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EFFICACY OF DIPHTHERIA AND TETANUS VACCINATION IN GAZA STRIP

فعالية التطعيم ضد الدفتيريا و التيتانوس في قطاع غزة

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DECLARATION

“I hereby declare that this manuscript is my own work and that, to the best of my knowledge and belief, it contains no material previously published, or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute, except where due acknowledgment has been made in the text".

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Date: 28-2-2006

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Abstract

Vaccination is a preventive strategy in fight against infectious diseases and it is one of the most effective weapons of health protection of the modern medicine. Hence, it is necessary to continuously monitor the efficacy of vaccination programs.

This study focused on the evaluation of effectiveness and usefulness of vaccination against diphtheria and tetanus in different age groups in Gaza Strip.

Blood samples were collected from 180 children below 12 years of age, 90 males and 90 females, children were classified into 3 age groups, (2-4y), (7-8y) and (11-12y).

Diphtheria and tetanus antitoxins were measured in serum samples using Enzyme Linked Immunosorbent Assay (ELISA).

The study showed that the efficacy of diphtheria and tetanus vaccination among children below 12 years in Gaza was 87.8% and 98.3%, respectively, with a significant difference in efficacy among age groups. The study showed also that the mean titer of antibodies varies significantly among age groups (0.239, 0.632 and 0.460 IU/ml for diphtheria) and (1.00, 2.62 and 1.20 IU/ml for tetanus). There was no significant difference between male and female in vaccine efficacy.

This study indicates that vaccination against tetanus is highly effective while it is less effective for diphtheria. Antibody titers in the vaccinated group were relatively low for both diphtheria and tetanus, which means that vaccine gives a short term protection, antibody level and vaccine efficacy decline over time and there is a need for Td booster dose.

Key words: diphtheria, tetanus, vaccination, efficacy, booster dose, DPT, DT, immunity, antitoxoid.

فعالية التطعيم ضد الدفتيريا و التيتانوس في قطاع غزة

الخلاصة

يعتبر التطعيم من الإجراءات الوقائية للحماية من الأمراض المعدية كما يعتبر أكثر الوسائل نجاعة في الحفاظ على الصحة لذلك يجب تقييم كفاءة جداول التطعيم بشكل مستمر.

تهدف هذه الدراسة إلى تقييم كفاءة برنامج تطعيم الأطفال للوقاية من مرضي الدفتيريا و التيتانوس في قطاع غزة.

تم جمع عينات الدم من 180 طفل دون سن 12 سنة ، 90 ذكور ، 90 إناث صنفت في ثلاث مجموعات عمرية مختلفة وهي (2-4) سنة ، و (7-8) سنة ، و (11-12) سنة وقد فحصت أمصال العينات التي أخذت من كل طفل لتحديد تركيز الأجسام المضادة لكل من الدفتيريا والتيتانوس وذلك باستخدام تقنية Enzyme Linked Immunosorbent Assay (ELISA)

أظهرت الدراسة أن كفاءة التطعيم كانت بنسبة 87.8% للدفتيريا و بنسبة 98.3 % للتيتانوس وقد كان معدل تركيز الأجسام المضادة للدفتيريا (0.239، 0.632، 0.460 وحدة / مل) و للتيتانوس (1.00 ، 2.62 ، 1.20 وحدة/ مل) للثلاث فئات العمرية على التوالي.

كما أشارت الدراسة إلى وجود فروقات ذات دلالة إحصائية في كفاءة التطعيم وفي معدل تركيز الأجسام المضادة بين الثلاث فئات العمرية، وعدم وجود فروقات ذات دلالة إحصائية في كفاءة التطعيم بين كلا الجنسين.

نستنتج من هذه الدراسة أن التطعيم للوقاية من التيتانوس ذو كفاءة عالية ، بينما تقل هذه الكفاءة في الدفتيريا ، كما أن معدل تركيز الأجسام المضادة لكلا اللقاحين كان منخفض. و هذا يعني أن التطعيم لا يزود الأطفال بمناعة طويلة الأجل ، كما أن معدل تركيز الأجسام المضادة يقل مع تقدم العمر وهناك حاجة لجرعة مقوِّية أخرى (booster dose) من كلا اللقاحين.

Dedication

To my father,

my mothers' pure spirit,

my husband and my child.

