EFFICACY OF VARIOUS INACTIVATING AGENTS IN THE PRODUCTION OF PERTUSSIS VACCINE

A PROJECT REPORT

Submitted by P-1832

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(INTERNAL EXAMINER)

(EXTERNAL EXAMINER)

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ABSTRACT

Whooping cough or pertussis is a disease of the upper respiratory tract caused by *Bordetella pertussis*. The major victims are young children and infants. Our project is mainly concerned with developing an efficient inactivation method in the production of pertussis vaccine.

Bordetella pertussis strain 134 was grown in a shaker flask for 48 hrs, various parameters such as agitation (130-160rpm), inoculum concentration (2%-5%) and temperature (34.5-37°C) which affects the growth of organisms were studied and the optimum factor at which maximum yield of organisms was recorded.

The harvest after checking for sterility and opacity was centrifuged at 8000 rpm with 40 ml of thiomersal and pellet was collected in normal saline. The harvest was then inactivated with inactivating agents such as heat (54-60°C for 10 minutes), formaldehyde (0.1ml-0.175ml at 37°C for 24hrs), glutaraldehyde (0.3-0.9ml for ten minutes at room temperature) and thiomersal (0.1-0.5ml for 24 hrs at 37°C) to reduce the toxicity of the toxins.

After inactivating the vaccine, it was checked for viability for 7 days in mice, then the mice weight gain was observed for 10-14 days. Then toxicity test was done in mice and the survival of the mice was observed for 7 days finally the potency of the toxins was determined by performing the potency test and observed the mice for 14 days to find the best inactivation method. TIP is more potent than any other vaccine but it was out ruled because of its toxicity values. GIP and FIP have similar potency values as of HIP but as FIP has the least toxicity value. It is identified as the safest inactivation method.

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PT	Pertussis Toxin
BG	Bordet and Gengou
FHA	Filamentous Haemagglutinin
DPT	Diphtheria Pertussis Tetanus
LPF	Lymphocytosis Promoting Factor
IU	International Unit
BCA	Bacto Casamino Acid
OD	Optical Density
MWGT	Mouse Weight Gain Test
TIP	Thiomersal Inactivated Pertussis
GIP	Glutaraldehyde Inactivated Pertussis
FIP	Formaldehyde Inactivated Pertussis
HIP	Heat Inactivated Pertussis
HLT	Heat Labile
SPA	Stromata Protective Antigen

1. INTRODUCTION

Pertussis is a bacterial respiratory infection caused by *Bordetella pertussis*, a gram negative bacillus. It is other wise called as whooping cough. Its major manifestation is a protracted cough illness that lasts many weeks. The disease is most severe in infants and in young children, many of them suffer from the intense paroxysmal coughing that terminates in an inspiratory whoop.

A disease known in Britain from the early 16th century as chyne-cough probably was *pertussis* and the terms whooping cough and chynecough, was grown by Rules Bordet and Octave Genou in (1906), and the first crude vaccines appeared soon thereafter.

Bordetella pertussis

Morphology

It is a minute, gram negative, non-acid fast and non-sporing coccobacilli. There are two motile and two non motile species. They are rod shaped coccobacillary or coccoid 0.3 – 0.5 μm, arranged single or in pairs or in small groups. They cannot be distinguished from members of the *Haemophilus* group, but long bacillary and thread-like forms are less common. In virulent strains of *B.pertussis* rod forms predominate, in avirulent strains coccobacilli predominate.

Pertussis [Whooping cough] is still a major health hazard in developing countries, especially for children below one year who are at the higher risk of getting the disease. The disease has been controlled in many countries with the use of killed whole cell perussis vaccine. The hazards of

the vaccine range from minor local to systemic reactions with rare incidences of convulsions, infantile spasms and more serious serological illness.

A subcellular acellular pertussis vaccine is highly desirable, but the problems of establishing the clinical efficacy of such a vaccine should not be underestimated. Recently, Japanese workers have developed a pertussis component vaccine with lower toxicity and fewer side effects. With this, the study is developed with the following objective.

OBJECTIVE:

1. Developing whole cell vaccine with reduced toxicity, more potent and stable.
2. To use different inactivating agents for the preparation of whole cell vaccine.
3. To study the various parameters like temperature, agitation, pH and media concentration which affect the toxicity and potency of vaccine.
4. To study the effect of different inactivating agents like formaldehyde, glutaraldehyde, thiomersal and heat on the potency and toxicity on pertussis vaccine.
5. To study the efficacy of the vaccine by
    a. Toxicity test
    b. Potency test
    c. Challenge test

LITERATURE REVIEW

2. LITERATURE REVIEW

Heat-labile toxin is a cell-associated, heat-sensitive((56°C) for 10 min) toxin, which can be isolated from the soluble cytoplasmic fraction of cell lysates of B.pertussis active HLT is a poor immunogen but treatment with formaldehyde will inactivate toxicity and provide a toxoid that is antigenic was described by Munoz *et al.,(*1959).Many other substances such as formaldehyde, glutaraldehyde, and thiomersal act as effective inactivating agents on *Bordetella pertussis* organism in vaccine production was done by Burrell *et al.,* (1948).

An improved extract pertussis vaccine included the stromata-protective antigen (SPA) was developed by Pillemer *et al.,* (1954) . The SPA was prepared from extracts of sonically disrupted *B.pertussis* cells absorbed on human erythrocytes. The vaccine was protective in the mouse potency assay showed clinical efficacy in the British Medical Research Council trials (Medical Research council, 1956).The effect of heat, merthiolate and formaldehyde on Histamine sensitizing factor and protective activity of soluble extracts from *Bordetella pertussis* was observed by Munoz J *et a*l., (1966). The influences of heat and formalin on the preparation of pertussis vaccine was studied by Yoshioka M *et al.*, (1967).

The stability of tetanus and pertussis component of DPT vaccine on exposure to different temperatures was studied by Virender kumar, *et al.*, (1982)..The effects of different inactivating agents on the potency, toxicity stability of pertussis vaccine was determined by Gupta.R.K *et al.*, (1987). The detoxification of *Bordetella pertussis* toxin with glutaraladehyde as an inactivating agent was studied by Iida T, *et al.*, (1987).

Formalin is an inactivating agent for the manufacture of pertussis vaccine and is also used for the detoxification of a cellular pertussis vaccine was recommended by Saran, *et al.*,(1989).Improved pertussis toxin production by adjusting the growth mediums ionic composition was studied by Frohlich BT *et al.*, (1995).

The genus Bordetella

According to the current taxonomy, there are three species in the genus. *Bordetella pertussis* is an etiologic agent of human pertussis and *Bordetella parapertussis* is responsible for a mild pertussis like disease in human and *Bordetella bronchiseptica* is primarily an animal pathogen that causes kennel cough in dog and atrophic rhinitis in swine.

2.1. Growth, Variation And Metabolism

The media required for the growth of *Bordetella pertussis* is known as BG Medium (Bordet Gengou medium). The media contains nicotinamide, glutathione, ascorbic acid, salts, a sulphydryl – containing aminoacid [cystine, cysteine], glutamic acid. The primary isolation of the whooping cough bacillus with glycol-potato-extract agar medium, without peptone but containing 50% blood was carried out by Bordet and Gengou, (1906.) The amount of blood in BG medium may be reduced to 15-20% there by making haemolysis around colonies easier to see.

Additives such as starch, charcoal, anion- exchange resins, blood cells, albumin or methyl-B-cylodextrin, used to absorb fatty acids which will

permit the typical growth of *B.Pertussis*. The use of a complete synthetic medium containing an adsorbant such as methyl-B-cyclodextrin will markedly improve the growth of typical *B.pertussis* in laboratory culture system.

When grown aerobically on BG agar at 35.5°C, *B.pertussis* produces a high convex glistening, translucent colony with an entire margin and a hazy zone of hemolysis. Cultures grown from small inoculum may not produce visible colonies for 48 to 72 hr. The organism is gram negative in stained smears and coccobacillary, measuring 0.2 to 0.8 μm pleomorphism, filaments and short chains may develop indegraded strains or in cultures with conditions that permit growth but restrict cell division. Leslie and Gardener in (1931) reported a four phase dissociation described as types I, II, III and IV. The changes from phase I to phase IV are associated with a loss of virulence and have been considered to reflect a series of loss of mutations and selection under the conditions of culture in artificial medium. Recent studies indicate that phase change is completely reversible and regulated by a single region of the chromosome, perhaps coding for a trans-acting gene product needed for the expression of the various virulent phase genes.

