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RESEARCH ARTICLE

IN- VITRO EVALUATION OF LIPOSOMAL SERODIAGNOSTIC FORMULATION ON BLOOD SAMPLES OF TYPHOID FEVER PATIENT

Abhinav Mishra[2], Brajesh Kumar Tiwari[1], Sidharth Singh[3] Dinesh Kumar Saraf[2], Varsha Sharma[2]

1. Department of Pharmaceutical Sciences, Dr. Harisingh Gour Central University, Sagar(M.P.) India.
2. Department of Zoology, Dr. Harisingh Gour Central University, Sagar(M.P.) India.
3. Department of Botani, Dr. Harisingh Gour Central University, Sagar(M.P.) India.

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Abstract

The current study evaluated the performance of Ty2 antigen and antimouse IgG conjugated liposomes test kit among patients presenting with fever at a primary care facility. Liposomes were prepared by the thin-film hydration technique followed by sonication. Ty2 antigen of typhoid virus, IgG and IgM antibodies was conjugated on the surface of Dye–loaded liposomes. The developed formulations were characterized on the basis of physicochemical parameters, such as morphology, particle size, polydispersity index (PDI), entrapment efficiency, and zeta potential. The *in vitro* release of Rhodamine from liposomal formulation was carried out in phosphate buffered saline (PBS) buffer (pH 7.4). Liposomal formulations were then evaluated for in-process antigen stability and storage stability. Blood samples of typhoid patients were collected from various Govt. Hospitals and typhoid fever was diagnosed based on clinical symptoms and signs, serological testing by the Widal test and blood culture. Liposome tagged with antigen of S. typhi method was developed as an alternative assay for the diagnosis of typhoid. The advantage of using antigen conjugated liposomes as compared to plain antigen is the higher sensitivity of the former.

1. Introduction

Typhoid fever is a distinctive acute systemic febrile infection that grounds to a sizeable morbidity. Human carriers are the only known reservoir of the disease that is spread through contaminated food and water. Early diagnosis and treatment can reduce the duration and complications of typhoid fever. The disease is caused by several serotypes of the Gram negative bacillus Salmonella typhi. Blood culture is generally recognized as the most useful diagnostic test for detecting Salmonella enterica serotype Typhi. However, a single blood culture is estimated to be only 50% to 80% sensitive [1-4], and the delay from specimen collection to diagnosis of 5 to 7 days. Moreover, blood culture is prohibitively expensive in most settings where typhoid fever is endemic. Therefore, diagnosis and treatment of typhoid fever in endemic and resource-constrained settings is commonly done on the basis of clinical presentation or a positive Widal test, which has poor specificity [5-8]. There is a compelling need to develop more accurate, reliable user-friendly rapid diagnostic assays for early detection typhoid fever.

To accomplish the unmet need for a reliable serological assay for typhoid fever, we have developed a novel liposomes based diagnostic kit. The assay or the diagnosis of the infection may be equally useful because of being cost effective, good sensitivity or specificity, as well as provide reproducibility when evaluated by different groups. The assay format employed fulfils all the different special requirements for use in resource-poor clinical settings. The assay is based on the reaction of specific antibodies in the sample with antigen or anti-antibody conjugated on the liposomes. The strategy is based on ligand-receptor interaction where the antibody in the patient's serum may interact with one or more antigens conjugated on liposomes and provides a more sensitive assay. In this study we

investigated its performance characteristics in a laboratory-based study of specimens collected in India. Serum samples collected from patients with clinical suspicion of typhoid fever were tested, and results were compared with those of the Widal test.

2. Material and method

2.1 Material

The antigen Ty2 of typhoid was obtained as a generous gift sample from Bharat biotech , Antibodies IgG and IgM was reaised in serum institute of india Puna, Phosphati-dylethanolamine (PE), cholesterol (CHO), rhodamine and all reagents used were obtained from Sigma (St. Louis, Missouri, USA). Phosphatidylcholine (PC), chloroform, methanol, and glutaraldehyde were purchased from Himedia (Mumbai, India). Blood samples were collected from patients specially from laboratory and Govt. Hospitals.

