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Research Article

IMMUNOEVALUATION OF TYPHOID ANTIGEN BY USING POTATO STARCH NATURAL BIODEGRADABLE POLYMER AS A SINGLE CONTACT USED AS AN ADJUVANT

L. NIRMALA*, VIJAYASHREENAYAK[1], DEECARAMAN[2]

Department of Industrial Biotechnology, Dr. MGR Educational & Research Institute Maduravoyal Chennai 600095, Tamil Nadu
Email: snwiar@yahoo.co.in

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ABSTRACT

The therapy of single administration of typhoid antigen based on potatostarch using as an adjuvant, In our research work, we have selected starch polymer as a model of immunomodulatory effect of vaccine of typhoid antigens as an single contact. According to WHO, typhoid is systemic infectious disease caused by genus *S.typhi* has been estimated that the typhoid fever endemicity among large populations and global emergence of multidrug resistant strains impose greater urgency on the evaluation of existing and new vaccines to prevent typhoid fever. Recently available recombinant vaccine was seems to be side effect and cost effective. The starch polymer in the form of microsphere was preferred in order to replace the alum to elicit sustained immune response because alum induces local granuloma and hypersensitivity reaction to some individual. We have employed emulsion crosslinking technique by using 0.5% ml glutaraldehyde as a crosslinking agent. The particle size was analyzed as 43.34 µm; the loading capacity was about 44.76% and the loading efficiency as about 75.17%, while the morphology was unchanged in the stability studies. *In vitro* studies were analyzed by SEM and compatibility studies were performed by IR α F.T spectroscopic analysis. The percentage of antigenically active typhoid antigens was found to be 91.5% by ELISA after entrapment with 8% of starch and 0.5% of stabilizing agent. Immunoprecipitation assay exhibited that the amount of antigen in the sample was found to be 51 µg and at the dilution of 1:50 showed agglutination factor (with the titer value of 50).

Keywords: Potato starch, Biodegradability, Immunomodulatory, Single contact, Antibody response

INTRODUCTION

The world spread use of vaccines over the last few decades has resulted in a reduction in the incidence of many infectious diseases in developed countries. Nevertheless, there are still significant challenges for vaccine developments including the need to make improvement in existing vaccine by making them safer, more immunogenic and to extend vaccine coverage in certain populations particularly in the developed world[1]. One of the most important issues in vaccinology is the need for new adjuvant in vaccine delivery system[2]. Most of the vaccines currently in development are based on purified subunits, recombinant molecule, synthetic peptides or nucleic acids which are often poorly immunogenic, expensive and produce adverse effect[3]. It is clear that new generation of vaccines will require better adjuvant delivery systems to induce optimal immune response.

Typhoid (cloudy) fever is a systemic infection, caused mainly by S. *typhi* found only in man, children. It is characterized by a continuous fever for 3-4 weeks, relative bradycardia, with involvement of lymphoid tissue and considerable constitutional symptoms[4]. Antibiotics resistance, particularly emergence of multidrug resistant (MDR) strains among salmonellae is also a rising concern and has recently been linked to antibiotic use in livestock[5]. Many S. *typhi* strains contain plasmids encoding resistance chloramphenicol, ampicillin and co-trimoxazole, the antibiotics that have been long used to treat enteric fever. In addition, resistance to ciprofloxacin also called nalidixic acid resistant S.*typhi* strain either chromosomally or plasmid encoded, has been observed in Asia[6]. The chemotherapy of typhoid is complicated by the need of multidrug regiments that need to be administered over long periods, poor patient compliance, toxicity, tachycardia, bradycardia, myalgia, hyperplasia, necrosis, enterocolitis, peyer's patches[7]. Extensive progressive efforts have been made to develop various microspheres as potential carriers to increase potentials out comes. the system under discussion employ either biodegradable polymer or system requiring removal after use and can release the drug either by membrane or matrix- controlled diffusion. Recent trends in potential carrier delivery have seen microencapsulation of pharmaceutical substances in biodegradable polymers as an emerging technology. Currently, biodegradable polymer representing class of ubiquitous material and are being shown in the macromolecules by the fabrication vaccine system[8]. Biodegradable polymer in the form of microsphere has shown the ideal perquisite for ideal microsphere carrier.