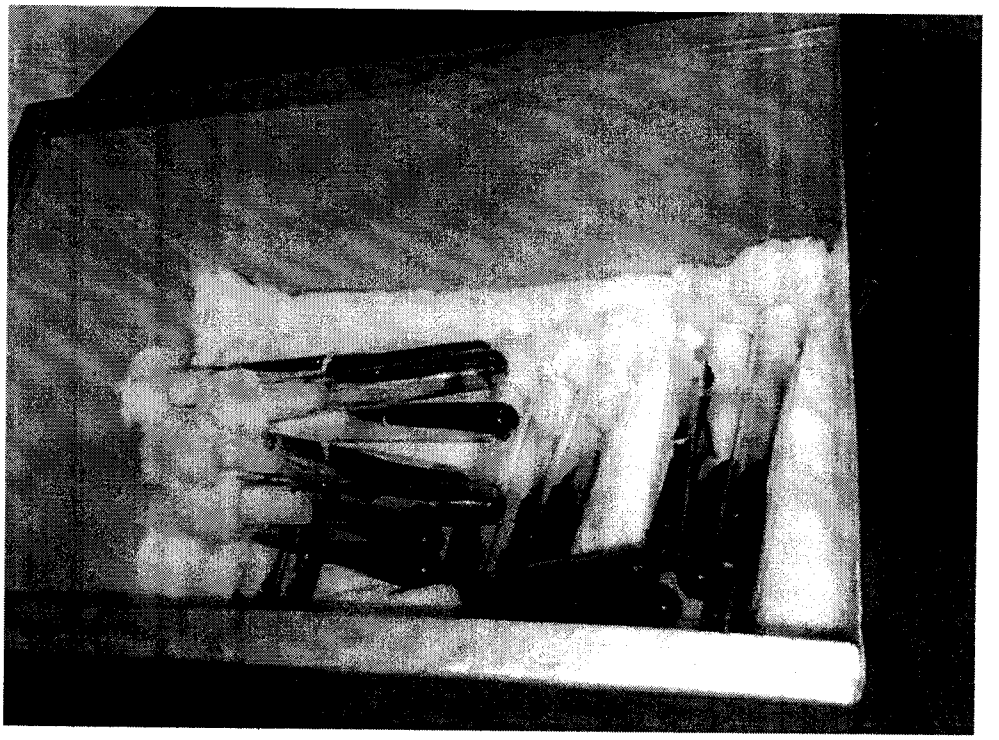

Figure 2.1 Bg Tubes

Natural habitat

The *Bordetella* are obligate respiratory tract pathogens of warm-blooded animals, including birds and so far it is not known having no reservoirs in the natural environment, nor do they have significant capacity survival outside of their hosts.

Antigenicity and biologically active components

Bordetella pertussis produces an array of biologically active components that plays an important role in host parasite relationship in pertussis. The key components of the *B.pertussis* organism are given.

Component		Biological activity
Pertactin	-	An outer membranes protein that promotes adhesion to ciliated respiratory epithelium
BRKA	-	An outer membrane protein that mediates adherence and resists complement.
Filamentous Haemagglutinin	-	Involved in attachment to ciliated respiratory epithelium
Pertussis toxin	-	A Secreted exotoxin that includes lymphocytosis sensitivity to histamine, pancreatic islet cell activation andimmune enhancement
Endotoxin	-	Contributes to fever and local reactions in animals and probably in humans
Tracheal cytotoxin	-	Causes ciliary stasis and cytopathic effects on tracheal mucosa.
Dermonectotic or heat labile toxin	-	Causes dermal necrosis and vasoconstriction in animals.
Adenylate cyclase	-	Inhibits phagocytic function

Table:2.1 The Key Components Of The B.Pertussis

Unlike the other Bordetella toxins described above, PT is restricted to *B.pertussis* and is thought to play a key role in the pathogenesis of whooping cough. However, although the modes of action of PT at the biochemical and cellular levels, have been defined, the actual role of PT in natural disease and in immunity to this is still not clearly studied. Culture media containing methylated-B-cyclodextrin stimulate the secretion of large quantities of extra cellular PT during growth of *B.pertussis*.

2.2. Structure of pertussis toxin

PTx is a hexameric protein with an A-B architecture similar to that of other related bacterial toxins, such as vibro choleral toxin and E.coli heat-labile enterotoxin. The A ["active"] protomer of PTx is composed of a single s subunit of 26,026 daltons; it is this enzymatic component of the molecule that confess many of its biological activities. The "B" [binding] oligomer is a complex pentamer containing subunits S_2 ,S_3, S_4 and S_5 in the rates of 1:1:2:1 respectively. B oligomer acts as the delivery platform for the S_1 subunit, contributing cell- receptor recognition functions to the molecule and permitting it to be internalized through the lipid membrane. These adhesion properties reside in the S_2 and S_3 subunits, which have significant amino acid sequence homology. The term pertussis toxin for the component of B.pertussis was proposed by Pittman., (1979).The variety of other names are histamine-sensitizing factor, leucocytosis and lympholytosis promoting factor (LPF), LPF-haemagglutinin, islets-activating factor, protein and pertusigen. In addition to its toxic activities, PT is expressed on the surface of *B.pertussis*.

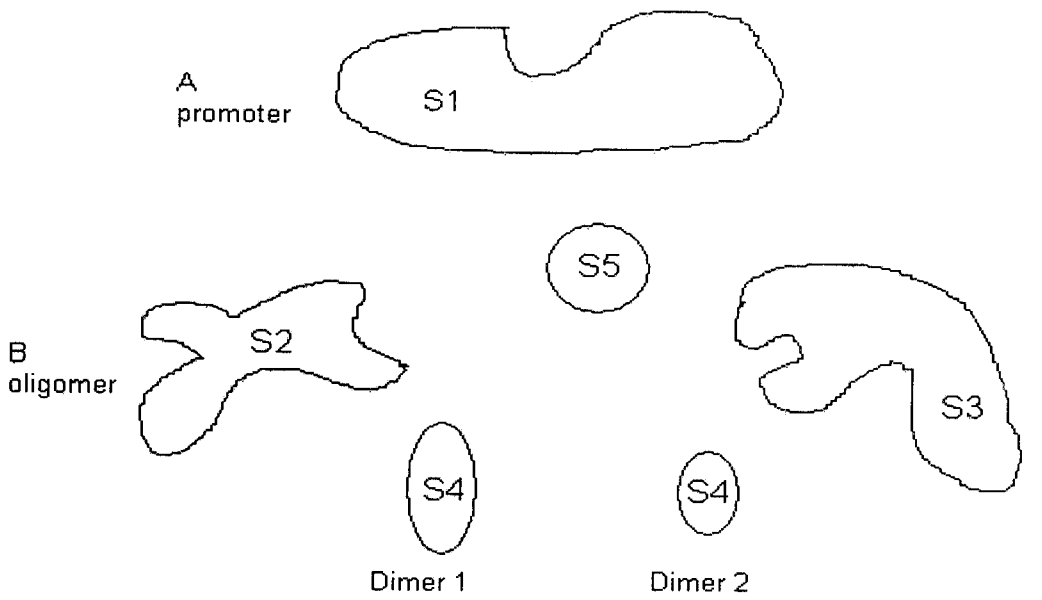

Figure 2.2 Structure Of Pertussis Toxin

However, each of these subunits appears to recognize structurally distinct cellular receptors, glycolipid moieties and glycol proteins for S_3. It is not apparent how of *Bordetella pertussis* or where and how it assembles its subunits into the holotoxin form. Tamura *et al(2002)* demonstrated that purified PTx could be dissociated into its component subunits and then reassembled *invitro*, perhaps in a Co-operative and Co-ordinated manner to that of cholera toxin. The tracheal cytotoxin from the culture supernatant of *B.pertussis* was isolated by Goldman *et al.,(*1982). The toxin is a small glycopeptide containing glutamic acid, alanine diaminopimelic acid andmuramic acid. The presence of both diaminopimelic acid and muramic acid suggests heat part of the toxin is derived from the peptidoglycon of the cell envelope

2.3. Transmission

Transmission occurs by inhalation of the bacterium in droplets released from an infectious person. The incubation period is 7 to 14 days. Once it enters inside the upper respiratory tract, the bacteria attach to the ciliated epithelial cells by producing adhesions factor called filamentous haemagglutinin, which recognizes a complementary molecules on the cells. After attachment, the bacteria synthesize several toxins that are responsible for the symptoms. The most important toxin is PT, which causes increased tissue susceptibility to histamine and serotonin, and an increased lymphocyte response.

Symptomatic Stages

***Pertussis* is divided in to three stages**

The catarrhal stage: It is named so because of the mucous membrane inflammation, which is insidious and resembles the common cold.

Paroxysmal stage: During this stage, the infected person tries to cough up the mucous secretion, by making 5 to 15 rapidly consecutive coughs followed by the characteristic whoop – a hurried deep inspiration.

The convalescent Stage: The catarrhal and paroxysmal stages last about 6 weeks. Final recovery may take several months.

2.4. Diagnosis

Laboratory diagnosis of pertussis is done by culture of the bacterium in BG medium, fluorescent antibody test, nasopharyngeal swabs and serological tests. The recent finding that an increase in serum IgA antibodies

to FHA is associated with infection ,but not with vaccination, may provide the laboratory tool for diagnosis of pertussis. The development of immunity takes place after initial infection.