2.2 Preparation of liposomes (UL)

Liposomes were prepared by the thin-film hydration technique followed by sonication for size reduction, as described elsewhere [9], with slight modifications. Briefly, a mixture containing 75 mg of phosphatidylcholine, 35 mg of phosphatidylethanolamine (PE), and 40 mg of cholesterol were dissolved in a 2-mL chloroform-methanol solution (6:1; v/v) and then dried to a thin film, using a rotary evaporator at 45°C. Subsequently, the film was hydrated in 5 mL of phosphate buffer (pH 7.4), containing Rhodamine (6.5 mg dye/mL) at 25°C for 30 minutes, followed by vigorous sonication (Soniweld; Imeco Ultrasonics, Mumbai, India; 40% pulsar ratio of a 950-W ultrasonic sonicator) for 2 minutes.

2.3 Preparation of Ty2 antigen and antimouse antibody complexed liposomes

The recombinant protein Ty2 antigen of typhoid virus and antimouse IgG antibody was separately complexed onto the surface of different liposomes by the method described by Tiwari et al. (2011) and (Nakano et al., 2001) [10-11] with minor modifications. Briefly, 0.6 mL of 2.5% (w/v) glutaraldehyde solution was, dropwise, added to 1 mL of a liposome suspension with gentle stirring (3,000 rpm; Remi, Mumbai, India) for 1 hour at 4°C. Excess of glutaraldehyde solution was removed by dialysis [molecular-weight cut-off (MWCO), 25 KDa; Sigma] in phosphate buffer (pH 7.4) at 4°C. Further, 0.2 mL of a 1-mg Ty2 antigen solution was added to the dialyzed liposomal formulation under gentle stirring (100 rpm; Remi) at 4°C. One hour later, 0.2 mL of a 3-M glycine-NaOH solution (pH 7.2) was added to block excess aldehyde groups on liposome surfaces and incubated for 6 hours at 4°C (Scheme 1). The unbound antigen was removed by gel filtration using sepahadex-100 mini column [12]. Similar method was followed for antimouse IgG/IgM antibody coupling to liposomes.

2.3 Characterization

The liposomal formulations were characterized for their shape and morphology by transmission electron microscopy (TEM) (JEM-200 CX; Jeol, Tokyo, Japan). Samples for microscopy were prepared by the conventional negative staining method, using 0.2% phosphotungstic acid. The grid was held horizontally to allow the molecular aggregates to settle and then tilted to 45◦ for a while to drain the excess fluid. Afterward, drop of phosphotungstic acid (pH 4) was added to the grid to give a negative stain. The grid was then kept aside for 20 s before removing excess stain as above. Specimens were air-dried before and examined using transmission electron microscopy (Philips Morgagni, Netherlands). Vesicle size, size distribution, and zeta potential were determined by a Malvern zetasizer (Nano ZS 90; Malvern Instruments, Malvern, UK) at 25ºC by diluting the liposomal dispersion to the appropriate volume with phosphate-buffered saline (PBS) (pH 7.4).

2.4 Entrapment efficiency

The unentrapped dye present in the prepared liposomal formulation was separated by Sephadex G-75 minicolumn, using the centrifugation technique [13]. The method was repeated thrice with a fresh syringe packed with gel each time. The vesicular fraction obtained after the separation was allowed to disrupt with a minimum amount of Triton X-100 (0.5%; w/v). The liberated dye was estimated by flourimetric estimation and the percent vesicular fraction of dye entrapment was determined.

2.5 In vitro release

The *in vitro* release of Rhodamine from liposomal formulation was carried out in phosphate buffered saline (PBS) buffer (pH 7.4). Vials containing 40 mg of liposomes, 5 mL of PBS (pH 7.4) and Tween-80 (0.02%, w/v) to reduce the absorption of the released protein as well as to prevent the particle aggregation and to improve their wettability, were incubated at 37°C in constant shaking mixer. At appropriate intervals 1.0 mL of release medium was collected by centrifugation (22,000*g* for 20 min) and replaced by 1.0 mL of fresh PBS (pH 7.4). The amount of released rhodamine was estimated by flourimetric estimation.

2.6 Storage stability

In present work, stability studies of formulations were carried out after storing the Ty2 antigen and antimouse IgG antibody conjugated formulations of liposomes, at 4 ± 1 and 28 ± 1 ˚C for 28 days in a screw-capped amber coloured glass bottle. The stored formulations were evaluated for change in size and percent residual rhodamine content after every 7 days. The size was measured by Malvern zetasizer and the percent residue rhodamine was determined, as reported for efficiency. The initial rhodamine content was considered as 100%.