The adjuvant effect was achieved as a consequence of the encapsulation of antigens in microparticles has been known for many years. The enhanced immunogenicity of particulates antigens is unsurprising, since pathogens are particulates of similar dimensions and the immune system has evolved to deal with these. Particulates delivery system presents multiple copies of antigens to the immune system and promote trapping and retention of antigen in local lymph node moreover particles are taken up by macrophages and dendrite cells leading to enhanced antigen penetration and the release of cytokines to promote the induction of an immune response[9]. Sustaining response for a longer duration including local mucosal immune response, generating antibody with increased avidity and neutralization capacity eliciting cytotoxic T lymphocyte (CTLs) enhances immune response.

This paper describes the use of microparticles as a potential adjuvant for Typhoid antigen. The immune responses of the antigen loaded microparticles were compared with the conventional typhoid vaccine. Starch can be modified through physical, chemical or enzymatic processes[10]. Cross linked starches have long been used as food additives because of their safety and low cost. Very low levels are used and these are approved by the FDA (CFR 172.892). Different covalent cross-linking agents have been used.[11]

MATERIALS AND METHODS

Sample of Typhoid antigen obtained from Serum Institute of India Guindy (Chennai), Organic solvent of AR. grade, Tween-80 (hi-media), Glutaraldehyde (spectrochemicals).

Preparation of potato starch

Starch (chemical formula (C6H10O5) n,) is a mixture of *amylose* and *amylopectin*. The former consists of long, unbranched chains of D-glucose residues connected by (α1→4) linkages. Potato was washed efficiently to remove dirt, fungi, and rotten spots. Rasping was performed to release the tuber cells and starch. The potato juice is generally rich in sugar and protein. So when opening the cells the juice is instantly exposed to air and reacts with the oxygen, forming coloured components, which may adhere to the starch. This can be reduced by adding sufficient amount of sulpurdioxide gas to maintain the juice and pulp light yellow. The sample was centrifuged at 5,000rpm and supernatant sample was collected, dried, and stored.

Preparation and characterization of microparticles

Typhoid vaccine encapsulated Potato starch microparticles were prepared by microencapsulation techniques (Emulsion crosslinking) Based upon the result obtained by changing various parameters such as effect of polymer concentration and effect of crosslinking agent concentrations various batches were prepared, to standardize an ideal batch. Starch solution (1% to 10% / ml) was prepared in a 250 ml beaker by continuous stirring for one hour at 5000 rpm. An emulsion was prepared by mixing the vegetable oil and toluene with two drops of Tween-80. To the gel 1ml of Typhoid antigen and 1 ml of 0.5% Glutaraldehyde was added and stirring is continued for one hour. Add 2 ml of gel containing typhoid antigen in to the emulsion with continuous stirring. At the end the precipitates were centrifuged, washed with various organic solvents' A white powder were obtained (microspheres) many batches were formulated, pooled and stored in a refrigerator at 4^0C.

The morphology and size distribution of dried microspheres was evaluated by Scanning Electron Microscopy and the size of the microparticles was determined by optical microscope using calibrated scale. The magnitude of loading Typhoid antigen in microparticles was performed by mixing with phosphate buffer saline (pH7.4). Under shaking at room temperature and kept for 3hour. The suspension was centrifuged at 4000 rpm for 15 min to remove free antigen. This process was analyzed by Lowry' method.[12]

$$\text{Loading capacity \%} = \frac{\text{Total amount of antigen} - \text{Free antigen}}{\text{Wt of microparticles}} \text{x } 100$$

$$\text{Loading eficiency \%} = \frac{\text{Total amount of antigen} - \text{Free antigen}}{\text{Total amount of antigen}} \text{x } 100$$

The stability of the formulation Typhoid antigen encapsulated starch microparticles and unloaded starch microparticles were determined over period of week. Both antigens loaded and unloaded micro particles were kept at 4^0C. At predetermined time intervals the samples were taken at 0, 1, 2, 3, 4, 5, 6, and 7 days. The morphology was determined by light microscope and the size was determined by using stage-eyepiece micrometer.