2.4.1 Treatment and Prevention

Treatment is with antibiotics namely erythromycin, tetracycline or chloramphenicol. Treatment reduce the severity of the disease when begin within a weeks of the onset of the paroxysmal cough. Prevention is with the DPT vaccine. Vaccination of children is recommended when they are 2 to 3 months old.

2.4.2 Pertussis vaccine

There are two types of Pertussis vaccine. They are

a) Whole cell vaccine
b) Acellular vaccine

2.4.2.1 Whole cell vaccine

Whole cell pertussis vaccine is a suspension of killed *B. pertussis* organisms. Strains capable of yielding vaccine of acceptable quality are selected and used. Four approved *Bordetella pertussis* strain 509, 134, 10536 and 19190 are being used in the production of pertussis vaccine. Two or more strains are selected in such a way that the final vaccine contains agglutinogens 1, 2and 3. The strains used in our production laboratory are approved by the WHO and National Control Authority. A killed

(inactivated) vaccine containing whole bacteria was developed by Fine and Clarkson, (1924).The trials were concluded in Faroc-Islands, United Kingdom by the British medical research council and also in the mice used in the laboratory control of the vaccine to produce a wide range of protective efficiency of whole-cell vaccines.

Present whole cell pertussis vaccines comprises a certain level of toxicity, in order to achieve an acceptable level of potency. DPT is inactivated by the methods used for the manufacture of pertussis vaccine, therefore, the toxicity of whole cells vaccines is probably due to endotoxin, which is present in all gram negative bacteria. It is also heat inactivated at 56°C. PT is also inactivated with chemical agents such as formaldehyde, glutaraldehyde, Thiomersal etc., A vaccine for clinical trial was developed by Pearl L Kendrick, (1933). She used freshly isolated strains of *B.pertussis* grown on BG medium containing sheep blood. Vaccine concentrates were inactivated with thiomersal plus storage at refrigerator temperatures rather inactivation by the harsh methods such as the heat or phenol.

2.4.2.2 Acelluar pertussis vaccines

Acellular pertussis vaccines was prepared using formaldehyde or glutaraldehyde contain a range of biologically active PT, with the quantity in some preparations equivalent to the amount demonstrated to be present in whole cell vaccine.

A acellular pertussis vaccine prepared from a trisodium phosphate extract *of B.Pertussis* cells, component of their DTP adsorbed vaccines. Acelluar vaccine appears to be safe and to be less reactogenic than whole cell vaccines. LPF plays an important role in the pertussis disease. LPF is inactivated by formalin. It is possible that toxoiding, with formalin can alter

the LPF molecule that it no longer functions as a protective antigen. If methods can be devised that efficiently and effectively modify the LPF molecule so that it is no longer toxic but retain its protective ability, acellular vaccines containing such toxoids could be safer and more potent.

The pertussis vaccine is rarely given alone. It is commonly used combined with diphtheria and tetanus toxoids along with adjuvants such as aluminium phosphate or hydroxide.

2.5 Parameters

The parameters such as pH, temperature, agitation, inoculum and ingredient variations, affects the growth pattern of *B.Pertussis* organisms.

2.5.1 Inactivation

The concentrated vaccine bottles are kept in a water bath at 56°C for 10 minutes with frequent shaking for heat inactivating the organism. There should be no growth of *B. pertussis* or of any other organisms. Heat-labile toxin is a cell-associated, heat-sensitive(56°C for 10 min) toxin, which can be isolated from the soluble cytoplasmic fraction of cell lysates of B.pertussis active HLT is a poor immunogen but treatment with formaldehyde will inactivate toxicity and pro vide a toxoid that is antigenic was described by Munoz *et al.,(*1959).

The toxin can also be inactivated by chemical methods such as formaldehyde, glutaraldehyde, thiomersal etc for use as killed vaccines in combination with DT.

2.5.1.1 Formaldehyde inactivation

To 10ml of the bacterial harvest of the four wheaton bottles 40% formaldehyde solution was added it the following concentration as 0.1m, 0.125%, 0.150%, 0.175% to make a final concentration of 0.1% of formaldehyde. After mixing, the bottle was held at 37°Cfor 24hr. That toxin was destructed by 0.3% formalin at 37°C occurred in 20 hours done by Evans and Maitland, (1937) and a toxoid was formed which was antigenic . Grinding destroyed some toxin when heat was produced by the process.

2.5.1.2 Glutaraldehyde Inactivation

40ml of the bacterial harvest was divided into the four parts 10ml each to each 50% glutaraldehyde solution was added in the following concentration as 0.3, 0.5, 0.7 and 0.9ml to make a final concentration of 0.05% glutaraldehyde. The suspension was held at room temperature (20 – 25^0C) for 10 minutes with frequent shaking. The vaccines by the action of glutaraldehyde on toxins of bacteria, viruses and allergens was prepared by Cameron.J *et al.*, (1983).The degree of protection afforded by glutaraldehyde –treated toxin, depended upon the conditions of the toxoiding treatment was reported by Robinson, *et al.*, (1985).

2.5.1.3 Thiomersal Inactivation

Thiomersal solution of the following concentration as 0.2, 0.3, 0.4 , 0.5 was added to each of the wheaton bottles containing 10ml of the bacterial harvest to provide a final concentration of 0.02% thiomersal. After shaking, the suspension was held at 37^0C for 24 hrs.

2.6 *Invivo* tests

3.6.1 Toxicity Test

This test is performed after inactivating the organisms by means of heat and chemicals such as formaldehyde. Glutaraldehyde, and thiomersal. After using these inactivating agents the sample is incubated for 7 days at room temperature to see the viability. If the viability test is passed i.e., if there is no growth in BG medium it shows that the organism get inactivated. Then the samples are injected into mice intraperitonially. After 7 days incubation at room temperature the mouse weight gain test was performed.

2.6.2 Potency test

The potency assay shall be performed by the intra cerebral mouse production test. The potency of each final bulk or pool shall be determined by a comparative assay in relation to that of the International Standard for pertussis vaccine or National standard for pertussis vaccine. The potency of the final bulk shall be not less than 4 IU in volume recommended as a single human dose.

2.7 Vaccine production

Fermentation method is employed for the production of both whole sale and acellular vaccine production.

3. MATERIALS AND METHODS

3.1.Media study-Preparation of Bordet Gengou Medium:

3.1.1 Base Medium:

Raw potatoes of 1000g was washed and peeled. It is again washed with distilled water and cut into slices. The sliced potatoes are suspended in distilled water along with 36g of NaCl and boiled for 45 minutes to 1 hour. It is then filtered through muslin cloth. The volume is made up to 2000ml with distilled water and the pH is adjusted to 7.2 with 50% NaOH. Protease peptone 80g and 80ml of glycerol are added 20g of agar is dissolved in 6000ml of distilled water by stirring for 30 min and filtered. The two solutions are mixed distributed in 200ml amounts in sterile plasma bottle and sterilized at 120-121°C for 30 min. The bottle are incubated for 78 hrs at 35°C and then shifted to cold room [+4°C] till further used.

3.1.2 Complete Medium

400ml of the base medium is cooled to 5-6°C and 150-200ml of defibrinated sheep blood is added to it. Mixed gently without frothing and distributed into tubes and plates. Incubated at 35°C for 24 hrs and then stored at 4-8°C.

