DISSERTATION

on

A COMPARATIVE STUDY OF WIDAL TEST AND IMMUNOCHROMATOGRAPHIC ASSAY FOR RAPID DIAGNOSIS OF TYPHOID FEVER AND MOLECULAR ANALYSIS OF PLASMID MEDIATED QUINOLONE RESISTANCE IN CLINICAL ISOLATES IN A TERTIARY CARE CENTRE

submitted in partial fulfillment of the requirements for the degree of

Doctor of MICROBIOLOGY (BRANCH-IV)

M.D. MICROBIOLOGY

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CHENNAI

DEPARTMENT OF MICROBIOLOGY
TIRUNELVELI MEDICAL COLLEGE
TIRUNELVELI
MAY 2019

BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled "**A COMPARATIVE STUDY OF WIDAL TEST AND IMMUNOCHROMATOGRAPHIC ASSAY FOR RAPID DIAGNOSIS OF TYPHOID FEVER AND MOLECULAR ANALYSIS OF PLASMID MEDIATED QUINOLONE RESISTANCE IN CLINICAL ISOLATES IN A TERTIARY CARE CENTRE**" submitted by **Dr.MAYA KUMAR** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

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DECLARATION

I solemnly declare that the dissertation titled **"A COMPARATIVE STUDY OF WIDAL TEST AND IMMUNOCHROMATOGRAPHIC ASSAY FOR RAPID DIAGNOSIS OF TYPHOID FEVER AND MOLECULAR ANALYSIS OF PLASMID MEDIATED QUINOLONE RESISTANCE IN CLINICAL ISOLATES IN A TERTIARY CARE CENTRE"** is done by me at Tirunelveli Medical College hospital, Tirunelveli. I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in or abroad.

The dissertation is submitted to The Tamilnadu Dr. M.G.R.Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

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Dear, Dr. MAYA KUMAR, MBBS., Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 10.03.2017

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1. TIREC Application Form ☐
2. Study Protocol ☐
3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
5. Investigator's Brochure ☐
6. Proposed Methods for Patient Accrual Proposed ☐
7. Curriculum Vitae of the Principal Investigator ☐
8. Insurance /Compensation Policy ☐
9. Investigator's Agreement with Sponsor ☐
10. Investigator's Undertaking ☐
11. DCGI/DGFT approval ☐
12. Clinical Trial Agreement (CTA) ☐
13. Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA) ☐
14. Clinical Trials Registry-India (CTRI) Registration ☐

THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
2. The date of commencement of study should be informed
3. A written request should be submitted 3weeks before for renewal / extension of the validity
4. An annual status report should be submitted.
5. The TIREC will monitor the study
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8. In the events of any protocol amendments, TIREC must be informed and the amendments should be highlighted in clear terms as follows:
   a) The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
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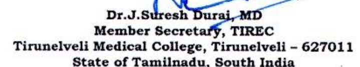

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This is certify that this dissertation work title **"A COMPARATIVE STUDY OF WIDAL TEST AND IMMUNOCHROMATOGRAPHIC ASSAY FOR RAPID DIAGNOSIS OF TYPHOID FEVER AND MOLECULAR ANALYSIS OF PLASMID MEDIATED QUINOLONE RESISTANCE IN CLINICAL ISOLATES IN A TERTIARY CARE CENTRE"** of the candidate **Dr.MAYA KUMAR** with registration Number **201614303** for the award of **M.D.** Degree in the branch of **MICROBIOLOGY(IV).** I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion page and result shows **17 percentage** of plagiarism in the dissertation.

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LIST OF ABBREVIATIONS

S. Typhi	*Salmonella* Typhi
CLSI	Clinical Laboratory Standards Institute
PCR	Polymerase chain Reaction
MDR	Multi – drug resistant
DNA	Deoxyribonucleic Acid
MIC	Minimum Inhibitory Concentration
MHA	Mueller Hinton Agar
µg	Microgram
Mac	MacConkey Agar
BAP	Blood Agar Plate
DCA	Deoxycholate Citrate Agar
XLD	Xylose Lysine Deoxycholate
H2S	Hydrogen sulphide
K/A	Alkaline / Acid
HCl	Hydrochloric acid
AIDS	Acquired Immuno Deficiency Syndrome
WHO	World Health Organisation
ELISA	Enzyme Linked Immunosorbent Assay
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESBL	Extended Spectrum Beta Lactamases
TSI	Triple Sugar Iron

INTRODUCTION

Typhoid fever is caused by *Salmonella* Typhi, through ingestion of contaminated food and water. It is a life threatening infection and continues to be a major public health problem, in the developing countries.

In 2015, there were 12.5 million new cases in the world. The disease is most common in India. In 2015, it was the cause 1,49,000 deaths worldwide – down from 1,81,000 in 1990 .

It is an endemic disease in the India , Bangladesh, South-East and Far-East Asia, the Middle East, Africa, Central and South America (Gillespie, 2003).The disease occurs in all age groups (Anggraini et al, 2004).

Case-fatality rates was 10% and has reduced to less than 1% with appropriate antibiotic therapy (WHO, 2000). The multidrug-resistant strains causing typhoid fever are associated with significant morbidity and mortality(Rahman et al, 2006).

An annual incidence rate of 493.5 cases per 100,000 person years has been reported from India.

In the early 19th centuryTyphoid fever became an important infectious disease. The incubation period is 3 to 21 days, it starts with fever, headache, mild abdominal pain and constipation, followed by appearance of rashes (Lesser & Miller, 2005; Gopalakrishnan et al, 2002).

Symptoms are mostly non-specific and indistinguishable from other febrile illnesses. Clinical severity varies and severe cases may lead to serious complications and sometimes even death. It occurs because of poor sanitation and lack of clean drinking water.

During the acute typhoid fever phase , specific antibody IgM is induced . It lasts for several weeks. It is then later replaced by IgG (Anggraini et al, 2004).

In the third week, a state of prolonged apathy, toxemia, delirium, disorientation and finally coma followed by diarrhea (Gopalakrishnan et al, 2002) . Patients carry the bacteria in their blood and intestinal tracts for a long period of time .Salmonella typhi lives only in human beings

Some patients continue to excrete the organism in their faeces or urine for more than 1 year and are called “Carriers”. Approximately 5% of infected persons become carriers (CDC, 2005).

Delayed diagnosis and appropriate therapy significantly increase the risk of serious morbidity and mortality(Bhutta, 1996). accurate diagnosis at an early stage is important for both diagnosis, and also to identify potential carrier, thus responsible for future typhoid outbreaks(Parker, 1990).

The definitive diagnosis depends on the isolation of *S.*Typhi bacteria from blood, bone marrow, rectal swab, urine or duodenal aspirate culture (Gasem et al, 1995 & Wain et al, 2001).

The Gold standard for diagnosis of Typhoid fever is still the blood culture. The positive rate is only 50–70%. Blood cultures are usually positive in about 80%(untreated cases) during the first week and declining 20%- 30% later in the course of the disease (Gillespie, 2003)

Since many a times low number of the organism causes severe disease and could be lower than 10 per ml of blood,blood cultures then show no growth in positive cases (Haque et al, 1999). Even though blood culture is gold standard,the test is highly specific but its

sensitivity is affected by prior antibiotic intake and stage of illness (House et al, 2001). And,the possibility of positive bacterial yield is variable.

Irrational and widespread use of antibiotics is the main reason for the low sensitivity of blood cultures.

Bone marrow culture has a higher sensitivity than blood culture. It's more of an invasive procedure(Farooqui et al, 1991 & Gasem et al, 1995). Bone marrow cultures is not practically feasible in mass public health screening in the field.

Stool and urine cultures is much lower in sensitivity and they become positive later than the first week of infection.

Widal test is being in use for more than a century in developing countries for diagnosis of typhoid fever. the sensitivity, and specificity is low and varies in different geographical regions.Fernend Widal described the Widal test in 1896.Detection of agglutinating antibodies against the O and H antigens of Salmonella typhi and paratyphi A and B is the principle of this test (Olopoenia& King 2000).This test is still used in developing countries extensively.

In developed countries, clinical application of the Widal test has diminished in recent years.The test, has suboptimal sensitivity and specificity (Olopoenia&King 2000, Parry et al, 1999; Rodrigues, 2003), resulting from negativity in early infection and failure to mount an immune response by certain individuals (Olopoenia& King 2000).

Poor specificity is due of pre-existing baseline antibodies in endemic areas and cross reaction with other Gram-negative infections and non-typhoidal salmonella and post typhoid vaccination

A paired sera is required for accurate interpretation (Olopoenia& King, 2000; Bhutta&Mansurali1999).Its value for early diagnosis of the diseases is limited (Haque et al, 1999). The test becomes reliably positive only during the second week of the disease.

The widal test lacks in both sensitivity and specificity. A single titer reading is lacking in reliability. It requires a paired sera showing fourfold rise in titre to prove positivity.It also requires more than 1week for the titre to buildup in the blood. Thus making this test,though widely used, not a reliable diagnostic tool .

These limitations have thus prompted the emergence for other newer serological tests like ELIZA, latex agglutination, coagglutination and the PCR (Haque et al, 1999; Jesudason et al, 1994; Mukherjee et al, 1993).

A serologic test based upon the presence of specific IgM antibodies to a specific 50-KDa outer membrane protein (OMP) antigen on S. typhi strains and is now commercially available. This test also detects IgG antibodies in serum (Choo et al, 1994).

Immunochromatographic test

The ICT is one such latest rapid diagnostic tool for typhoid fever.Immunochromatographic assay is an economical, and reliable serodiagnostic test recently available commercially and studied in many endemic areas with promising reports of higher sensitivity and specificity.

Typhipoint, Tubex, IgM-dipstick, etc., which directly detectIgM or IgG antibodies against specific *S.* Typhi antigens have been developed. These IgM antibodies against *S.*Typhi can be detected as early as within 4–5 days of fever . Initial studies done in Asian countries like India Malaysia, Indonesia, Philippines, Pakistan, And Bangladesh have shown favourable results in sensitivity (73–95%) and specificity (68–95%) of these tests.

DOT EIA has a sensitivity and specificity of 70-100% and 43-90% respectively (Khan *et al,* 2002, Bhutta&Mansurali 1999). The IgM levels reveals acute typhoid fever in the early phase of infection, while the detection of both IgG and IgM suggests acute typhoid in the middle of the infection.

IgG antibodies persists for more that 2 years following a typhoid infection (Saha *et al,* 1999).

The detection of only specific IgG , will not differentiate between acute and chroniccases. Furthermore, false positive results because of previous infection may occur. The IgG positivity may also occur in the event of current reinfections.

In reinfections there is a secondary immune response with a significant increase of IgG over IgM, such that the later cannot be detected and its effect is masked. To solve this problem is to enable the detection of IgM by ensuring that it is unmasked (Bhutta 1996).

Studies using a modified test, Typhidot M, shows that inactivation of IgG removes the competitive binding and allows the access of the antigen to the specific IgM . The Typhidot M that detects only IgM antibodies of *Salmonella* Typhi is more specific in detecting acute illnesses in few studies (Hatta *et al,* 2002, Choo *etal,* 1999).

This ICT test is simple, rapid, early to diagnose and higher positive predictive values. The test become positive as early as in the first week of the infection.The results can be read visually and available within one hour and requires no special skill to perform.(Ismail *et al*; Choo *et al,* 1994)

This study is undertaken to evaluate the immunochromatographic assay for its usefulness in patients of typhoid fever presenting to our hospital in terms of their reliability, economical and rapid diagnostic value .Thus , facilitating early rapid diagnosis and timely effective management thereby reducing the morbidity , mortality and carrier state due to typhoid fever.

Definitive treatment of typhoid fever (enteric fever) is based on susceptibility to specific antibiotics. The general principle of antimicrobial treatment is that intermediate susceptibility is considered as equivalent to resistance. Previously the drug of choice was chloramphenicol. Now-a-days, its no longer in common use because of the side effects, a high rate relapse and widespread bacterial resistance due to overt abuse of the antibiotic.

Commonly prescribed antibiotics include : Ciprofloxacin,Azithromycin and Ceftriaxone.The changing trends in the antibiotic susceptibility patterns for *S.*Typhi and high endemicity of the infection in india , continuous monitoring for drug resistance is necessary.The overt abuse of quinolones has led to the emergence of *Salmonella* strains with resistance to these drugs. (Riyaz chungathu et al,2015).

This present study is being done to find out the susceptibility profile of *S.typhi* isolates and the detection of PMQR in typhoidal *Salmonellae* in our tertiary care center, which also caters to a large out-patient population for providing suitable guidelines for the treatment of this potentially fatal disease.

AIMS AND OBJECTIVES

1. To isolate *Salmonella* from blood culture and to determine the antibiotic susceptibility pattern by using disc diffusion method.
2. To detect IgM/ IgG antibodies to *Salmonella typhi* specific antigen by Immunochromatographic test.
3. To assess the reliability of Immunochromatographic IgM / IgG test in early diagnosis of typhoid fever.
4. To compare the usefulness of Immunochromatographic test and widal test in the diagnosis of typhoid fever.
5. To evaluate the PMQR pattern in the clinical isolates in our setup by molecular analysis.

REVIEW OF LITERATURE

Typhoid fever is characterized by a prolonged fever, bacteremia without endothelial or endocardial involvement.the bacteria invades and multiplies within the mononuclear phagocytic cells of the liver, spleen, lymph nodes, and Peyers patches (Brusch, 2006).

It is potentially a fatal disease if untreated. It is caused by the bacteria *Salmonella* Typhi(Kidgell *et al,* 2002).

1. Bacteriology

1.1 Definition of Genus *Salmonella*

Salmonella belongs to the Family Enterobacteriaceae.It consists of a large group of genetically similar organisms. (Baker S, Dougan G.et al 2007) .A major group of the organisms causing clinical disease in animals and humans is caused by serovars within the *Salmonella enterica* subspecies. They cause from local gastroenteritis to a fatal diseases. The clinical outcome of *Salmonella* infection depends on the causative serovar , the infected host and its immunological status(Costa LF, Paixao 2012;93(1) [PubMed]. Some *Salmonella* serovars are responsible for large outbreaks of gastroenteritis associated with contaminated meat or processed food (Nyachuba DG et al.,(2010))

Table 1: Classification of Salmonella

***Salmonella* species and subspecies**	**No. of serotypes within subspecies**	**Usual habitat**
S. enterica subsp. *enterica* (I)	1,454	Warm-blooded animals
S. enterica subsp. *salamae* (II)	489	Cold-blooded animals and the environment
S.enterica subsp. *arizonae* (IIIa)	94	Cold-blooded animals and the environment
S. enterica subsp. *diarizonae*(IIIb)	324	Cold-blooded animals and the environment
S. enterica subsp. *houtenae* (IV)	70	Cold-blooded animals and the environment
S. enterica subsp. *indica* (VI)	12	Cold-blooded animals and the environment
S. bongori (V)	20	Cold-blooded animals and the environment

In contrast, *Salmonella* serovarsTyphi and Paratyphi cause systemic disease in humans that could be fatal if not appropriately treated (Parry CM, Hien TT et al)

Salmonella serovars causing gastroenteritis are also able to cause serious systemic disease in individuals with a primary or acquired immune deficiency and rapidly turn fatal. (Gordon MA et al;2008)

1.2 . NOMENCLATURE ANDTAXONOMY

Dr. Daniel Salmon ,discovered the organism Salmonella,hence named after him.The genus Salmonella initially included organisms that gave rise to a certain type of illness in humans and animals and were related to one another antigenically. Later more emphasis was placed on biochemical activity than antigenic structure in their definition.

It comprises of two species:

I. Salmonella enteric , and

II. Salmonella bongori.

There are six subspecies of S. enterica, the most important of which is S.enterica subsp. enterica (subspecies I) which includes the typhoid and paratyphoid bacilli andis responsible for widespread disease in humans.

Other five subspecies (II–VI)are : *salmae,arizonae,diarizonae,houtenae,indica* .

Genus *Salmonella* are currently divided into 2,463 serovars (Popoff*et al*, 2000).They are proved as genetically belonging to a single species (Crosa*et al*, 1973).

Kauffmann described 150 serotypes' in the 6th edition of Bergey's Manual Kauffmann, 1948.In 1952, he described three species, which could be differentiated by biochemical tests (Kauffmann & Edwards, 1952).

In 1982, about 88*Salmonella* reference strains belonging to be subgenera I-IV by Kauffman (Le Minor *et al*, 1982a) were described. Le Minor *et al.*(Le Minor *et al*, 1982) proposed to

combine four Salmonella subgenus strains into a single species, namely *Salmonella choleraesuis.*

Le Minor *et al*. intended to place all *Salmonella* serovarsin the *Salmonella choleraesuis subsp. Choleraesuis*.

However, an official proposal to place *S. typhi, S. Typhimurium* and *S. Enteritidis* as subjective synonyms of *Salmonella choleraesuis subsp. choleraesuis*, did not appear in a Validation List in IJSB..

Thus*S.typhi, S. Typhimurium* and *S. Enteritidis* remained as validly named species.

S. Typhi*, S. Typhimurium* and *S. Enteritidis* were included in the *S. enterica. Salmonella enteric* originally was used by Edwards& Ewing (1963).

Thus, currently, five species and six subspecies of *S. choleraesuis*. The species name of *Salmonella typhi* is still valid although this species is genetically identical to *S.choleraesuis subsp*. choleraesuis because of their high DNA-DNA similarity value (Crosa*et al*,1973; Stoleru *et al*, 1976

1.3 . Morphology

The morphology of salmonellae is similar to that ofother enterobacteria. With the exception of Gallinarum-Pullorum, they are motile by peritrichous flagella.Nonmotile strains may be isolated from clinical cases.Most salmonellae form common fimbriae and most fimbriate strains (80 percent) possess type-1 fimbriaeassociated with mannose-sensitive adhesive properties(Duguid et al. ; Duguid and Old). Thesefimbriae are composed of fimbrillin subunits (MW c.21 kDa) containing a high proportion (40 percent) ofhydrophobic amino acids. Failure to form type-1fimbriae is the rule in some serotypes, e.g. Paratyphi A,Gallinarum-Pullorum and is unusual in others, e.g. ParatyphiB, Typhi and Typhimurium (Duguid et al. 1975;Duguid and Old 1980, 1994). Strains of Gallinarum-Pullorumform type-2 fimbriae which are morphologicallyand antigenically like type-1 fimbriae but nonadhesive

(Duguid et al. 1966; Crichton et al. 1989; Duguid and Old 1994).

Some strains of serotypes such as Enteritidis andTyphimurium that are usually type-1 fimbriate produce thin fimbriae (<3 nm diameter) the presence of which renders them O-inagglutinable (Rohde et al. 1975).Originally described as nonadhesive, they agglutinatetannic acid-treated red blood cells (Adegbola et al..1983)and behave like type-3 fimbriae commonly presentin Klebsielleae and Proteeae (Old and Adegbola 1985)

1.4.Antigenic structure

The various antigenic types of *Salmonella* were organized by White in 1934 intoa classification scheme that was useful in epidemiological study and typing ofGenus Salmonella (Joklik, 1992).They have subdivided *Salmonella* into 2,463 serovarscontaining different combination of antigens.The antigens used to define serologic types of *Salmonella* include (Old et al.,1996)

Surface antigens

a) O- antigen(German for 0hne meaning without)**:** The presence and proper chain length distribution of the O-antigenpolysaccharide are essential for serum resistance of *Salmonella* Typhi but not for invasion of epithelial cells (Hoare *et al,* 2006).

b**) H antigen**(german Hauch meaning breath or mist) : Flagellar antigen made up of protein flagellin.More immunogenic.Appears late in infection.

c) Vi antigen(Vi for Virulence)**:** The capsular polysaccharide Vi antigen (ViCPS) is an essentialvirulence factor and also a protective antigen of *Salmonella typhi*(Tang *et al*, 2003). The Vi antigen is found in all clinical isolates from patients with acute typhoidinfection. It protects *S.* Typhiagainst complement mediate lysis as well as phagocytosis(Kossack*et al,* 1981).

d) Fimbrial antigen: *S.* Typhisynthesizes type IV pili and such pili are important inadherence to or invasion of human intestinal cells (Zhang *et al*, 2000).

e) Outer membrane protein (OMP): 55 kDa outermembraneprotein from short-chain fatty acids induces apoptosis in macrophages and thus acts as a virulence factor (Chander *et al*, 2006)

Kauffman -White classification

The **Kauffmann–White classification** or **Kauffman and White classification** scheme is a system that classifies thegenus *Salmonella* into serotypes, based on surface antigens. It is named after Philip Bruce White and Fritz Kauffmann.

First the "O" antigen type is determined based on oligosaccharides associated with lipopolysaccharide. Then the "H" antigen is determined based on flagellar proteins.

Since *Salmonella* typically exhibit 'phase variation' between two motile phenotypes,different "H" antigens may be expressed. Salmonella that can express only one "H" antigen phase consequently have motile and non-motile phenotypes and are termed monophasic, whilst isolates that lack any "H" antigen expression are termed non-motile.

Pathogenic strains of *Salmonella* Typhi, *Salmonella* Paratyphi C, and *Salmonella* Dublin carry the capsular "Vi" antigen , which is a special subtype of the capsule's "K" antigen (from the German word *Kapsel* meaning Capsule).

Primary subdivision is into O serogroups, each of which shares a common somatic antigen.O serogroups were formerlydesignated by letters of the alphabet and some groups were subdivided into subgroups. serogroups are defined by the 67 O antigens .(Lindberg and Le Minor ; Popoff and Le Minor).

The serotypes classified in the scheme include members of all subspecies and of S. bongori. An antigenic scheme was developed independently from the Kauffmann–White scheme for the characterization of salmonellae of subspp. arizonae and diarizonae when they were considered to belong to a separate genus (Arizona) distinct from Salmonella.

It is convenient and sensible to designate the serotypes of all subspecies of Salmonella by one set of symbols. Hence, the ‘Arizona’ formulae have been deleted from the scheme (Popoff and Le Minor).

In the Kauffmann–White scheme, only antigens of diagnostic value are cited.

For most purposes a simplified version of the scheme, based on only 12 O, 18 H sera, and the Vi serum, gives sufficient information for routine diagnostic identifications.

The distribution of 2399 known salmonella serotypes among the different subspecies is: enterica (1 416), salamae (477), arizonae (94), diarizonae (317), houtenae (66), indica (10), and S. bongori (19) (Popoff et al. 1995).

The majority (96.5 percent) of the serotypes of subsp. enterica belong to 6 O serogroups (O4–O1,3,19) and the rest are scattered throughout the remaining serogroups. Most of the serotypes from subspecies other than enterica belong to higher O serogroups O11–67, some of which also contain serotypes of enterica.

Table 2: Kauffmamann white classification

Group, Serogroup Serotype	O Antigen	H Antigens Phase1	Phase 2
Group A, Serogroup 2			
S. paratyphi A	1,2,12	a	-
Group B, Serogroup 4			
S. paratyphi B	1,4,5,12	b	1,2
S. derby	1,4,5,12	f.g	(1,2)*
S. typhimurium	1,4,5,12	i	1,2
Group C, Serogroup 7	6,7	c	1,5
S. choleraesuis	6,7,(Vi)*	c	1,5
S. paratyphi C			
Group D, serogroup, 9			
S. typhi	9,12,(Vi)*	d	-
S. enteritidis	1,9,12	g.m	-
S. pullorum-gallinarum	1,9,12	-	-
Group E, Serogroup 3,10			
S. weltevreden	3,10	r	z_6
S. anatum	3,10	e.h	1.6
Group G, serogroup 13,22	13,22	z	1,6
S. Poona	1,13,23	z_{29}	-
S. cubana			

Serotypes in subspp. arizonae and houtenae and S. bongori are monophasic.

1.6 .Cultural characters and growth requirements

Salmonellae grow over a wide temperature range from 7° to 48°C, at pH 4–8 and at water activities above 0.93(Baird-Parker 1991). *S.*Typhigrows luxuriantly in all ordinary culture media. It grows best under aerobic conditions, but may also grow anaerobically. The temperature range for the growth of *S.* Typhi is from 4° to 40°C; the optimum being 37°C (34). *S.*Typhi can survive about a week in sewage contaminated water and remains viable in fecal materials for 1-2 weeks.(Smith AC.et al)Some serotypes of salmonellae, particularly

Paratyphi B, produce mucoid colonies best developed at low temperature, low humidity, and high osmolarity(Anderson and Rogers 1963). The presence of a mucoid surface layer, the M antigen of Kauffmann, inhibits Oand H agglutinability. Because the M antigen is identicalin all salmonella serotypes and is like the colanic acid materials of other Enterobacteriaceae, it is unimportant diagnostically. The 'mucoid-wall test' is positive with most strains of Paratyphi B that cause enteric fever(Kauffmann 1966).

1.6.1. On nutrient agar and blood agar

The colonies of most strains are moderately large, 2-3 mm in diameter after 24hrs at37ºC. They are grey-white, moist circular discs with a smooth convex surface andfull edge. Non-virulent, rough strains (S-R variation) form opaque granular colonieswith an irregular surface and indented edge.

1.6.2 Peptone water and nutrient broth

Most strains show abundant growth with uniform turbidity. A thin surface pellicleforms on prolonged incubation. Rough (R) variants, which have a hydrophobic surface, tend to autoagglutinate and produce a granular deposit and a thick surface pellicle.