The swelling ratio of the starch microparticles was determined as the percentage of particle size change after incubation in the phosphate buffer (pH 7.4). Wt of microparticles with similar diameter were chosen. Diameter of the beads was measured before and after incubating in phosphate buffer (pH 7.4) for 12hours under optical microscope. The swelling percent was calculated as follow:

$$\text{\% Swelling ratio} = \frac{\text{Diameter in swollen state} - \text{Diameter in dry state}}{\text{Diameter in dry state}} \text{x } 100$$

Compatibility study was performed by Infrared Spectroscopy and the integrity of antigen was studied by SDS PAGE and ELISA

***In vitro* release profile**

The 200 mg of the Typhoid vaccines micro particles were taken in a 250ml conical flask. To this 50ml of phosphate buffer (pH7.4) was added. The flask was kept in the shake cum incubator. The shaker was adjusted to 80 horizontal strokes per min at 37 ^{0}C. From this 1ml of solution was taken in test tube and fresh phosphate buffer was added immediately in the flask. This was repeated at various pre determined time intervals 2hrs, 4hrs, 6hrs, 8hrs, 10hrs, 12hrs, 14hrs, 16rsh, 18hr and 20hrs. The collected samples were centrifuged and supernatant solution analyzed by Bronsted Lowry's method.

Immunogenicity studies

Immunoprecipitation test

The simplest precipitation reaction was carried out in solution in test tubes for a quantitation estimation of the amount of antibody in a serum, was carried out by mixing different dilutions of the antigen to the same volume of the antiserum, incubating the mixture first mixture at 37^0C for about an hour, and then at 4^0C for a longer period. The materials in each tube series were then centrifuged and supernatant was tested for excess antibody or antigen. Then the precipitate was dissolved in NaOH (0.1), and optical density was measured of solution at 280 nm. The amount of antigen was calculated.

Hemagglutination test

Widal test is a serological method used in the diagnosis of enteric fever (Typhoid and Para typhoid fever) is caused by organism belonging from the genus S. *Typhi.* 1ml of sample was taken in to 10 ml of PBS From above 5 ml saline and 0.4 ml 0f sample was taken and dilution was made. (1:12.5 dilutions).8 test tubes were arranged and 0.2 ml was taken in 1 & 2 tubes from the master dilution. 0.2 ml of saline was added in tubes

Bleeding of immunized Wister rats was done by Retro orbital plexus by using a capillary tube. The samples were pooled and serum was separated. The serum sampled was analyzed by ELISA. The potency of typhoid vaccine encapsulated starch microparticles was tested by measuring the specific antibody by immunoprecipitation assay. The Ig titer was found by immunoprecipatation assay with Nephelometric end point.

RESULTS AND DISCUSSION

SEM analysis

Scanning electron microscopy was performed in order to assess the morphology and size of the micropaticles formulation of typhoid antigen encapsulated starch microparticles. The antigen unloaded microparticles was found to be irregular in size and shape (Fig.1) while the antigen loaded starch microparticles are homogenous and shown to be more or less with spherical geometry (Fig.2). By differential analysis it was found that the loading of antigen into the microparticles and the size of the microparticles were greatly influenced by stabilizing agent and the polymer concentration (Table 1 & 2). The loaded particles become smooth surfaced with reduced size compared to unloaded particles (Table 3). The average size of microparticles was found to be 43.34 µm (Table 4).