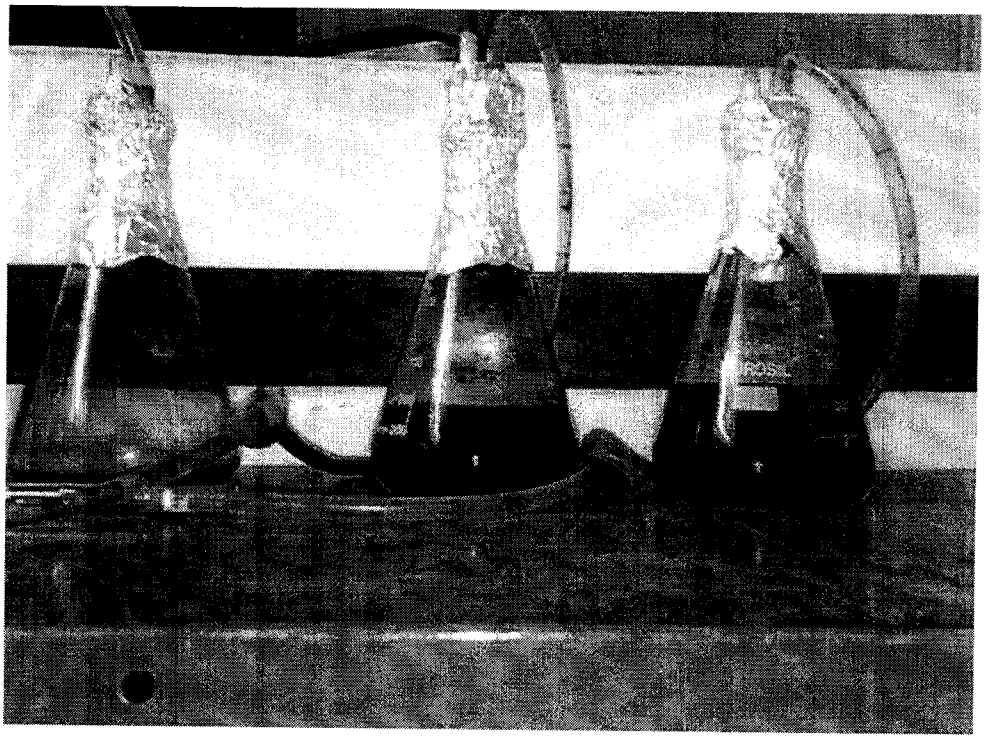

Figure 3.1 DE-FIBRINATED SHEEP BLOOD

3.1.3Preparation of B_2 Medium

BCA 1800gm is weighed and taken in a 300 liter capacity double jacketed kettled ad dissolved with dis.H_2O. To this 1500gm of L-Glutamic acid is added and dissolved well. 750gm of NaCl was weighed and dissolved in distilled water and added to the kettle. 30gm of MgO_4, 150gm of KH_2PO_4 and $CuSO_4$ are dissolved separately in sterile distilled water and added one by one to the kettle to the mixture 1500g of yeast extract, 450g of soluble starch are added. The volume was made up to 300 liter with sterile distilled water and the pH was adjusted to 7.2 with 50% NaOH. The solution was filtered using china cloth and the bottles were sterilized at 120-121°C for 30 minutes. The bottles are incubated for 48hrs.

3.2. Aeration and agitation

Shaker flask cultivation method or alternative cultivation method

The shaker flask cultivation was generally the simplest method and an alternative small scale cultivation method also employed in our project studies for the cultivation of *B.pertussis*. The shaker flask cultivation method is generally simplest round – bottom flask submerged batch culture technique. So using of 10 litres rotary shaker or bottles are used for *B. pertussis* growth that must have an adequate air supply which satisfies rotation at a suitable angle. The method was being determined by the facilities available and the size of the unit adopted. The method described below for bottles is that of spargers.

The bottle is assembled, medium added, sterilized and inoculated. It should contain eight litres of medium. An inoculum containing an adequate number of organisms is introduced into the bottle, the contents of which should be at 35°C and aeration through a sparger at the rate of three litres per minute is started. Growth with aeration is allowed to continue for 30 hours or until maximum growth has been attained. Duration of growth will be 36 – 48 hrs / 35°C.

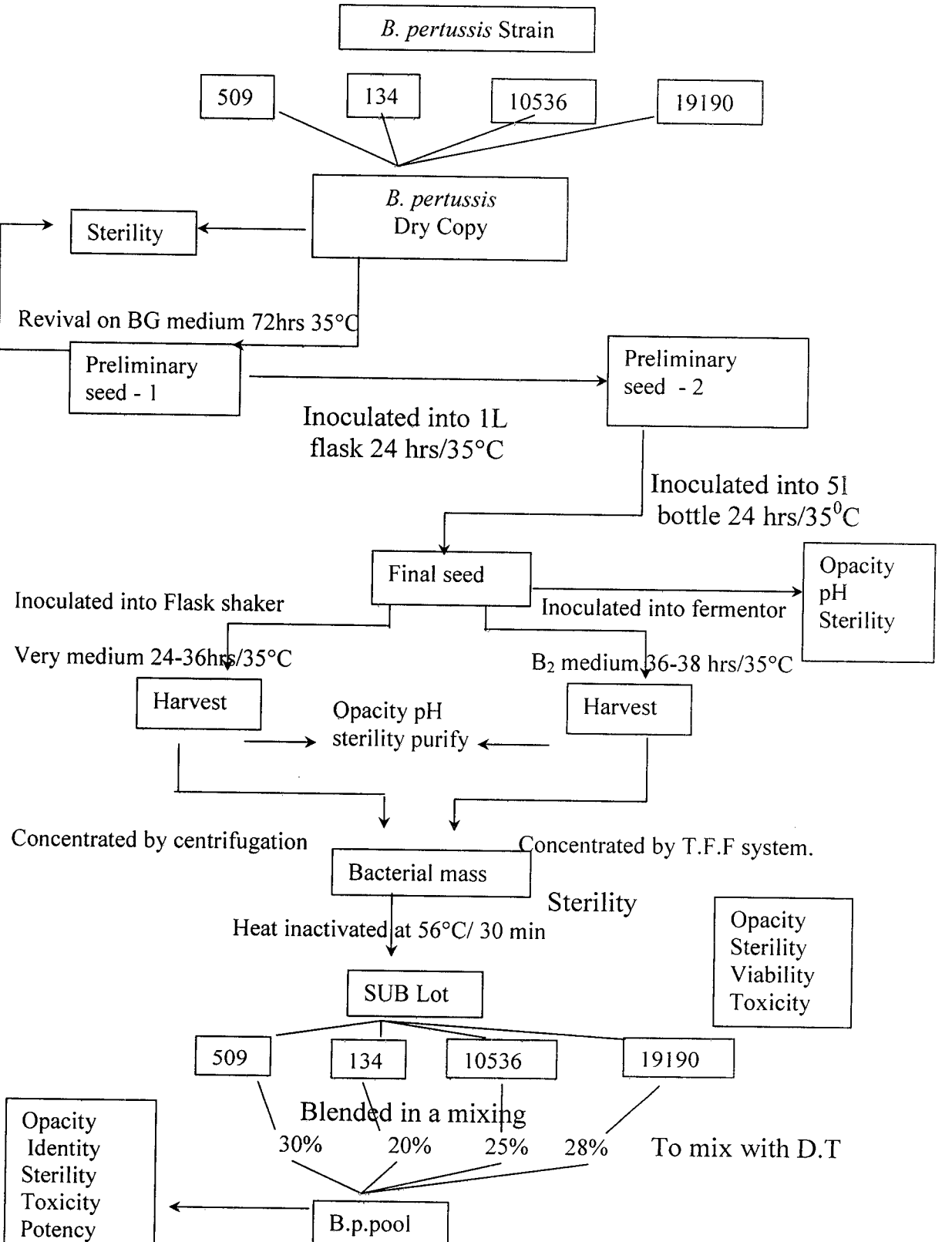

Table 3.1 Production Of Pertussis Vaccine

3.3. Shaker Flask Aeration

The shaker – flask is generally the simplest vessel for submerged agitation batch culture. This technique was first developed by Kluyver and Perquin (1933) has since been extended to the culture of all types, plants and animal tissue cells.

Rotary shakers are generally preferred to the reciprocating shaker because rotary shaking avoids the splashing which throws the organism out of the liquid and causes it to adhere to the walls above the liquid level an advantage particularly marked with filamentous mould cultures.

Siliconing the walls of the flask is often effective in preventing aeration of biomass on the walls. Both reciprocating and rotary shakers normally have throws in the range 25 to 50mm, that is the maximum distance moved by a point on the flask. Reciprocation is usually about 100 cycles / min and in rotary shaking the range is about 150 to 300 rpm.

3.3.1 Inoculum

Inoculum is one of the parameters that is altered in various cone of strain 509 such as 2%, 3% and 5% in the media to be inoculated to see the various growth stages to get high yield of *Bordetella pertussis*. Here the organism is inoculated in this concentrations and kept in a shaker to see the growth of organisms after 24 and 48 hours.

3.3.2 Temperature

Temperature is also one of the important parameters in the growth of *B. Pertussis*. The strain used here for varying the temperature is 509. This strain is inoculated into the 1litre conical flask containing B_2 – medium and

the flask was kept on shaker at various temperatures such as 34.5°C, 35°C, 37°C and the results were observed after 24 and 48 hrs to see the growth.