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LIST OF ABBREVIATIONS

BCG	Bacillus Calmette-Guerin
C. diphtheria	*Corynebacterium diphtheria*
C.tetani	*Clostridium Tetani*
DPT	Diphtheria-pertussis-tetanus vaccine
DTaP	Diphtheria tetanus acellular pertussis
DTwP	Diphtheria Tetanus whole cell Pertussis
ELISA	Enzyme Linked Immunosorbent Assay
EMR	Eastern Mediterranean Region
eIPV	enhanced inactivated poliovirus
EPI	Expanded Program for Immunization
FDA	Food and Drug Administration
FT	Full-term infants
GMT	Geometric mean titer
HA	Passive hemagglutination test
HAI	Haemoagglutination inhibition test
HB	Hepatitis B vaccine
HBsAb	Hepatitis B surface antibody
Hib	*Haemophilus influenzae* type b
Hib-PRP	*Haemophilus influenzae* type b polyribosylribitol phosphate
HRP	Horseradish peroxidase
IPV	Intramuscular Polio Vaccine
IU	International units
Lf unit	Flocculation unit
MHC	Major histocompatibility complex
MMR	Measles Mumps Rubella vaccine
NIS	Newly Independent States
PRP	polyribosylribitol phosphate
PT	Preterm infants

OD	Optical Density
OPV	Oral polio vaccine
RIA	Radioimmunoassay
Td	Preparation of diphtheria and tetanus toxoids with a low amount of diphtheria toxoid, for adolescents and adults
TdaP	Tetanus diphtheria acellular pertussis
TMB	Tetramethylbenzidine
ToBI	Toxin Binding Inhibition test
TT	Tetanus Toxoid
WHO	World Health Organization

1.1. Background of the problem

Although diphtheria and tetanus infectious diseases occur rarely in the world, the outbreak of diphtheria in the former Soviet Union (1990-1993) is a reminder that even a well controlled infection can reemerge when herd immunity is not maintained, but recent large epidemics of diphtheria in several eastern European countries have again drawn attention to this forgotten disease [1].

Changes in the epidemiology of diphtheria occur worldwide. A large proportion of adults in many industrialized and developing countries are now susceptible to diphtheria. Vaccine-induced immunity wanes over time unless periodic booster is given or exposure to toxigenic *C. diphtheriae* occurs. Immunity gap in adults coupled with large numbers of susceptible children creates the potential for new extensive epidemics. Epidemic emergencies may not be long in coming in countries experiencing rapid industrialization or undergoing sociopolitical instability where many of the factors thought to be important in producing epidemic such as mass population movements and difficult hygienic and economic conditions are present. The continuous circulation of toxigenic *C. diphtheriae* emphasizes the need to be aware of epidemiological features, clinical signs, and symptoms of diphtheria in vaccine era so that cases can be rapidly diagnosed and treated, and further public health measures can be taken to contain this serious disease [1].

Diphtheria is an acute, toxin-mediated disease caused by *C. diphtheriae,* which is an aerobic gram-positive bacillus. Toxin production (toxigenicity) occurs only when the bacillus is itself infected (lysogenized) by a specific virus (bacteriophage) carrying the genetic information for the toxin (tox gene), only toxigenic strains can cause severe disease [2].

Like other respiratory infections, transmission is increased in over-crowded and poor socio-economic conditions. In temperate climates, prior to vaccination, respiratory diphtheria commonly affected preschool and school-age children [3]. Susceptible persons may acquire toxigenic diphtheria bacilli in the

nasopharynx [2].

The organism produces a toxin that inhibits cellular protein synthesis and is responsible for local tissue destruction and membrane formation. The toxin produced at the site of the membrane is absorbed into the bloodstream and then distributed to the tissues of the body. The toxin is responsible for the major complications of myocarditis and neuritis and can also cause low platelet counts (thrombocytopenia) and proteinuria. Clinical disease associated with non-toxin-producing strains is generally milder. While rare severe cases have been reported, these may actually have been caused by toxigenic strains which were not detected due to inadequate culture sampling [2].

Large epidemics occurred in Europe during and after the Second World War, with an estimated one million cases and 50,000 deaths in 1943. Nasal diphtheria may be mild and chronic carriage of the organism frequently occurs; asymptomatic infections are common. A cutaneous form of diphtheria is common in tropical countries, and may be important in transmission [4].