1.6.3 Diferential and Selective solid media

Selective culture media must be used to recover the *Salmonella* from clinical specimensthat potentially harbour mixed bacteria.The following can be used as differential and selective media for the isolation of*Salmonella* from different specimens:

a)MacConkey agar

After 18-24 h at **37ºC,colourless colonies**, 1-3 mm indiameter, and easily distinguished from the pink-red colonies of lactose-fermenting organisms.

b) Brilliant green MacConkey agar

This makes it an excellent selective as well as differential medium for *salmonellae*except *S. typhi*, which doesn't grow well. *Salmonellae* produce low convex, palegreentranslucent

colonies, 1-3 mm in diameter. Lactose-fermenting bacteria, and somerare strains of *Salmonella* serotypes, produce blue-purple colonies.

c) Deoxycholate-citrate agar (DCA)

This medium is superior to MacConkey agar as far as isolation of *S.* Typhi isconcerned. The colonies are of the same size or slightly smaller in size than those onMacConkey agar. They are pale, nearly colourless, smooth, shiny and translucent.

After 48 hours of incubation the colonies may show black centre, surrounded by a zoneof clearance.

d) Wilson and Blair.s brilliant-green bismuth sulphite agar (BBSA)

The medium is particularly useful in the isolation of *S.* Typhi. The cultures need to beexamined after 24 h, and then again after 48 h. Closely packed small (about 1 mmdiameter) colonies may take up the dye from the medium and appear green or palebrown . *Salmonellae* ,which produce H2S, shows larger discrete colonies with a black centre and a clear edge surrounded by a metallic sheen.

e) Xylose lysine deoxycholate agar (XLD)

Salmonella and *Shigella* grow well in this media. Both form red colonies. Butsalmonellae are distinguished from *Shigellae* as they produce H2S which reacts withferric ammonium citrate in the medium to produce black centres in the red colonies.

The colonies of *Shigellae*are red without any black centre.

f) Salmonella - Shigella agar (SS agar)

SS agar is highly selective medium formulated to inhibit the growth of most coliformorganisms and permit the growth of species of *Salmonella* and *Shigella.* The high bile salts concentration and sodium citrate inhibit all gram-positive bacteria and many gram-negative organisms, including coliforms. Lactose is the sole carbohydrate and neutral red is the indicator for acid detection. Sodium thiosulfate is a source of sulphur. H2S production is

indicated by theblack precipitate formed with ferric citrate. High selectivity of SS agar permits use ofheavy inoculum.

1.6.4 Enrichment media

These are liquid media used to assist the isolation of salmonellae from faeces, sewage,foodstuffs and other materials containing a mixed bacterial flora. The enriched cultureis plated on selective and/or differential media, usually after 24 hours incubation.

1.6.5 Tetrathionate broth

It can be used with or without addition of brilliant green which increases the selectivelyof this medium to most of the *Salmonellae* but makes it inhibitory to *S. typhi*and*Shigellae.*

1.6.6 Selenite F broth

It is the most commonly used enrichment medium for specimens that may containeither *Salmonellae* or *Shigellae*. It is excellent for Typhi and Dublin . It is not suitableparticularly for isolation of *S. paratyphi*A and *S. choleraesuis*.This broth is found to be more efficient than other enrichment media for the isolation ofsalmonellae from faeces, water and foodstuffs[62] (Old, 1996).

1.7 Biochemical reactions

Most strains of *Salmonella* closely resemble each other biochemically, although thereare a number of exceptions; no organism should be excluded on the basis of a singletest. The usual reactions include:

(1) fermentation of glucose, maltose, mannitol and sorbitol with the production of acid and gas;

(2) absence of fermentation of salicin, sucrose and adonitol;

(3) failure to produce indole, to hydrolyse urea or to deaminate phenylalanine; and

(4) a positive methyl-red reaction and a negative Voges-Proskauer reaction (Old, 1996).

Salmonella like *Shigella* can be presumptively identified biochemically using TSI(Triple sugar iron) agar medium and individual biochemical tests (Cheesbrough,2000).

TSI: This medium is used to help identify salmonella following isolation on a selectivemedium *Salmonella* produce:- pink-red (alkaline) slope and yellow (acid) butt, indicating fermentation ofglucose but not lactose.

- Cracks in the medium if serotype produce gas from glucose fermentation

(*S*. Typhi does not produce gas).

- Blackening in the medium due to H_2S , unless serotype does not produce H_2S,*S.* Paratyphi A- abundant H_2S production.

*S.*Typhi produces only a speck of H_2S.

Summary of biochemical reactions of *Salmonella* (most serotype) (Cheesbergh).

▪ Urease and indole negative

▪ Lactose negative

▪ Gas produce from glucose fermentation (*S.* Typhi does not produce gas).

▪ Citrate positive (*S.* Typhiand *S.* ParatyphiA are citrate negative)

▪ Lysine decarboxylase (LDC) positive (*S.* Paratyphi A is LDC negative)

▪ Beta- galactosidase (ONPG) negative.

2.0 Pathogenesis

The infectious dose of *S.* Typhi varies between 1000 and 1 million.Virulence of the organism depends upon strains. (Parry *et al,* 2002).Vi-positive strains are more virulent than Vi-negative strains .*Salmonella* crosses the gastric acid barrier to reach the small intestine. The bacteria adhere to mucosal cells of smallintestine and then invade the mucosa.

The site of the internalization of *S.* Typhi is the M cells(specialized epithelial cells overlying Peyers patches)and its transport to the underlying lymphoid tissue. The bacteria passes to the

intestinal lymphoid follicles and the draining mesenteric lymph nodes. The reticuloendothelial cells of the liver and spleen are invaded(Parry *et al*, 2002).

2.1 Host-Cell Entry of *S. typhi*

2.1.1Attachment to Host Cells

Salmonella typhi targets intestinal epithelial cells (enterocytes) attaches to host cell by using long, hair-like filaments known as fimbriae. Fimbriae are coated with receptor specific adhesins that recognize and bind to only certain types of sites on the surface of target cells (Baulmer *et al*, 1996). And these receptors are found only on certain target cells, great specificity of attachment is achieved (Ohl and Miller, 2001).

2.1.2 Invasion of Host Cells

A complex process known as bacterial mediated endocytosis is utilized by the *Salmonella* (Ohl and Miller, 2001). The bacteria translocate virulence proteins across its cell membranes. This is accomplished by the formation of Type III Secretion Systems (TTSSs). The virulent proteins, now in extracellular space, are transferred across the host-cell membrane by a series of translocase proteins.Two virulent proteins, SopE and SptP are key players are involved in enterocyte invasion by *S*. Typhi(Sah*e etal*, 2000).

These proteins are part of the Rho family of G-proteins. They regulate the actincytoskeleton and gene regulation and rearrangement via phosphorylation of lysophosphatidic acid .(Sah*e et al*, 2000).

The protein SptP reconstitutes the brush border surface of the enterocyte. There are no drugs that will target these complex pathways of salmonella invasion of intestinal epithelial cells or systemic infection. The metabolic pathways of the bacteria antibiotics targets these pathways (Ohl and Miller,2001).

2.3 Persistence in Host Cells

2.3.1 Metabolism of *S*.Typhi. Most antibiotics are targeted toward oneor more specific enzymes in *S.Typhi* that catalyze these important reactions whichultimately kills the bacteria (kelly *et al*, 2004).

2.3.2 Host Cell Dependent Pathways for Systemic Infection

Several virulence factors are present to survive in a microbicidal environment. *S*. Typhiafter invading enterocytes and M-Cells, triggers macrophage activity due to its

- antigenic factors . Bacteria will be phagocytosed in the submucousal region of the intestine and macrophage act as a vector for transport to reticuloendothelial organs . *S*.Typhievades the humoral immune response. It protects *S*. Typhi from the internal environment of the macrophage which contains hydrolytic enzymes, antimicrobial peptides, and other factors.

To survive, the bacteria utilize the following strategies:

- The bacterial lipid membrane is modified by the lipopolysaccharide by altering membrane fluidity and surface charge density(Ohl and Miller, 2001).

  Resistance to antimicrobial peptide insertion occurs which is used by phagocytes to disrupt bacterial cell membranes.
- *S*. Typhi, produces several enzymes including homocysteine that deactivates nitric oxide radicals in the phagocyte(Fang, 1997).
- Essential metabolites (purines, aromatic amino acids, etc.) are produced in the nutrient deprived environment of the phagocyte.*S*. Typhi produces several proteins .These interfere with the maturation of a phagocyte, preventing the fusion between phagosome and lysosome.

2.3.3 Virulence Factors Mediated by PhoP and PhoQ

In *Salmonella* Typhi there is a simple two component regulatory system.There are two proteins, a sensor kinase and an effector polypeptide, they controls the expression of the genes for virulence due to which the bacteria is able to survive in the phagocyte.

Environmental signals activate the sensor kinase that phosphorylates the effector protein, which acts as a transcriptional regulator in the bacteria (Perraud*et al*).

In the case of *S.*Typhi, the sensorkinase , PhoQ, is activated upon environmental signals unique to the internal environment of the phagocyte, including low pH and deficient nutrient levels.

After activation, phosphorylation of the cytosolic effector molecule PhoP will occur,

and acts as the transcriptional regulator for the virulence genes.

The "pag and prg" , are such important genes which mediate such virulence factors(Miller *et al*).

3.0 Immune response

The nature of protective immunity for typhoid in man is not well understood.

It is well documented in the literature that humans as well as experimental animals

Salmonella infection causes activation of both humoral and also cell-mediatedimmune responses (CMIR) (McGhee &Kiyono).

These immune responses are correlated with the clinical picture and specific CMIR protection against typhoidfever (Sarma*et al*).

2.9.1 Cell mediated immune response (CMIR)

The CMIR in typhoid fever develops during the second week of illness in uncomplicated cases and is often negative in complicated cases (Sarma*et al*).

Cell-mediated immunity as assessed by the "leucocyte migration inhibition test" (LMI),

and developed in all cases with typhoid fever. Positive LMI was evident in the firstweek of the illness and was positive in some patients was still after a year since infection.

It also developed at the end of 3 weeks in TAB vaccinated subjects (Dham& Thompson). Positive LMT is associated with good clinical recovery (Sarma*et al*).

Few studies have shown that, iron-regulated outer-membrane protein (IROMPs)expressed by *S.* Typhiinduce a CMIR against infection through Th1and Th2 type cells (Sood *et al*). The cellular immune response induced by IROMPs resulted in an enhanced DTH (delayed-type hypersensitivity response) and exhibited a significant increase the ratio of CD4+/CD8+ cells and increased production of interleukin (IL)-2- and interferon (IFN)- in early period and in the later period of the study, increased production of IL-4 producing cells.

The increase in the lymphocytes in PPs (Peyer's patch) might have caused the increase in the sIgA . Therefore, it is speculated that immunization with IROMPs may evoke a peripheral aswell as mucosal immunity against *S.* Typhi infection(Sood*et al*).

In uncomplicated cases of typhoid fever, an intact CMIR was found (Rajagoplan*et al,*). The ratio of T lymphocyte were altered in the complicated cases as compared to uncomplicated cases.

CMIR become depressed in complicated patients with typhoid fever. The imbalance within the subsets of T lymphocytes may be responsible for the depressed state of CMIR in complicated cases of typhoid fever.

CMIR so emerges as the cardinal point for recovery in typhoid fever rather thanthe specific antibodies(Rajagoplan*et al,* 1982; Sarma*et al*, 1977).

2.9.2 Humoral immune response

The humoral response to *Salmonella* Typhiis important method providing immunity againsttyphoid fever, as with killed cell vaccines and component vaccines (outer membrane proteins, Vi antigen) (Aron *et al*, 1993).

Antibodies to *S.* TyphiO, H, and Vi appear to mediate protectionagainst *S.* typhiinfection by acting in conjunction with other adaptive responses, or serves as a surrogate for the presence ofmore prevailing protective immune responses e.g., in cell-mediated immunity (Sztein,2007).

Anti-O-polysaccharide chain antibody titres are lower during the first week and then increases in the third week of the infection.*S.* Typhi IROMPs have immunogenic potential and they stimulate antibody-mediated protection at systemic and mucosal levels (Sood*et al*,2005).

Both antibody and cell-mediated immune(CMI) responses are considered to be important,in protection against salmonella Typhi.

Susceptibility to chemical agents

- Many observations on the resistance of salmonellae to different chemical substances have been derived from attempts to devise enrichment and selective media for the isolation of salmonellae from samples containing other coliform bacilli. The use of dyes, particularly those of the triphenylmethane group, as inhibitors has been well defined since the early 1900s when malachite green and later brilliant green were used.
- Brilliant green-containing media are useful for the isolation of most serotypes other than Typhi. RV medium , a modification by Vassiliadis et al. in1981 of the original Rappaport medium, in which the concentration of malachite green was reduced to 0.004 percent w/v, is considered superior to other enrichment media.
- Tetrathionate broth, especially the modification of Kauffmann–Muller containing brilliant green and 0.018 M tetrathionate, is useful for a wide range of serotypes including Dublin, but not for *S.*Typhi which is best recovered through selenite broth.

- Many salmonellae multiply at 43ºC and incubation of enrichment media such as selenite or RV broths at that temperature is being recommended for the isolation of salmonellae. Solid media containing bile salts or pure sodium deoxycholate, often in the presence of other inhibitory substances, are generally used as selective plating media.
- Bismuth sulfite agar, often recommended for Typhi, is perhaps even more useful for the detection of lactose fermenting salmonellae. Many other media based on the action of yet other inhibitory substances have been devised though few are in use today. For a discussion of the use and choice of these media and modifications of those described above, the authoritative review of Fricker in1987 is invaluable.

Background and history of typhoid fever

Typhoid fever was not recognised as a separate clinical entity until the late 19th century and was often confused with other prolonged febrile illness such as typhus fever of rickettsial origin.

Typhos, in Greek means “smoke” and typhus fever got its name from smoke that was believed to be the cause. Typhoid means typhus-like and thus the name given to this disease.

Although typhoid fever was first discovered by Willis in 1643 (Collier Encyclopedia 1989), it was mistakenly understood to be typhus fever for a long time. Gerhard in 1837 (Collier.sEncyclopedia 1989), distinguished the two illnesses and coined the name “Typhoid fever” meaning Typhus like fever.

The causative organism was visualized in tissue sections from Peyer’s patches and spleens of infected patients by Eberth in 1880 and named it as *Salmonella* Typhosum and was grown in pure culture by Gaffky in 1884 (Topley and Wilson 1990).

The significance of water contamination in the spread of the disease was first recognized by Budd in 1856 (Topley and Wilson 1990).His observations provided one of the greatest milestone in the development of hygiene.

He proved that the disease was infectious and spread can happen through infected patients faeces. It was also shown that milk and water played important role in the transmission of typhoid fever.

In 1907, the famous Mary Mallon was the first identified carrier of typhoid fever. She has thus been called as" TYPHOID MARY". Mary gave up working as a cook ,but returned as a cook again under a false name. She was again identified after another typhoid outbreak and detained in quarantine where she died after 26years due to pneumonia.

An effective vaccine was developed by Almroth Edward Wright in 1897. Antibiotics were introduced in clinical practice in 1948, greatly reducing mortality (Lesser & Miller, 2005). The rate of typhoid infection has begun to reduce in the first half of 20th century.

<u>Epidemiology</u>

2.6.1 Typhoid Fever Global Scenario

Typhoid fever still remains to be a global health problem. It is endemic in areas of poor sanitation and unsafe water supply is unsafe. More than 33 million people suffer from typhoid globally every year.

One third of the cases get complicated if untreated resulting in deaths in 75% of these.The case fatality rate is 10% in inappropriately treated cases. 5% of surviving patients become chronic carriers (Park, 2011). The only reservoirs are humans for this infection (Black, 1985; Stroffolini , 1992; Egoz, 1998; Mermin, 1999) . Hospital based studies will underestimate true incidence because the major portion of patients, 60%-90%, are treated as outpatients.

Humans are the only natural host and reservoir. The infection is transmitted by feco-oral transmission. Shellfish taken from contaminated water, and raw fruit and vegetables fertilized with sewage,and on occasions ice-creams have been sources of past outbreaks (WHO, 2003).

The inoculum size and the type of vehicle in which the organisms are ingested greatly influence both the attack rate and the incubation period (WHO,2003)

About 1%-5% of patients become chronic carriers, harbouring *S.*Typhiin the gallbladder (WHO, 2003). Chronic typhoid carriers status are responsible for the endemicity and frequent outbreaks of the disease in the region. In patients with billiary, gastrointestinal and other related diseases higher chances of carrier states are recognised (Vaishnavi*et al,* 2005).

The worldwide yearly incidence was estimated at approximately 12 million cases for 2010 . The case fatality rate remains at about 1% such that about 130,000 typhoid deaths occur every year. Antibiotic resistance is a challenge is becoming increasingly problematic with the spread of the multi-drug resistant strains.

An outbreak of nearly 6000 cases of multi-drug resistant typhoid fever in Nepal, which was traced to municipal water supplies highlights the global importance of this infection (Lewis *et al,* 2005).

INDIAN SCENARIO

In india typhoid is the 5th most common communicable disease.Emergence of multi-drug resistant strains are posing a more threatening picture. Latest upgraded drugs are proving to be ineffective against these resistant strains of typhoid bacteria.

The disease is endemic in almost all parts of the country with periodic outbreaks.

At Safdarjang hospital, Delhi was undertaken between January 1999 and December 2003 estimating age related epidemiological , clinical and microbiological characteristics in

enteric fever cases, which showed that more than 24% of cases were in children up to 5 years of age (Walia*et al.,* 2006). In 1992, about 143,52,980 cases with 735 deaths were reported. Hospital-based studies and outbreak reports from India indicate that enteric fever is a major public health problem in this country, with *Salmonella* Typhi the most common etiologic agent but with an apparently increasing number of cases due to *S*. Paratyphi *A*(Kanungo, 2008).

In the second week of the illness the patient becomes toxic and apathetic with sustained highfever. The abdomen is slightly distended and splenomegaly is common (Gillespie, 2003). Relative bradycardia is not a consistent feature. Rose spots are reported in 5.3% of cases mostly missed in the dark-skinned patients. These rose spots are small blanching erythematous maculopapular lesions typically on the abdomen and chest. Melanesian typhoid patients may develop purpuric macules that do not blanch (Parry *et al,* 2002).

With the onset of third week the patient become more toxic and fever persist and adelirious confusional state sets in (Typhoid states). Abdominal distention becomes pronounced, with scanty bowl sounds. Diarrhoea is common, with liquid, foul green yellow stools. The patient becomes weak with a feeble pulse and rapid breathing; crackling sound on ascultation may be heard over the lung basesDevastating toxaemia, myocarditis, intestinal haemorrhage or perforation leads to death. Considerable weight loss is common.

During fourth week, the fever, mental state and abdominal distention slowly improve but intestinal complication is still a possibility.Convalescence is usually a slow process (Gillespie, 2003). The tongue is covered with a thick furry white to brown coating that spares the bright red tip and edges. The classic description of doughy feeling on palpation on abdomen is due to the bowl filled with air and fluid especially in the ileo-caecal region commonly designated as “Caecal gurgling”.

Atypical manifestations observed such as burning micturition with normal urine examination, diarrhoea and encephalopathy during the first week, isolated hepatomegaly, pneumonitis and bone marrow depression (Dutta *et al,* 2001).

The fever persists for two weeks or more and may delay the clinical suspicion of the disease. over the following 2 to 3 weeks. Convalescence may last for 3-4 months. On appropriate antibiotic treatment the fever gradually falls over 3-4 days. The severity of the disease is also influenced by the delay,if any in the initiation of appropriate treatment.

The individuals infected with multi-drug-resistant (MDR) isolates of *S.* Typhi suffer from more severe form of the disease (Parry *et al*, 2002).

2.12.1 Typhoid fever in children

Typhoid fever, affects all age groups, the classical features
described are usually absent in children. Fever was the most common presentingsymptom and diarrhoea was more common than constipation. Unproductive cough,hepatomegaly and splenomegaly are present. Rose spots and relative bradycardia wererarely observed (Yap &Puthuchery, 1991).

2.13 Complications

Typhoid fever is a multi-system disease involving all the organs, leading to aprolonged illness, systemic complications and high mortality rate if inappropriately managed.

Some of the complications associated with high morbidity and mortality are as follows.

Intestinal perforation and bleeding

The intestinal bleeding and ileal perforations, arising from necrosis of Peyer's patches in the terminal ileum is the most serious complication in Typhoid fever .The majority of typhoid fever patients develop perforation nwithin the first 2 weeks of the illness .

Risk factors for enteric perforation in patients with typhoid fever are –a short duration of symptoms,inadequate antimicrobial therapy prior to admission,in males, and leukopenia (Hosoglu *et al,* 2004).

Typhoid encephalopathy

Typhoid encephalopathy is often accompanied by shock,and is associated with a high incidence of mortality. Patients may display the 'Typhoid Facies' - a thin, flushed face with a staring,apathetic expression.

Mental apathy may progress to an agitated delirium, frequently accompanied by tremor of the hands, tremulous speech and ataxiac gait. Muttering delirium, twitching of the fingers and wrists agitation and plucking at bedclothes (carphology), and a staring, unrousable stupor (coma vigil) (Parry *et al,* 2002).

Hepatobilliary manifestation

Acute cholecystitis is a common complication; however, classical symptomof acute cholecytitis may be absent. *Salmonella* infection has been reported to beassociated with increased incidence of gallstones. In about1% of patients with typhoid fever, which usually occurs as a result of typhoid hepatitis (hepatomegaly with raised transaminases), septicaemia, or liver abscesses (Joshi,2001), followed by jaundice is seen.

Complications of typhoid fever in children

It includes -

An icteric hepatitis, bone marrow suppression, paralytic ileus, myocarditis, psychosis, cholecystitis, pneumonia, haemolysis, and syndrome of inappropriate release of antidiuretic hormone (SIADH) ,osteomyelitis,peritonitis (Malik, 2002).

Relapse

Relapse occurs in 5% to 10% of patients, usually 2 to 3 weeks after defervescence. There is usually an afebrile period between the first and second episode of fever which

maybe a few days to a few weeks (Joshi, 2001).The illness is of mild intensity than the original attack and the relapse *S.* Typhi isolate the susceptibility pattern is same as in the first attack (Parry, 2002).

Carrier

It is reported that 1% to 5% of those infected become chronic carriers (WHO, 2003) andcarrier status persists throughout the lifetime of the person.

Carriers of *S.* Typhiare

- "Convalescent Carriers"- who excrete the organism for a limited period of time after apparent cure, and
- "Chronic Carriers" - in whom persistent excretion of *S.* Typhiinstool or urine can be detected a year after clinical illness. Chronic faecal carriers aremore common than chronic urinary carriers (Singh, 2001).

This is also responsible for deaths due to hepatobiliary carcinoma.

Chronic carriers give no prior history of typhoid fever in up to 25% of cases. Faecalcarriage is more frequent in individuals with gallbladder disease and is most commonin women over 40 (Vaishnavi *et al*, 2005). Chronic carriage carries an increased risk of carcinoma of the gallbladder, pancreas and large bowel (Parry *et al,* 2002). Urinary carriage is associated with schistosomiasis and nephrolithiasis.

Review of diagnostic methods

Laboratory diagnosis of typhoid fever relies on

- The isolation and the identification of *S.* Typhifrom a suitable clinical specimen like blood, stool, urine, bone marrow, duodenalaspirate by culture.
- The detection of *S.*Typhi specific antibodies and antigen byserological test and identify DNA by PCR (Pearson & Guerrant, 1995).

Recent advances in immunochemistry has provided different new approaches. Amongthem Dot enzyme immunoassay (EIA) and Immunochromatographic test (ICT) are of major serologic importance.

2.16.1 Isolation of the organism

S. Typhican be isolated from patients of typhoid fever if blood, stool, rose spot, and bone marrow aspirates are cultured at the appropriate time (Gilman *et al,* 1975). *S.*Typhimaximally isolated from blood in the first week of disease, from feces in thesecond and following weeks from urine.

The various culture methods available are:

A. Blood culture;
B. Clot culture;
C. Faeces culture;
D. Bone marrow culture;
E. Urineculture;
F. Bile culture/ Duodenal aspirates culture.

A . Blood culture

This is the method of choice .It has the advantage of showing not only that the patient is infected with the bacillus but thatthe infection is active and is almost certainly responsible for the disease (Parker, 1990). Though it is gold standard, the result of blood culture is variable. The blood cultures are usually positive in about 80% of untreated patients,during the first week and declines thereafter to 20%- 30% in the course of the disease (Gillespie, 2003). The cause for such low yeild is due to very low numbers of bacteria causing a severe disease which may be less than 10 per ml. of blood (Haque*et al*, 1999). The bacterial detection by blood culture is influenced by the culture medium , the number of bacteriain circulation, the blood collection time and volume collected, the host's immune response

system,and the intracellular character of these bacteria (Gavirla-Ruizi and Cardona-Castro, 1995).

Sufficient volumes of medium should be used in blood culture to avoid negative results . A study suggests that a minimum of 50 ml of medium was adequate for 8 ml of blood, presumably because of very low degrees of bacteremia in some patients (Watson, 1978).