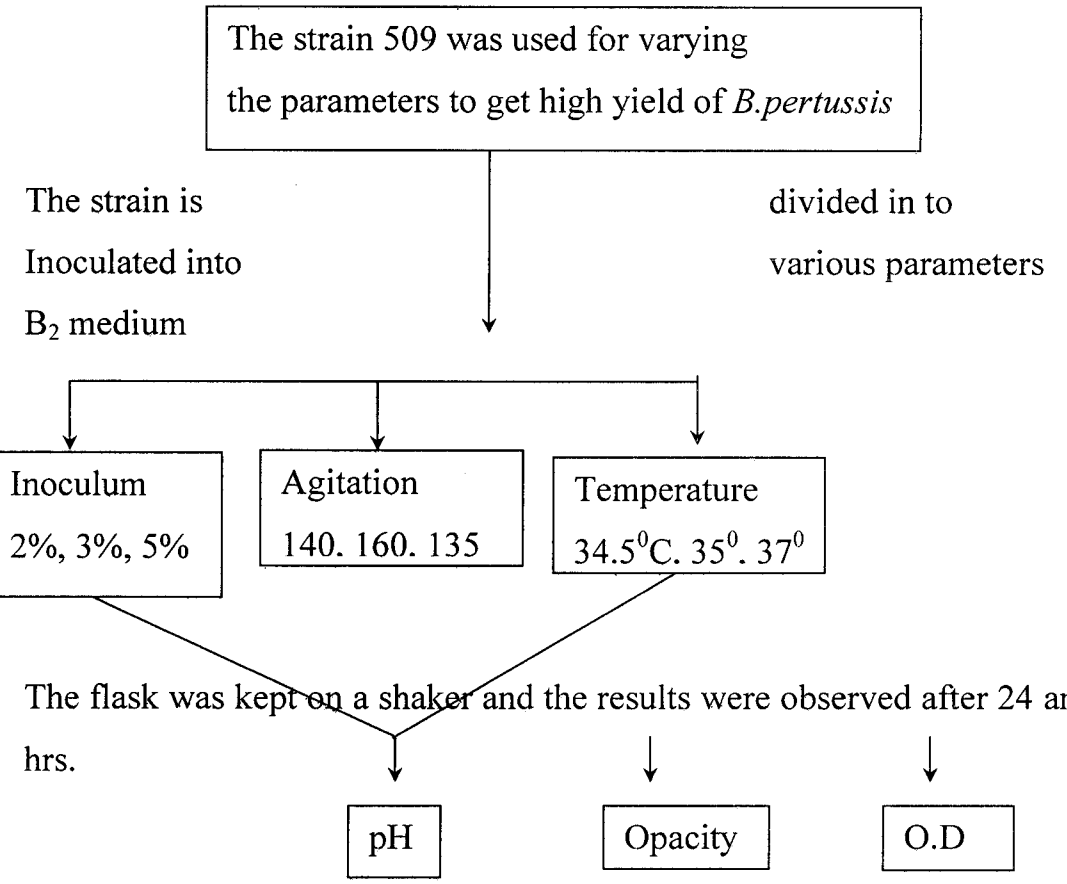

Table 3.2 Varied Parameters

3.3.3 Agitation

Agitation is also one of the important parameters that plays an important role in the growth of *B. pertussis*. Here the 509 strain inoculated in 1 litre flask was kept on a shaker at 140 rpm, 160 rpm and 135 rpm and the results were observed after 24 and 48 hrs to see at which rpm the growth was maximum.

3.3.4 Ingredients

The media used for the growth of *B. pertussis* was B_2 – medium. The B2 medium consists of various ingredients that induce the growth of an organism. These ingredients are altered to various concentrations to see their growth. Thus the three ingredients namely Bacto casamino acid [BCA], starch and yeast extract was altered in the medium to get high yield of an organism. These three ingredients are altered in the media and the strain 509 is inoculated to see the growth. The standard concentration of these three ingredients are BCA – 6 g, soluble starch 1.50 g yeast extract 4.6 g.

The above ingredients are altered in the medium. These ingredients are increased by 1 in 3 gm. When increased 2 g each the pH was 8.0 after 48 hrs and the opacity was 55 which showed a good yield and the medium was sterile.In order to know which one of the three ingredients influence the growth rate, the individual ingredients are altered in the media.

3.4 .Gram Staining

The most direct method of measuring growth rate is to check the size or mass of organisms at various times (Brock, 1971). Microscopy is used to visualize the increase in the cell size. The procedure is tedious especially with slow growing organism. Staining can reveal the size of the organism and at the same time differential staining can differentiate the Gram positive and Gram negative bacteria. Hence, Gram staining is prepared. Gram staining procedure involved the use of two dyes such as crystal violet [the primary stain], the saffranin [the secondary stain] a mordant – gram's iodine and a de-colourizer - acetone (95%0 Gram-positive forms retain the violet colour of the primary stairs and the Gram negative forms did not

Saffranin was employed to stain the gram – negative forms, and they appear pink in colour. The mordant seems to form an insoluble complex and fix the colour to the bacterial cell. The lipid content present in the cell wall of bacterial is readily dissolved by the action of ethyl alcohol Aneja, (1996). A thin emulsion of the culture with sterile saline was made on the clear glass slide. The emulsion was dried and heat fixed. Then the slide was washed with 1:600 acetic acid. The bacterial film was then covered in iodine solution for 1 minute and 30 seconds, washed with alcohol or spirit and then treated with fresh alcohol until the excess violet colour was removed. Counter stained with weak, Carbol-fuchsin for 5-15 seconds. Then the slide was washed, dried and examined.

3.5 Reagents required

Acetic acid

1 ml of acetic acid is dissolved in 599ml of distilled water.

Methyl violet

0.5gm of methyl violet dissolved in 100ml of distilled water and is filtered in filter paper.

Lugol's iodine

1gm of iodine and 2gm of potassium iodide in 100ml distilled water. Filter through the filter paper.

Spirit or alcohol

10ml of 95% alcohol was dissolved in 10ml distilled water.

Carbol fuchsin (Strong)

0.5 gm of basic fuchsin in 100ml of distilled water. Add 2.5gm of crystal and mix well and then filter through filter paper. Add 10ml of rectified spirit. Dilute 1ml in 20ml of distilled water.

3.6 Sterility test

Samples of vaccine are inoculated into 4 bottles of thioglycollate medium and incubated at 35^0C for 15 days. The test is passed with found sterile at the expiry of 14 days observation period . After completion of this test, the vaccine bottles are pooled into test, the vaccine bottles are pooled into 10 l bottles strainwise and labelled as lots.

3.7 Brown's opacity method (Brown 1010)

This method essentially consists in comparing the opacity of the bacterial suspension with that of a series of narrow tubes containing different series of dilutions of barium sulfate suspended in 1% of sodium citrate. The tubes were shaken well before use for making comparison the bacterial suspension were placed in tubes of similar dimensions to the standards. If the sample were dense they were diluted to a suitable value and the tubes were kept in good light upon a clearly printed paper. The opacity of the sample and the brown's tubes were compared by rolling the two tubes from side to side and raising them slightly from the surface of the paper. The number of the dilution, which correctly matches with the bacterial suspension, was taken as the result. The result were recovered giving the dilutions and the number on the opacity tubes.

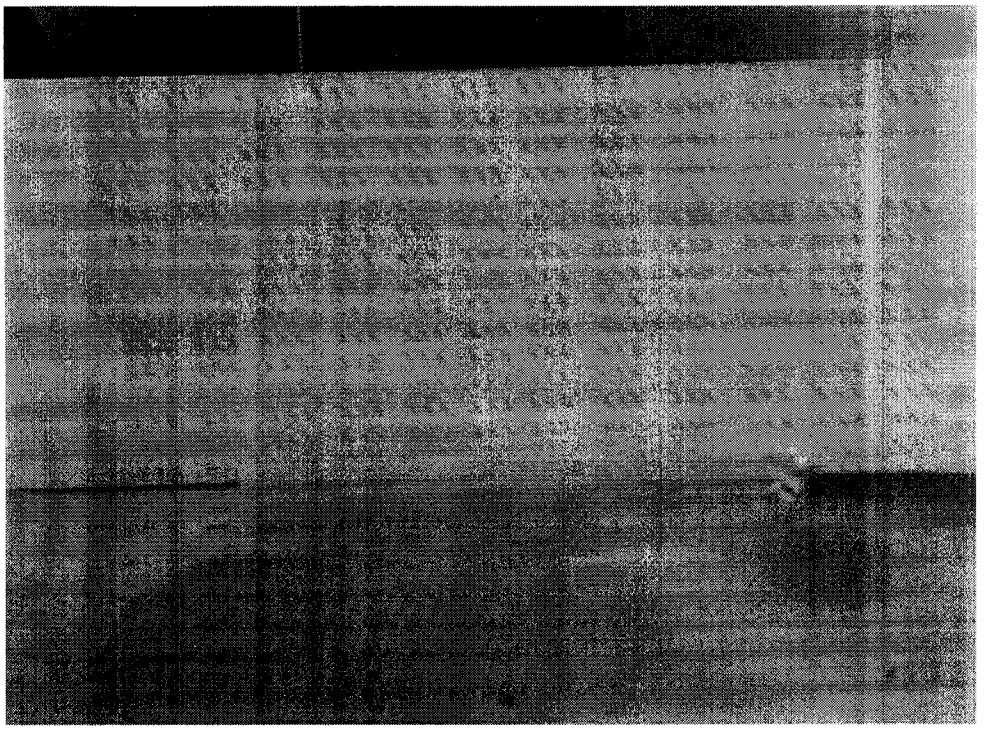

Figure 3.2 Printed Sheet For The Measurement Of Opacity

3.8 The turbidity measurement of the sample by the photoelectric colorimeter

Bacterial population or amount of growth can be determined by measuring turbidity or optical density of a both culture. The more turbid the suspension the less light will be transmitted through it. Since turbidity is directly proportional to the number of cells this property is used as an indicator of bacterial concentration in the sample Aneja (1996). Turbidity is quantified with the help of a colorimeter. It transmits a beam of light at a single wavelength through the liquid to reach the photo electric cell and the amount of light energy transmitted through the suspension is measured as percentage of transmission on the colorimeter as 0% - 100%. The density of cell suspension is expressed as absorbance or optical density, which is directly proportional to the cell concentration. The instrument used was photoelectric colorimeter model AE-11N, Japan. The colorimeter was

switched on and the correct filter fixed to obtain a wavelength of 540nm (green filter).