Recently, a major epidemic of diphtheria occurred in countries of the former Soviet Union beginning in 1990. By 1994, the epidemic had affected all the 15 Newly Independent States (NIS). More than 157,000 cases and more than 5,000 deaths were reported. In the six years from 1990 through 1995, the NIS accounted for more than 90 % of all diphtheria cases reported to the WHO from the entire world. In some NIS countries, up to 80% of the epidemic diphtheria cases have been recorded among adults. The outbreak and the age distribution of cases are believed to be due to several factors, including a lack of routine immunization of adults in these countries [2].

In contrast to the majority of diseases caused by microbes and against which vaccination affords protection, the possibility of eradicating the tetanus microbe is not practicable. The bacterium is ubiquitous, especially in the soil, thus we shall always have to depend upon vaccination to avoid tetanus [5].

Tetanus is an acute, often fatal, disease caused by an exotoxin produced by *C. tetani*. It is characterized by generalized rigidity and convulsive spasms of

skeletal muscles. The muscle stiffness usually involves the jaw (lockjaw) and neck and then becomes generalized [6].

C. tetani is a slender, gram-positive, anaerobic rod that may develop a terminal spore, giving it a drumstick appearance. The organism is sensitive to heat and cannot survive in the presence of oxygen. The spores, in contrast, are very resistant to heat and the usual antiseptics. They can survive autoclaving at 121°C for 10-15 minutes. The spores are also relatively resistant to phenol and other chemical agents. The spores are widely distributed in soil and in the intestine and feces of horses, sheep, cattle, dogs, cats, rats, guinea pigs, and chickens [6].

C. tetani usually enters the body through a wound. In the presence of anaerobic (low oxygen) conditions, the spores germinate. Toxins are produced, and disseminated via blood and lymphatics. Toxins act at several sites within the central nervous system, including peripheral motor end plates, spinal cord, brain, and sympathetic nervous system. The typical clinical manifestations of tetanus are caused when tetanus toxin interferes with release of neurotransmitters, blocking inhibitor impulses. This leads to unopposed muscle contraction and spasm. Seizures may occur, and the autonomic nervous system may also be affected [6].

1.2. Diphtheria and tetanus in the Eastern Mediterranean (EMR)

Since the introduction of diphtheria and tetanus vaccination in the EMR, there is gradual decrease in the incidence of both diseases in this region; table 1 shows the epidemiology of them since 1990 to 2004.

Table 1: Reported annual morbidity due to EPI target diseases in the EMR, 1990-1994, 2003-2004

Disease	**1990** [7]	**1991** [8]	**1992** [9]	**1993** [10]	**1994** [11]	**2003** [12]	**2004** [13]
Neonatal tetanus	4 907	5 209	4 122	3 335	3 152	1458	910
tetanus	5247	1924	1 434	1486	989	664	199
Diphtheria	3 763	1 464	1 047	404	312	329	145

The highest number reported in the Eastern Mediterranean of adult tetanus cases in 2003 was in Egypt in which 407 cases were reported. [12]

1.3. Vaccination schedule

For immunization there is no simple and universal schedule for immunization, the choice of an appropriate schedule depends on the epidemiological pattern of diphtheria. In developing countries where the reservoir of *C. diphtheriae* is still large and natural immunity plays a significant role in protection against the dangerous, pharyngeal form of the disease, the first priority is to ensure high coverage of infants with the primary series of three doses of DPT vaccine. Priority should be given to achieving at least 90% coverage [4].

In developing countries which have already achieved high coverage with three doses of DPT vaccine in children under one year of age, the policy of using a booster dose of DPT vaccine at the end of the second year of age and/or a dose of DT or Td at school entry should depend on the pattern of diphtheria and the availability of the vaccines. If diphtheria poses a significant health problem in preschool or school-age children, supplementary doses of

diphtheria toxoid may be warranted. Data from serological studies which show declining antibody levels may serve as a valuable guide in deciding when booster doses are warranted [4].

The use of DT or Td vaccine at school entry or leaving must be important for providing anti-tetanus immunity for these ages. Health authorities need to consider the time required to deliver these additional vaccine doses and balance this against the time needed for other services. The cost of additional doses should also be considered [4].

In developed countries, primary immunization usually-consists of three doses of DPT vaccine, given at intervals of 4 or more weeks, beginning at 2 or 3 months of age, and reinforced by a fourth dose given in the second year of life. The policy of using booster doses of vaccines containing diphtheria (and tetanus) toxoid varies considerably. In some countries, booster doses of DPT vaccine are given above the age of 3 years (Hungary, and United States). In many other countries, booster doses of DT vaccine are given at primary school entry and at school leaving. Many countries, however, give only monovalent tetanus toxoid to older school children [4].