3. In vitro Study

3.1 Patients and specimens

The blood samples of Typhoid patients were collected from Govt. Hospital District Sagar, Govt. District Hospital Damoh, Govt TB hospital Sagar, Jabalpur Medical collages, Chitanya Hospital, Hemant pathology and Dixit Pathology of Sagar Distt, after the consultation and advice of physician. With each sample name of patient address, age, period from which he/she suffered from fever, economic status were noted. Eligible patients were enrolled on the basis of continuous high-grade fever, toxic appearance and the presence of constitutional symptoms. A total of 205 patients (mean age 21 years, range 7-55) were enrolled in the study. The mean duration of illness at first consultation was 6 days (range 4-15). Clinical specimens were collected on the day of hospital admission or during the first few days thereafter. In addition one or two follow-up samples were collected from 138 of these patients who were diagnosed with typhoid fever. The follow-up samples were collected approximately 1 and 2 weeks after first presentation. The duration of hospitalization was less than 1 week for most patients, and in these cases the collection of follow-up samples was attempted during home visits. The collection of follow-up samples was not attempted for patients with a diagnosis other than typhoid fever. Informed consent was obtained from all patients or from their parents/guardians. Typhoid fever was diagnosed based on clinical symptoms and signs, serological testing in the Widal test and blood culture.

3.2 Culture

Blood culture was performed by inoculation of 5ml freshly collected blood in 15 ml ox bile broth and incubation for 24h at 37◦C. A 1 ml culture sample was then plated on Salmonella Shigella agar. After incubation for 24 h at 37 ◦C, colonies were examined by Gram staining and tested biochemically and serologically to identify *Salmonella*-positive cultures. Culture medium containing ox bile was used as this supplement is reported to improve the sensitivity of manual culture systems [14].

3.3 Widal test

The Widal O antigen test was performed by mixing one drop of the O antigen suspension (Murex Biotech Ltd, Dartford, UK) with one drop of two-fold serial dilutions (1:20—1:1280) of the serum sample and incubating for 4 h at 50 ˚C. A titre ≥1:320 was considered consistent with typhoid fever. The assay was performed according to routine laboratory procedures in test tubes, as this was found more convenient than making dilutions on a plate.

3.4 Slide Drop Test

The Widal test has been used very extensively in the serodiagnosis of typhoid fever and, in developing countries, remains the only practical test available.

Serum samples for the test were collected from patients. In two separate a 96 well plates, the first plate was tagged with Dye loaded liposomes conjugated with IgM (100 µl), second plate was tagged with Dye loaded liposomes conjugated with anti-IgG antibody (100 µl). To each well 200 µl of serum samples of various human volunteers was added. After all the tubes have been treated with specific antigen suspensions, the 96 well plate is placed in a thermostatically controlled water bath maintained at 37^{o}C for overnight incubation.

The result of slide drop test performed by our group was compared with the sample widal test of Typhoid.(Fig.5)

4. Results and Discussion

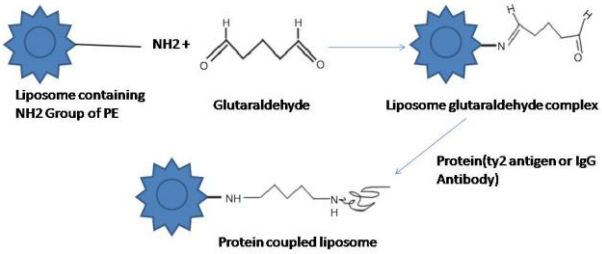
4.1 Preparation and characterization of liposomal vaccine formulations

The liposomes were prepared by reverse phase evaporation method since it is a simple and convenient method to prepare liposomes and associated with good entrapment efficiency.

4.2 Preparation of Ty2 antigen and antimouse IgG/IgM antibody complexed liposomes

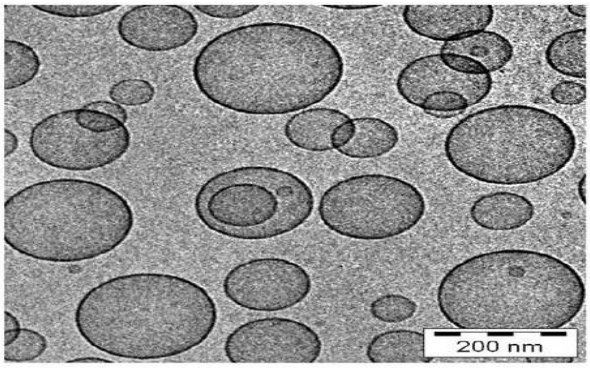
The liposomal formulation was subsequently separately conjugated with Ty2 antigen protein and antimouse IgG antibody to enhance specificity. The complexation of Ty2 antigen and antimouse IgG antibody on to the surface of liposomes was done by glutaraldehyde coupling method. The method of glutraldehyde coupling is fairly good method to facilitate conjugation of protein on the surface of liposomes and has been used many times conjugation of proteins on the surface [18-19] for protein coupling on liposome surfaces and results in a fairly high coupling efficiency.