If whole blood is to be cultured, it is essential to prevent bactericidal effects of serumeither by enough dilution of the sample in an sufficient volume of the medium or byinhibition of the serum bactericidal factors. Sodium polyanethol sulfonate (SPS) (Liquoid)and bile salt act as the inhibitors of this bactericidal effect (Parker, 1990). SPS in concentration of 0.025% to 0.03% is the best anticoagulant for blood.

In addition to its anticoagulants properties, SPS also has anti-complementary and anti-phagocytic , and interferes with the activity of some antimicrobial agents, notable amino glycosides (Forbes *et al*, 2002).

A study indicates that SPS helps in early recovery of *S.* Typhi *and S.* Paratyphi A bacteria fromblood cultures (Escamilla *et al*, 1985).Also taking culture on several occasions may improve the yield (Le & Hoffman, 1999).

Trypticase soya broth, bile broth or glucose broth, brain heart infusion broth are commonly used medium for conventional methods of blood culture. The media is incubated aerobically at37^{o} C. Subculture should be done on MacConkey agar, blood agar media daily for 1week (Watson, 1978).

Modern automated blood culture techniques allows the bacteriological confirmationof typhoid fever in a high number of cases. They employ equipment thatautomatically detect any early sign of bacterial growth in a special blood culture bottle(Collee & Marr, 1996). A

92% isolation rate with the Bactec 460 radiometric system using a blood: broth ratio of 1:6 was found in a study (Duthie& French, 1990).

B . Clot culture

Blood clot from which serum has been removed often gives a positive result when asimilar volume of whole blood yields no growth (Parker, 1990). A method of clotculture with streptokinase has been recommended (Watson, 1956). Blood is taken fromthe vein in the usual way and 8-ml quantities are, allowed to clot in a sterile screwcappedUniversal containers. The separated serum is removed. The medium usedconsists of a Wilson &Blair agar slope in a 100ml bottle to which is added 15 ml.of streptokinase bile salt broth. Streptokinase causes rapid clot lysis releasing the bacteria trapped in the clot. The cultures are then incubated. Positive results may beobtained in less than 24 hours (Watson, 1956). Clot culture is more sensitive than bloodculture.An isolation rate 92% is reported.The clot technique has many advantages overconventional whole blood culture, both in reliability and in cost (Watson, 1978).

2.16.4 Culture of the Mononuclear Cell-Platelet Fraction of Blood

The low concentration of *S.* Typh*i* cells in the blood of patients with typhoid fever, <10bacteria per ml, undoubtedly contributes to the moderate sensitivity of blood culture.

By this method blood from typhoid patients is subjected to density gradientcentrifugation, virtually all *S.* Typhicells are in the fraction containing onlymononuclear cells (MNC) and platelets. Colonies of *S.* Typhi were present in allmononuclear cell-platelet layer-positive cultures within 18 hours of plating and wereidentified within an additional 10 min by a coagglutination technique. In contrast,identification of all positive cultures by conventional blood culture required 3 days(Rubin *et al,* 1990).

2.16.5 Bone marrow culture

Bone marrow aspirates are known to yield a higher rate of positive cultures in typhoidthan peripheral blood (Gilman *et al,* 1975; Farooqui *et al*, 1991)). Bone marrow culturemay give a positive result when blood culture fails, particularly in patients admitted tohospital while on antibiotic treatment. Unlike blood culture bone marrow culture ishighly sensitive (90%) (Lesser & Miller, 2005) despite 5 days antibiotic treatment(Gasem*et al,* 1995). A study report shows that the concentrations of *S.* Typhiin thebone marrow are considerably higher than in peripheral blood (Wain *et al,* 2001). In thebone marrow there are over 10 times more bacteria than in peripheral blood. It appearslikely therefore that a large-volume blood culture (>10 ml) would be needed to matchthe positivity rate of a 1-ml bone marrow culture (Wain *et al,* 2001). This should be true particularly for patients who have been previously treated or who present late in the illness.

2.16.6 Duodenal string-capsule culture (DSCC)

The string capsule device is a useful and simple method for culturing duodenalcontents for the presence of *S.* Typhi. This capsule device is useful as a simpleout patient method for defining typhoid carriers (Gilman & Hornick, 1976). The stringcapsule device is made of a length of nylon yarn that is coiled onto a weighted gelatincapsule. A thread is attached to the yarn protrudes from the capsule and allows for the yarn to uncoil when the capsule is swallowed and is also retrieval of the yarn from the intestine. The final 12 inches of bile-stained string is cut off and dropped into seleniteF broth. 24 hrs post incubation at 37 C, the broth is streaked on MacConkey agar (Gilman &Hornick, 1976).

The sensitivity of duodenal string-capsule culture (DSCC) is compared to that ofbone-marrow-aspirate culture (BMAC), single 3-ml blood culture (BC), and rectal swabculture (RSC) for isolating *S.*Typhi and *S.*Paratyphi Type Afrom patients with typhoid and

paratyphoid fever, when the DSCC was positive in 57.6%, RSCin 35.6%, BC in 54.2%, and BMAC in 85.6%. The sensitivity of DSCC was improvedby an additional 4.7% when it is daily subcultured for seven days. There is no added advantage of DSCC over the combination of RSC and BC and is inferior in sensitivity to theBMAC method . In the inability to do BMAC, the addition of the DSCC to BC and RSC can be expected to improve the isolation rate by greater than 17%, to at least85% (Hoffman *et al,* 1984).

2.16.7 Stool Culture

Stool specimen is collected in a sterile wide mouthed container. Specimensare preferably processed within 2 hours from collection. In the case of delays thespecimen should be stored in a refrigerator at 4°C or in a cool box with freezer packs.

The sensitivity of stool culture depends on the amount of feaces cultured. The positivity rate increases with the duration of the illness.

Rectal swabs should be avoided as these are less successful. Stool cultures are positive in 30% of patients with acute enteric fever (Parry *et al,* 2002). For the detection of carriers, because of irregular shedding of *salmonella,* it is advised to examine several samples.

2.16.8 Urine culture

Urine cultures are not recommended for diagnosis in view of poor sensitivity (Parry *etal,* 2002; Gilman *et al,* 1975). Bacteria are not excreted continuously and thereforeseveral specimens may need to be cultured before organisms can be isolated(Chessbrough, 2000).

2.16.9 Serodiagnosis of typhoid fever

Widal test

Fernend Widal first described the WIDAL TEST in 1896. It detects agglutinating antibodiesagainst the O and H antigens of *S.* Typhiand H antigens of Paratyphi A and B(Olopoenia & King, 2000). The "O" antigen is the somatic antigen of *S.*Typhi.

It is common in *Salmonella paratyphi A, paratyphi B*, other *Salmonella* species andother members of the Enterobacteriaceae family (Rodrigues, 2003). Antibodies againstthe O antigen are predominantly IgM, rise early and also disappear early in the illness(Rodrigues, 2003). The H antigens are flagellar antigens of *Salmonella* Typhi*,* Paratyphi
A and Paratyphi *B.* Antibodies to H antigens are both IgM and IgG, rise late in theillness and persist for a longer period of time (Olopoenia & King 2000, Rodrigues, 2003).

Anti -O antibodies appear on day 6-8 and anti-H antibodies on days 10-12 after the onsetof the disease. The test is usually performed on the serum at first contact with thepatient. A convalescent serum should preferably also be collected so that pairedtitration can be performed.Conventionally, a positive Widal test result means demonstration of rising titers inpaired blood samples 7- 10 days apart (Olopoenia & King, 2000). Thiscriteria is purely of academic interest. Decisions about antibiotic therapy cannot wait for results from two samples. The antibiotics however, dampen the immune response and thus prevent a rising titre even in truly infected individuals. Therapeutic decisions are thus, generally based on results of a single acute sample. In endemic areas, baseline anti-O and anti- H antibodies are present in the population owing to repeated subclinical infections with *S.* Typhi/Paratyphi*,* infections and other tropical diseases such as dengue and malaria (Olopoenia & King, 2000; Parry*et al*, 1999). These antibody titres vary with age, socio economic status, type of geographicareas and prior immunization with the TAB vaccine.

Interpreting the results of the Widal test, both H and O antibodies are to betaken into account. The predictive value of O and H antibodies for diagnosis of enteric fever is contraversial. For practical purpose and for optimal result the test should be done after 5-7 days of fever by tube method and level of both H and O antibodies of 1 in 160 dilution (four fold rise) should be taken as cut off value for diagnosis.

The Widal test as a diagnostic modality has suboptimal sensitivity and specificity(Olopoenia & King, 2000; Parry *et al,* 1999; Rodrigues , 2003). It can be negative in up 30% of culture proven cases of typhoid fever. Suboptimal sensitivity results fromnegativity in early infection, prior antibiotic therapy and inabilty mount an immuneresponse (Olopoenia & King, 2000). Low specificity is a consequence of pre-existing baseline antibodies in endemic areas, cross reaction with other Gram-negative infections and non-typhoidal salmonella, anamnestic reactions in unrelated infections and prior TAB or oral typhoid
vaccination.

Despite these drawbacks, the Widal test may be the only test available incertain regions for diagnosis of enteric. In Vietnam, using a cut-off of>1/200 for the O agglutinin or >1/100 for H agglutinin test are performed on acute-phaseserum .This Widal test could correctly diagnose 74% of blood culture positive typhoidfever, however 14% results would be false positive and 10% false negative (Parry *et al,*1999). It is important to realize the limitations of the Widal test and interpret theresults carefully in light of endemic titres so that both over diagnosis and underdiagnosis of typhoid fever and the resulting consequences can be avoided.

Other serological tests

In view of these limitations of the Widal test ,a need for a cheap and rapid diagnosticmethod , several new alternative serologic tests have been developed. Theseinclude rapid dipstick assays, dot enzyme immuno-assays and agglutination inhibitiontests (Olsen *et al*, 2004).

2.16.10 Antibody detection:

DOT Enzyme Immunoassay (EIA) test

A dot enzyme immunoassay that detects IgG and IgM antibodies against the somatic (O), flagellar (H) or capsular (Vi) antigen of *Salmonella* Typhi is commercially available (Gasem *et al,* 2002).The sensitivity and specificity of this test has been reported to be 70-100% and
43-90% respectively (Khan *et al*, 2002, Bhutta & Mansurali 1999). This testoffers simplicity, speed, early diagnosis and high negative and positive predictivevalues. The detection of IgM reveals acute typhoid in the early phase of infection, whilethe detection of both IgG and IgM suggests acute typhoid in the middle of theinfection. In areas of high endemicity the detection of specific IgG increase. IgG can persist for more that 2 years after typhoid infection the detection of specific IgG can not differentiatebetween acute and convalescent cases(Saha *et al,* 1999),. Furthermore, false positive results due to previous infection may occur.

On the other hand IgG positivity may also occur in the case of current reinfection. In cases of reinfection there is a secondary boosted immuneresponse with increase of IgG over IgM, such that the later can not bedetected and its effect masked.

The original test modified by inactivating the total IgG in the serum samples have shown that inactivation of IgG removes competitive binding and allows the access of the antigen to the specific IgM when present.

The Typhidot M that detects only IgM antibodies of *Salmonella* Typhihas been reportedto be slightly more specific in a couple of studies (Hatta*et al*, 2002; Choo *et al,* 1999).

IDL Tubex test

The Tubex test is easy to perform and takes approximately 2 minutes time (Lim *et al,*1998). The test is based on detecting antibodies to a single antigen in *S.* Typhionly. TheO9 antigen used is very specific found in only sero group D *salmonellae*. Apositive result

suggests only a non specific *salmonellae* infection. Infection by other serotypes like *S.* ParatyphiA give negative result. This test detects IgM antibodies but not IgG ,thus is helpful in the diagnosis of current infections.

IgM dipstick test

This test is based upon on the binding of the *S.* Typhispecific IgM antibodies to thelipopolysaccharide (LPS) antigen of S.Typhi and the staining of the bound antibodies by ananti-human IgM antibody conjugated to colloidal dye particles. This test is usefulin places where culture facilities are not available.It can be performed without formaltraining and without any of the specialized equipments. The specific antibodies appear a week after the onset of symptoms ,so the sensitivity of this test increases only with time (Hatta *et al*, 2002).

2.16.11 Antigen detection tests

Enzyme immuno-assay, counter-immune electrophoresis and co-agglutination tests detect serum or urinary somatic/ flagellar /Vi antigens of *Salmonella* Typhi (Fadeel *et al,* 2004; Kalhan *et al,* 1999). The sensitivity of detecting Vi antigen has been found to be superior to somatic and flagellar antigen and has been reported to be around 50-100% in different studies (Rao *et al,* 1999; Fadeel *et al,* 2004; Kalhan *et al,*1999).

Specificity estimates are reported to vary from 25% -90%. The suboptimal and variable sensitivity and specificity , inability to detect *Salmonella* Paratyphiinfection and Vi antigen negative strains of *S.* Typhiare serious limitations of the Vi antigen detection tests.

Immunochromatographic Assay

Immunochromatographic assay (ICA) is a widely used technology in the held of rapid detection(W. C. Mak et .al ,2015.) Traditional colloidal gold-based immunochromatographic assay (CG-ICA) has four advantages: short period of time required to acquire test results,

user-friendly format, and long-term stability over a wide range of climates, and relatively inexpensive.(Y. Xie, Y. H. Wu, et.al, 2016).

Immunochromatographic qualitative test assay is performed in a test device, which shows a colour band when there is IgM in serum against coated antigen. Pink purplish coloured lines which confirms the result as a positive test result. It was compared with positive control, which was also coated in respective test devices.

An alternative approach for rapid detection of S. typhi with an immunochromatographic strip test was successfully developed (P. Preechakasedkit et al.). This work (LOD: 1.14 × 105 cfu mL−1, 15 min) provided a lower LOD and shorter analysis time than a dot blot immunoassay (LOD: 8.88 × 106 cfu mL−1, 110 min). This proposed method was also successfully applied to the detection of S. typhi in spiked normal human serum within 15 min using only one step and had accuracy and specificity of 100%. This strip test can be kept until 30 days at room temperature to detect S. Typhi effectively. Therefore, the immunochromatographic strip test offers an attractive method for the detection of S. Typhi in clinical and point-of-care diagnosis.

2.16.12 Molecular methods

PCR as a diagnostic modality for typhoid fever was first evaluated in 1993 by Song, *et al*. They successfully amplified the flagellin gene of *S.* Typhi in all cases of culture proven typhoid fever and from none of the healthy controls (Song *et al,* 1993). Some patients with culture negative typhoid fever were PCR positive signifying that PCR diagnosis of typhoid may be superior to cultures. A handful of studies have reported PCR methods successfully targeting the flagellin gene, somatic gene, Vi antigen gene, 5S-23S spacer region of the ribosomal RNA gene, invA gene and hilA gene of *S.* Typhi for diagnosis of typhoid fever (Haque *et al,* 1999). These studies have reported excellent sensitivity and specificity.The time for diagnosis has been less than 24 hours. The Clinical utility of PCR tests has been

inadequately evaluated. Performance of the test in individuals with febrile illnesses other than typhoid, in those with past history of typhoid, carriers of *S.* Typhi, and those vaccinated with typhoid vaccine is not known. Patients with aclinical diagnosis of typhoid fever who are culture negative but PCR positive may infact be false positives. The tests claim to detect as few as 10 organisms, in typhoid fever the median bacteremia is 0.3 CFU/ml of blood (Wain *et al,* 2001). Using small volumes of blood for DNA extraction may significantly lower the sensitivity of these tests. The cost and requirement for sophisticated instruments is also a potential drawback of this molecular method of diagnosis.

2.17 Treatment of typhoid fever

Typhoid fever is a severe systemic infectious disease. Treatment with appropriateantibiotics is essential for recovery. Since 1990s *S.* Typhihas developedresistance to first line drugs (chloramphenicol ,cotrimoxazole and ampicillin) and are known as Multi-Drug Resistant typhoid fever (MDRTF). There are some reports of re-emergence of fully susceptible strain to first line drugs (Bhatia *et al,* 2006). Unless antibiotic sensitivity testing shows the organisms to be fully susceptible to first line drugs they are not advocated for empirical therapy in typhoid.

2.17.1 Chloramphenicol

Chloramphenicol , has been the "Gold Standard " therapy since its introduction in 1948. Treatment with chloramphenical reduces typhoid fever mortality from approximately 20% to 1% and fever duration from 14-28 days to 3-5 days (Lesser & Miller, 2005).

Re-emergence of sensitivity of *Salmonella enteric* serovar Typhi to chloramphenicol and a study results in India show that a high sensitivity of *Salmonella enteric* serovar Typhi to chloramphenicol (96%) (Bhatia *et al*, 2006).

2.17.2 Cotrimoxazole

Cotrimoxazole is a first line drug for typhoid fever but resistance is an increasingproblem. In adults the effective dose is 800 mg of sulfa and 160 mg of trimetoprim every 12 hours for 15 days.

2.17.3 Fluroquinolones

Fluoroquinolones are widely regarded as the most effective drug for the treatment oftyphoid fever (Bhutta *et al,* 1999). But unfortunately, some strains of *S.* Typhihaveshown reduced susceptibility to fluoroquinolones (Parry *et al*, 2007).

Ciprofloxacin, ofloxacin, perfloxacin are common fluoroquinolonesproved to be effective and used in adults. Ciprofloxacin is usually given orally 500mg twice daily for 14 days, but there are report that course of seven days may beadequate (Gillespie, 2003). Fluoroquinolones have the advantage of lower stool carrier rates compared to the first line drugs. Fluoroquinolones are not approved in India to be used under 18 years of age unless the child is resistant to all other recommended antibiotics and is suffering from life threatening infection.

There is now considerable amount of evidence from the long term use of fluroquinolonesin children that neither they cause bone or joint toxicity nor impairment of growth (Kaundo *et al,* 2006).

The fluroquinolone drugs are highly effective against multi-resistant strains and trialshave shown this in comparison with parenteral and oral ceftriaxone and cefixime (Gillespie, 2003).

2.17.4 Third generation cephalosporins

Fluoroquinolones resistance has led to third generation cephalosporins being used in treatment reports of resistance to these antibiotics also have followed. Of the third generation cephalosporins oral, cefixime has been widely used in children (Matsumoto *et al,* 2001).

Among the third generation cephalosporins the injectable forms like, ceftriaxone, cefotaxime and cefoperazone are used, of which ceftriaxone is most convenient.

Oral cefixime is used in a dose of 15-20 mg per kg per day in two divided doses. Parenteral third generation cephalosprins such as ceftriaxone 50-75 mg per kg per day in one or two doses; cefotaxime 40-80 mg per kg per day in two or three doses and cefoperazone 50-100 mg per kg per day in two doses are used.

A short, 5-day course of ceftriaxone is a useful alternative to conventional 14-daychloramphenicol therapy in both children and adults (Islam *et al*, 1993).

2.17.5 Other Antibiotics

In cases of uncomplicated typhoid oral third generation cephalosporin e.g., cefiximeis the drug of choice. If by the end of 5 days there is no clinical improvement and the culture report is inconclusive a second line drug is added e.g. azithromycin or any other drug effective against *S.* Typhi depending upon the sensitivity pattern of the area (Kaundu *et al,* 2006)

2.17.6 Role of Steroids

Glucocorticoids in the management of infectious diseases remains controversial, although experimental data obtained both in vitro and in experimental infections in animals provide evidence of a beneficial effect of such treatment. The use in the treatment of severe typhoid fever has been shown to be beneficial (Lesser &Miller, 2005).

A study from Jakarta showed a significant reduction in mortality in patients with severe typhoid fever (i.e. CNS symptoms, shock, disseminated intravascular coagulation), Dexamethasone (3mg/kg as a loading dose over 30 min,followed by 1mg/kg every 6h for 24h to 48h) used along with parenteral antimicrobials appears to have reduced mortality(Lesser & Miller, 2005).

2.17.7 Treatment of complications

Drug of choice in complicated cases is parenteral third generation cephalosporine.g. ceftriaxone. In cases of sever life threatening infections of typhoid fluroquinolones , Aztreonam and imepenem may also be used (Kaundu *et al,* 2006).

2.18 Drug resistance in typhoid fever (ICDDR.B, 2007)

Antibiotic resistance is an important problem in Asia and in Africa due to easy over the counter availability and hence the abuse of antibiotics. Susceptibility patterns of S. Typhi strains showed high rates of multidrug resistance (MDR) in Asia and in Africa, with rates of 40% of isolates from India, 70% from Pakistan, 40% from Bangladesh, and 77% from Vietnam. A non-plasmid-mediated fluoroquinolone-resistant *S.* Paratyphi A was reported from India. In addition, resistance of S. Typhito nalidix acidhave been reported in asia.

Resistance of S. Paratyphi *A* to nalidixic acid was also reported in South China. Usually, the resistance to nalidixic acid is considered a marker for reduced susceptibility to fluoroquinolones (Kadhiravan *et al,* 2005). It is still not known if the Nalidixic acid resistence in *S*. ParatyphiA has a clinical profile similar to resistance of S. Typhito nalidixic acid.

Antibiotic Resistance

The classic presentation with a slow step-ladder rise in fever and toxicity is rarely seen with the in adverent use of antibiotics. However, the rising antibiotic resistance has been associated with increased severity of illness and related complications.

Chloramphenicol resistant S.typhi first emerged in UK in 1950 and subsequently in Greece and Israel. Later Chloramphenicol resistance was reported worldwide, including India.The plasmid encoded Chloramphenicol resistance was first observed in early 1970 followed by epidemics in Central America. In 1972, chloramphenicol resistant S.typhi strains became a major problem in causing outbreaks worldwide.(Jaspal Kaur et al)

Multi-drug resistant S.typhi strains emerged in south east Asia in 1980. This multi-drug resistance is highly transmissible.In 1987 multi-drug resistant strains of S.typhi were first reported in Pakisthan. In India MDR strains of S.typhi were first reported in 1990 and an outbreak called Dombivali fever reported in Mumbai. There were sporadic reports of multi drug resistant S.typhi strains all over India. (Jaspal Kaur et al)(Syed Ahmed Zaki ,Sunil karande)

In UK fluoroquinolone resistant strain of *S*.typhi emerged in 1992. Das et al in 2000 found *S*.typhi strains developed resistance to ciprofloxacin in Orissa.Over a period of seven years S.typhi strains gradually developed resistance to fluoroquinolones.

Saha et al and Kumar et al in 2007 declared third generation cephalosporins also developed resistant to S.typhi strains.(Jaspal Kaur et al) (Harriet Ugboko and Nandita De et al)

Mechanism of Antimicrobial resistance:

Typhoidal Salmonella exhibits antimicrobial resistance by two factors

i) Acquisition of foreign genes via plasmids

ii) Mutation on chromosomes.

Resistance can be achieved by horizontal acquisition of resistant genes, mobilized via insertion sequences, transposons, and conjugative plasmids. The antimicrobial resistance can occur by recombination of foreign DNA into the chromosome or by mutations in different chromosome loci.(Harriet Ugboko and Nandita De)

Plasmid mediated resistance :

Antimicrobial resistance in S.typhi are either plasmid mediated resistance or chromosomal mediated resistance. Plasmids of incompatibility group are important vectors of antibiotic resistance in S.typhi. Plasmid mediated resistance often code for enzymes that destroy or modify the drugs. Plasmid associated genes have been implicated in resistance to

chloramphenicol, aminoglycosides, penicillins, cephalosporins, tetracyclines and sulphonamides.(skov R, Matuschek E, Sjölund-Karlsson M)

The mechanism of drug resistance mediated by acquisition of R plasmids are due to

i) Inactivation of the drug

ii) reduced membrane permeability

iii) modification of drug site

iv) rapid efflux of antibiotic

i) Inactivation of drug :

This is the common cause of resistance that inactivates antimicrobials. The enzyme beta lactamase present in various bacteria is best known example for inactivation of the drug. Enzyme beta lactamase cause hydrolysis of beta lactam ring of penicillin and cephalosporins. The initial strains of antibiotic resistant *S.*Typhi carried chloramphenicol acety (Harriet Ugboko and Nandita De et al)l transferase type I, which encodes an enzyme that inactivates chloramphenicol via acetylation. Datta et al reported that S.typhi has acquired R plasmid in the intestine of human beings from other enteric bacteria. Chloramphenicol resistant S.typhi have emerged due to acquisition of R plasmid whichencodes the enzyme acetyl transferase that inactivates chloramphenicol.(Harriet Ugboko and Nandita De)(Jaspal Kaur et al) The genes which are responsible for resistance to ampicillin and cotrimoxazole are dihydrofolate reductase type VII and TEM -1 Beta lactamase. 4 A single plasmid has been known to code for the multidrug (Ruchi Girotra1, Reetika Dawar2, Ruby naz1)

resistance and this plasmid belongs to the incompatibility group, H I1 and this is highly transmissible.

ii) Reduced membrane permeability :

The pathogens by preventing the entrance of the drug they become resistant. (Syed Ahmed Zaki ,Sunil karande) The new genetic information changes the nature of proteins in

the membrane, which leads to the alteration in the membrane permeability.Such an alteration cause a change in the membrane transport system

pores, and hence the antibiotics can no longer cross that membrane.*Salmonella* typhi, exhibited resistance to tetracycline, quinolones and some aminoglycosides by this mechanism.

iii) Modification of target site :

The mechanism of drug resistance in S.typhi occurs when the target enzyme or the cellular structure of the pathogen gets modified, so that it is no longer susceptible to the drug.

iv) Rapid extrusion or efflux of the antibiotic :

Many pathogens develop resistance to antibiotics by pumping out the drug from within the cell. These pathogens have plasma membrane translocases, often called efflux pumps that expels the drug.Because they are non-specific and can pump many different drugs including quinolones, these transport proteins are called multi-drug resistance pumps. Resistance to sulfonamides is mediated by a plasmid encoded transport system that actively expels the drug out of the cell. (eucast.org/clinical_breakpoints)

The genes like plasmid mediated beta lactamases, tetracycline –resistance genes, and aminoglycoside modifying enzymes are organized on transposons.