The immunity level acquired in infancy and early childhood should be maintained through properly timed booster doses of DT or Td vaccine. Td vaccine should be used for older children or adolescents leaving primary or secondary schools [4].

Immunization schedule in Gaza Strip [14]

In Gaza Strip the approved vaccination schedule which is applied in the primary health care centers is the expanded program for immunization (EPI) of WHO, this program is shown in table 2.

Table 2: Vaccination schedule in Gaza Strip

Age / Vaccine	HB	BCG	DPT	OPV	IPV	Td	MMR	DT	Measles	Rubella
1 day	■	■								
1 month	■				■					
2 months			■	■	■					
4 months			■	■						
6 months	■		■	■						
9 months									■	
12 months			■	■						
15 months							■			
6 years		■		■				■		
12 years										**Girls only**
15 years						■				

1.4. Aim of the study

Since there is no previous study to evaluate diphtheria and tetanus vaccination program in Gaza, this study aims at assessing the efficacy of diphtheria and tetanus vaccines in children after the four essential doses of DPT, and after the DT booster dose which is given at the school entry age, to evaluate the immunogenicity of this dose and to follow up the duration of immunity by measuring the titer after 4 years. This could be achieved by the determination of tetanus and diphtheria antibody level after vaccination and to compare this level with the approved level which gives protection against these diseases.

1.5. Significance

Measurement of diphtheria and tetanus antitoxioid titer among DPT vaccinated children and after the DT booster dose which will enable us to detect:

- If this level is protective according to the recommendation of WHO.

- The duration of protection within the first 3 years following DPT vaccination.
- The immunogenity and effectiveness of DT vaccine

1.6. Statement of the problem

- Do all children until 12 years have antibody level more than the protective level according to the recommendation of WHO?
- What is the percentage of children with antibody titer less than the protective level?
- Is there a difference among age groups?
- Is there a difference in antibody titers before and after the DT booster dose?
- Is there a need for another Td booster dose?
- Is there a difference in titer between male and female?

1.7. Specific objectives

- Measurement of diphtheria and tetanus antibody titers.
- Find antibody titers less than the protective levels
- Duration of immunity to diphtheria and tetanus.
- Compare between male and female.

1.8. Delimitation

The study was confined to specific groups of children in Gaza strip and covers the period from May to July 2005.

2.1. Historical background

The concept of immunization was based on the observation that those who survived certain diseases often failed to develop the disease a second time. The practice of inoculating material from smallpox pustules for the prevention of the disease (variolation) was practiced in China, India and Presia long before it was introduced into Europe. Lady Mary Wortely Montague (1689-1762), wife of the British Ambassador at Constantiople, saw variolation carried out by Turkish women, and credited with its popularization in England [15]. Edward Jenner at (1798) showed that inoculating people with fluid obtained from the skin lesions of cows who where infected with cowpox virus protected them from the highly infectious and fatal disease, smallpox. This process came to be called vaccination. Jenners theory was advanced by the work of other scientist, including Louis Pasteur, Benjamin Waterhouse and Thomas Cimsdale. Pasteur who defined the concept of virulence, showed that virulence may be altered during growth in suboptimal conditions or in unrelated host, and the organism become attenuated, but retain the capacity to stimulate the immune system [16], this concept led to the use of attenuation as a mean of vaccination [15].

In 1888, Roux and Yersin noted that the diphtheria bacillus produced an exotoxin in liquid culture, and showed that the disease could be reproduced by injection of the bacteria free medium of a diphtheria culture and two years later, Von Behring and Kitasato developed a diphtheria antitoxin in horses and demonstrated that serum from an individual exposed to the toxin protected against exposure to the same toxin [17].

As shown small pox vaccine was the first vaccine used at1798 followed by other vaccines against several diseases such as rabies, plague, diphtheria, pertussis, BCG (tuberculosis), tetanus, yellow fever, polio, measles, mumps, rubella and hepatitis B virus. Table 3 shows the date of introduction the first generation of vaccines for use in humans.

Table 3: The date of introduction of the first generation of vaccines for use in humans [18].