Scheme1, Preparation of protein coupled liposomes

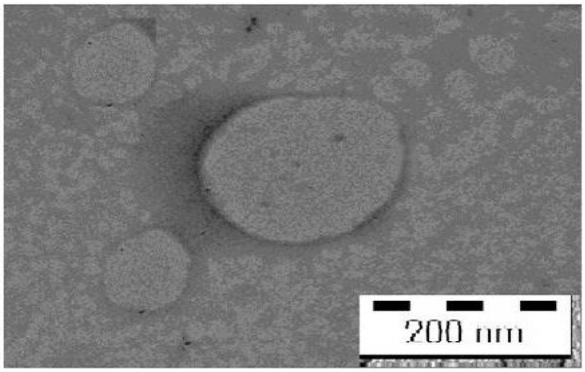


Schem 1. Preparation of Protein Coupled Liposome

Figure 1, TEM Images for Plain and Protyein coupled liposomes



A. Plain liposomes



B. Protein coupled liposomes

Figure 2, Cumulative in vitro dye released

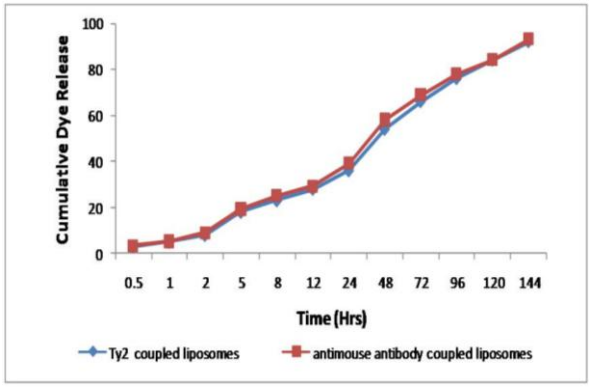


Figure 3, Average vesicle size of various liposomal formulations at 4 ± 1°C and 28 ± 1°C. Values represent mean ± SD (*n* = 4.

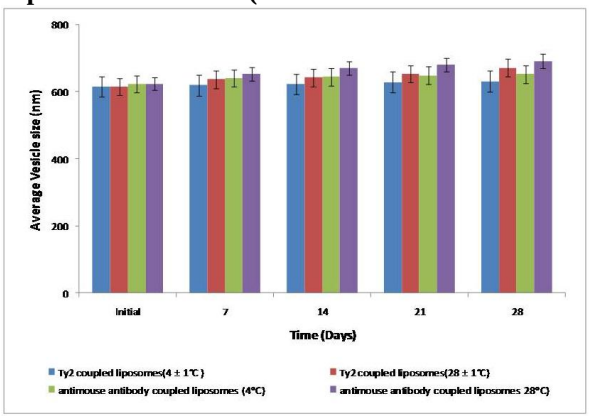


Figure 4. Percent residual dye content from various liposomal formulations at 4 ± 1°C and 28 ± 1°C. Values represent mean ± SD (*n* = 4.

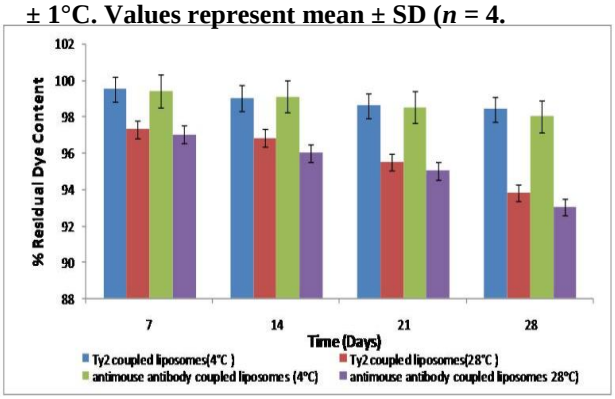
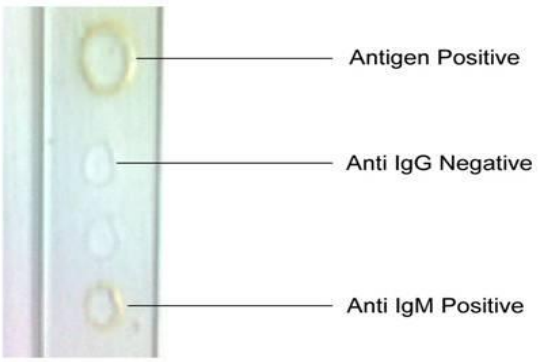
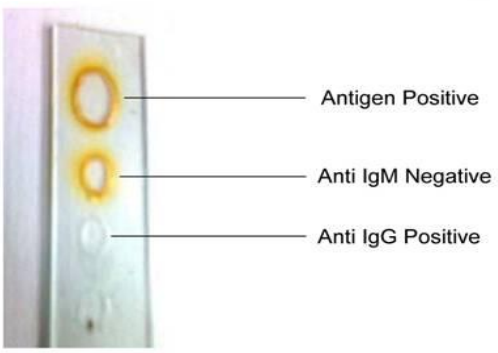