The optical density value of the samples, after appropriate dilution with physiological saline were measured. Sterile both, was used as a blank and the reading was adjusted to 100% transmittance. The growth of the culture is observed as the change in the optical density with the time. The O.D is directly proportional to the cell concentration when cell number increases there will be an increase in the optical density.

3.9 Measurement of pH

An accurate and practical method for measuring pH involves the use of a pH meter David plummer, (1990). The pH meter is a potentiometer that measures the potential development between a glass electrode and a reference electrode. In modern instruments the two electrodes are frequently combined into one electrode Becker *et al,* (1996).

The pH meter used was digital pH meter model LT 120, Hyderabad, India. The electrode was rinsed with distilled water dried and the pH of the samples were recorded.

3.10 Inactivation and detoxification of *B.pertussis* organisms

The *B.pertussis* strain 134 was grown in B_2 medium at 35^0C for 42-48h in 10 l flask loaded on shakers. The bacterial mass in each flask was separated by centrifugation and suspended in 250ml of normal physiological saline without preservative. The harvest from one flask represented one batch in this study. Each batch was examined for purity, opacity and agglutinogens. Batches of *B.pertussis* 134, showing weakly positive (t) agglutinogens, and thus suspected of having low potencies were deliberately

selected in order to observe the effect of inactivating agents on low potency batches.

Each batch was adjusted to a concentration of 160 x 10^9 organism/ml in normal physiological saline, except for one batch which contained 128 x 10^9 organisms/ml . A volume of 250 ml of each batch was used for the various inactivation procedures so that the volume in every inactivation procedure was under uniform conditions.

Bacterial harvests were divided into four parts containing 40ml each. The parts of the harvest were inactivated each by one of the following inactivating agents under specified conditions.

3.10.1 Heat Inactivation

40ml of the harvest was divided into four parts and poured into four different wheaton bottles each containing 10ml. The bottles were heated at 54^0C, 56^0C, 58^0C and 60^0C for 10 minutes in a water bath. The vaccine was shaked frequently during heating. After heating, the bottle was cooled in running water as quickly as possible.

3.10.2 Formaldehyde inactivation

To 10ml of the bacterial harvest of the four wheaton bottles 40% formaldehyde solution was added it the following concentration as 0.1ml, 0.125, 0.150, 0.175ml to make a final concentration of 0.1% of formaldehyde. After mixing, the bottle was held at 37°Cfor 24hr.

3.10.3 Glutaraldehyde Inactivation

40ml of the bacterial harvest was divided into the four parts 10ml each to each 50% glutaraldehyde solution was added in the following

concentration as 0.3, 0.5, 0.7 and 0.9ml to make a final concentration of 0.05% glutaraldehyde. The suspension was held at room temperature (20 – 25^{0}C) for 10 minutes with frequent shaking.

3.10.4 Thiomersal Inactivation

Thiomersal solution of the following concentration as 0.2, 0.3, 0.4 , 0.5ml was added to each of the wheaton bottles containing 10ml of the bacterial harvest to provide a final concentration of 0.02% thiomersal. After shaking, the suspension was held at 37^{0}C for 24 hrs.

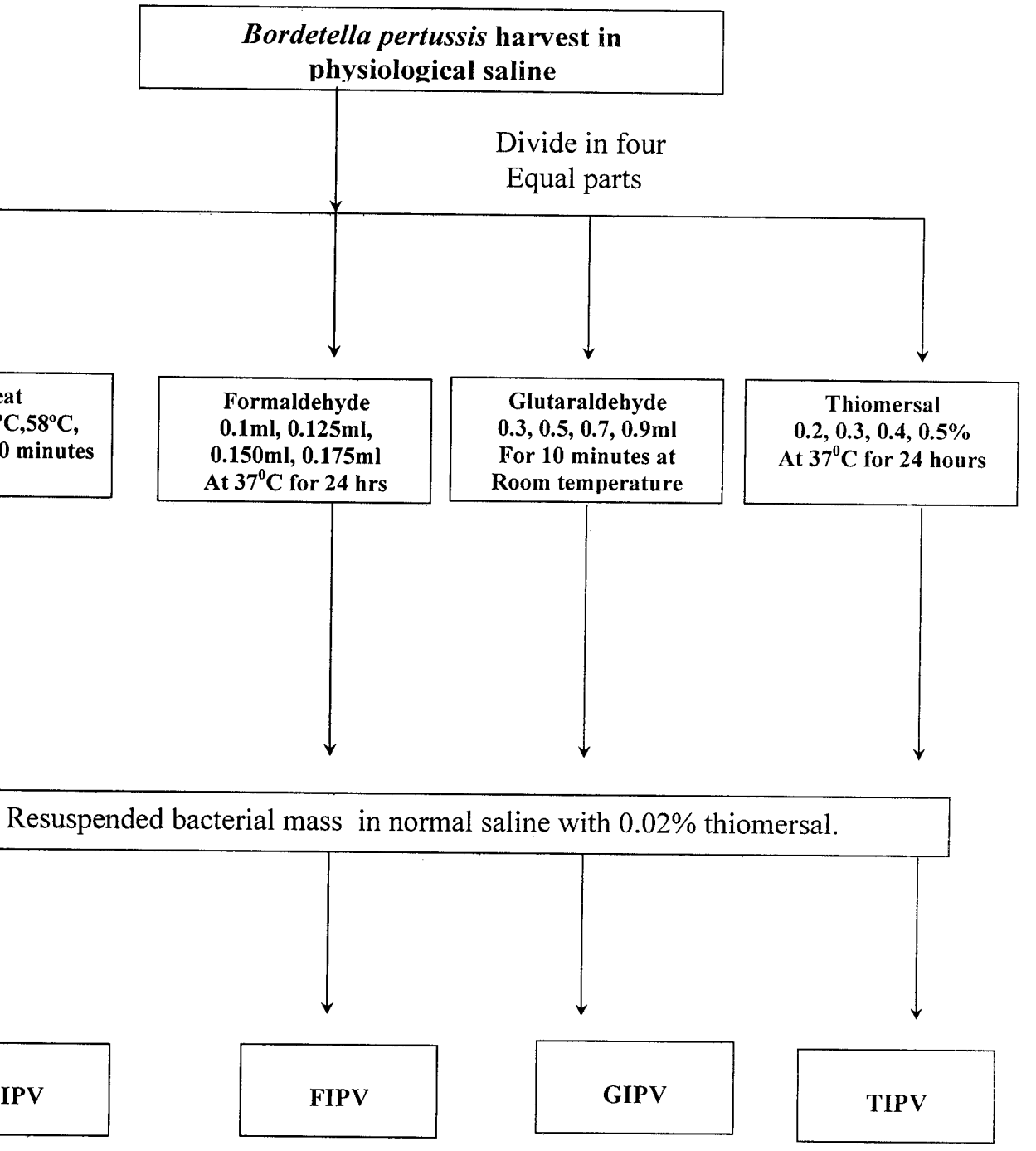

Table 3.3 Various Inactivating Agent

3.11 Test for mouse toxicity (mouse weight – gain test)

Ten healthy mice, each weighing 14-16gm are used before inoculation. They should have access to food and water for not less than two hours and continuously throughout the test period after inoculation the total weight of the mice is noted before inoculation and each mouse is injected with 0.5ml of the vaccine which contains not less than half of the largest volume recommended as a single human dose. A control group is inoculated with saline. The group weight of the mice is noted on the third and seventh day after inoculation. The vaccine is considered non – toxic, of