1798	Smallpox	1935	Yellow Fever
1885	Rabies	1955	Injectable Polio Vaccine (IPV)
1897	Plague	1962	Oral Polio vaccine (OPV)
1923	Diphtheria	1964	Measles
1926	Pertussis	1967	Mumps
1927	BCG (tuberculosis)	1970	Rubella
1927	Tetanus	1981	Hepatitis B virus

2.2. Types of immunization

There are two major types of immunization

2.2.1. Active immunization

Active immunization is induced when an immunocompetent host develops an immune response as the result of exposure to an immunogen [15]. Usually, both humoral and cell mediated responses are evoked, and the immunogen is recognized and eliminated. Active immunization can be induced by natural or artificial means. Natural active immunization occurs when a host is exposed to a pathogen, and develops immunity against it. This immunity gives protection when re-exposed to the same pathogen. By contrast, artificial active immunization involves administration of a vaccine that contains a killed or avirulent form or an immunogenic component of a pathogen, designed to elicit protective immunity. On subsequent exposure to the infectious pathogen, it is recognized and eliminated, thereby affording protection against the disease [15].

2.2.1.1 Types of active immunization

2.2.1.1.1. Inactivated whole organism vaccines

Most bacterial vaccines are of this type. They include pertussis, cholera, typhoid and plague vaccines which are produced by killing the microorganisms with heat or by treatment with chemicals such as formaldehyde or phenol [15]. Non-living virus vaccines are available for the prevention of diseases caused by influenza, poliomyelitis (Salk) and rabies. These vaccines are prepared from viruses that grow in eggs; a continuous monkey kidney cell line, or human diploid fibroblasts, virus is then inactivated with formalin [19]. Although the infectivity of the pathogen is destroyed by these treatments, much of their antigenic integrity remains [15].

2.2.1.1.2. Whole organism attenuated vaccine

Live vaccines are derived from a wild, or disease causing, virus or bacterium. The wild virus or bacterium is attenuated, or weakened, in a laboratory, usually by repeated culturing [20] in cell culture prepared from an unnatural host, leading to the emergence of mutants [19]. Although a number of viral vaccines are attenuated, such as, measles, mumps, rubella, vaccinia, varicella, yellow fever, influenza and oral polio. The only bacterial vaccines in this category are the bacillus Calmette-Guerin (BCG) strain of *Mycobacterium bovis*, used to vaccinate against tuberculosis and oral typhoid vaccine [20].

A relatively small dose of virus or bacteria is given, which replicates in the body and creates enough virus or bacteria to stimulate an immune response which resembles the natural infection including humoral and cell mediated immunity, while inactivated vaccines are not alive and can't replicate, they induce only humoral immunity and the protective immune response develops after the second or third dose [20].

2.2.1.1.3 Toxoids

Toxins can be inactivated to make harmless toxoids which are used for vaccination. Administration of toxoids prepared from inactivated tetanus, botulism or diphtheria toxins elicit antibody response that neutralizes infection [21].

The toxicity is removed by the treatment with formalin, and the inactivated toxin always adsorbed to alum. Adjuvant evokes high titers of antitoxic IgG antibodies [15]. Toxoids are effective despite the fact that natural infection dose not always confer long-lasting immunity, presumably because the amount of toxin produced in infection may not be sufficient to elicit a strong immune response [21].

2.2.1.1.4. Polysaccharide vaccines

Polysaccharide vaccines are a unique type of inactivated subunit vaccine composed of long chains of polysaccharides [20]. Pure polysaccharide vaccines are available for *Streptococcus pneumonia, Neisseria meningitides and Salmonella typhi*. Humoral immunity is important for protection against encapsulated pathogens, which are usually poorly immunogenic. Another problem with polysaccharide vaccines is that young children tend not to mount antibody responses to polysaccharide antigens [21]. An efficient way of overcoming this problem is to chemically conjugate bacterial polysaccharide to protein carriers, which provides immunogen that can be recognized by antigen-specific cells, thus avoiding a T-cell independent response. By using this approach, various conjugate vaccines have been developed against *Haemophilus Influenzae,* an important cause of serious childhood chest infections and meningitis, and these are now widely applied [22].

