
<td>2609-Mumbai poultry</td>
<td>663-Kolkata pig</td>
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<td></td>
<td>Same as haemolysin type II</td>
<td>1986-Kolkata fish</td>
<td>3256-Ranchi Fish</td>
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<td></td>
<td>of S. Gallinarum</td>
<td>2603-Mumbai poultry</td>
<td>3257-Ranchi Fish</td>
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<td>2609-Mumbai meat</td>
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<td>All but goat</td>
<td>2598-Mumbai meat</td>
<td>651-Kolkata rat</td>
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<td>3274-Ranchi fish</td>
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<td>2601-Mumbai poultry</td>
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<tr>
<td>IV</td>
<td>All but human blood group A</td>
<td>723-Bareilly food</td>
<td>2403-Bareilly man</td>
<td>3271-Ranchi fish</td>
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<td>2599-Mumbai poultry</td>
<td>1984-Kolkata fish</td>
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<td>All but cattle and sheep</td>
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<td>801-Agartala calf</td>
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<td>VII</td>
<td>All but cattle</td>
<td>3266-Ranchi fish</td>
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<td>VIII</td>
<td>All but cattle and human</td>
<td>2610-Mumbai poultry</td>
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<td></td>
<td>blood group A</td>
<td>855-Kolkata dog</td>
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<tr>
<td>IX</td>
<td>All but human blood group O</td>
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<tr>
<td>XI</td>
<td>All but rabbit and cattle</td>
<td>923-Mathura sewage</td>
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</table>

All haemolysed guinea pig, horse and human B group erythrocytes

comparatively less adhesion ability in comparison to their counterparts that are non-hemolytic but mostly it appeared to be a serovar related trait governed by some other genes. In adhesion assay, isolation of only 298 to 730 Salmonella from about a million mononuclear cells exposed to 10 million Salmonella indicated that either there are very few monocytes having receptors for Salmonella or only few Salmonella produces ligands to attach on blood mononuclear cells. Presence of receptors only on few mononuclear cells may be explained on the basis of heterogeneous population of the cells. Only a fraction of MNCs develop into macrophages and some times take long time to develop the potential and thus many remain unable to provide anchor to Salmonella. Many of the S. Paratyphi B, those adhered to mononuclear cells, could not invade into cells as only 26 to 40% Salmonella got internalized and detected in intracellular position in invasion assays. The observations revealed that either some of adhered Salmonella lack the invasion potential which is highly improbable in a isogenic population or some of the mononuclear cells though provide anchorage to Salmonella may not be permitting invasion probably due to lack of phagocytic potential or S. Paratyphi B lacks ability to invade sheep MNCs or the phagocytized Salmonella get readily killed after phagocytosis. Contrary to S. Paratyphi B, most of the S. Abortusequi adhered to MNCs became intracellular in short period of time and survived therein to increase their number. Thus, the most probable cause of isolation of a very few S. Paratyphi B from MNCs appears to be rapid killing of it in phagosomes and it became more evident with the assays on intracellular survival of S. Paratyphi B. After 18 hr of incubation, only 33 to 67% (12-25% of the invaded) S. Paratyphi B could be detected intracellular. Though, there is lot of controversy with regard to intracellular multiplication of Salmonella, observations on S. Abortusequi in sheep MNCs revealed that there is
strains into MNCs indicated that the haemolysins of Salmonella associated with a bit better ability of invasion of hemolytic Salmonella being phagocyted and help intracellular bacteria to survive better. As far as role of earlier known haemolysins of Salmonella is concerned, intracellular survival of hemolytic strains has been reported better than the non-hemolytic ones. The contrasting finding of this study may be either due to some differences in design of the study or difference in the haemolysins detected in this study than those in earlier studies. The most probable reason for contrasting findings appears to be the basic difference in haemolysins detected under this study than those were detected earlier.

The haemolysins produced by Salmonella neither match with streptolysin O nor with related haemolysins produced by a number of bacteria as these do not lyse unwashed erythrocytes and have a defined range of susceptible washed erythrocytes. On the other hand, streptolysin O and related haemolysins are not usually restricted by the source of erythrocytes and are equally active on unwashed and washed erythrocytes. Salmolysins of this study neither relates to phospholipase C, which also lyse cells of all types. Very narrow zone of haemolysis on blood agar made of a few types of erythrocytes led to origin of suspicion that the salmolysins may be contact haemolysins. However, contact haemolysins are known to support the pathogen to survive intracellularly in macrophages, while the salmolysins detected under the present study had negative correlation with intracellular survival as all 3 strains, hemolytic for sheep RBCs, survived in less numbers in sheep MNCs than non-hemolytic strains of similar serovars, discussed earlier, moreover contact haemolysin expression did not require use of washed erythrocytes.