Chromosome mediated resistance :

Chromosomal resistance is by the mutation in the gene that codes for the target of the drug on the cell or the transport system in the membrane that controls the drug's uptake. The irrational use of the antibiotic has led to the emergence of chromosomal mediated drug resistance phenomenon against fluoroquinolones. This has been attributed to a single point mutation in the quinolone resistance determining region of topoisomerase gene gyrA, which encodes DNA gyrase. The fluoroquinolones target DNA gyrase and topoisomerase IV.These are bacterial enzymes that are responsible for the uncoiling and recoiling of bacterial DNA

for transcription. Salmonella typhi most commonly develops fluoroquinolone resistance through specific mutations in gyrA and par C which codes for DNA gyrase and topoisomerase IV,respectively.Partial resistance is due to a single point mutation in gyrA gene. When asecond gyrA point mutation is added, the resistance increases. However, a mutation in parC along with mutation in gyrA confers full invivo resistance. The risk of relapse after bacterial clearance is higher in both partially and fully resistant strains than fully susceptible strains.34

The quinolones that are used in the treatment of enteric fever are ciprofloxacin, levofloxacin, ofloxacin and gatifloxacin. In gatifloxacin and moxifloxacin the primary target is gyrA gene, and for ciprofloxacin and levofloxacin it is the parC gene.This explains the varied pattern ofsusceptibilities, so all the fluoroquinolones should be tested individually. (Jaspal Kaur et al)Resistance to trimethoprim is due to mutations in the chromosomal gene that encodes the enzyme dihydrofolate reductase. The resistance to sulfonamides is mediated by a chromosomal mutation in the gene encoding for the target enzyme dihydropteroate synthetase, that reduces the binding affinity of the drug.

Extensive use of Cephalosporins leads to the development of resistance by producing Extended Spectrum Beta Lactamases. *S*.typhi produces a variety of ESBL types like TEM, SHV, CTXM enzymes.TEM types of ESBL was first discovered in a patient called Temonieria and hence named as TEM. SHV was named so because they are sulfhydryl variable.CTX-M are ESBL s that have the tendency to hydrolyse Cefotaxime.

MATERIALS AND METHODS

The present study was undertaken at the Department of Microbiology, Tirunelveli Medical College for a period of one year from June 2017– July 2018.

- This was a prospective cross sectional study.

1.cases

One hundred clinically suspected typhoid fever cases were selected on the basis of following inclusion criteria -

Inclusion criteria (Butler & Scheld, 2004)

i) Fever for ≥ 3 days, with no obvious focus of infection

ii) Abdominal discomfort- constipation or loose motions

iii) Coated tongue, toxic look

iv) Hepatomegaly, splenomegaly

v) Relative bradycardia , rose spot etc.

Exclusion criteria

i) Persons who are immunized with typhoid vaccines.

ii) Persons suffering from fever other than typhoid

2.Ethical clearance

The study was started after getting ethical committee clearance from the institution

3.Informed consent

Informed consent was obtained from all patients included in the study.

4.Proforma:

The proforma was filled with the details like name, age, sex, ward, clinical diagnosis, risk factors, undergone any surgery, duration of hospital stay and other parameters significant to the present study.

5. Sample storage

The isolated Gram negative bacilli were sub-cultured on to nutrient agar slope and stored at +2˚C to 8˚C. The isolates were sub-cultured every fortnight.

METHODOLOGY

Specimen collection: Blood was taken for both culture & serological tests.

Procedure of collection of blood and separation of serum

Patients who met the criteria were asked to give informed consent and answer a brief questionnaire about clinical signs and symptoms, antimicrobial treatment, and history of typhoid fever and vaccination.

Blood sample was collected from each patient for culture and serological test.

Single sample of venous blood (preferably antecubital) was collected from each patient with sterile disposable syringe and needle .

Disinfection of the selected venipuncture site with 70% alcohol in a expanding circular scrubs was done from the centre to the periphery of the needle insertion site followed by 2% tincture of iodine which was allowed to dry for one minute (Forbes *et al*, 2002; Chessbrough, 2000).

At least 7 ml of blood from each adult patient were collected from single venepuncture. After removing the syringe and needle from the venipuncture site the sampling needle was discarded and replaced by a sterile needle.

The top of the rubber stoppers of the blood culture bottle were disinfected with 70% alcohol and 5ml of collected blood were injected immediately into the culture bottle. Rest 2 ml of blood from each sample were taken in a clean dry test tube for separation of serum.

Tubes containing 2 ml of blood was kept at room temperature for one hour to allow clotting of blood and then it was centrifuged at 1500 rpm for 15 minute. Serum was separated and kept in a sterile eppendorf's tube at -20C until further use.

Procedure of conventional blood culture method

Blood culture was done by conventional method using bile broth .5 ml of collected blood was inoculated immediately into 50 ml of bile broth (which was brought to room

temperature 30 minutes before inoculation) respectively. The inoculated bottle was inverted 3-5 times to mix blood with broth. Inoculated culture bottle was incubated at 37 ˚C aerobically.

Bottles were examined visually daily. Growth was usually indicated by haemolysis of red blood cells, gas bubbles in the medium or turbidity in the broth. When macroscopic evidence of growth was apparent, a Gram-stained smear of an air dried drop of medium was done.

In addition , blind subculture from conventional bottle after the first 24 hours of incubation was done onto MacConkey agar ,Nutrient agar and Blood agar plates.

The uncontaminated bacterial culture was taken using a sterile loop. The inoculated loop was further streaked over the surface of agar media. In between each streaking, the loop was heated in the flame of a bunsen burner. After the streaking of inoculum on agar, the plates were incubated 37 ˚C.

Subcultures were done after 48 hours and 7 days on macconkey agar .

The organisms were identified by their colony morphology, Gram staining methods, motility test and following biochemical reactions with suitable controls.

Salmonella typhi was identified by the presence of :

Smooth, colourless and moist colonies in Nutrient agar Smooth, non-lactose fermenting colonies in MacConkey agar.

Non hemolytic greyish white colonies in Blood agar.

Gram Staining: Salmonella species appear as gram negative rods

Motility :Motile by hanging drop method.

Catalase : Positive

Oxidase:Negative

Indole test:Negative

Citrate test:Negative

Urease test:Negative

Triple sugar iron test:Alkaline slant/Acid slant with speck of H_2S.

Nitrate reduction test: Positive(nitrate is reduced to nitrite)

Methyl red test: Positive

voges-Proskauer:Negative

Sugar fermentation tests:Glucose,maltose,mannitol,mannose,trehalose,xylose,sorbitol fermented

Decarboxylation test:Lysine is decarboxylated.

2. ANTIBIOTIC SUSCEPTIBILITY TESTING:

All the isolates were subjected to antibiotic susceptibility testing by Kirby Bauer disc diffusion method according to CLSI guidelines 28th edition.

<u>Kirby-Bauer's disc diffusion method</u>:

About 3-5 colonies of the test organism was picked up with sterile loop and suspended in peptone water and incubated at 37°C for 2 hours. The turbidity of the suspension was adjusted to 0.5 McFarland's standard (1.5 X 10^8 CFU/mL) using Wickerham's chart. It was then spread on the surface of a cation-adjusted Mueller-Hinton agar (MHA) plate using sterile cotton swab. The panel of antibiotic discs i.e chloramphenicol (30 µg),amoxicillin (10 µg), cotrimoxazole (1.25/23.75 µg),ciprofloxacin (5 µ g) ,ceftriaxone (30 µg) azithromycin (15 µg) and pefloxacin (5 µg) was used for isolates defined as per CLSI guidelines 28th edition. It was incubated at 37˚C for 18-24 hours. The zone size was recorded and interpreted as per the CLSI guidelines.

Serological tests

Antibody detection by Widal agglutination test

Widal Test Kit

Manufactured by: Tulip Diagnostic (P) Ltd, Date of manufacturing: September 2017, Date of Expiry: February 2019, Storage Temperature: 4-8 °C. Tydal contains ready to use concentrated, smooth antigen suspensions of the bacilli; S. typhi 'O', S. typhi 'H', S. paratyphi 'AH', S. paratyphi 'BH', polyspecific positive control , reactive with these antigens. The H agglutinable suspension of bacteria is prepared by adding 0.1 per cent formalin to a 24 hour broth culture or saline suspension of an agar culture. For preparation of O suspension of bacteria, the bacillus is cultured on phenol agar (1:800). The growth is scraped off in a small volume of saline and mixed with 20 times its volume of absolute alcohol. it is then heated in a water bath at 40°-50°C for 30 minutes, centrifuged and the deposit resuspended in saline to the appropriate density. Chloroform in then added as a preservative. S. typhi 901, O and H strains, are used for preparation of antigens. Each batch of prepared antigen is compared with a standard.

Procedure of Widal test

Rapid slide methods

1. The glass slide supplied in the kit was cleaned well and dried.
2. The circles (1, 2, 3, 4, 5 and 6) in the test card were labelled as O, H, AH, BH, Negative control and Positive control
3. A drop of undiluted test serum was placed in each of the four labelled circle (1, 2, 3 and 4) ie O, H, AH and BH and a drop of Negative control serum in circle 5 and Positive control in circle 6.
4. A drop of antigen O, H, AH and BH were placed in circle 1, 2, 3, and 4 respectively and no antigen in circle 5 and O/H antigen in circle 6.

5. The content of each circle was mixed with a separate wooden applicator stick and spread to fill the whole area of the individual circle.

The test card was rocked for a minute and observed for agglutination.

Interpretation

For agglutination visualised within 1 minute, tube test was done for the quantitative estimation of the titre of the antibody.

***II. Quantitative tube test*:**

1. A set of 8 clean dry test tubes (Kahn tubes) were taken and labelled as 1, 2,3, 4, 5, 6, 7 and 8 for O antibody detection.

- Similarly, 3 sets of 8 test tubes were taken and labelled as 1, 2…8.

The serum samples were diluted as follows:

- 1.9 ml of isotonic saline was pipetted in tube No.1 of all sets
- To each of the remaining tubes (2 to 8) 1.0 ml of isotonic saline was added.
- To the tube No.1 tube in each row 0.1 ml of the serum sample to be tested was added and mixed well.
- 1.0 ml of the diluted serum was transferred from tube no.1 to tube no.2 and mix well.
- 1.0 ml of the diluted sample from tube no.2 was transferred to tube no.3 and mixed well. This serial dilution was continued till tube no.7 in each set.
- 1.0 ml of the diluted serum was discarded from tube No.7 of each set.

- Tube No.8 in all the sets, served as a saline control. The following dilution of the serum sample was achieved in each set as follows: Tube No. : 1 2 3 4 5 6 7 8 (control) Dilutions 1:20 1:40 1:80 1:160 1:320 1:640 1:1280.

4. A drop of appropriate widal test antigen was added to all the test tubes

5. Mixed well and was incubated at 37°C for 16-20 hours and examined for agglutination.

6. Antibody titre with the highest dilution of serum showed clear agglutination.

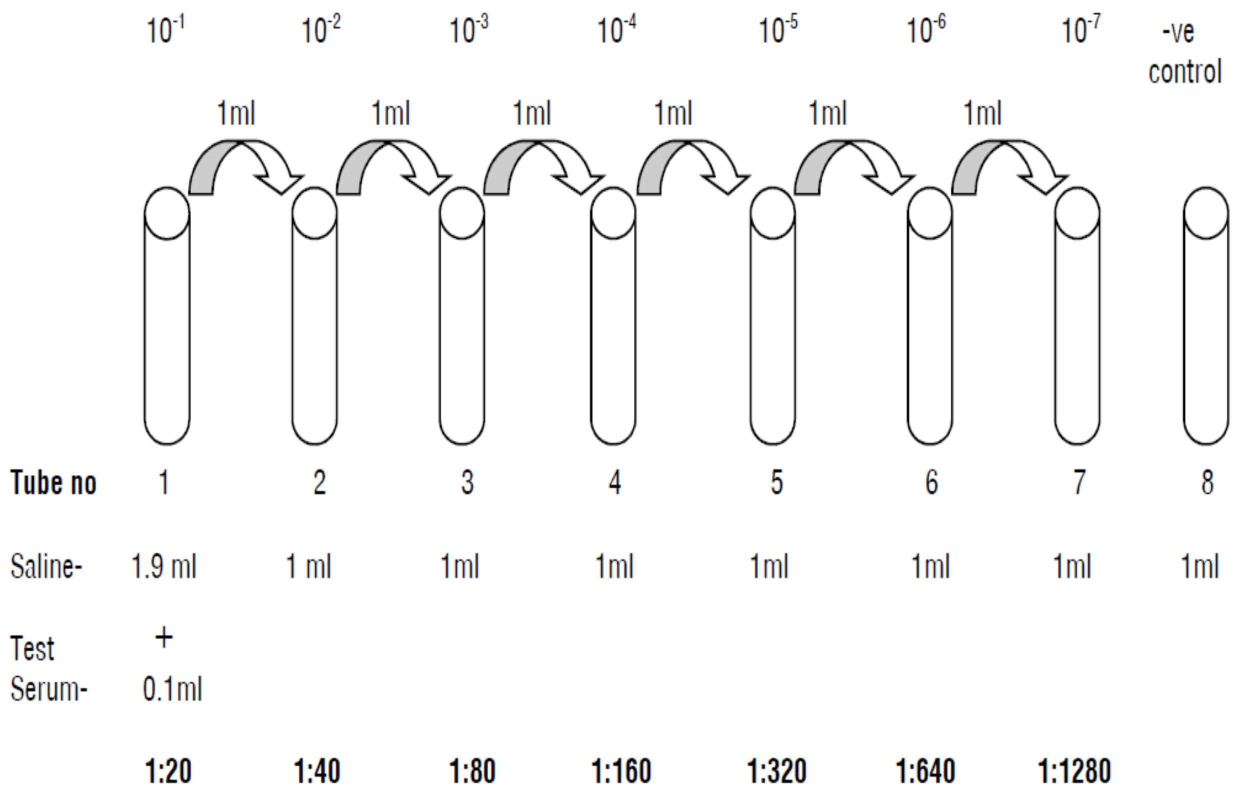


Table 3: Result interpretation of Widal test

Test tube	1	2	3	4	5	6	7	8
Dilution	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280	Control (saline)

Result interpretation of Widal test:

- Antibody titre greater than 1 : 80 was considered significant and suggested positive for Salmonella infection.
- Low titres are seen often in normal individuals.

d) ***IMMUNOCHROMATOGRAPHIC TEST*** :

Lateral flow immunoassay test was done on serum by using Rapid typhoid IgG /IgM test device kit. This test is a qualitative antibody detection test with total assay time of 15 minutes.

The test cassette consists of

1) a burgundy coloured conjugate pad containing recombinant H antigen and O antigen conjugated with colloidal gold (HO conjugates) and rabbit IgG-gold conjugates.

2) a nitrocellulose membrane strip containing two bands G and M bands and a control band (C band).

The M band is precoated with monoclonal anti-human IgM for the detection of IgM anti-S.Typhi. G band is precoated with reagents for the detection of IgG antiboidies. C band is precoated with goat anti rabbit IgG. IgM antibodies if present in patient serum, will bind to HO conjugates.

The immunocomplex is then captured on the membrane by the pre coated anti-human IgM antibody, forming a burgundy coloured M band, indicates positive test result. IgG antibodies if present in patient serum, will bind to HO conjugates.

The imunocomplex is then captured by the precoated reagents on the membrane, forming an burgundrycoloured G band, indicating positive test result. Absence of M and G bands suggests negative test

Procedure:

Serum samples were added to the sample well followed by adding the supplied diluents. The positive control forms a colored band in the test and control line. Any test sample showing similar or darker bands was defined as positive. The absence of any visible band was considered as a negative test result.

Molecular identification of Plasmid mediated quinolone resistance Gene

Material & Methods:

PureFast® Bacterial DNA minispin purification kit [Kit contains Lysozyme, Lysozyme digestion buffer, Proteinase-K, Binding buffer, Wash Buffer-1, Wash Buffer-2, Spin columns with collection tube and elution buffer. HELINI 2X ReDdye PCR Master Mix, Agarose gel electrophoresis consumables and qnrA and qnrB Primers are from HELINI Biomolecules, Chennai, India.

2X Master Mix: It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM $MgCl_2$, 1µl of 10mM dNTPs mix and RedDye PCR additives.

Agarose gel electrophoresis: Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide are from HELINI Biomolecules, Chennai.

PCR: HELINI Ready to use QnrA gene Primer mix - 5µl/reaction PCR Product: 400bp

HELINI Ready to use QnrB gene Primer mix - 5µl/reaction PCR Product: 430bp

Bacterial DNA Purification

1. 1ml of overnight culture was centrifuged at 6000rpm for 5min
2. Supernatant was discarded
3. Pellet was suspended in 0.2ml PBS.
4. 180µl of Lysozyme digestion buffer and 20µl of Lysozyme [10mg/ml] was then added.

5 . And incubated at 37°c for 15mins.

6. 400µl of Binding buffer, 5µl of internal control template and 20µl of Proteinase K was added, and mixed well by inverting several times.

7. Then incubated at 56ºC for 15min.

8. 300µl of Ethanol was added and mixed well.

9. Entire sample was transfered into the PureFast® spin column and Centrifuged for 1 min. The flow-through was discarded and the column placed back into the same collection tube.

10. Added 500µl Wash buffer-1 to the PureFast® spin column. Centrifuged for 30-60 seconds and discarded the flow-through. Placed the column back into the same collection tube.

11. Added 500µl Wash buffer-2 to the PureFast® spin column. Centrifuged for 30-60 seconds and discarded the flow-through. Placed the column back into the same collection tube.

12. Discarded the flow-through and centrifuged for an additional 1 min. This step was essential to avoid residual ethanol.

13. Transferred the PureFast® spin column into a fresh 1.5 ml micro-centrifuge tube.

14. Added 100µl of Elution Buffer to the center of PureFast® spin column membrane.

15. Incubated for 1 min at room temperature and centrifuge for 2 min.

16. Discarded the column and stored the purified DNA at -20°C. Quality and Quantity of extracted DNA is checked by loading in 1% agarose gel and 5µl of extracted DNA used for PCR amplification.

PCR Procedure:

1. Reactions were set up as follows;

Components Quantity

HELINI Red dye PCR Master mix	10µl
HELINI Ready to use - Primer Mix	5µl
Purified Bacterial DNA	5µl
Total volume	20µl

2. Mixed gently and spin down briefly.

3. Placed into PCR machine and programmed it as follows;

Table 4: PCR Amplification

Initial Denaturation	:	95ºC for 5 min
Denaturation	:	94ºC for 30sec
Annealing	:	58ºC for 30sec
35 cycles Extension	:	72ºC for 30sec
Final extension	:	72º C for 5 min

Loading:

1. Prepared 2% agarose gel. [2gm of agarose in 100ml of 1X TAE buffer]

2. Electrophoresis was run at 50V till the dye reaches three fourth distances and observe the bands in UV Transilluminator

<u>Agarose gel electrophoresis</u>:

1. Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven)

2. When the agarose gel temperature was around 60ºC, added 5µl of Ethidium bromide.

3.Then poured warm agarose solution slowly into the gel platform.

4. Kept the gel set undisturbed till the agarose solidifies.

5. Poured 1XTAE buffer into submarine gel tank.

6. Carefully placed the gel platform into a tank. Maintained the tank buffer level 0.5cm above than the gel.

7. PCR Samples were loaded after mixing with gel-loading dye along with 10µl HELINI 100bp DNA Ladder. [100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp and 1500bp]

8. Then electrophoresis done at 50V till the dye reaches three fourth distance of the gel.

9. Gel viewed in UV Transilluminator and observed the bands pattern.

5. RESULTS

5.1. STUDY DESCRIPTION

This study was conducted at the Department of Microbiology, Tirunelveli Medical College. A total of 100 samples were collected from patients having clinical features suggestive of typhoid fever.

These samples were subjected to widal test, blood culture and immunochromatography test. Antimicrobial susceptibility testing by disc diffusion method was done for the blood culture isolates for the following antibiotics: Ampicillin, Chloramphenicol, Cotrimoxazole, Nalidixic acid, Ciprofloxacin, Azithromycin, pefloxacin and Ceftriaxone. Those isolates found resistant to pefloxacin was subjected to PCR assay for the detection of PMQR genes such as qnrA and qnrB.

5.2. STATISTICAL ANALYSIS

Data collected were entered in Microsoft Excel and analysed using statistical analysis software Statistical Package for Social Services (SPSS) v.16. Appropriate descriptive and inferential statistics were calculated. p values <0.05 were considered to be statistically significant.

5.3. AGE AND SEX DISTRIBUTION

Among the 100 samples suggestive of typhoid, 59 blood samples were from male and 41 samples from female. The age of the patients ranged from a minimum of 16 years to a maximum of 74 years. Most of the isolates (39%) were from patients aged between 31 and 45 years. The mean age of the patients was found to be 38.9 years with a SD of 17.8 years. The table below shows the age and sex distribution of the patients.

Table 5 Age and Sex Distribution of the Patients

Age of the patients	Sex of the patients		Number of samples (N=100)
	Male	Female	
16 years to 30 years	20 (64.5%)	11 (35.5%)	31 (31%)
31 years to 45 years	20 (51.3%)	19 (48.7%)	39 (39%)
46 years to 60 years	14 (60.9%)	9 (39.1%)	23 (23%)
61 years to 75 years	5 (71.4%)	2 (28.6%)	7 (7%)
Total	59 (59%)	41 (41%)	100

Chart1: Age and Sex Distribution of the Patients

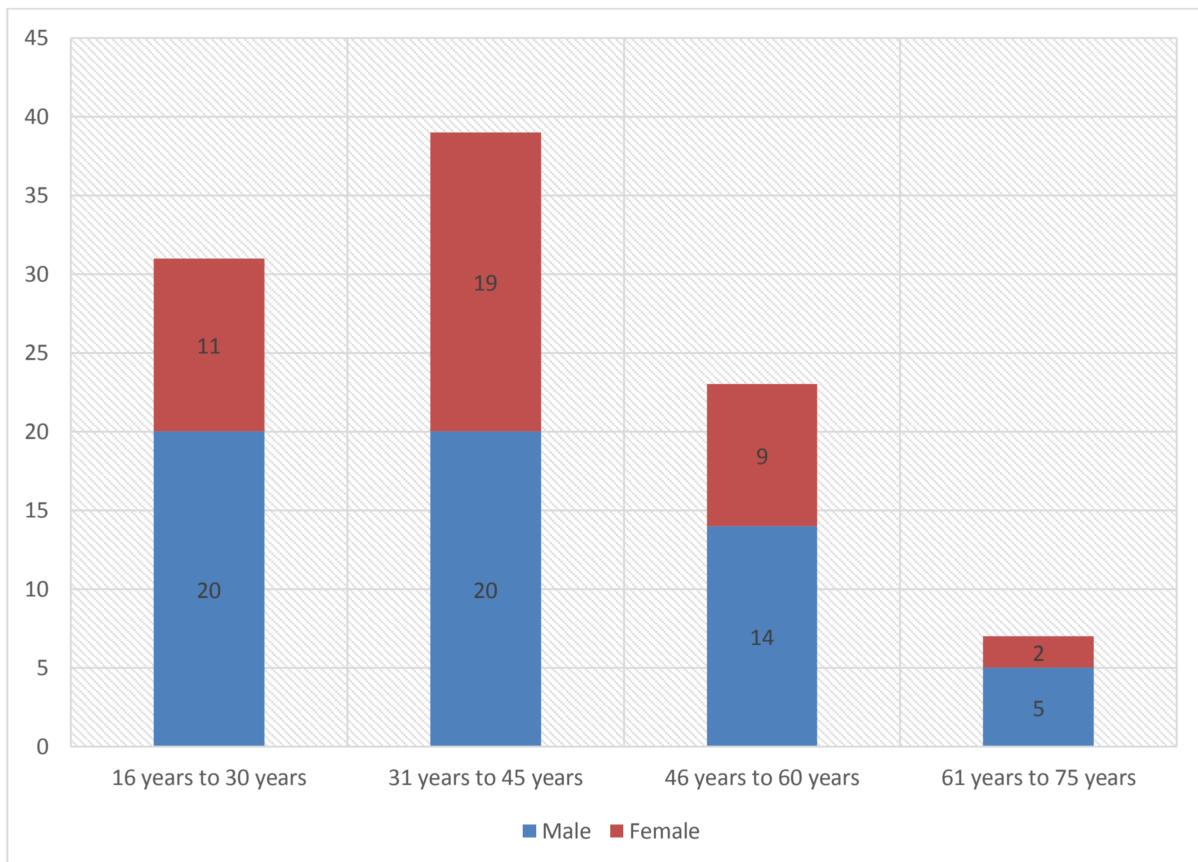
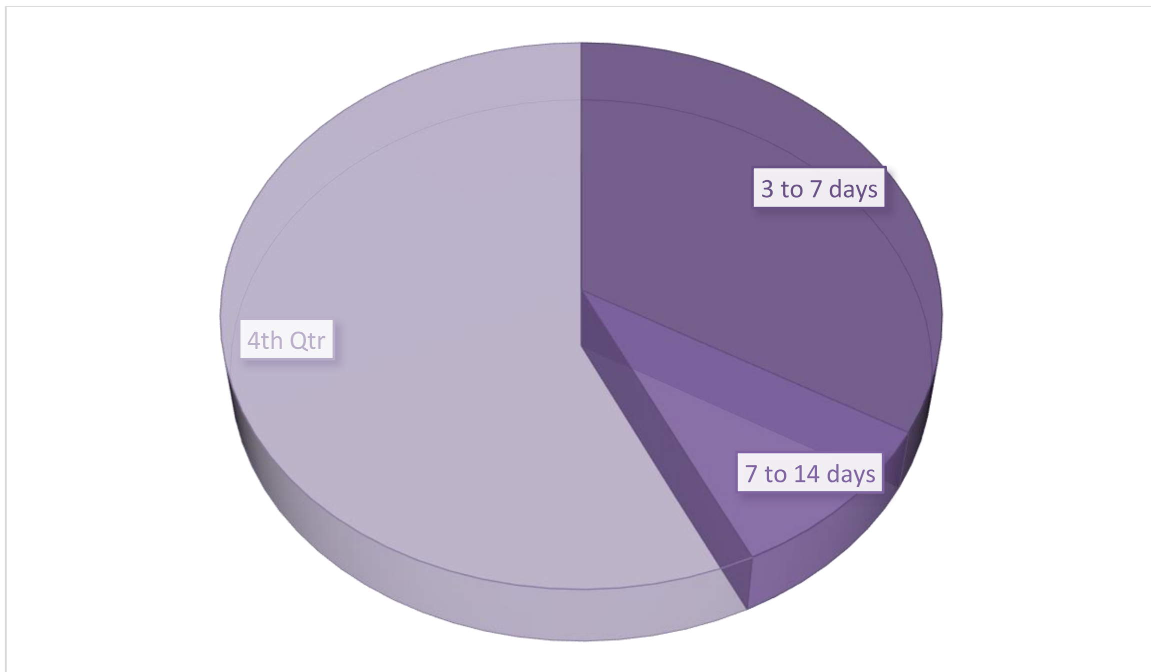


Table 6 Duration of Fever Among Study Groups

Duration	Positives
3-7 days	70(70%)
7-14 days	20(20%)
> 14 days	10(10%)

Out of 100 samples 70% of samples were collected from patients with fever of about a week duration.Remaining patients were in the 2nd week and later.