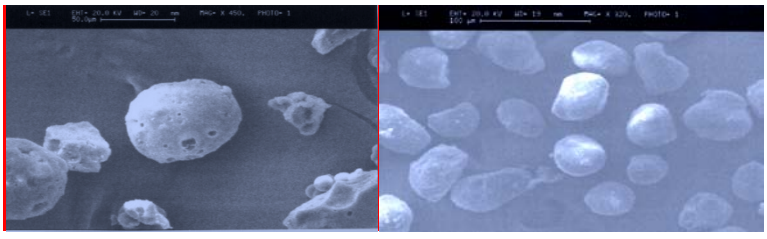


Fig. 1: Antigen unloaded microparticles

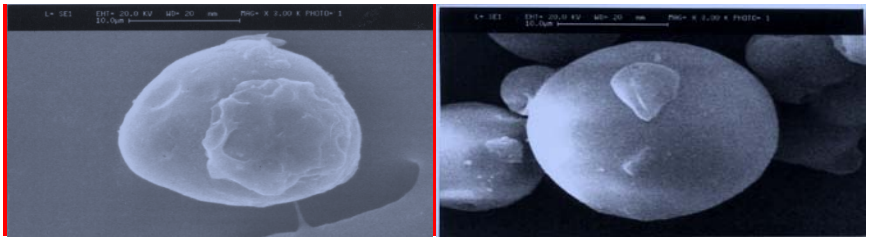


Fig. 2: Antigen loaded micropartlcles

Table 1: The effect of the polymer concentration by using 0.5% of glutaraldehyde as a stabilizing agent

Polymer	Polymer conc.	Stabilizing agent	Conc. of stabilizing agent (%)	Size & Shape
Starch	1%	Glutaraldehyde	0.5	Micro particles are not formed
	2%			Micro particles are not formed
	3%			Micro particles are not formed
	4%			Micro particles are not formed
	5%			Micro particles are not formed
	6%			Micro particles are not formed
	7%			Micro particles are not formed
	8%			Micro particles are formed
	9%			Clumping occurs

Table 2: The effect of the cross linking (stabling agent) concentration

Polymer	Polymer concentration	Stabilizing Agent	Concentration of stabilizing agent	Size & Shape
Starch	0.8	Glutaraldehyde	0.1%	Micro particles are not formed
			0.2%	Micro particles are not formed
			0.3%	Micro particles are formed but unstable
			0.4%	Micro particles are formed but not homogeneous
			0.5%	Smooth particles are formed

Table 3: Effect of loading on microparticle formulation

Characteristic antigen Encapsulated starch micro Particles	Size		Comments
	Before loading	After loading	
Typhoid antigen encapsulated starch micro particles	76 µm	10-50 µm	Size reduced, smooth micro particles are formed
Dummy batch	80 µm	20-45 µm	Size reduced, smooth micro particles are formed.

Table 4: Physical characteristic of prepared micro particles

Batch	Particle Size (µm)	Loading capacity (%)	Loading efficiency (%)	Swelling ratio
Dummy Microparticles	45.21± 0.088	-	-	3.61± 0.14
Antigen loaded Micro particles	43.34 ± 0.094	44.76 ± 041%	75.17 ± 1.56%	3.27 ± 1.24

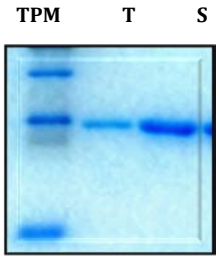


Fig. 3: SDS page gel electrophoresis

The loading capacity was about 44.76% and the loading efficiency as about 75.17 %(Table 4). Regarding stabilities studies, the morphology of typhoid antigen encapsulated starch microparticles were not changed at 4^{0}C and room temperature but not in 50^{0}C. The water uptake of the microparticles after 24h of incubation leads to bursting of the microparticles. Therefore, it is presumed that water penetrates in to the microparticles, and dissolves the stabilizing agent.