2.2.1.1.5. Synthetic peptide vaccines

Synthetic peptide vaccines are designed using the knowledge of the amino acid sequence of the protein antigen that elicits a protective immune response. In theory, synthetic peptide vaccines have the advantage that highly purified peptides may be made in large quantities and their simpler antigenic composition may afford protection with fewer side effects [21]. A problem in this type of vaccine is that peptides are not strongly immunogenic and it is particularly difficult to generate MHC class I- specific responses by *in vivo* immunization with peptides. One approach to solve the problem is to integrate peptides by genetic engineering into carrier proteins with a viral vector, such as hepatitis B core antigen, which are then processed *in vivo* through natural antigen- processing pathway [22]. A second possible technique is the use of immune stimulatory complexes which are lipid carriers that act as adjuvants but have minimal toxicity. They seem to load peptides and proteins into the cell cytoplasm allowing MHC class-I restricted T-cell responses to peptides to develop [22], or by use of multiple repeating peptides to enhance immunogenicity [23]. Another disadvantage of peptide as vaccines is the configuration and recognition of peptides by immunoresponsive cells [24]. Peptides usually present as linear determinants and are recognized by T cells in the context of MHC molecules and B cells, but the latter also recognize conformational determinants. Thus, the generation of B cell response against a protective conformational determinant may not be possible [19].

2.2.1.1.6. Anti-idiotype antibody vaccines

An antibody (idiotype) has idiotopes which are the hypervariable domains of the antibody molecule that serve as the paratope or antigen combining site. Therefore, the idiotopes of the antibody is the mirror image of the epitope or antigenic determinant that it binds, when an antibody (AB1) used to generate anti-idiotype antibody (AB2) the latter will structurally mimic the epitope, this is described as internal image of the epitope likewise, when an anti-idiotype antibody (AB2) is used to generate anti-idiotopes antibody (AB3) the latter recognized the AB2 idiotope because it is the mirror image of the epitope, similar to that of AB1 [25] since the idiotopes of anti-idiotypic antibodies could be used in vaccine preparation as a substitute for the protective epitope(s) of pathogen. Anti-idiotypic antibodies are especially useful when the epitope(s) of the pathogen is difficult to identify or synthesize [15]. Anti-idiotype antibody vaccines are safe and free from the adverse side effects associated with vaccine preparations that contain whole cell pathogen or its components. A major disadvantage of anti-idiotype antibodies in vaccine preparation is that their immunogenicity is weak. Experimental anti-idiotype vaccines have been developed for Hepatitis B [24].

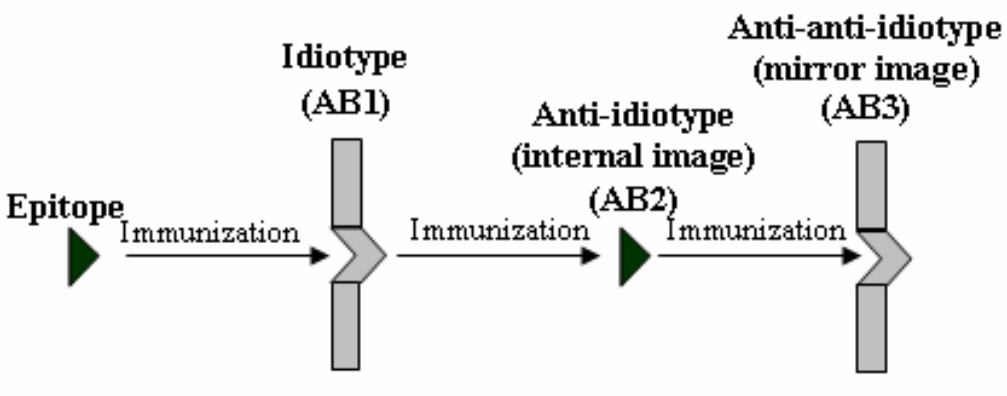

Figure 1: Anti-idioptype antibody vaccines production.

2.2.1.1.7. DNA immunization

Recombinant DNA technology provides the means for expressing protein antigens in large amounts for vaccine use [21]. The gene that encodes the protective antigen is identified, and then the gene is introduced into a suitable vector in which the desired recombinant protein is expressed in large quantities and then purified by chemical methods.

Types of vectors:

1- Viral and bacterial vectors

The gene that encodes the protective antigen is inserted into the genome of the attenuated microorganism, the inserted gene together with genome of the microorganism is transcribed and translated and the desired protective antigen along with other proteins of the microorganism is expressed. These vaccines elicit humoral and cell mediated immune response [25]. Several viruses may be used for this purpose such as vaccinia, adenovirus and bacteria such as *Salmonella typhimurium*, *Escherichia coli* and *Bacillus Calmette-Guerin* [21].