Chart 2: Pie chart showing duration of fever among study groups



5.4. BLOOD CULTURE

5.4.1. Positive blood culture distribution.

Out of the 100 samples, 14 samples were found to be positive for *Salmonella* Typhi by blood culture (8 – male, 6 – female). Most of the isolates (39%) were from patients aged between 31 and 45 years. No sample was positive in the age group more than 60 years. Blood culture did not show any significant association with age and gender of the patients (p value > 0.05).

Chart3 Blood culture results for Salmonella typhi

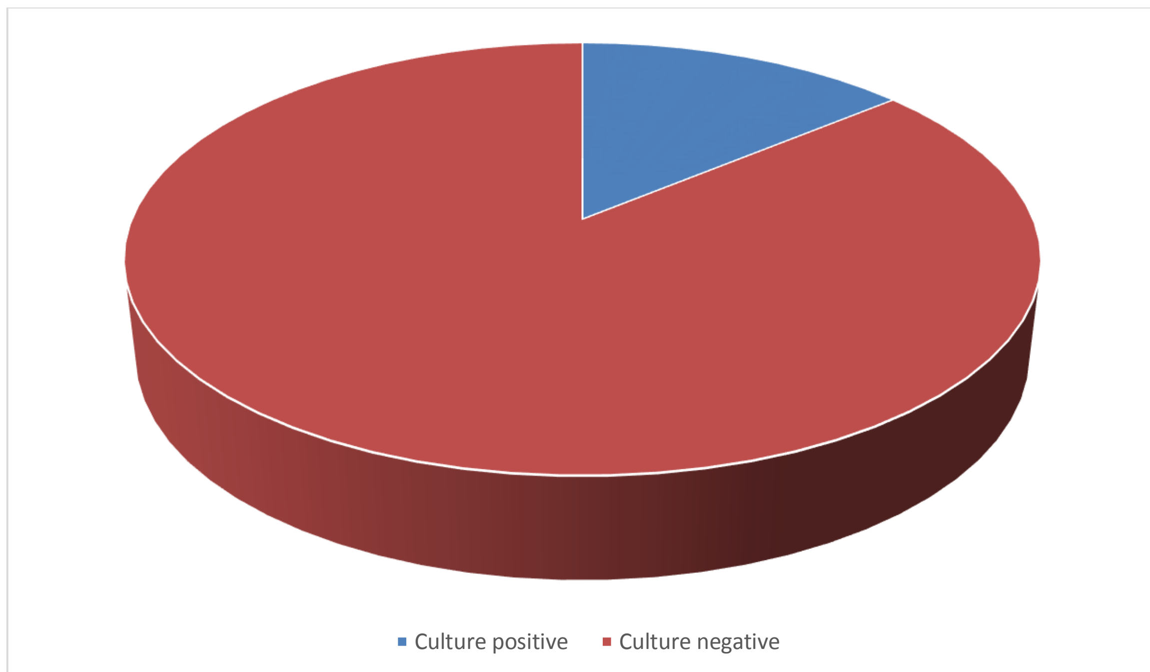
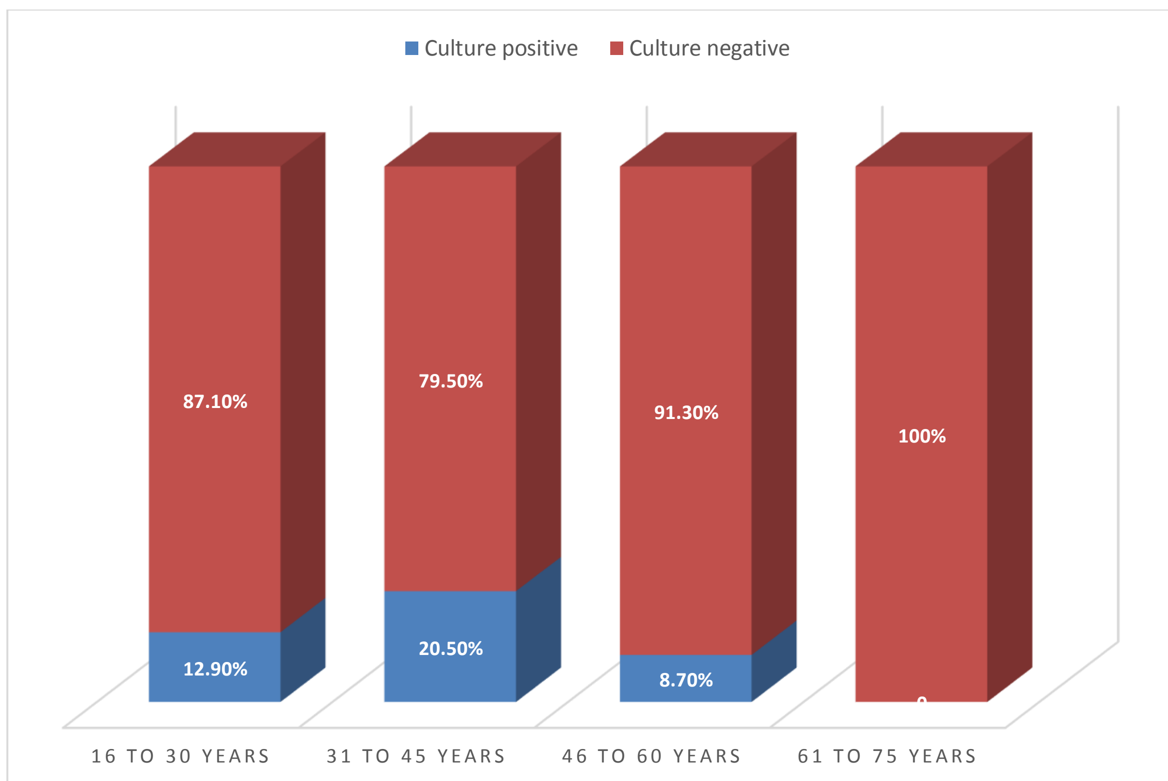


Table 7: Blood culture results among different age groups

Age group	Blood culture		Total
	Positive	**Negative**	
16 to 30 years	4 (28.6)	27 (31.4%)	31
31 to 45 years	8 (57.1%)	31 (36%)	39
46 to 60 years	2 (14.3%)	21 (24.4%)	23
More than 60 years	0	7 (8.1%)	7
Total	**14**	**86**	**100**

Chart4 Blood culture results among different age groups



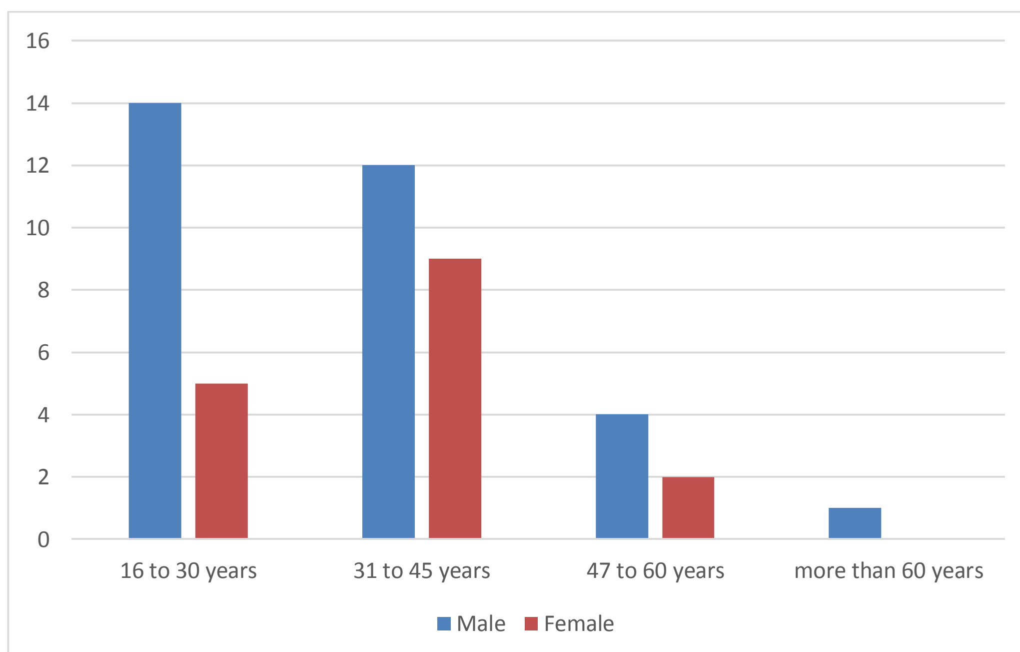
5.4.2.Interpretation of Widal test results

Blood culture showed only 14 samples to be positive for S.typhi, whereas widal test showed 47 samples to be positive. Out of the 47 samples, 31 were from males and 16 were from females.

Table 8. Age and Gender-Based Distribution of Widal test Positive Results

Age of the patients	Sex of the patients		Number of positive samples (N=47)
	Male	Female	
16 years to 30 years	14 (73.69%)	5 (26.31%)	19
31 years to 45 years	12 (57%)	9 (43%)	21
46 years to 60 years	4 (66.7%)	2 (33.3%)	6
61 years to 75 years	1 (100%)	0	1
Total	31 (66%)	16 (34%)	47

Chart 5: Age and Gender Distribution of Blood Culture Positive Results



5.4.2.1 Widal positive cases and duration of fever

Significant co relation has seen between widal positive cases and duration of fever.Patients with history of fever of more than a week showed maximum (97.86%)widal positivity.

Table 9. Relationship with Widal positive results and duration of fever

Duration	< 7 days	7-10 days	> 10 days
Positive	1(2.12%)	27(57.44%)	19(40.42%)
Negative	46(97.88%)	20(42.56%)	28(59.58%)

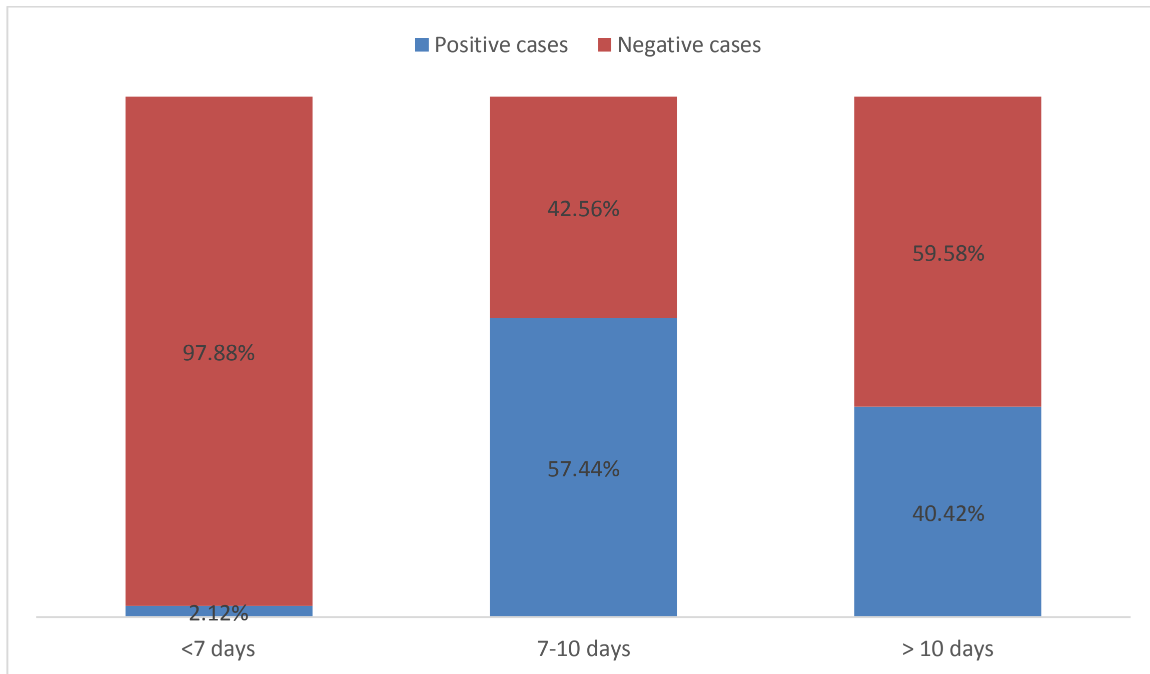
54.2.2 .Widal positive titre with duration of fever

In this study there was a significant relationship between the Salmonella Typhi antibody titre and the duration of fever. There was an increase in the titre in patients with fever of more than a week.

Table 10: Widal positive titre with duration of fever

Duration of illness	Widal titre TO/TH	Positive cases	Negative cases
<7 days	80/80	1(2.12%)	46(97.88%)
7-10 days	160/80	27(57.44%)	20(42.56%)
> 10 days	320/320	19(40.42%)	28(59.58%)

Chart 6:Relationship between Widal positive cases and duration of fever



5.4.3. Immunochromatographic assay

Immunochromatography assay showed 26% samples to be positive for IgM and 6% samples to be positive for IgG. None of the samples were positive for both IgG and IgM.

Table 11 . Comparison of duration of fever with ICT positive results

Duration	< 7 days	7-10 days	> 10 days
Positive	10	12	4
Negative	16	14	22

Out of the 26 positive ICT IgM cases the number of positive cases appear to gradually decrease as the duration of fever at presentation increases.

5.4.4. Blood culture Vs Widal test

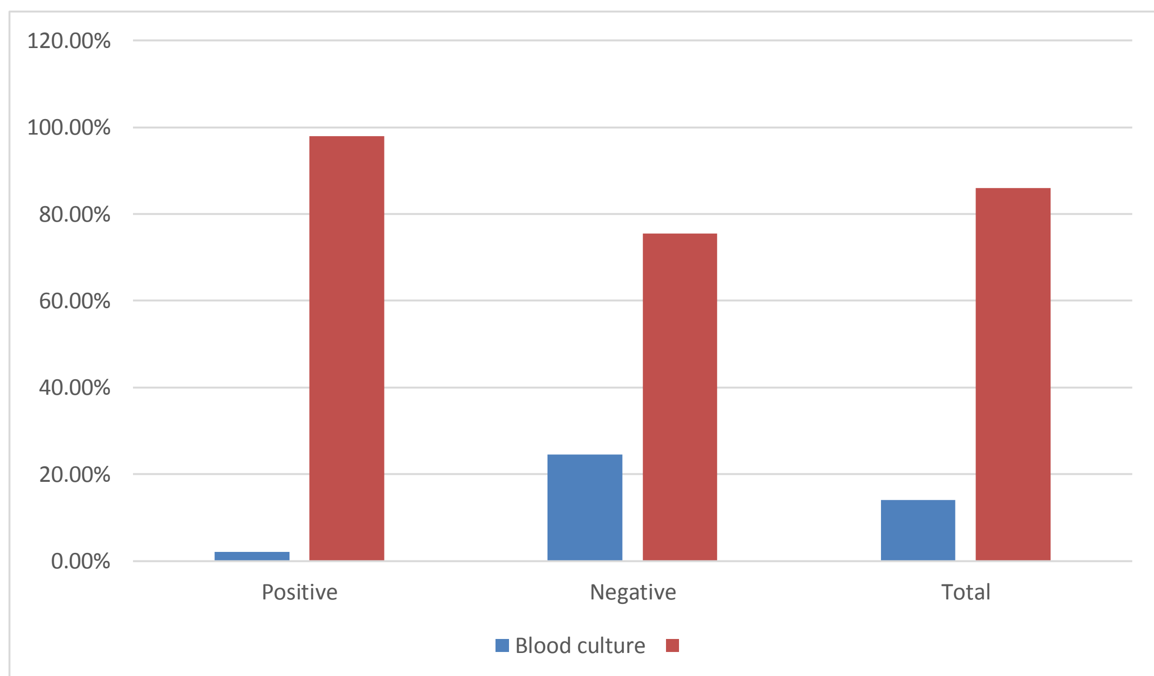
Out of 47 positive samples for widal test, only one sample was positive for blood culture (True positivity rate or PPV– 2.1%). Out 53 negative samples for Widal test, 40

samples were negative for blood culture (True negativity rate or NPV– 75.4%). There is a statistically significant difference in the results of blood culture and Widal test, p value – 0.001, Chi square test)

Table 12. Comparison of blood culture and Widal test results

Widal test	Blood culture		Total (N=100)
	Positive	Negative	
Positive	1 (2.1%)	46 (97.9%)	47
Negative	13 (24.5%)	40 (75.5%)	53
Total	14 (14%)	86 (86%)	100

Chart: 7Comparison of blood culture and Widal test results



5.4.5. Blood culture Vs Immunochromatography assay (IgM)

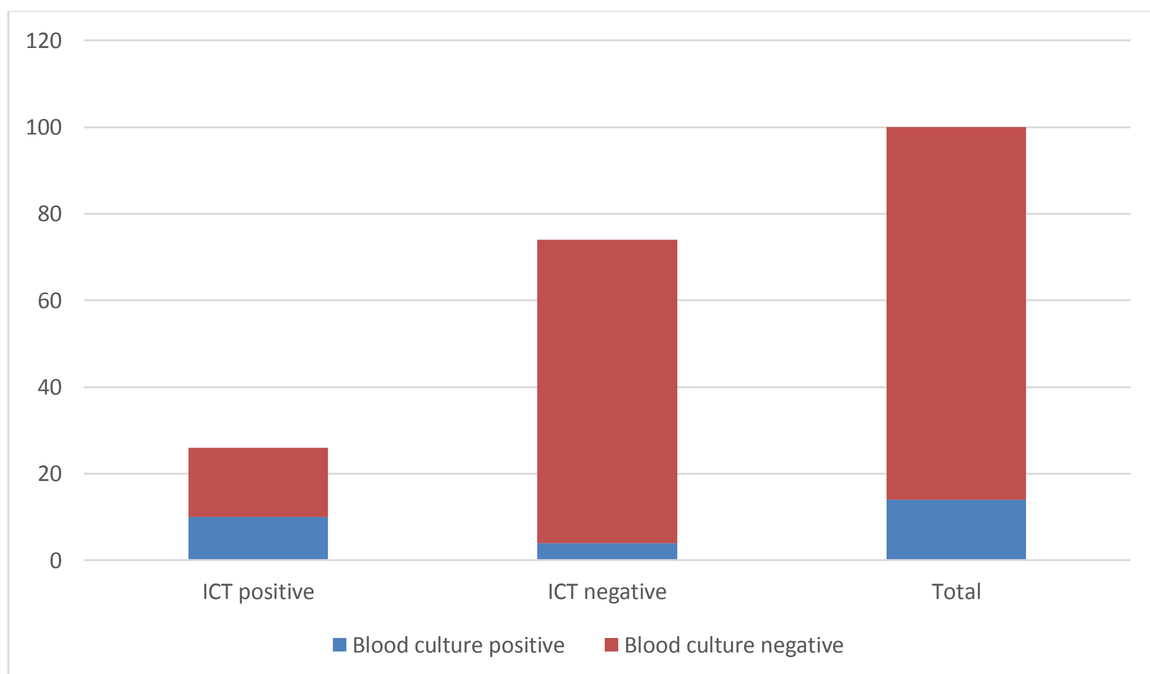
Out of 26 samples positive for IgM, 10 samples were positive for blood culture (True positivity rate or PPV– 38.5%). Out of 74 samples negative for IgM, 70 samples were found

to be negative for blood culture (True negativity rate or NPV – 94.6%). There exista statistically significant difference in the blood culture results and Immunochromatography assay, p value < 0.001, Fishers exact test

Table 13. Comparison of blood culture and ICT test results

Immunochromaography assay (IgM)	Blood culture		Total (N=100)
	Positive	Negative	
Positive	10 (38.5%)	16 (61.5%)	26
Negative	4 (5.4%)	70 (94.6%)	74
Total	14	86	100

Chart 8: ***Comparison of blood culture and ICT test results***



5.4.5. Comparison of Widal test and Immunochromatography

Chart 9 shows that Widal test yielded more positive results than Immunochromatography assay both in males and females. However, the true positivity rate is much lower in Widal test (2.1%) when compared to Immunochromatography (38.5%).

The sensitivity and specificity of the widal test is very much low when compared to Immunochromatography assay (Widal test: Sensitivity-7%, Specificity-46.5%; Immunochromatography assay: Sensitivity-71.4%, Specificity-81.4%).