Haemolysins of Salmonella strains in this study bears some relation to enterohaemolysins of VTEC, which also lyse only washed erythrocytes. Further similarities for Salmonella haemolysins can be found in multiple haemolysins of Serpulina hyodysenteriae, which greatly vary in activity against different types of erythrocytes and do not play any major role as determinant of pathogenicity. Further, Leptospira strains produce many different types of sphingomyelinas with varied range of haemolysis and are major determinants of pathogenicity that have also been used for grouping of Leptospira strains into many different haemolysin types as used for Hly typing of different Salmonella serovars in this study.
Considering the zoonotic significance and food borne nature of *Salmonella*, it is important to find simple methods to profile them to understand the epidemiology of the disease. In general, serotyping alone is insufficient for epidemiological purpose. Therefore, many methods, viz. molecular typing, biotyping, phage typing, sensitivity to bacteriocins, antibiograms etc. have been used from time to time. Kapperud et al. revealed that even simpler techniques as plasmid profiling, phage typing and biotyping work as better epidemiological marker than the more complex ones. With the results obtained in the present study, it may be concluded that haemolytic range of *Salmonella* haemolysin(s) varies greatly and can be used for haemolysin typing of *Salmonella* as another simple epidemiological tool for future. However, as epidemiological tool, haemolysins seems to be useful only for host adapted or host restricted *Salmonella* serovars and those serovars usually considered important for causing zoonotic infection or food borne infection had no or little variability in their haemolytic range on erythrocytes used in this study. The study suggested that haemolysins may be having some relation with host range of *Salmonella* as none of the S. Abortusequi (a equine adapted serovar) lysed horse erythrocytes and variability was evident towards haemolysis of washed erythrocytes of human in human adapted *Salmonella* Paratyphi B strains. The findings suggested that studies on haemolysins may reveal a lot about their differentiation into different groups adapted to different hosts.

All the *Salmonella* belonging to wide host range group haemolysed all the 9 types of erythrocytes irrespective of place and source of isolation. The results revealed that conserved gene(s) present on chromosome may encode haemolysin(s) of wide host range *Salmonella*. Moreover, it is also evident from the earlier studies on plasmids of S. Paratyphi B and S. Abortusequi strains, that the absence, presence and number of plasmids had no effect on hemolytic potential. Results also suggested that there might be some mechanism, related to variation in hemolytic pattern of *Salmonella* conferring them the ability of being host specific as many of the S. Paratyphi B and all of the S. Abortusequi strains failed to lyse human and horse erythrocytes, respectively. Furthermore, there was variation in lysis of erythrocytes from human beings of different blood groups but not in lysis of RBCs of a specific blood group from different individuals (data not shown). Therefore, further studies on haemolysins of *Salmonella* may enlighten on evolutionary process of host restricted *Salmonella* and also on susceptibility of hosts of different blood groups to *Salmonella* of different serovars.

Use of haemolysin typing as an epidemiological tool may not be having value for zoonotic *Salmonella* as all of them belonged to Type I (lysing all the 9 types of erythrocytes). However, as the host adaptation increases number of types of erythrocytes lysed decreases. As 14 strains of a less host specific S. Anatum causing keel disease in ducks could be divided into only 3 Hly types. However, 9 isolates of S. Gallinarum, a comparatively more hostrestricted *Salmonella* from the same source i.e. buffen could be classified into 6 Hly types.

All of the 32 rough strains in the study lysed all types of erythrocytes similar to isolates of zoonotic *Salmonella* serovars and belonged to Hly type I. Thus, the study indicated that either most of the rough strains in nature originates from zoonotic *Salmonella* or the strains possessing potential to lyse all types of erythrocytes have more tendency to become rough, or on conversion of a *Salmonella* to rough it acquires ability to lyse all types of the erythrocytes, the dilemma can only be resolved using isogenic strains.

Isolates of S. Paratyphi B (41 strains), a human host adapted serovar, and 45 isolates of S. Abortusequi (an equine pathogen) could be classified into 11 and 9 Hly types, respectively. All S. Paratyphi B haemolysed ≥7 types of erythrocytes while except a reference strain none of the S. Abortusequi isolates could lyse >6 types of erythrocytes. However, similar to zoonotic strains under study, source and place of isolation seems to have no relation with Hly types indicating that strains of a Hly type may be widely distributed in a locality in different hosts. It further potentiates the belief that hly gene(s) may be conserved in nature and this conservation if related to pathogenicity may affect the outcome of disease depending on Hly type rather than the source of infection. Results further suggested that Hly typing can be used as a stable marker to trace the leads of an outbreak that is little affected by source, place and time of isolation as evident from S. Abortusequi data (Table 2). Strains of same Hly types persisted for long on different farms in a locality.

It can be concluded that hemolytic activity of different strains on washed RBC’s of different origin could subdivide host restricted *Salmonella* into number of Hly types. The different hemolytic patterns of *Salmonella* strains may be either due to the production of different types of haemolysins or due to difference in quantity of haemolysin produced as erythrocytes of some animal species may be more
susceptible than those of the others as postulated earlier for *Klebsiella* haemolysins. Hly types have little or no correlation with pathogenicity potential of strains in mouse assay model but still not clear with regards to infection in natural host. Further studies are required to establish degree of conservation and multiplicity or variability of *Salmonella* hly gene(s) and relation of pathogenicity to expression of these genes in specific hosts.

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