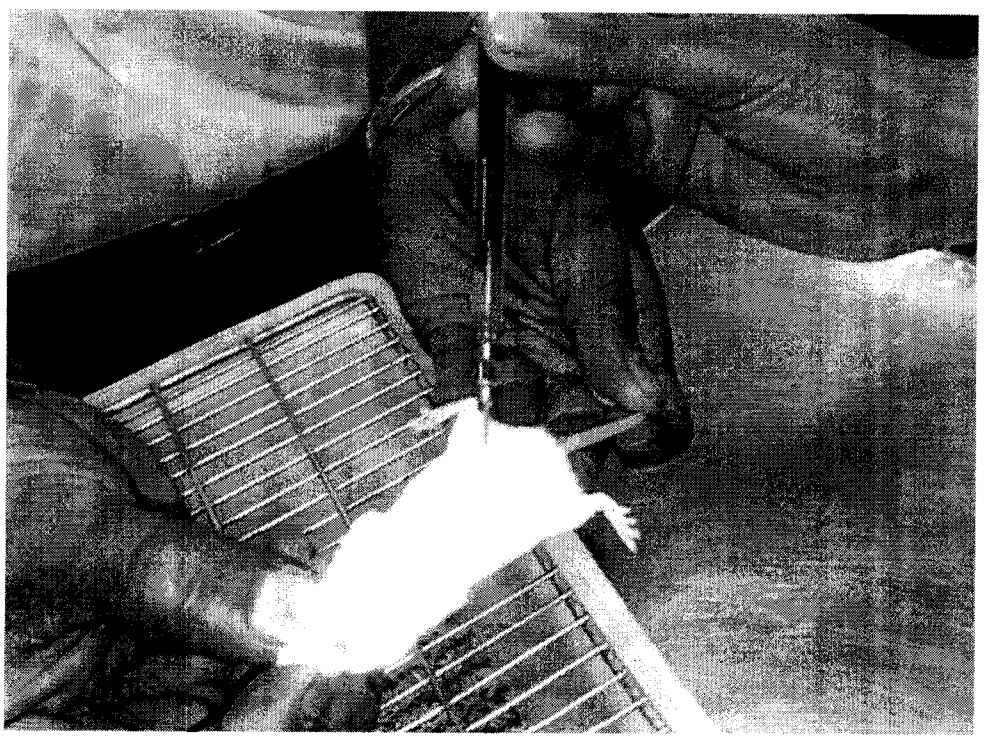

Figure 3.3 Intra-Peritoneal Injection

- These is no vaccine related to death.
- At the end of 72 hrs, the group weight is not less than that at the time of injection and At the end of seven days the gain in weight is not less than 60% of that of control group.

4. RESULT

In fermentor during the production of vaccine the parameters such as pH, temperature, agitation and the concentration of ingredients such as starch ,BCA, yeast are altered and the results were summarized.

4.1Effect of Parameters

4.1.1 Effect of Temperature

Maximum opacity of the harvest was seen only at temperature 35^0C. Hence the optimum temperature for the growth of *Bordetella Pertussis* is found to be 35^0C.

4.1.2 Effect of pH

Maximum opacity of the harvest was seen only at neutral pH values (pH 7.0) the optimum being pH 7.2. pH values affect the cell wall composition of *Bordetella Pertussis* inturn affect its growth. Thus maximum growth was observed at pH 7.2.

4.1.3 Effect of agitation

Increased growth of the organism was found at 140 rpm. Opacity of the harvest and turbidity found at 540nm indicates marked increased in growth of the organism at 140 rpm.

4.1.4 Effect of inoculum

At 2% and 3% of the inoculum high growth of the organism was seen at 48 hours. Thus they utilize sufficient nutrients for their growth.

4.2 Effect of inactivating agents

The effect of heat (56°C for 10 min) formaldehyde (0,15% at 37^0 c for 24hrs) glutaraldehyde (0.05% at room temperature for 10 min) thiomersal (0.02% at 37^0 c for 24hrs) when used as inactivating agents in the preparation of pertussis suspension, was studied with regard to potency, toxicity and stability. Five batches of *Bordetella Pertussis* strains were used for the study. The thiomersal inactivated pertussis (TIP) preparation was 1.5-2 times more potent than the heat inactivated pertussis (HIP) preparation.

The potency values of the formaldehyde inactivated pertussis (FIP) and glutaraldehyde inactivated pertussis (GIP) preparations were similar to those of the HIP preparation. The FIP preparation was the least toxic showing maximum weight gain in the mouse weight-gain test [MGWT], while the TIP preparation did not pass the MWGT. The weight gain of GIP is greater than that of HIP preparation. The potency of pertussis component in the vaccine was stable at 4-8^0C and 25^0C for three months for all types of pertussis vaccine. The inactivating agents used in the manufacture of pertussis preparations had no effect on the stability of the vaccine.

4.3 *INVIVO* TESTS

Weight of the mice	14 – 16 g
No. of animal	10
Dose	10 IU/0.5 ml
Route of Inoculation	Intraperitoneal
Period of test	7 days
Date of inoculation	MAR 2nd 2007

Table 4.1 Specific Toxicity test

RESULT

At the end of 72 h the total weight of the group was not less than it was before the injection;

At the end of the 7 days, the average weight gain for mouse was not less than 60 % of that of the control group of mice.

Not more than 5 % of the total number of injected mice died.

Sample used	Pertussis lot 68/06
Strain used	134
Route of injection	Intra peritoneal
Volume	0.5ml/mice, Guinea pig
Observation :	7 days
Test result	Passed

Result: After 7 days of inoculation all guinea pig and mice should survive.

Table 4.2 Abnormal Toxicity Test

Immunization Date	MAR 9th 2007
Weight of mice	14 – 16 g
No. of mice	16
Route	Intaperitoneal
Dosage	0.5 ml
Date of completion	MAR 23rd 2007
Reference vaccine used	PII working standard B – 8
Unitage of reference	8 Iu/ml

Table 4.3 Potency Test

Date	MAR 23rd 2007
Volume	0.03ml
No. of organisms	50,000
Route	Intracerebral
Dose	285 LD_{50}
Observation	75% survival

Table 4.4 Challenge Test

Inoculum	Incubation (hrs)	Agitation (rpm)	Temperature (°C)	Opacity (IU)	O.D	pH
1 2%	24 hrs	160	36°	$30*10^9$	1.1	7.6
2	24 hrs	160	36°	$30*10^9$	1.1	7.5
1 3%	24 hrs	160	36°	$55*10^9$	1.5	7.8
2	24 hrs	160	36°	$11*10^9$	1.5	7.8
1 2%	48 hrs	160	36°	$65*10^9$	1.6	7.9
2	48 hrs	160	36°	$65*10^9$	1.6	7.9
1 3%	48 hrs	160	36°	$120*10^9$	2.1	8.0
2	48 hrs	160	36°	$120*10^9$	2.1	8.0
1 4%	24 hrs	170	37°	$70*10^9$	1.7	8.1
2	24 hrs	170	37°	$70*10^9$	1.7	8.1
1 5%	24 hrs	170	37°	$85*10^9$	1.75	8.1
2	24hrs	170	37°	$85*10^9$	1.75	8.1
1 4%	48 hrs	170	37°	$140*10^9$	2.3	8.2
2	48 hrs	170	37°	$140*10^9$	2.3	8.2
1 5%	48 hrs	170	37°	$170*10^9$	2.45	8.35
2	48hrs	170	37°	$170*10^9$	2.45	8.35

Table 4.5 Results for Altered Parameters

oculum	Incubation (hrs)	Agitation (rpm)	Temperature (°C)	Opacity (IU)	O.D	pH
1 2%	24 hrs	140	35°	$40*10^9$	1.2	7.6
2	24 hrs	140	35°	$40*10^9$	1.2	7.5
1 3%	24 hrs	140	35°	$60*10^9$	1.6	7.8
2	24 hrs	140	35°	$60*10^9$	1.6	7.8
1 2%	48 hrs	140	35°	$90*10^9$	1.8	7.9
2	48 hrs	140	35°	$90*10^9$	1.8	7.9
1 3%	48 hrs	140	35°	$140*10^9$	2.3	8.0
2	48 hrs	140	35°	$140*10^9$	2.3	8.0
1 4%	24 hrs	150	35.5°	$80*10^9$	1.7	7.9
2	24 hrs	150	35.5°	$80*10^9$	1.7	7.9
1 5%	24 hrs	150	35.5°	$100*10^9$	1.8	7.9
2	24 hrs	150	35.5°	$100*10^9$	1.8	7.9
1 4%	48 hrs	150	35.5°	$150*10^9$	2.3	8.0
2	48 hrs	150	35.5°	$150*10^9$	2.3	8.0
1 5%	48 hrs	150	35.5°	$195*10^9$	2.5	8.2
2	48 hrs	150	35.5°	$195*10^9$	2.5	8.2

Table 4.5 Results For Altered Parameters

aration	Batch No-Average weight gain per mouse(g)									
	1/134		2/134		3/134		4/134		5/134	
	At Day		At Day		At Day		At Day		At Day	
	3	7	3	7	3	7	3	7	3	7
	0	1.5	0	1.0	0	1.0	0	1.6	0.2	1.7
	0	2.0	0.5	2.0	0.3	1.8	0.2	2.0	0.7	2.2
	0.1	1.6	0	1.3	0	1.0	0	1.6	0.2	1.8
	-0.3	1.4	-0.2	0.7	-0.7	0.3	-0.3	0.9	-0.3	1.2

Table 4.6 Mouse Weight Gain Of Pertussis Preparations Made With Different Inactivating Agents Using *Bordetella Pertussis* Strain 134