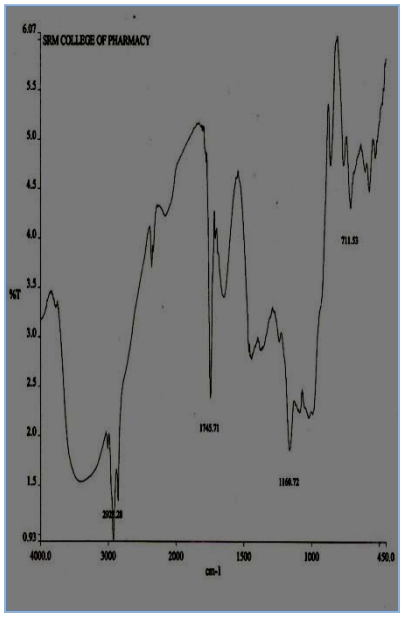
SDS page gel electrophoresis

SDS gel electrophoresis reveals that as the pore size of the microparticles increases the antigen release also enhanced which lead to the bursting effect. The integrity of the entrapped antigen was evaluated by SDS- PAGE and ELISA. The band intensity shows the nature of antigen release from the microparticles and found that there were no damage occurs during microencapsulation (Fig.3). The percentage of antigenically active typhoid antigens was found to be 91.5% by ELISA after entrapment with 8% of starch and 0.5% of stabilizing agent (Glutaraldehyde).

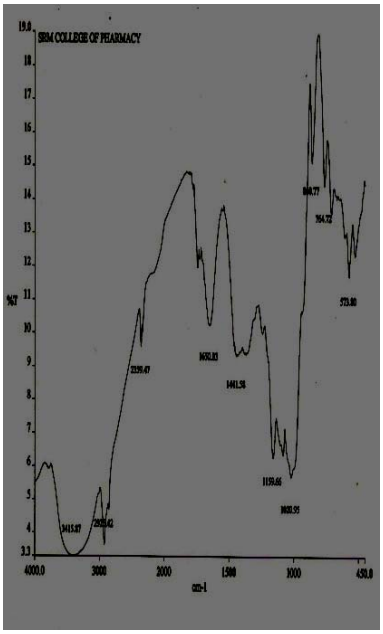
The above picture shows that the TPM – Typhoid marker protein, T test Antigen S- Conventional antigen the band intensity of antigen entrapped potato starch microparticles

Compatibility studies using IR spectrum

The IR spectrum of Typhoid antigen encapsulated starch microparticles, indicates that the antigen loaded microparticles and antigen unloaded with the characteristics peaks observed in 3415 cm-1, 1650 cm-1, 1159 cm-1 (Fig.4 a & b).



Antigen unloaded microparticles



Antigen loaded microparticles

Fig. 4: Graphical representation of ir spectropic analysis

In vitro release studies

It shows the gradual release of entrapped antigen and peak release was observed on (2 to 20 hrs). The release was pulsated for 6 hrs and this was confirmed by the Bronsted Lowry's methods. Based on the percentage of total protein and antigenic active protein, the release from microparticles was considered to be sustained antigenic release for a prolonged period (Fig. 5). Large amount of typhoid antigen was incorporated during the microencapsulation process, which increase the loading capacity. Some of earlier researcher reported that the molecular weight of the polymer had a great impact on the formulation of microencapsulation. However, further studies with some modification are needed to get better formulation to release active typhoid antigen.