Chart 9 Comparison of positive results by blood culture, Widal test and Immunochromatogaphy (IgM)

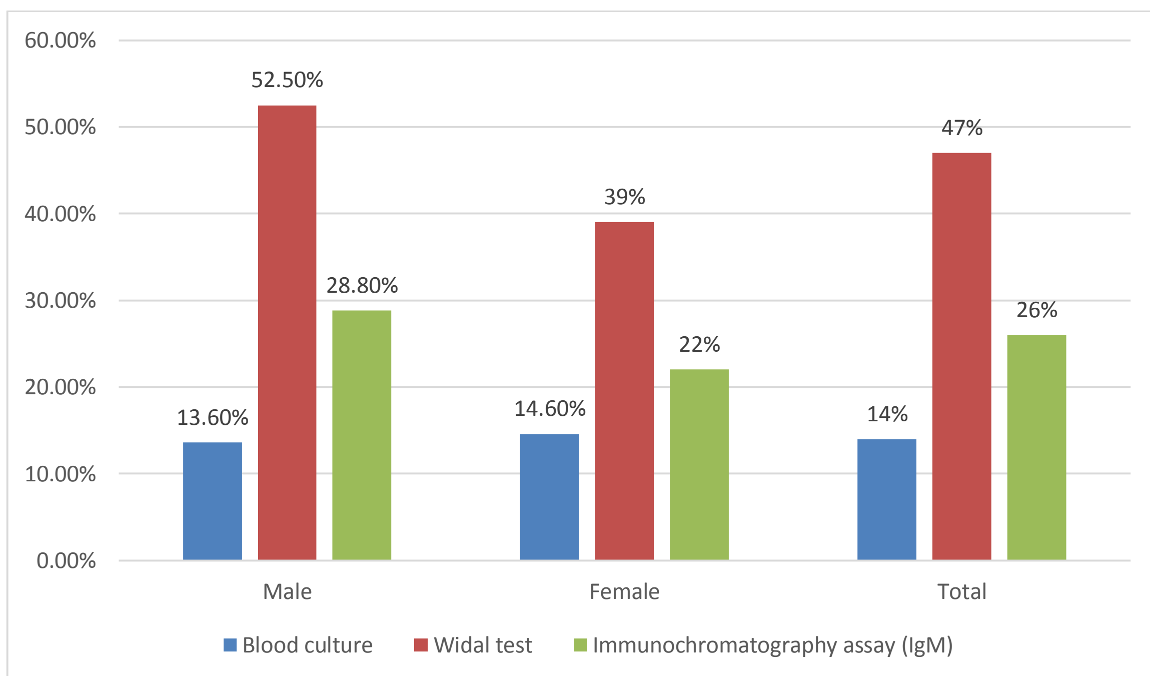
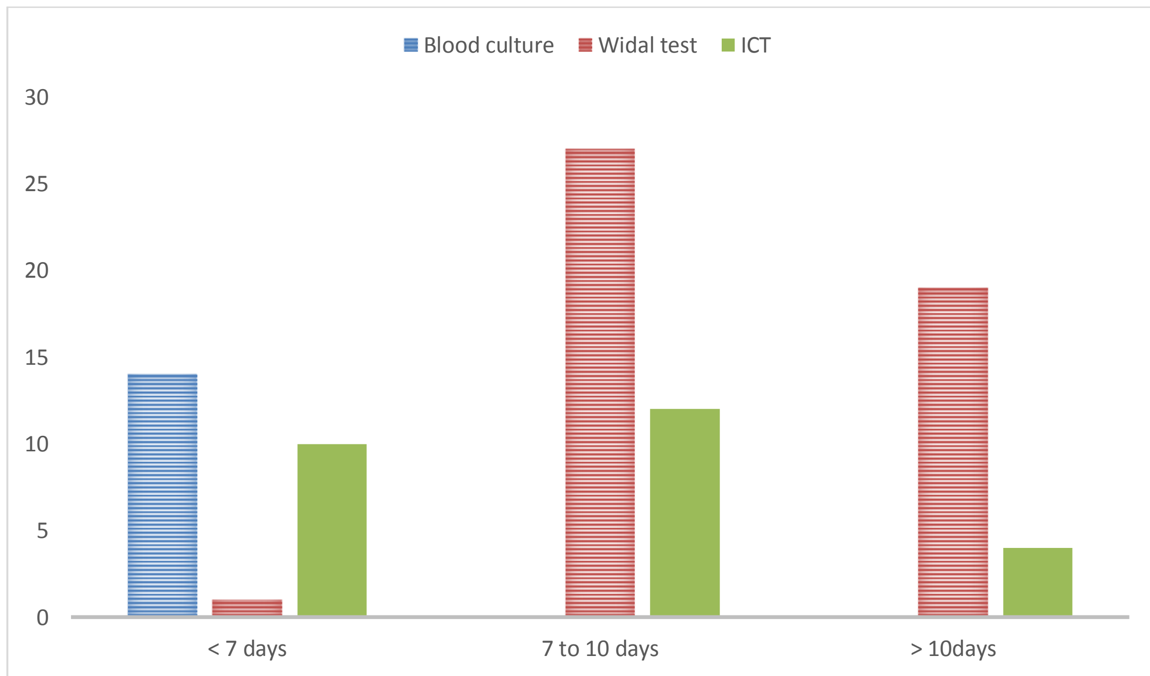


Table 14.Comparison of blood culture and widal test and ICT with fever duration

	< 7days	7-10 days	>10 days
Blood Culture	14	NIL	NIL
Widal Test	1	27	19
ICT	10	12	4

Chart 10: Comparison of positive results by various methods with duration of fever



5.5. ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE ISOLATES

5.5.1. Disc diffusion method

The table below shows the antibiotic susceptibility pattern of the Salmonella isolates by disc diffusion method. Highest prevalence of resistance was observed against Ampicillin (85.7%) followed by Chloramphenicol (74.3%) and Cotrimoxazole (74.3%) On the other hand, Azithromycin was found to be sensitive for 85.7% of isolates, followed by Ciprofloxacin (57.1%) and Pefloxacin (50%).

Table 15 Antimicrobial sensitivity pattern of the Salmonella isolates by disc diffusion method (N=100)

Antibiotic	Sensitive isolates (%)	Resistant isolates (%)
Chloramphenicol	4(25.7%)	10 (74.3%)
Ampcillin	2 (14.3%)	12 (85.7%)
Cotrimoxazole	4(25.7%)	10(74.3%)
Ciprofloxacin	8 (57.1%)	6 (42.9%)
Ceftriaxone	6 (42.9%)	8 (57.1%)
Azithromycin	12 (85.7%)	2 (14.3%)
Pefloxacin	7 (50%)	7 (50%)
Nalidixic acid	6 (42.9%)	8 (57.1%)

It could be seen from above table that only 50% of the isolates were sensitive to Pefloxacin.

Chart11 Antimicrobial susceptibility pattern of the isolates (n=14)

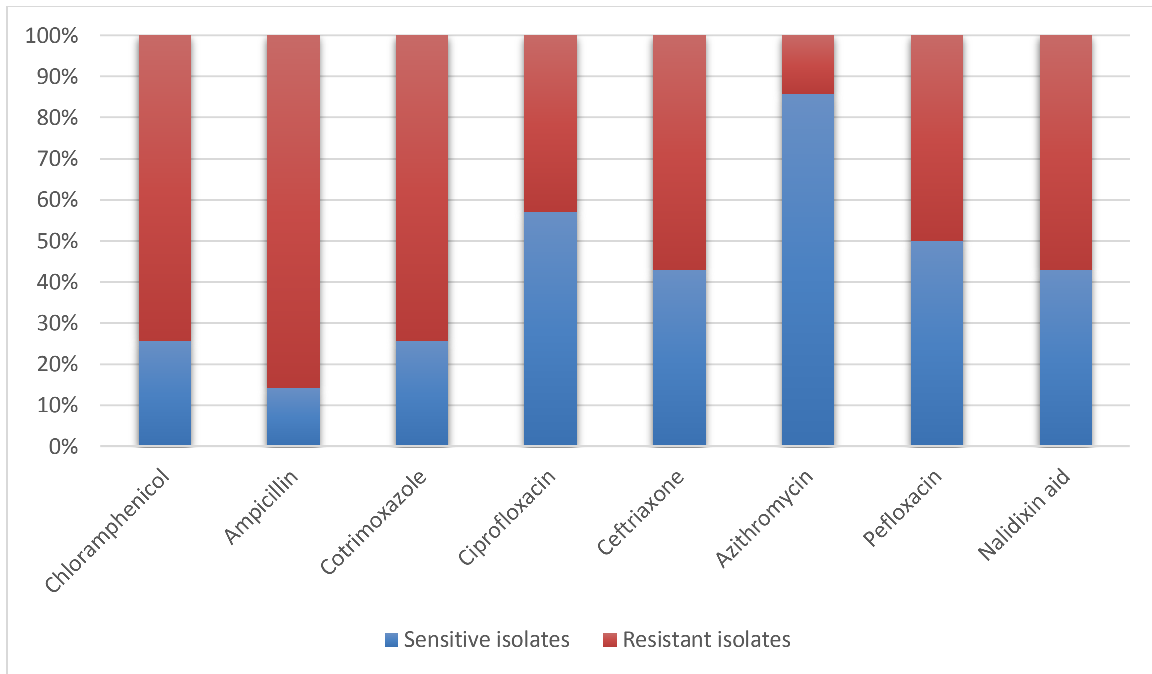
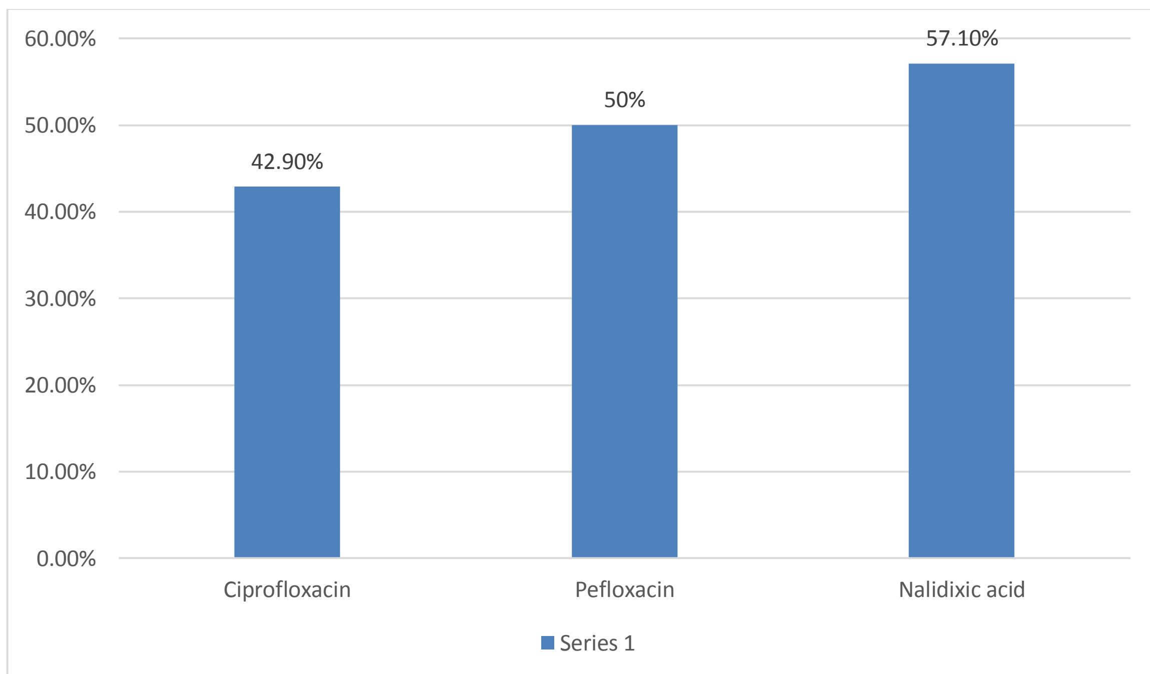


Chart 12**Comparison of resistance to nalidixic acid, pefloxacin and ciprofloxacin is shown below.**



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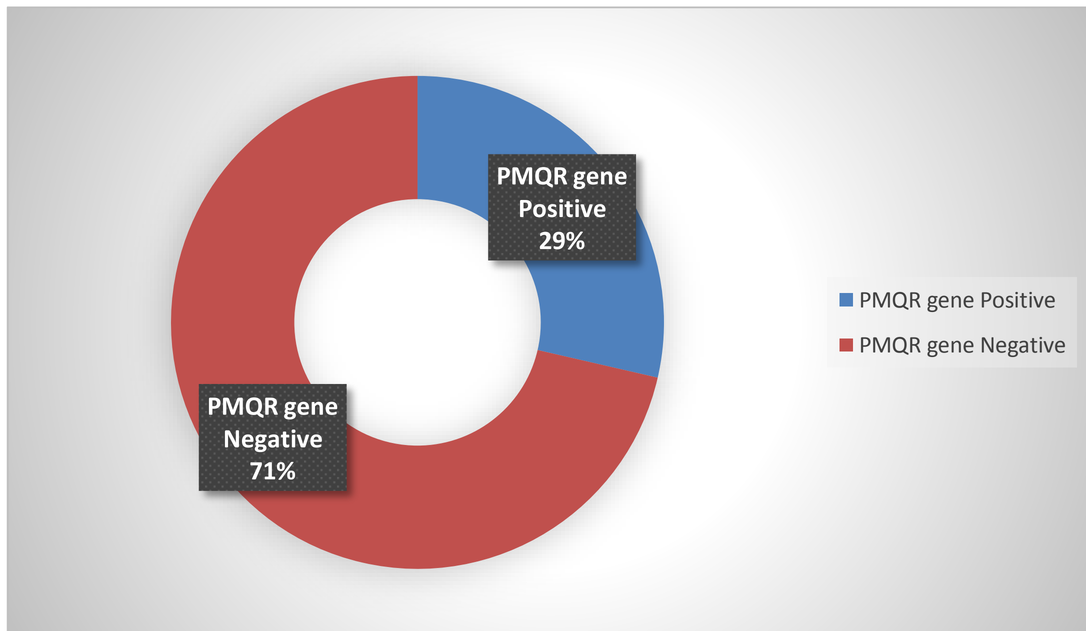
5.7. SCREENING FOR Plasmid Mediated Quinolone Resistant (PMQR) GENE

All the seven fluoroquinolone resistant isolates were subjected to screening for PMQR gene using molecular methods. It was found that only 2 isolates were positive for PMQR gene and remaining 5 were negative for PMQR gene.

Table 16 PCR test for detection of PMQR gene

PCR test result	Number (n = 7)	Percentage (%)
PMQR gene positive	2	28.5%
PMQR gene negative	5	71.4%
Total	7	100

Chart13: Results of PCR assay



DISCUSSION

This study was conducted in the Department of Microbiology, Tirunelveli Medical College. A total of 100 samples were collected from patients having clinical features suggestive of typhoid fever.

The gold standard test in diagnosis of typhoid fever is blood culture but its isolation is difficult (Hafasa A *et al,2013)*.So its role as a diagnostic parameter is low. The major reason for low isolation is the widespread use of antibiotics in the endemic areas and the small quantities of salmonella typhi (i.e., <15 organisms/ml) typically present in blood (Sudeepa kumar *et al*,2013) .

Hence, the physicians has to depend on Widal test and other rapid methods like immunochromatographic test (ICT) or ELISA to confirm the diagnosis.

Widal test has a very low sensitivity, specificity and a low positive predictive value. This again changes with the geographical areas.

Immunochromatographic test (ICT) is a rapid serological test for the diagnosis of typhoid fever. However, its usefulness in terms of specificity and sensitivity as compared to widal test has not been studied so far in our region. The studies which has been conducted in other parts of India and Asia has given good results. In view of this, the present study was conducted to know its utility and effectiveness in terms of diagnostic yields as compared to widal test .

In the present study, among 100 clinically suspected typhoid cases 59% were males and 41% were females (Table 1).This finding were similar to that of Roxas & Mendoza (1989) with 56% males and 44% females .The age of the patients ranged from a minimum of 16 years to a maximum of 74 years. Most of the isolates (39%) were from patients aged between 31 and 45 years .This is similar with the studies of Riyaz chungathu et al (2015) , Varsha Gupta et al (2013)

A study done by Butler *et al* (1991) also showed that infection rate is slightly higher in male population, because men are more in the habitof travelling more for work and more frequently exposed to outdoor food and water that may be contaminated and also males are more likely to report in hospitals. Health education and awareness regarding food and personal hygiene will bring this number down. This is comparable with the other studies of Shoora shetty Manohar Rudresh et al (2015) and Sarika Jain et al (2012).

Table 17:Age and Gender wise distribution

STUDY	MALE : FEMALE
Wain J et al (1998)	1 : 1
Yaramis A et al (2001)	1.4 : 1
Retnosari S et al.(2008)	1 : 2.3
Olsen SJ et al (2004)	1.05: 1
Chirag S et al (2005)	1.28 : 1
Dr .Balakrishna T.P (2010)	2.81 : 1
Present study	1.41 : 1

Table Showing the sex ratio in different studies among the Suspected cases Study series.

In this study 100 clinically suspected fever cases with fever of ≥ 3 days has been included 70% of cases presented with fever of 3-7 days and 30% were having fever of more than a week duration(Table 6). Isolation of *Salmonella* is possible in the earlier days of disease and antibiotic intake will be less during this period.

To compare the antibody level it was better to test the samples of patients presenting later into the week .This was comparable with the studies of Raveesh PM, et al.

In this study from blood samples of 100 febrile patients clinically suggestive of typhoid fever S.typhi was isolated. 14 samples (14%) were positive for *S. typhi* and hence bacteriologically proven typhoid fever or “true positive cases”(Table 7) . The remaining 86 patients were

culture negative . Similar culture findings were also reported by Hossain *et al* (2001) from Bangladesh of 16.67%. But ,Saha *et al* (2001) from Bangladesh and Jesudasson & Sivakumar from India reported an isolation rate of 8.40% and 6.92% respectively,which was even lower.

The overt abuse of antibiotics and it being difficult to obtain large enough volume of blood for the culture is the main cause for low isolation rate. As seen with the studies by Parande MA *et al*(2011) and Walia M, Kalaivani M, et al.

The Widal test is still the widely used serological test for Typhoid fever.Here the antibody against antigens O and H are detected . In this study, Widal test was carried out for all the clinically proven typhoid cases (Table 8). The cut off value of Widal test was considered as 1:80 for both TO and TH.

In our study about 57.44% cases with fever of more than a week showed an antibody titre of ≥ 160(Table 9).A study done by Shukla *et al*. (1997) also found that 44.2% had TO titre of ≥160 in single sample collected from patients suspected to have typhoid in an endemic area of South India. Second specimens are often not sent to the laboratory to verify the rising titre. It is possible that the Widal test would have performed better if paired sera were tested to demonstrate the rising titers. Patients rarely return for follow-up once treated so that obtaining paired sera in a routine clinical setting is unlikely.Clinicians cannot wait for results from two samples hence widely rely on “positive” Widal test done on a single serum sample.

In the present study 30% of samples were collected from patients with fever of >7 days.In such patients antibody titre was found to be ≥320.This is due to the increase in antibody titre as the duration of fever increases.

The incidence of false negative Widal test among the bacteriologically proven cases of this study was 13(24.5%)(Table 12). This findings were similar to when comparedwith findings

of Sudeepa Kumar M et al, 11.3% (Saha *et al*) and 6.9% in Malaysian populations (Malik, 2001).

In our study, among clinically suspected 100 typhoid fever cases 53 cases were both blood culture and Widal test negative (Table 12).Out of 47 positive samples for widal test, only one sample was positive for blood culture (True positivity rate – 2.1%). Out of 53 negative samples for Widal test, 13 samples were negative for blood culture (True negativity rate – 24.5%).This correlates with findings of Olopoenia & King, 2000; Parry *et al*; Rodrigues, 2003). Suboptimal sensitivity is due to prior antibiotic therapy and failure to mount an immune response by certain individuals (Olopoenia & King, 2000).The IgM antibody starts appearing later into the first week.

The sensitivity, specificity, Positive predictive Value and Negative predictive Value of Widal test were 7%,46.5%,2.1%and 75.4%.These values are in concordance with studies published by Sherwal et al.

Widal test has a low sensitivity, specificity and low PPV, but it has good NPV which indicates that negative Widal test result have a good indication for the absence of the disease.

In our study immunochromatographic test was evaluated for its usefulness in patients of typhoid fever presenting to our hospital and observed that it has a sensitivity of 71.4% and specificity of 81.4%, which was higher than that of widal test (sensitivity-7% and specificity-46.5%) and comparable to the studies done elsewhere in India and outside.

ICT (typhifast) had a comparable sensitivity of 94% and specificity of 77%, while widal test had a sensitivity and specificity of 63% and 83% only in a study conducted in Pakistan.The effectiveness of ICT in early diagnosis of typhoid fever patients was also studied in two different studies in Malaysia. Its sensitivity and specificity was reported as

90.3% and 91.9% respectively in the first study, and was significantly higher. The second study, also showed a sensitivity and specificity of 98% and 76.6% respectively.

Out of 26 samples positive for ICT, 10 samples were positive for blood culture (True positivity rate – 38.5%)(Table 13). Out of 74 samples negative for ICT only 4 samples were found to be positive for blood culture (True negativity rate – 94.6%). IgG antibody are not considered as comparison because long-term persistence of the IgG antibody after exposure to typhoid infection or vaccination.

In this study we have compared the relative diagnostic accuracy of widal test with a rapid immunochromatographic test (ICT) taking blood culture positive cases as relative standard.

Results show that ICT with sensitivity of 71.4% and specificity of 81.4% being comparibly superior to that of widal test and is further both rapid and cost effective.Thus making it an ideal alternate , economically and a reliable diagnostic tool in our setup to be considered.

Table 18: Comparison of ICT results from different studies

Studies	**Sensitivity (%)**	**Specificity(%)**	**PPV(%)**	**NPV(%)**
Narayanappa et al.	92.6	37.5	48.7	88.8
Sherwal et al	92	87.5	92	NA
Gopalakrishnan et al.	82	68.1	57.7	90.1
Yadav et al.	90	100	100	93
Beig et al. [	90	100	100	92.1
Bukhari et al.	93	87	NA	NA
Udayakumar et al	81.7	84.6	69.8	91.4

In developing countries,emperical antibiotic therapy is started before investigations due to the high cost of investigations, resulting in poor culture results and misuse and abuse of antibiotics. widal test is positive only after first week, thereby ICT gives a clue towards the diagnosis at the possible earliest.

Salmonella diarrhoea is generally self-limiting, and antimicrobials are usually not required for treatment. But in case of invasive infections if not treated properly, enteric fever carries a mortality rate of 30%. Appropriate antibiotic treatments reduces this mortality rate to as low as 0.5%.

Multidrug resistant strains(resistant to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (co-trimoxazole),are present since 1980s and 1990s.

The fluoroquinolone (FQ), ciprofloxacin has become the first-line drug for treatment, especially since the global emergence of *S.* Typhi isolates that are multidrug resistant (MDR). However, this switchover to ciprofloxacin has led to a subsequent increase in the occurrence of typhoidal salmonellae resistant to this ciprofloxacin.(Sherwal et al. And Rahman M, Siddique AK).By the year 2000, clinical failure to ciprofloxacin was reported due to the nalidixic acid resistant *S.* Typhi (NARST) phenotypes causing enteric fever. Since these strains were reported as ciprofloxacin susceptible by the laboratories as per the CLSI guidelines available that time, ciprofloxacin continued to be the first line of treatment.

CLSI revised the susceptibility breakpoints of ciprofloxacin interpretative criteria in 2012 where the MIC breakpoints were lowered from ≤1 to ≤0.06 μg/ml and zone diameter increased from ≥21 to ≥31 mm which has changed the ciprofloxacin susceptibility percentage in the strains from about 90% to 8%. Similar results are reported from other regions where the impact of revised CLSI guidelines resulted in susceptibility change from 95% to 3%.

The culture-positive cases increased again during that time. Following ciprofloxacin clinical failures and reports of NARST phenotypes causing enteric fever increased, cephalosporins

(ceftriaxone) became the choice of antibiotic to treat enteric fever. In the recent past, 3rd generation cephalosporins have gained importance for the treatment of enteric fever because of their pharmacodynamic properties and the very low prevalence of resistance to these agents. Ceftriaxone administered either intravenously or intra-muscularly and cefixime which is administered orally are both effective in typhoid fever and are commonly used in the treatment. Cefotaxime and cefoperazone are other treatment options.

There were reports of gradual increase of mean MIC of the cephalosporins in the *S*. Typhi isolates from different parts of the world which later became obvious with clinical failures with ceftriaxone treatment of *S. Typhi* infections.(Sarika jain et al .,2013), Muzamil Mahdi Abdel Hamid (2017), which shows that S.typhi strains havedeveloped low level of resistance to ceftriaxone .

Extended spectrum cephalosporin resistance in Salmonella strains is usually due to the production of plasmid mediated extended-spectrum β-lactamases or AmpC β-lactamases.There is gradual increase in the use of 3rd generation cephalosporins and declining ciprofloxacin usage during that time and the culture-positive case decreased subsequently after 2009. In absence of facilities for blood cultures in the health care centers, the empirical antibiotics used to treat all fevers in the community could be responsible for only the complicated cases presenting to the tertiary care centres.

Treatment with ceftriaxone has started to show poor clinical response, an increase in the resistance proportion of culture-positive cases can be seen after 2014(Jaspal Kaur et al).

In the present study 42.9% of isolates were sensitive for ceftriazone and 57.1% resistant.

Studies of Sarika jain et al (2013),Muzamil Mahdi Abdel Hamid (2017) also shows that S.Typhi strains have developed low level of resistance to ceftriaxone.

This can be attributed to the facts like inappropriate prescription and irrational use of these drugs by clinicians.

Azithromycin has been used for uncomplicated cases of enteric fever since 1992 though there were no laboratory criteria for susceptibility determination. CLSI in 2015 addedazithromycin MIC and disk diffusion criteria for *S*. Typhi and *S*. Paratyphi A.(Gopalakrishan V, Sekhar WY , Soo EH *et al*)

We tested azithromycin susceptibility in strains and found that 85.7% of *S*.Typhi were susceptible to azithromycin as per current CLSI guidelines. Azithromycin susceptibility is promising in *S*. Typhi.

The preferred test for assessing fluoroquinolone susceptibility or resistance in Salmonella species is a ciprofloxacin MIC test. If MIC test cannot be done for ciprofloxacin, levofloxacin or ofloxacin , pefloxacin disk diffusion may be used as surrogate test to predict ciprofloxacin susceptibility (CLSI 28th edition)

In the present study regional differences in antibiotic sensitivity against *S*. Typhi were observed. Most isolates were sensitive to azithromycin, ciprofloxacin and pefloxacin. Although previous studies showed the growing resistance of ciprofloxacin, but we found both ciprofloxacin resistant and susceptible samples, 57.1%were susceptible to ciprofloxacin. And the other 42.9% of the samples were resistant to it. Among them 50% were susceptible to pefloxacin According to CLSI guidelines 28th edition (2018) ciprofloxacin can be reported as susceptible or resistant based on the pefloxacin test result if MIC test was not conducted in the case of ciprofloxacin.