Date	Day	Test vaccine [Weight]	Saline control [Weight]
16/02	0	148 g	148 g
17/02	1	149 g	156 g
18/02	2	152 g	160 g
19/02	3	163 g	170 g
20/02	4	169 g	178 g
21/02	5	169 g	178 g
22/02	6	173 g	181 g
23/02	7	176 g	184 g

Table 4.7 Mouse Weight Gain Test

Name of the animal	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Remarks**
G.Pig	✓	✓	✓	✓	✓	✓	✓	
G.Pig	✓	✓	✓	✓	✓	✓	✓	
Mouse 1	✓	✓	✓	✓	✓	✓	✓	Passed
Mouse 2	✓	✓	✓	✓	✓	✓	✓	
Mouse 3	✓	✓	✓	✓	✓	✓	✓	
Mouse 4	✓	✓	✓	✓	✓	✓	✓	
Mouse 5	✓	✓	✓	✓	✓	✓	✓	

Table 4.8 Toxicity Test Observation [✓For Survival]

Vaccine No.	Dilution ml	Observation in days	Survival ratio	Values
Ref. vaccine	1/8 1/40 1/200	14 days	13/16 5/16 1/16	ED_{50} 0.0216 ml
TIP	1/8 1/40 1/200	14 days	14/16 7/16 2/16	ED_{50} 0.0140 ml
FIP	1/8 1/40 1/200	14 days	13/16 7/16 1/16	ED_{50} 0.0223 ml
GIP	1/8 1/40 1/200	14 days	12/16 8/16 2/16	ED_{50} 0.0234 ml

Ref. vaccine : ED_{50} = 0.0216 ml

Potency unit= 8 IU / ml

TIP : ED_{50} = 0.0140 mL

Potency unit = 12.34 IU/ml

Table 4.9 Result For The Potency Test

Challenge: LD_{50} (Lethal Dose)

Vaccine	Observation	Survival Ratio	Values
50,000	14 + 14 days	0/10	
10,000	(28 days)	0/10	LD_{50} = 333
2000		1/9	Organisms/ml
400		2/8	
80		6/4	

Result: Test Process

LD_{50} = 333 organisms

Table 4.10 Result For The Challenge Test

The vaccines passes the requirements for potency if the result of a statistically valid test shows that the estimated potency of the vaccine is not less than 4.0 IU/ml in the volume recommended for a single human dose

5. CONCLUSION

The currently available whole-cell pertussis vaccines compromise a certain level of toxicity to achieve a sufficient level of potency. In the present study, it has been observed that the various inactivating agents used in the manufacture of pertussis suspension have variable effects on the potency and toxicity of the preparation.

The potency of the TIP preparation was greater than that of the other preparations, confirming earlier reports that thiomersal does not affect the potency of the pertussis vaccine. Sarart found that the ATP vaccine to be more potent than the HIP vaccine. The potencies of the FIP preparation were slightly lower than those of the HIP preparation. (Tables 1 and 2) but the differences were not statistically significant and observed no deleterious effect of formalin on potency, although some workers have reported loss of potency in formal treated vaccines,Formalin is recommended as an inactivating agent for the manufacture of pertussis vaccine and is also used for the detoxification of acellular pertussis vaccine. The potency of GIP preparation was similar to that of the FIP and HIP preparation.

In the MWGT, mice treated with the FIP preparation showed the maximum weight gain at three and seven days after inoculation which confirms previous finding. The TIP preparation, although a highly potent suspension, did not pass the WHO MWGT compared with the HIP preparation.

In the stability study, it was observed that the potency of the pertussis component in the DTP vaccine was stable at 4-8 and 25^0C for three months, at 35^0C, the loss in potency varied from 54 to 65% after three months. In this study, all types of pertussis vaccines contained thiomersal as a preservative and it had already been reported that thiomersal preserved

vaccine was more stable than vaccine without preservative. Hence it was concluded that the pertussis vaccine preserved with thiomersal was quite stable and the inactivating agent used in the manufacture of the pertussis suspension has no effect on the stability of such vaccine.

A variable effect of glutaraldehyde on potency was found by Relyveld *et al.(1983),* when different concentrations of the reagent were used. The concentration of glutaraldehyde used in this study was the same used by Munoz *et al.*,(1959) for the detoxification of pertussigen. In this study the time of treatment with glutaraldehyde was reduced from 2hrs to ten minutes. *B.pertussis* organisms were completely inactivated in 10minutes.

On the basis of the toxicity of the TIP preparation thiomersal have not been found to be better inactivating agents under the given conditions. Thus it appears worthwhile to use formaldehyde for inactivation in the preparation of a safer pertussis suspension for the adsorbed DPT vaccine

APPENDICES

1. B_2 Medium

BCA 1800*g*

L-glutamic Acid 1500*g*

Nacl 750*g*

Mgo_4 30g

KH_2Po_4 150g

$FeSo_4$ 3.75*g*

$CaCl_2$ 3.0*g*

$CuSo_4$ 0.15*g*

Yeast 1500*g*

Soluble Starch 450*g*

Distilled Water 300*litre*

PH adjusted to 7.2 with 50% NaOH

2. B-G Medium

Potato 1000*g*

Nacl 36*g*

Peptone 80*g*

Glycerol 80*ml*

Agar 200*g*

Sheep Blood 200*ml*

Distilled H_2O 10*litre*

PH adjusted to 7.2 with 50% NaOH

3. Nutrient Agar

Meat(beef)extract 10*g*

Peptone 10*g*

Nacl 5*g*

Distilled Water 1000*ml*

Agar 2*g*

4. Gram Staining Reagents

i) Acetic Acid

1*ml* of acetic acid is dissolved in 599*ml* of distilled water.

ii) Methyl Violet

0.5*gm* of Methyl Violet is dissolved in 100*ml* of distilled water and is filtered in filter paper

iii) Lugol's Iodine

1*gm* of iodine and 2*gm* of potassium iodide in 100*ml* distilled water.

iv) Spirit or Alcohol

10*ml* of 95% alcohol was added to 100*ml* distilled water.

v) Carbol Fuchsin (strong)

0.5*g* of carbol fuchsin in 100*ml* of distilled water.

5. Inactivating Agents

i) Formaldehyde (40%)

40*ml* of formaldehyde is made upto 100*ml* with distilled water.

ii) Glutaraldehyde (50%)

50*ml* of glutaraldehyde is made upto 100*ml* with distilled water.

iii) Thiomersal (1%)

1*ml* of thiomersal is made upto 100*ml* with distilled water.

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KUMARAGURU COLLEGE OF TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

EFFICACY OF VARIOUS INACTICVATING AGENTS IN THE PRODUCTION OF *PERTUSSIS* VACCINE

Project By

-Kamalnath Manoharan 71203204010

Guided By

-Dr.R.Baskar(Assistant Professor)

Date of Evaluation-02.05.2007

ABSTRACT

Whooping cough or pertussis is a disease of the upper respiratory tract caused by *Bordetella pertussis*. The major victims are young children and infants. Our project is mainly concerned with developing an efficient inactivation method in the production of pertussis vaccine.

Bordetella pertussis strain 134 was grown in a shaker flask for 48 hrs, various parameters such as agitation (130-160rpm), inoculum concentration (2%-5%) and temperature (34.5-37°C) which affects the growth of organisms were studied and the optimum factor at which maximum yield of organisms was recorded.

The harvest after checking for sterility and opacity was centrifuged at 8000 rpm with 40 ml of thiomersal and pellet was collected in normal saline. The harvest was then inactivated with inactivating agents such as heat (54-

60°C for 10 minutes), formaldehyde (0.1ml-0.175ml at 37°C for 24hrs), glutaraldehyde (0.3-0.9ml for ten minutes at room temperature) and thiomersal (0.1-0.5ml for 24 hrs at 37°C) to reduce the toxicity of the toxins.

After inactivating the vaccine, it was checked for viability for 7 days in mice, then the mice weight gain was observed for 10-14 days. Then toxicity test was done in mice and the survival of the mice was observed for 7 days finally the potency of the toxins was determined by performing the potency test and observed the mice for 14 days to find the best inactivation method. TIP is more potent than any other vaccine but it was out ruled because of its toxicity values. GIP and FIP have similar potency values as of HIP but as FIP has the least toxicity value. It is identified as the safest inactivation method.

KUMARAGURU COLLEGE OF TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

EFFICACY OF VARIOUS INACTICVATING AGENTS IN THE PRODUCTION OF *PERTUSSIS* VACCINE

Project By

-Sajjeev Jagannnathan 71203204023

Guided By

-Dr.P.Rajasekaran(Professor and Head)

Date of Evaluation-02.05.2007

ABSTRACT

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KUMARAGURU COLLEGE OF TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

EFFICACY OF VARIOUS INACTICVATING AGENTS IN THE PRODUCTION OF *PERTUSSIS* VACCINE

Project By

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Guided By

-Mr.S.Shanmugam(Senior Lecturer)

Date of Evaluation-02.05.2007

ABSTRACT

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