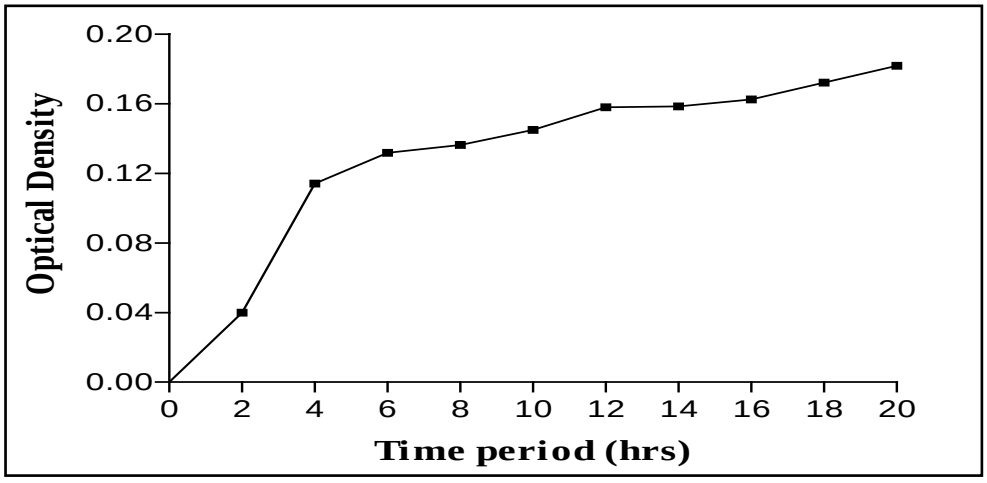


Fig. 5: Time period (hrs) Vs percentage release of antigen (O D) Each point indicates that the antigen release the microparticles for prolong period

Table 5: Hem agglutination test

Master dilution	1:12.5
2nd dilution	1:25
3rd dilution	1:50
4th dilution	1:100
5th dilution	1:200
6th dilution	1:400
7th dilution	1:800

Immunogenicity studies

The principle has been extended for the detection of antigens. The antigen may be attached to red blood cell surface and antibodies presented to these cells. The cell was precipitated. Agglutination tests are useful for assaying the amount of antibody present in a sample. The dilution ratio of 1: 50 was showed agglutination factor than the titer value is 50. The amount of antigen in the sample was found to be 5l µg by immunoprecipitation assay

Hem agglutination test

The principle has been extended for thedetection of antigens. The antigen may be attached to red blood cell surface or antibodies can bepresented to these cells. Agglutination tests are useful forassaying the amount of antibody present in a sample. Forthis, agglutination test was carried out withblood sampleby precipitating the RBC. The dilution ratio of 1: 50(Table 5) showed agglutination factor and thetiter value was 50. The amount of antigen assessed byimmunoprecipitation assay was found to be 5l µg.

In vivo immune response

Based on the release of antigen from starch microparticles in vitro, further release of antigen was confirmed in vivo in the Wister rat, where it was capable of forming antigen depot and the antigen was released slowly from the site of injection. Generally, in animalsystem, small microparticles can be directly tak up into macrophages by phagocytes. However, larger micro particles need to undergo biodegradation before phagocytosis that can occur; at this stage, microparticles can be covered with one or several layers ofmacrophages as a consequence of the wound healingr esponse to injected particles. The immunoglobulin (Fig.6, 7, 8) shows the increased level of IgA, IgM, IgG on 15thand 35thdays.In immunoglobulin titer values, the IgA shows the moderate responses (Fig. 7 & 8) where as IgG shows the moderate effect till15th and 35th day (Fig. 7), IgM was increased in conventional typhoid antigen(Fig. 8). IgE was increased in 35th day. Anti-typhoid antigen shows the decreased effect in the antigen loaded potato starch micropaticles.