Out of the 14 Salmonella isolates, all were found to be multi drug resistant (resistant to three or more antibiotics). Nalidixic acid was found to be sensitive in 42.9% of the isolates, whereas 57.1% were found to be nalidixic acid resistant.

The Continuous use of antibiotics in India can be a reason for high occurrence of resistance among the isolates in this study. Inconclusive diagnosis, over dependence on antibiotics can be causes for this.

In our study highest prevalence of resistance was observed against Ampicillin (85.7%) followed by Chloramphenicol (74.3%), Cotrimoxazole (74.3%). On the other hand, Azithromycin was found to be sensitive for 85.7% of isolates, followed by Ciprofloxacin (57.1%) and Pefloxacin (50%).

All the seven fluoroquinolone resistant isolates were subjected to screening for Plasmid mediated quinolone resistant gene (qnr A and qnr B) using molecular methods. It was found that 2 isolates were positive for qnrA gene and remaining 5 were negative for both qnrA and qnrB gene, since plasmid mediated resistance is not the only mechanism, mutation in QRDR of DNA gyrase and topoisomerase or efflux pump mechanism may also be responsible . However, search for other mutations and/or efflux pump mechanisms was beyond the scope of this study.

CONCLUSION

Typhoid fever continues to be a major public health problem and the emergence of antimicrobial resistance by *Salmonellae* Typhi adds to the complexity in treating the patients .

Though blood culture is gold standard, its non availaility in rural areas, being time consuming and positivity mainly in early period,limits its utility in our region. Widal test is less sensitive and takes 24 hours for the results. Immunochromatographic test is reliable and easy to perform and detects antibodies by the end of 1st week.So thereby making Immunochromatographic test a better option in the diagnosis of typhoid fever in rural set up and endemic areas. Our results thus supports the fact and suggests that laboratories could easily employ this rapid, economical test with no expert technical skill requirement,apt for our region.

In order to decrease the incidence of typhoid in tropical countries we can adopt measures such as proper sanitation, safe drinking water, newer vaccines, and so on, early diagnosis and treatment and ICT could come in handy at the diagnostic laboratories.

ICT is a highly sensitive and specific test in diagnosing typhoid fever. It is a rapid, easy to perform, more reliable test for typhoid fever as compared to widal test and can be useful in early and appropriate institution of therapy . However, a larger prospective study would be required to fully evaluate the usefulness of this test in countries endemic to typhoid fever.

Rapid ICT tests offer increased sensitivity, rapidity, early diagnosis and simplicity over blood culture and Widal test, and can be used as a reliable alternate diagnostic tool to the most commonly used serological tests. Positive Salmonella-IgM tests among blood culture negative patients should always be correlated with clinical picture of the patient. Many a times, blood culture may be negative due to other reasons like prior intake of antibiotics, low volume of blood taken for culture. However, culture isolation of *Salmonella* remains

essential, especially for antibiotic susceptibility testing and these serological tests should be used in conjunction with culture for the early diagnosis of enteric fever.

With regards to the present antibiotic of choice for typhoid fever, this study clearly implies that the earlier experienced resistance pattern for fluroquinolones has reversed and the sensitivity increased thereby making it again the choice of treatment in this region.

SUMMARY

The present study was carried out in Tirunelveli Medical College and Hospital,Tirunelveli for a period of one year from June 2017 to July 2018. A total of100 clinically suspected enteric fever cases blood samples were subjected for blood culture,widal test and Immunochromatographic test. Antibiotic sensitivity pattern of the isolates were analysed along with plasmid mediated quinolone resistance in the same.

- Enteric fever was predominantly seen among males (59%) than females(41%) .
- 39% of enteric fever was seen in adults in the age group of 31 – 45yrs and 23% in age group of 46-60 yrs.
- A total of 14 Salmonella Typhi isolates were isolated from 100 blood culture samples
- A total of 47 samples were positive for widal test. Out of this only one sample was positive for blood culture (True positivity rate – 2.1% and True negativity rate – 75.4%).
- A total of 26 samples were positive for immunochromatographic test(IgM).
- Out of 26 samples positive for IgM, 10 samples were positive for blood culture (True positivity rate – 38.5%).
- Out of 74 samples negative for IgM, only 4 samples were found to be positive for blood culture (True negativity rate – 94.6%).
- All isolates of S.typhi was found to be Multi-drug resistant.
- S.typhi showed 50% resistance to Pefloxacin and 42.9% resistance to Ciprofloxacin
- Salmonella typhi showed 42.9% sensitivity to Ceftriaxone and Nalidixic acid.
- S. Typhi A showed 74.3% resistant to Chloramphenicol ,
- 85.7% resistant to Ampicillin , 74.3% resistant to Cotrimoxazole.

- Molecular characterisation was done, to determine whether fluoroquinolone resistance was plasmid mediated .
- The two resistant isolates of S.Typhi were found to be positive for plasmid mediated quinolone resistance gene qnr A. No mutations were detected in qnrB gene.

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ANNEXURE– 1

1.Nutrient agar medium:

Composition

Ingredients gram/liter

Peptic digest of Animal Tissue 5.00

Sodium Chloride 5.00

Beef Extract 1.50

Yeast Extract 1.50

Agar 15.00

Twenty-eight grams of dehydrated nutrient agar medium was added to 1000 ml ofcold distilled water in a flask and boiled to dissolve the medium completely. Themedium was then sterilized in an autoclave at 121^0C and 15 lbs pressure for 15minutes. The sterile media were stored in a refrigerator at 4^0C for future use.

2. MacConkey agar medium:

Composition - Ingredients gram/liter

Peptone 19.0

Lactose 10.0

NaCl 5.0

Na- Deoxycholate 1.0

Neutral Red 0.03

Crystal Violet 0.001

Agar 15.0

Fifty-two grams of dehydrated MacConkey agar medium was suspended in 1000 ml of cold distilled water and boiled to dissolve the medium completely. The solution wasthen sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes.

3. Blood agar medium

Composition

Ingredients gram/liter

Heart infusion 500.00

Tryptose 10.00

Sodium chloride 5.00

Agar 15.00

Forty grams of the dehydrated blood agar medium was suspended in 1000 ml colddistilled water in a flask and boiled to dissolve the medium completely. It was thensterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes. The autoclavedmaterials were allowed to cool to a temperature of 45^0C in a water bath. Defibrinated5-10% sheep blood was then added to the medium aseptically and distributed tosterile petridishes. Sterile media was stored in refrigerator at 4^0C for future use.

4.Triple sugar agar medium

Composition	**Ingredients Gms / Litre**
Beef extract	3.000
Peptone	20.000
Yeast extract	3.000

Lactose	10.000
Sucrose	10.000
Dextrose monohydrate	1.000
Ferrous sulphate	0.200
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	

Suspend 64.42 grams of dehydrated medium in 1000 ml distilled water. Heat to boiling. Dissolve the medium completely. Mix well and distribute into test tubes and Sterilize by maintaining at 10lbs pressure (115°C) for 30 minutes. Allow the medium to set in sloped form with a butt about 2.5cm long.

5. Muller Hinton agar medium

Composition

Ingredients gram/liter

Beef dehytrated infusion 300

Casein hydrolysate 17.50

Starch agar 17.00

Agar 17.00

Thirty-eight grams of dehydrated Mueller Hinton agar medium was suspended in1000 ml cold distilled water and boiled to dissolve the medium completely. Thesolution was then sterilized by autoclaving at 121^{0}C and 15 lbs pressure for 15minutes. The autoclaved media was stored in the refrigerator and used later.

6. McFarland Standard (0.5):

Reagents:

Sulphuric acid,1%: To 100 ml of distilled water,1 ml of conc.sulphuric acid is added.Barium chloride, 1.175%: To 100 ml of distilled water, 1.175gm of barium chloride is added and mixed well.

To prepare McFarland 0.5 standards:

To 85 ml of 1% conc.sulphuric acid, 0.5 ml of Barium chloride is added in a flask while constantly swirling the flask. Bring to 100 ml with 1% conc.sulphuric acid. Aliquot in test tubes and cap tubes tightly. Store in the dark at room temperature for 3 months or longer.

PROFORMA

Name : S/O, W/O:

Age / Sex : Address:

Complaints:

History of Present Illness :

Past History:

Treatment History:

Examination: Well / Moderately / ill built Temp: F

Pallor : Yes / No HR/PR /Min

Icterus : Yes / No RR: /Min

Pedal edema: Yes / No

CVS.

RS

P/A: : Hepatospleenomegaly + / -

INVISTIGATION: CBC:

WBC- NEUT % LYM %

RBC- Hb%-

PLT RBS:

Blood Culture:

Others:

Column1	ae	Column2	Column3	Column4	I C T			ANTIBIOTIC SENSITIVITY PATTERN								P C R	Fever	Duration	
serial no.	age	sex	blood culture	widal test	IgM	IgG	IgM+IgG	chloramphenicol	ampicillin	cotrimoxazole	ciprofloxacin	ceftriaxazone	azithromycin	pefloxacin	naldixic acid	qnrA&B	3-7Days	7-10Days	>10 days
1	28yrs	2	2	2	1	2	2										1	2	2
2	20yrs	1	2	2	2	2	2										2	1	2
3	16yrs	1	2	2	1	2	2										1	2	2
4	29yrs	1	2	1	2	2	1										2	1	2
5	53yrs	1	2	2	2	2	2										1	2	2
6	61yrs	1	2	1	2	2	1										2	1	2
7	28yrs	2	2	2	2	2	2										2	1	2
8	32yrs	2	2	1	2	2	1										1	2	2
9	44yrs	1	2	1	1	2	2										2	2	1
10	22yrs	1	1	2	1	2	2	2	2	2	2	1	1	2	2	2	1	2	2
11	19yrs	1	2	1	1	2	2										1	2	2
12	20yrs	1	2	2	2	2	2										1	2	2
13	24yrs	2	2	1	2	1	2										1	2	2
14	18yrs	2	2	2	2	2	1										1	2	1
15	33yrs	2	2	2	2	2	1										1	2	2
16	22yrs	1	2	1	1	2	2										2	2	2
17	18yrs	2	1	2	1	2	2	2	1	1	2	1	1	2	2	2	1	2	2
18	35yrs	2	2	2	2	2	1										1	2	1
19	18yrs	2	2	1	1	2	2										1	2	2
20	16yrs	1	2	1	1	2	2										2	1	2
21	29yrs	1	2	1	2	2	1										1	1	2
22	32yrs	2	1	2	1	2	2	2	1	1	2	1	1	2	2	2	1	2	2
23	45yrs	1	2	2	2	2	2										2	2	2
24	28yrs	1	2	1	2	1	2										2	2	2
25	44yrs	2	2	2	2	2	2										2	2	2
26	56yrs	1	2	1	2	1	2										1	2	1
27	44yrs	2	1	2	1	2	2	2	2	1	1	2	1	1	1		1	2	2
28	33yrs	2	2	2	2	2	2										1	2	2
29	48yrs	2	2	2	2	2	2										1	2	2
30	54yrs	2	2	2	2	2	2										2	1	2
31	54yrs	1	2	2	2	2	2										2	1	2
32	42yrs	1	2	1	1	2	2										1	1	2
33	36yrs	2	2	1	2	1	2										2	2	1
34	32yrs	1	2	1	2	2	1										2	1	2
35	38yrs	2	1	2	1	2	2	2	2	2	1	2	1	1	1		2	1	2
36	25yrs	1	1	2	1	2	2	2	2	2	2	1	1	2	2	2	1	2	2
37	28yrs	1	2	1	1	2	2										2	1	2
38	24yrs	2	2	2	2	2	2										2	2	1
39	25yrs	2	2	1	1	2	2										2	1	2
40	36yrs	2	1	2	1	2	2	1	2	2	1	2	1	1	1		1	2	2
41	55yrs	1	2	2	2	2	2										2	1	2
42	58yrs	2	2	2	2	2	2										2	2	1
43	63yrs	1	2	2	2	2	2										1	2	2
44	72yrs	2	2	2	2	2	2										2	1	2
45	51yrs	1	2	1	2	2	2										2	2	1
46	49yrs	1	1	2	1	2	2	2	2	2	1	2	1	2	1		1	2	2
47	67yrs	1	2	2	2	2	2										1	2	2
48	68yrs	2	2	2	2	2	2										1	2	2
49	55yrs	1	2	2	1	2	2										1	2	2
50	41yrs	1	2	1	1	2	2										2	2	1
51	22yrs	1	2	1	2	1	2										2	1	2
52	32yrs	2	2	2	2	2	1										1	2	2
53	53yrs	1	2	2	2	2	2										2	1	2
54	43yrs	1	2	2	2	2	2										1	2	2
55	74yrs	1	2	2	2	2	2										1	2	2
56	54yrs	2	2	2	2	2	2										1	2	2
57	42yrs	1	2	2	2	2	2										1	2	2
58	47yrs	1	2	2	2	2	2										1	2	2
59	38yrs	2	2	1	2	2	1										1	2	2
60	48yrs	1	1	1	1	2	2	1	2	1	1	2	1	1	2		1	2	2
61	46yrs	1	2	2	2	2	2										1	2	2
62	48yrs	1	2	2	2	2	2										1	2	2

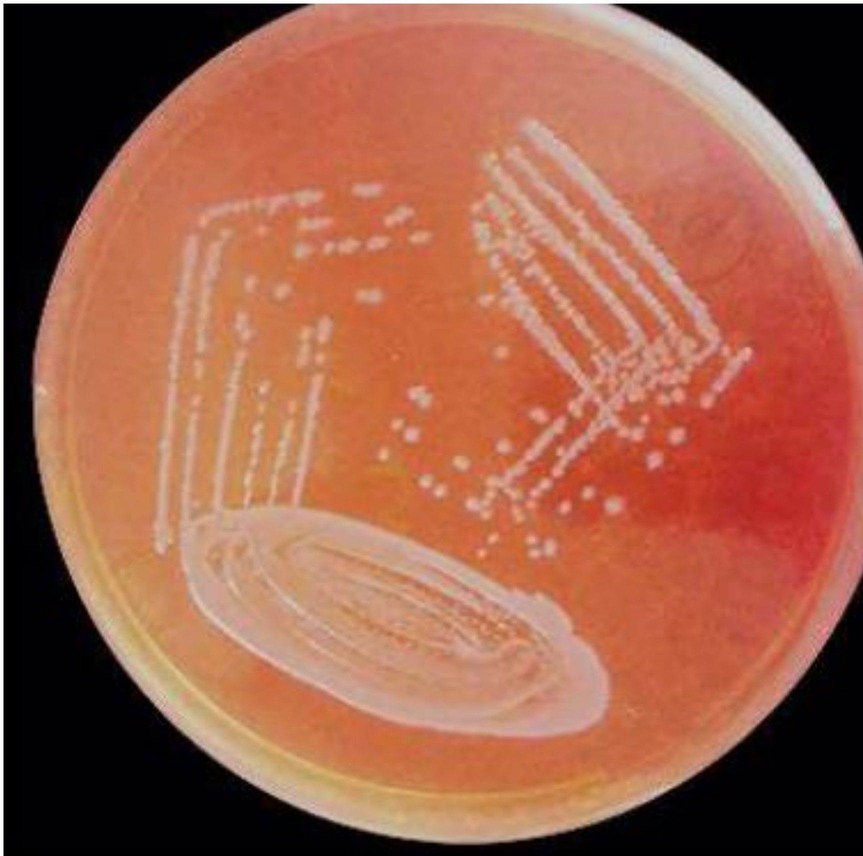
serial no.	age	sex	blood culture	widal test	IgM	IgG	IgM+IgG	chloramphenicol	ampicillin	cotrimoxazole	ciprofloxacin	ceftriaxazone	azithromycin	pefloxacin	naldixic acid	qnrA&B	3-7Days	7-10Days	>10 days
63	28yrs	1	2	1	2	2	1										2	1	2
64	36yrs	1	2	1	1	2	2										2	2	1
65	36yrs	2	2	1	1	2	2										2	1	2
66	39yrs	2	2	1	2	2	2										1	2	2
67	30yrs	2	2	2	2	2	1										1	2	2
68	20yrs	1	1	2	2	2	2	2	1	2	1	2	2	1	1		1	2	2
69	40yrs	1	2	1	2	2	2										1	2	2
70	43yrs	1	2	2	1	2	2										1	2	2
71	27yrs	1	2	1	2	1	2										1	2	2
72	38yrs	2	2	1	2	2	1										1	2	2
73	29yrs	1	2	1	2	2	1										1	2	2
74	19yrs	1	2	1	2	2	1										1	2	2
75	29yrs	1	2	1	2	2	1										1	2	2
76	38yrs	1	1	2	2	2	2	2	2	2	1	2	1	1	2		1	2	2
77	47yrs	2	2	2	2	2	2										1	2	2
78	45yrs	1	1	2	1	2	2	1	2	2	1	2	1	1	1		1	1	2
79	56yrs	1	2	2	2	2	2										1	2	2
80	45yrs	1	2	2	2	2	2										1	2	2
81	34yrs	2	1	2	2	2	2	2	1	2	2	1	1	2	2	1	1	2	2
82	38yrs	2	2	1	2	2	2										1	2	2
83	27yrs	1	2	1	2	2	1										1	2	2
84	46yrs	2	2	1	2	2	1										1	2	2
85	54yrs	2	2	2	2	2	2										1	2	2
86	43yrs	1	2	1	2	2	1										1	2	2
87	34yrs	2	2	1	2	2	1										1	2	2
88	23yrs	2	2	1	2	2	2										1	2	2
89	32yrs	1	2	1	2	2	1										1	2	2
90	33yrs	2	2	1	2	2	1										1	2	2
91	35yrs	1	2	1	2	2	2										1	2	2
92	33yrs	1	2	1	2	2	1										1	2	2
93	56yrs	1	2	1	2	2	1										1	2	2
94	57yrs	2	2	2	2	2	2										1	2	2
95	58yrs	2	2	1	2	2	1										1	2	2
96	39yrs	1	2	1	1	2	2										1	2	2
97	45yrs	1	1	2	2	2	2	1	2		2	1	2	2	2	2	1	2	2
98	34yrs	1	2	1	2	2	2										2	1	2
99	24yrs	2	2	1	2	2	1										2	1	2
100	65yrs	1	2	2	2	2	2										2	1	2

MALE=1, FEMALE=2
ve=1 , negative = 2
ınochromatographic test
ymerase chain reaction
positive =1 Negative=2
positive =1 Negative=2

DSITIVE =1 NEGATIVE=2

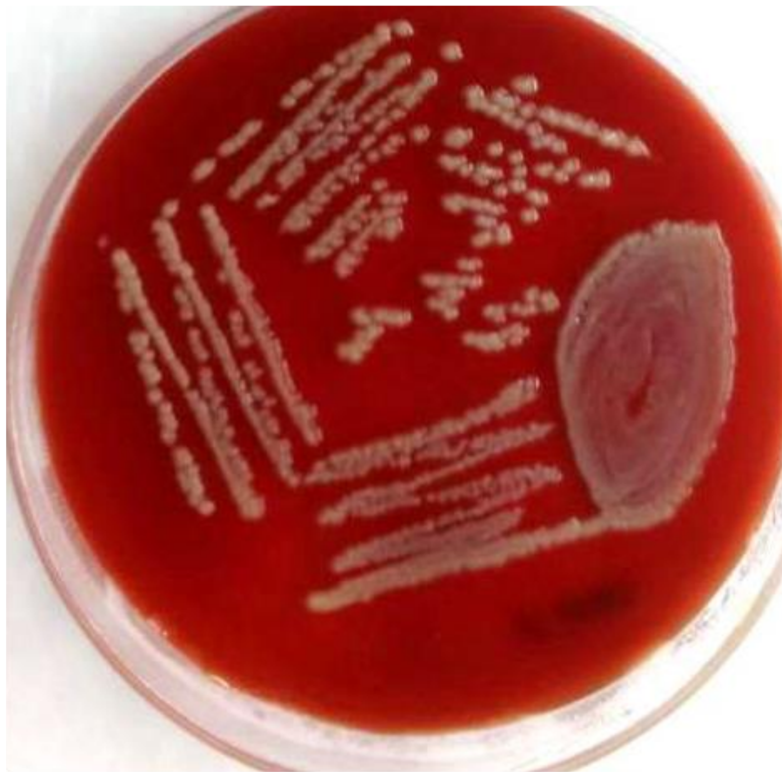
0

Mac Conkey agar – Non lactose fermenting colonies of *Salmonella* Typhi



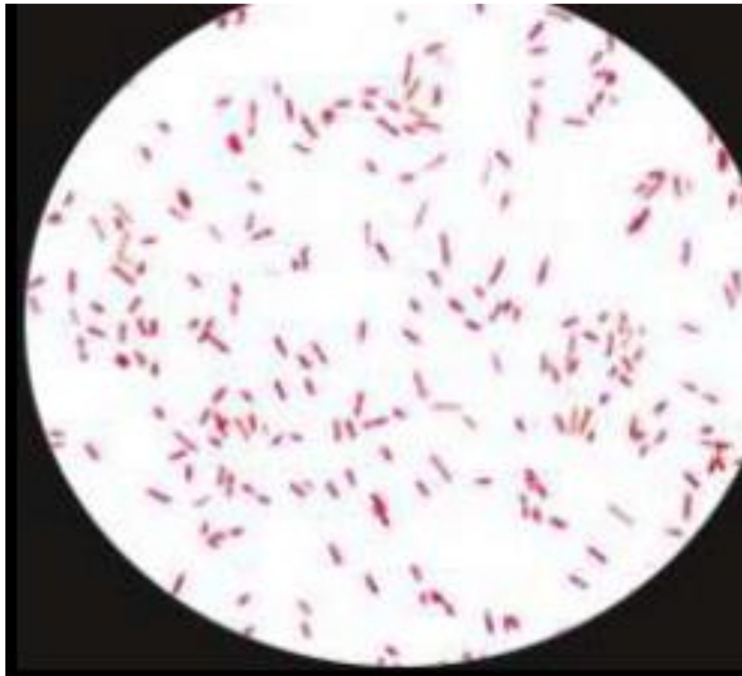
Nutrient agar plate - *Salmonella* Typhi colonies

Blood Agar plate - *Salmonella* Typhi greyish white colonies



Deoxycholate citrate Agar - *Salmonella* Typhi with black head colonies

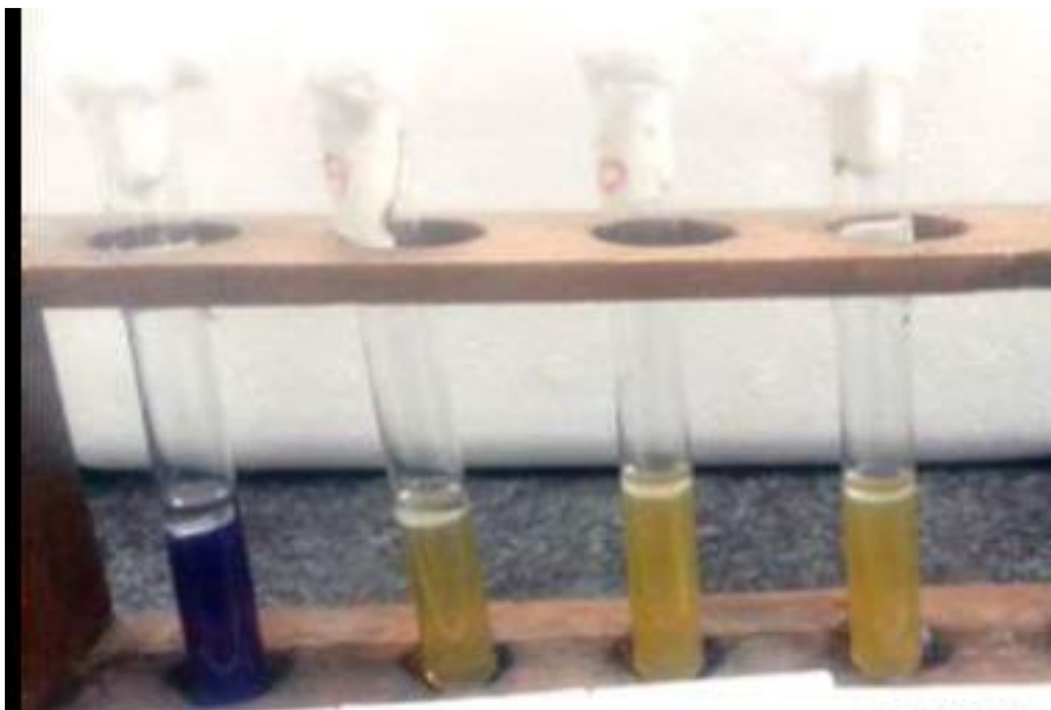
Grama Stain – Gram Negative bacilli



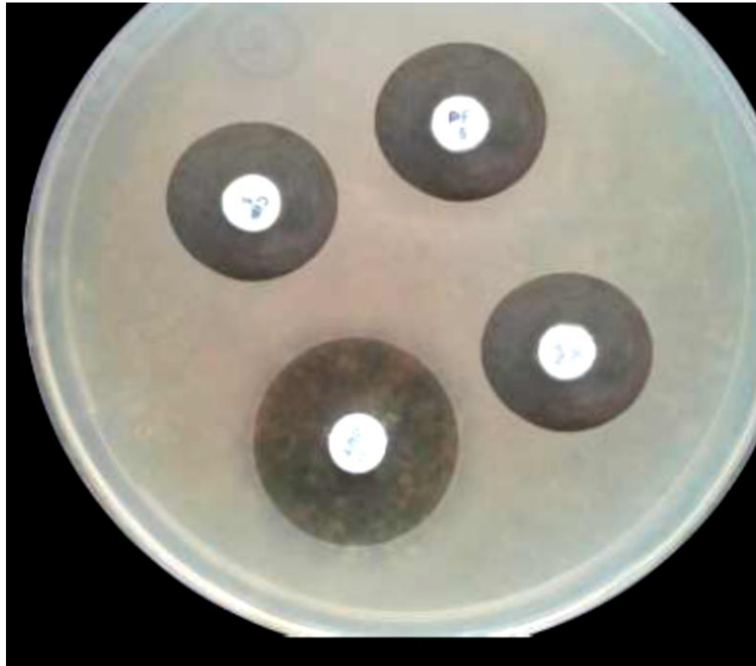
Biochemical reactions of *Salmonella* Typhi Triple sugar Iron – Alkaline / Acid with speck of H_2S