Figure 5. Slide Drop Test.

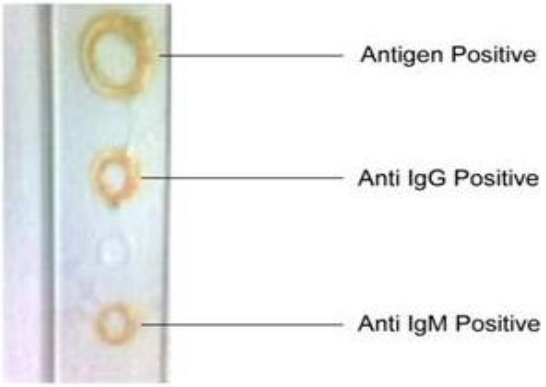


a. Typhoid Positive Test on Slide



b. Slide Showing Positive but other Antigen Negative

c. All three slides showing the Typhoid results of adhered anti-IgM, anti IgG and antigen on liposome with the plasma.



4.3 Characterization

The shape and surface morphology of the antigen-coupled liposomes were evaluated by photomicrography and TEM (Figure 1). The photomicrographs and TEM images of liposomes clearly depict a spherical shape of Ty2 antigen and antimouse IgG/IgM antibody coupled liposoems (Figure 1).

A very low polydispersity index of less than 0.2 was recorded for all the formulations indicate narrow size distribution of vesicles (Table 1). The encapsulation efficiency was expressed as percentage of rhodamine which was added initially for loading of vesicles. The average vesicle size and entrapment efficiency of Ty2 antigen coupled liposomes was found to be 615±12nm and 59.2±1.2%. Similarly average vesicle size and entrapment efficiency of Ty2 antigen coupled liposomes was found to be 624±15nm and 59.8±1.4%. Zeta potential of Ty2 antigen and antimouse antibody-coupled optimized liposomes was found to be -35.12± 0.68 and -37.22± 0.52mV respectively.

4.5 In vitro release

In vitro dye release study was conducted with uncoupled as well as Ty2 antigen coupled liposomes. The rhodamine release pattern from various liposomes is shown in Figure 2. In case of Ty2 antigen complexed liposomal formulation rhodamine release was found to be 92.23±2.10% at the end of 144 h where as in the case of antimouse antibody conjugated liposomal formulations the release was found to be 93.12 ±1.72% (Figure 2). The release patterns were found to be typically biphasic with an initial burst release, which may be attributed to the release of surface associated dye, followed by a slower release phase which may be accounted for slow diffusion of entrapped dye from inner aqueous layers into the release medium [20-21]

4.6 Stability study

Stability studies of the liposomal formulations were carried out in order to assess the ability of the developed formulation system to withstand environmental stress as well as to retain the stability of the entrapped antigen. The storage stability of formulations, based on liposomes, is of great concern, as it is the major restraint in the development of clinically acceptable marketed preparations. The selected dye loaded formulations, were stored in tightly closed amber-colored bottles at 4 ± 1°C and at the elevated temperature of 28 ± 1°C for 28 days and were analyzed weekly for antigen integrity. Only slight changes in mean vesicle diameter were observed on storage for liposomes at 4 ± 1°C and a slight increase on storage at 28 ± 1°C, suggesting that the developed coupled lipid vesicles were a quite stable delivery system (Figure 3). This stability may be attributed to the presence of charge, which, through electrostatic repulsion, causes the retardation of fusion and aggregation.