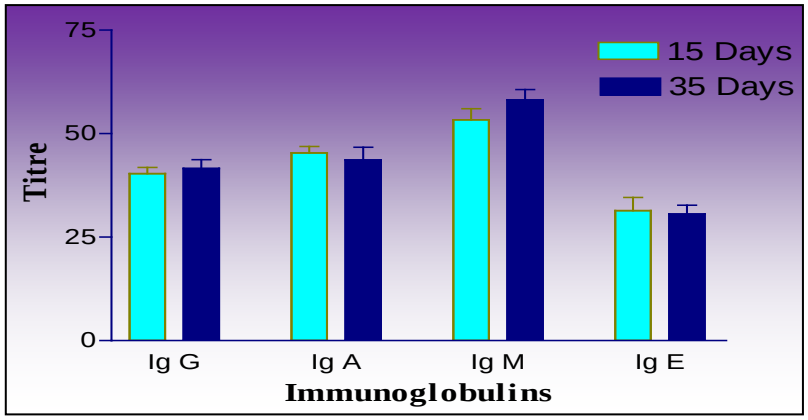


Fig. 6: Immunoglobulin titre

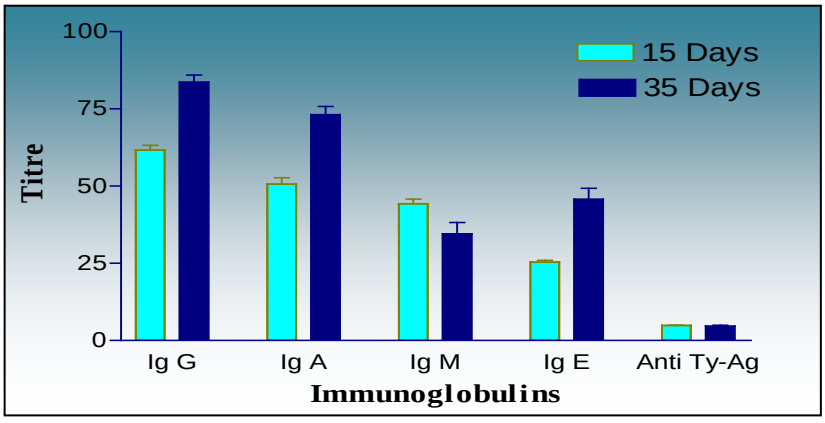


Fig. 7: Immunoglobulin titer Typhoid Potato Starch (IgG,IgA,IgM,IgE, AntiTy-Ag)

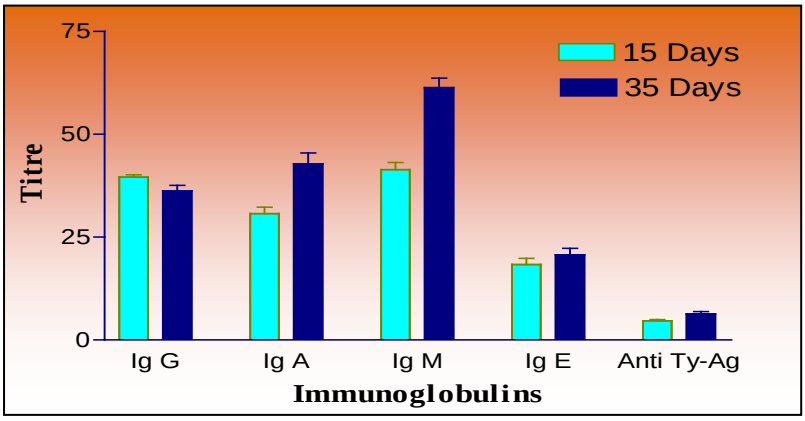


Fig. 8: Immunoglobulin titer (Conventional Typhoid Vaccine)

CONCLUSION

In this research work, mainly focused towards the enhance immune response by using natural biodegradable polymer mainly on polysaccharides derivatives like starch, due to its versality, biodegradability, biocompatibility, as an adjuvant to increase the efficacy.

The commonly used Immunization schedule required multiple injection dose often leads to dropouts among subjects to be immunized causing failure of protection. Therefore development of control delivery system for *S.typhi*, which could induce the desire antibody response from a single injection at IM.

The polymeric microparticles of Starch were successfully formulated by using encapsulation technique. The size of the vaccine loaded microparticles was found to be 43 µm. The immunogenicity studies were carried out by Heamagglutination test and Immunoprecipitation test for the antigen and antibody. The enhance immunogenicity of Typhoid vaccine loaded potato starch microparticles was determined by antibody induction method. This shows the good immune response till 35th day. However it is clear that more detailed investigation are necessary to clarify the effect of matrix polymer on antigen stability and enhanced immunogenicity during microspheres preparation and antigen releasing procedures and the effect of immunization of animals with microspheres formulation.

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