Sugar Fermentation of *Salmonella* Typhi

Lysine Decarboxylated in *Salmonella* Typhi

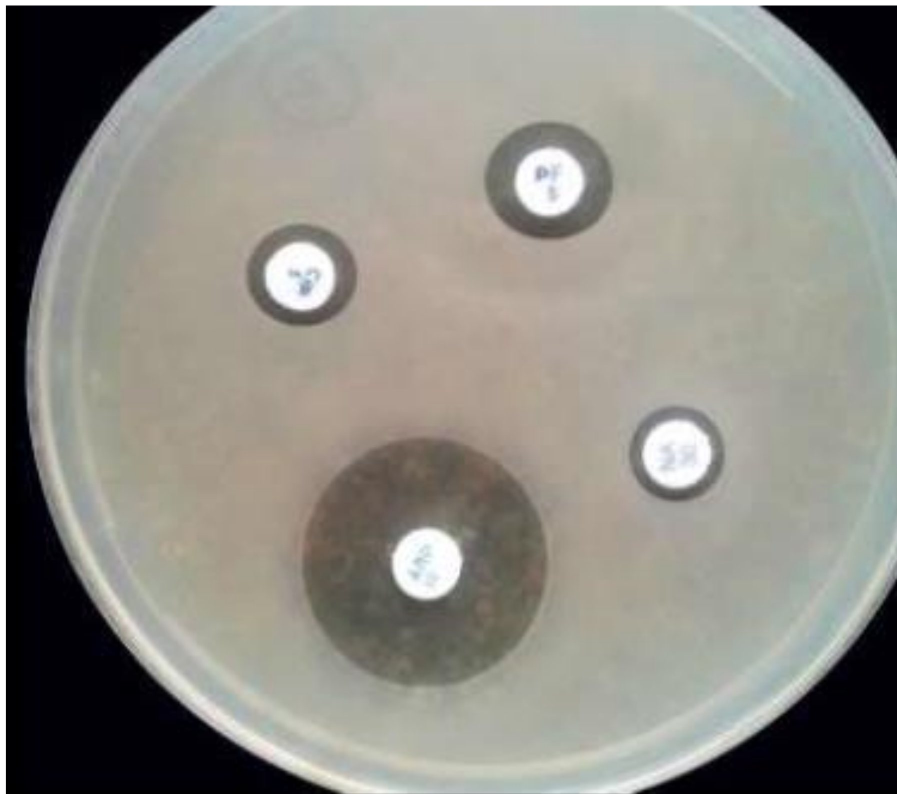


Mueller Hinton Agar – AST by Kirby Bauer method

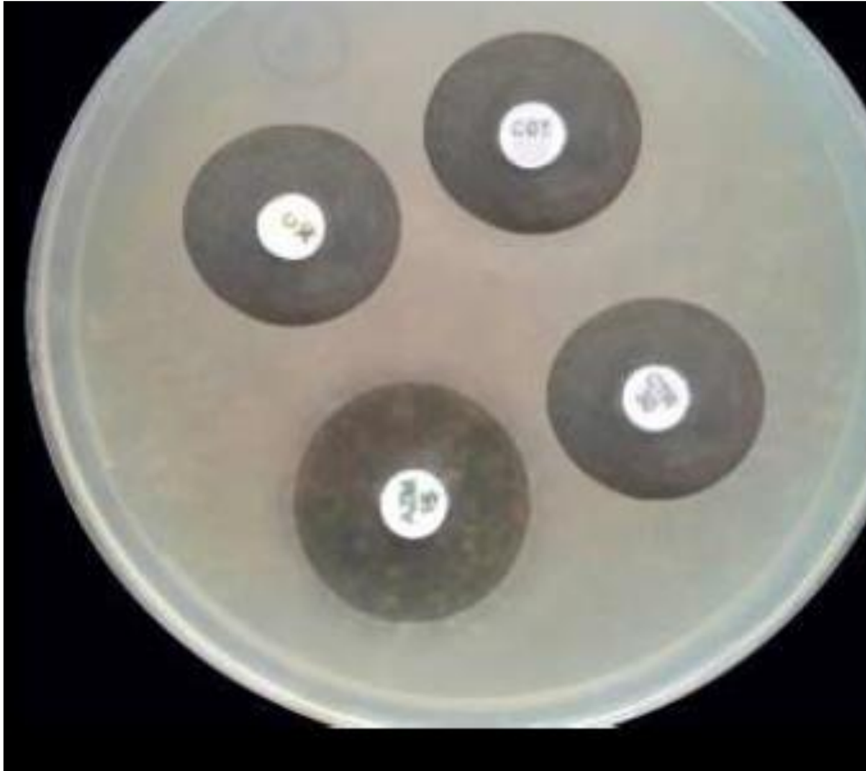


Salmonella Typhi sensitive to ciprofloxacin, Pefloxacin, Nalidixic Acid

***Salmonella* Typhi resistant to ciprofloxacin, Pefloxacin, Nalidixic Acid**

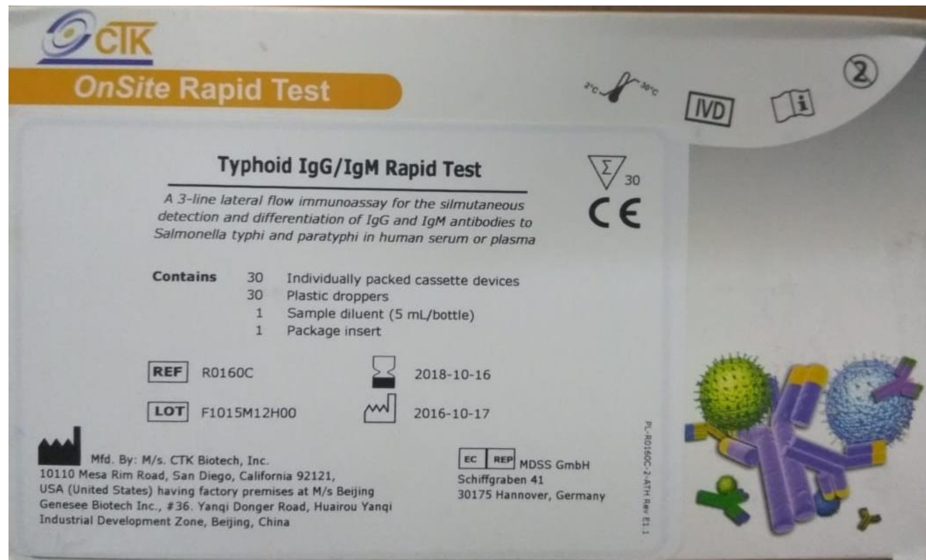


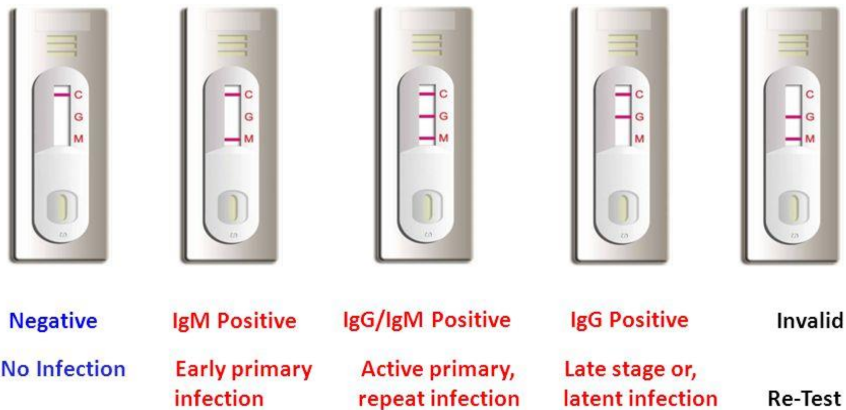
***Salmonella* Typhi sensitive to Chloramphenicol, Cotrimoxazole, Ceftriazome, Azithromycin**



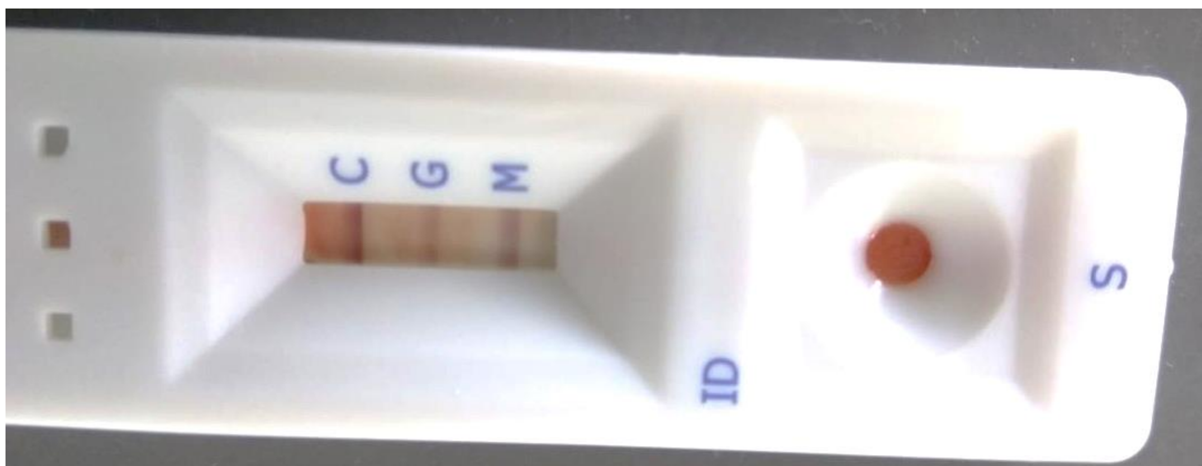
Typhoid IgG / IgM Rapid Test

Test Result Interpretation

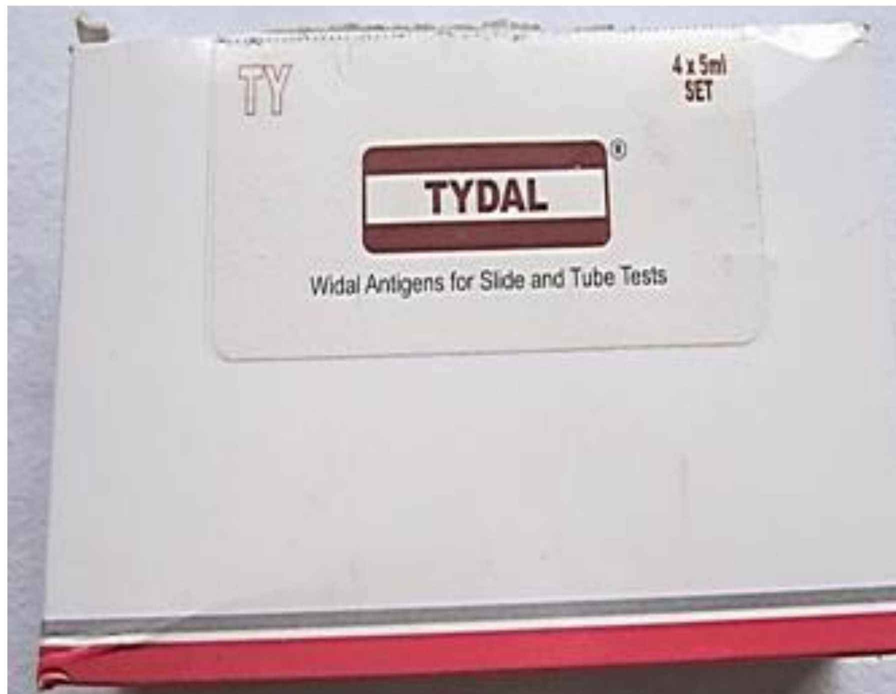




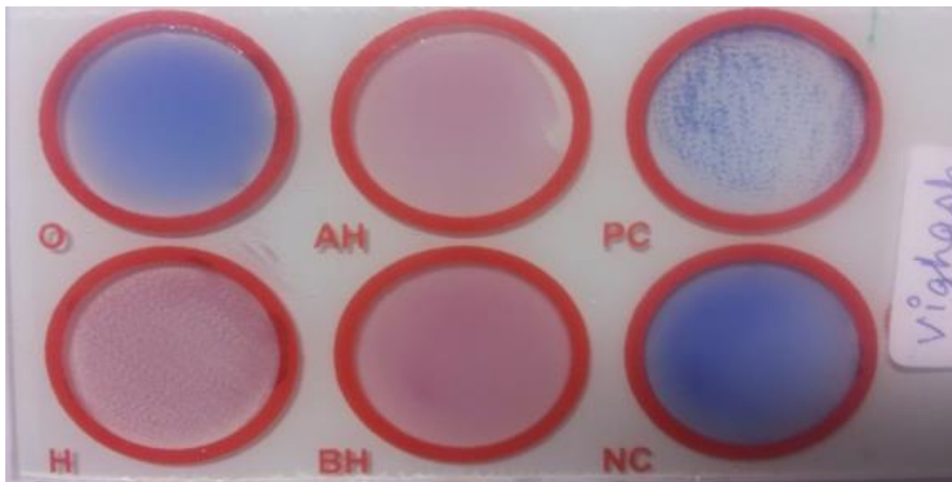
Negative	IgM Positive	IgG/IgM Positive	IgG Positive	Invalid
No Infection	Early primary infection	Active primary, repeat infection	Late stage or, latent infection	Re-Test



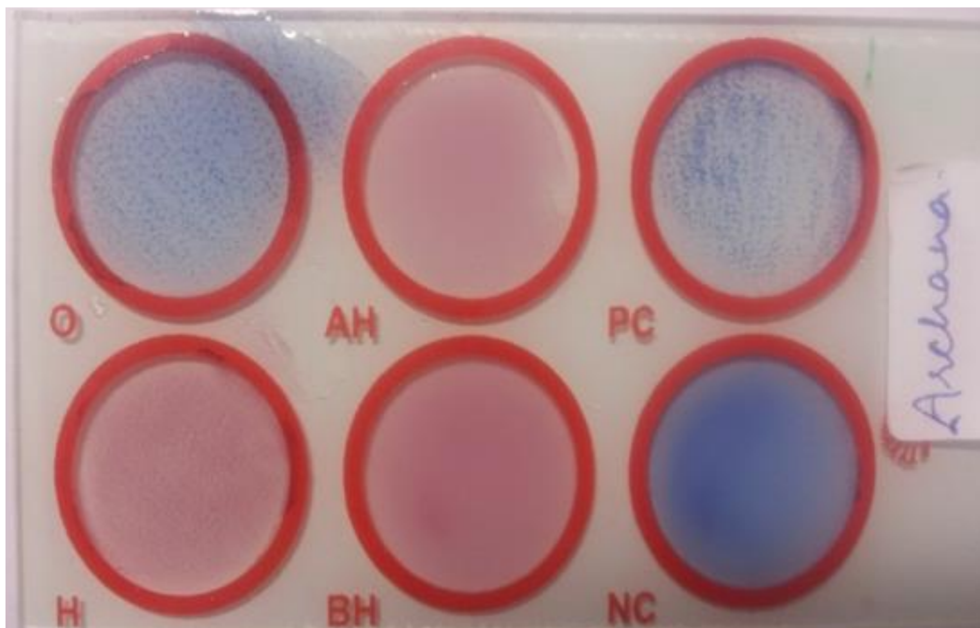
Widal Kit



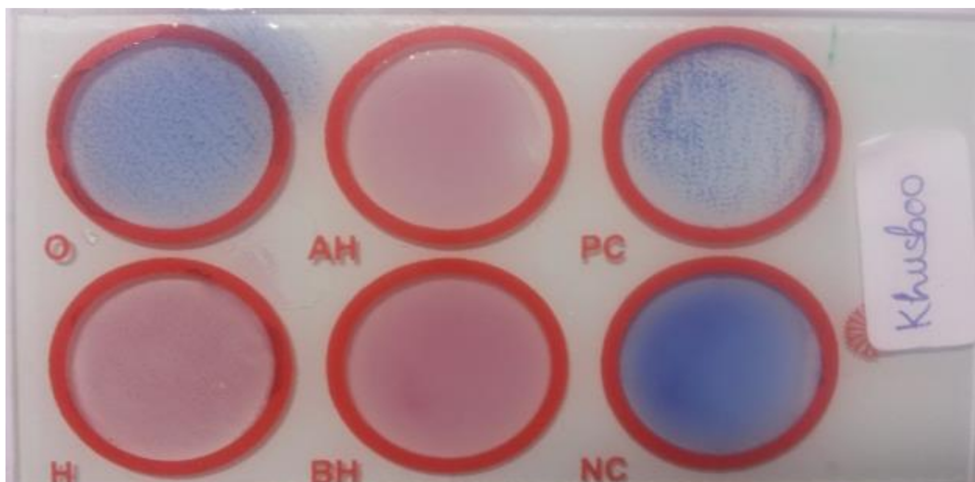
Widal Slide Test

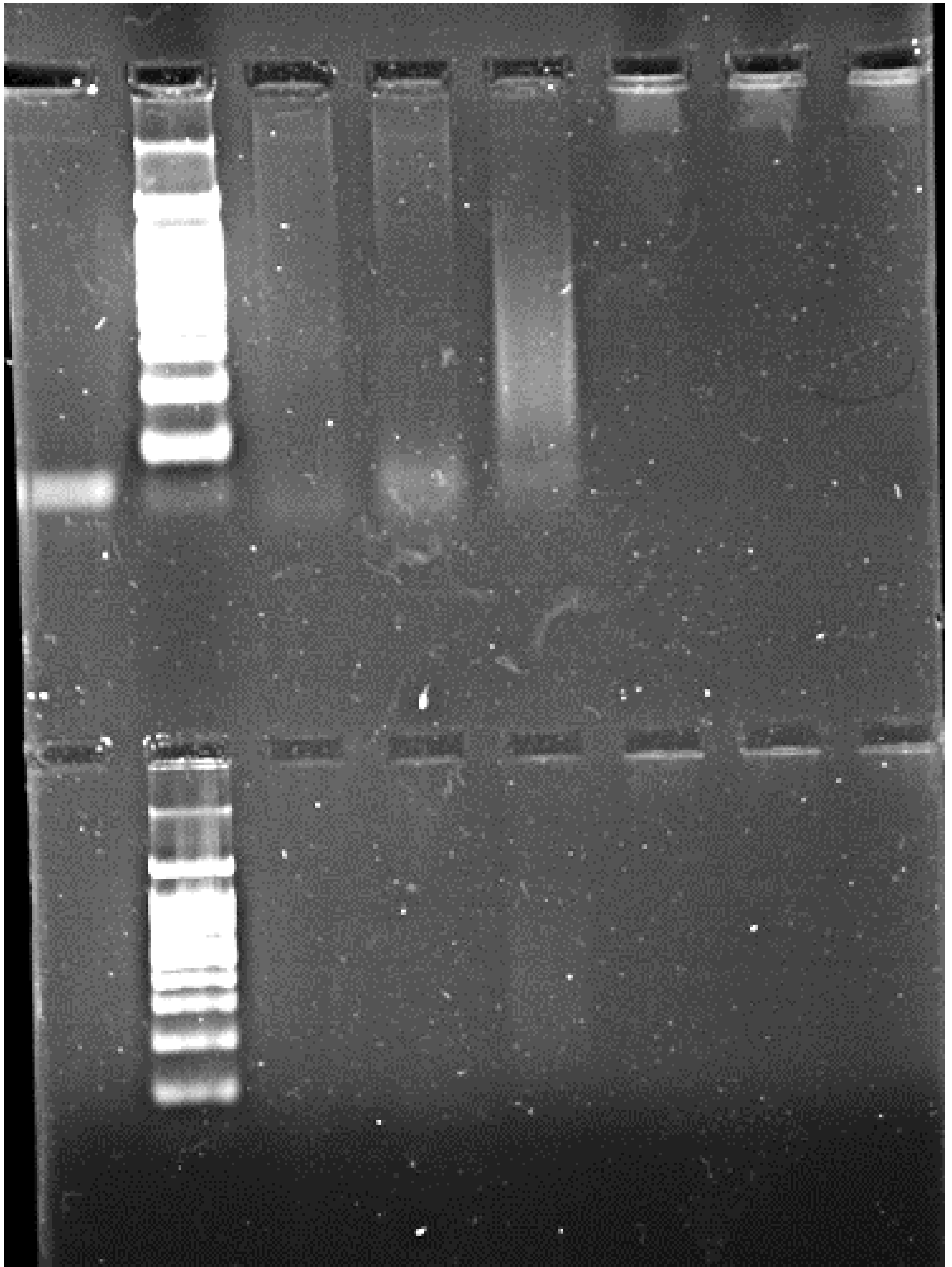


Widal Slide Test Positive

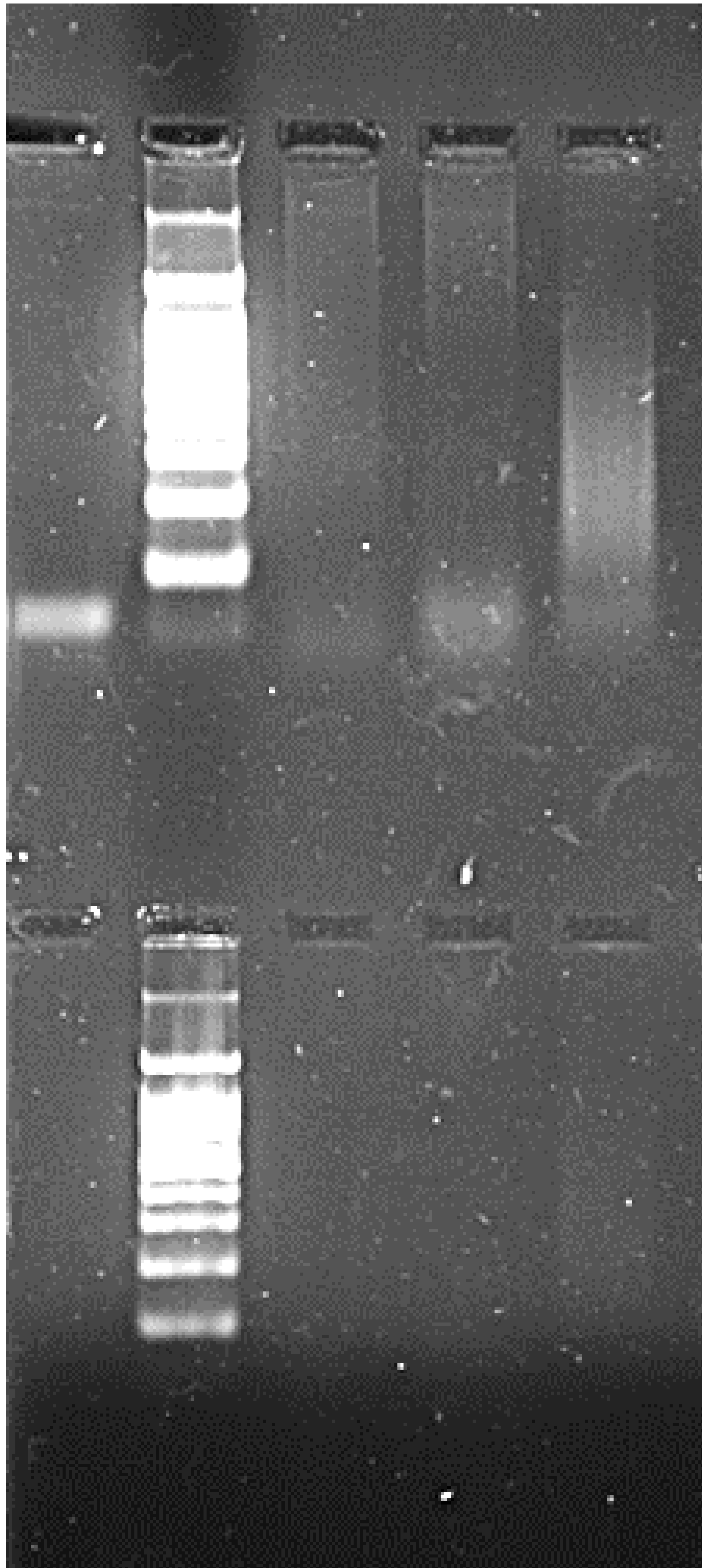


Widal Slide Test Negative





PMQR PCR



PMQR PCR