By considering the initial dye content as 100%, it was observed that after 28 days at 28 ± 1°C and 4 ± 1°C, 93.8 ± 0.15% and 98.41 ± 0.32% dye remained encapsulated in Ty2 antigen complexed liposomal vesicles respectively. Similarly rhodamine after 28 days at 28 ± 1°C and 4 ± 1°C, 92.81 ± 0.32% and 98.07 ± 0.19% dye remained encapsulated in antimouse antibody complexed liposomal vesicles respectively (Figure 4). Thus, from the results obtained, it could be concluded that for better stability, the formulation should be stored only at refrigerated conditions.

***4.7 In vitro* Study**

The typhoid fever requires prompt medical attention as it may lead to sever health consequences and may be fatal. In South Asia the disease is endemic and a major cause of morbidity and mortality. Each year many thousand deaths are accounted for this life threatening disease around the World over. In ample number of cases clinical symptoms and sign of typhoid fever are non specific and may be confusing as Green 2003 states the need for a simple, rapid and affordable point-of-care test for typhoid fever is imperative. Widal test, a rapid serological test has certain limitations like the problem in antigen standardization, false positively and sensitivity etc.

Liposome tagged with antigen of S. typhi method was developed as an alternative assay for the diagnosis of typhoid. The advantage of using antigen conjugated liposomes as compared to plain antigen is the higher sensitivity of the former. The antigen conjugated liposomes can detect the disease even in

elderly patients where the number of antibodies produced may be markedly less. When color is produced by incubation of serum with Dye loaded liposomes conjugated with IgG/IgM, this indicates early infection. Moreover, when color is produced by incubation of serum with Dye loaded liposomes conjugated with anti-IgG antibody, this indicates 40 days of infection.

Standard serum of healthy non-infected person was taken as control (100% transmission) with anti IgM, anti IgG and liposome tagged with Ty2 antigen. The 100% transmission for the entire test was fixed with this standard serum. Since a standard volume of anti IgM, IgG tagged liposome and serum had been reacted, the percent transmission of the reacted well is the indication of positive result with the intensity of the colour, Blood samples of the little over 210 patients had been collected from different hospitals who were clinically diagnosed as typhoid and confirmed by Widal test.

Table-1:

Formulation	Average vesicle size	Entrapment efficiency	Zeta potential (mV)	PDI
Uncoupled liposomes	615±12nm	59.2±1.2%	-35.12± 0.68	0.172±0.025
Ty21a coupled liposomes	624±15nm	59.8±1.4%	-37.22± 0.52	0.161±0.021

Characterization of various liposomal formulations

Table 2 : Samples tested of various age group patients by slide drop test and compared with widal test results

S. No.	Specimen Used	No. of Sera	Slide Drop Test		Widal Test	
			+ve	-ve	+ve	-ve
1.	Children (10-15 yrs)	21	18	03	17	04
2.	Young (17-25 yrs)	18	16	02	15	03
3.	Adult (27-40 yrs)	37	33	04	31	06
4.	Old (45-60 yrs)	29	22	07	22	07
5.	Female (22-35 yrs)	33	22	11	22	11

A study of table -2 shows that in all 112 patients had been confirmly found to have typhoid by slide drop test while 107 did show Widal positive test (Table 2). 26 patients were declared typhoid negative by slide drop test as opposed to 31 patients by Widal test. The negative results were again confirmed by using other serodiagnostic techniques. These result show slide drop test better than Widal test. The sensitivity of the present test for positivity although ranges between 84.2 - 88.9 %, but it is higher in samples collected from Govt. hospitals. This can be interpreted with the patient's community and lower economic status. In Government hospitals patients report after having been suffered for long time hence a strong sensitivity was observed in them. The sensitivity for negativity falls in the range of 77 – 87%. The slide drop test for diagnosis of typhoid in Widal plus culture positive cases is 90%, in Ty2 vaccinated cases is 87%, a little more 88.7% in Ty2 non vaccinated persons and it is highest in normal healthy persons. Thus slide drop test doses show high sensitivity for positive and negative results. This test has also been found to be effective in detecting positive and negative results in children of 10-15 years age group.

5. Conclusion

The current study evaluated the performance of Ty2 antigen and antimouse IgM conjugated liposomes test kit among patients presenting with fever at a primary care facility. The tests were conducted in India had reported good sensitivity and positive predictive value of test among patients identified through an active community surveillance for fever, when evaluated among febrile patients with known etiology

of culture confirmed typhoid. The result can yield useful information to the clinician and provide clear diagnoses for febrile patients. However further studies are needed to evaluate the clinical utility of the liposomes based diagnostic kit in different clinical settings and under different epidemiological conditions.

6. Disclosure

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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