2- Plasmid vectors

Vaccination with a plasmid encoding the DNA sequence for a protective antigen linked to a strong mammalian promoter can elicit an immune response to the expressed protein [21]. The plasmid vectors are taken up, usually by muscle cells, at the site of inoculation; they remain extrachromosomal within these cells and are transcribed and translated into the desired immunogen. The proteins expressed by nucleic acid vaccination are usually processed and presented by MHC class I pathway, and elicit cytotoxic T cell responses. Helper T cell and antigen specific humoral responses are also elicited [26].

DNA vaccines have a group of advantages such as the absence of infection risk, greater stability relative to protein vaccines and could be useful for immunizing young children who still have maternal Abs [21]. The feasibility of DNA immunization has now been demonstrated against several viral, bacterial, and protozoal infections in laboratory animals, several DNA vaccines are undergoing testing in humans to determine their usefulness in prevention or treatment of HIV, malaria and Hepatitis B. However, no DNA vaccines are currently used in humans [21].

2.2.1.1.8. Edible vaccines

The idea of edible vaccines, resulting from the expression of antigenic proterins in transgenic plants, comes from Arntzen and colleagues. In the first instance, the gene for HBsAg was inserted into cells of tobacco plants, and these produced antigen that was immunogenic on injection. The heat-labile enterotoxin of *E.coli* has been engineered into potatoes, which accumulate 1 mg per raw potato. The virus capsid antigen of Norwalk virus had been similarly engineered, and 5-g samples were fed to mice, which produced serum and secretory antibodies. Currently, efforts is being directed at the genetic engineering of bananas. The long term hope is the possibility of a multisubunit vaccine, incuding an oral adjuvant, which could be eaten, and could be cheap and acceptable in a third world setting [27].

2.2.2. Passive immunization

Passive immunization is the administration of preformed antibodies produced by another individual or animal. This method of immunization provides protection against a pathogen or toxin without the need for a course of immunization The most common reason for the passive immunization of healthy

immunocompetent individuals is exposure to a toxin or poison [15], passive immunization can occur naturally as is the case during transfer of antibodies through the colostrum or therapeutically when performed antibody is administered for the prophylaxis or therapy of infectious disease [21] such as diphtheria, botulinum and tetanus toxins, after known or presumed exposure to rabies [15]. Immunocompromized or immunodeficient individuals, who cannot mount humoral response, may also be given passive immunization. Children with hypogammaglobulinemia, individuals with AIDS, patients receiving chemotherapy, and organ transplant recipients receiving immunosupressive therapy cannot respond appropriately to natural infection or active immunization and, therefore may require passive immunization and supportive immunoglobulin transfusion to prevent severe infection [15].

A major disadvantage of passive immunization is its brief period of effectiveness, transfused immunoglobuins have a short half-life (typically 25 days for IgG) and passive immunization does not evoke immunological memory in the recipient [15].

2.3. Diphtheria and tetanus toxoids

Diphtheria and tetanus toxoids are prepared by treating a cell-free purified preparation of toxin with formaldehyde thereby converting it into harmless toxoid, which is poor immunogen and for use as a vaccine it is usually adsorbed onto an adjuvant either aluminum phosphate or aluminum hydroxide [28].

2.3.1. Diphtheria toxoid

Beginning in the early 1900s, prophylaxis was attempted with toxin-antitoxin mixtures. Toxoid was developed around 1921, but was not widely used

until the early 1930s. It was incorporated with tetanus toxoid and pertussis vaccine and became routinely used in the 1940s [2]**.**

2.3.2. Tetanus toxoid

Tetanus toxoid was first produced in 1924. Tetanus toxoid immunizations were used extensively in the armed services during World War II. Tetanus cases among this population dropped from 70 in World War I (13.4/100,000 wounds and injuries) to 12 in World War II (0.44/100,000). Of the 12 cases, half had received no prior toxoid. Tetanus toxoid consists of a formaldehyde-treated toxin. There are two types of toxoid available — adsorbed (aluminum salt precipitated) toxoid and fluid toxoid. Although the rates of seroconversion are about equal, the adsorbed toxoid is preferred because the antitoxin response reaches higher titers and is longer lasting than the fluid toxoid [6].

Single antigen diphtheria toxoid is not available [2], while tetanus toxoid is available as a single antigen preparation, combined with diphtheria as pediatric DT or adult Td, and with both diphtheria toxoid and acellular pertussis vaccine as DTaP. Pediatric formulations (DT and DTaP) contain a similar amount of tetanus (5 lf* units) toxoid as adult Td, but contain 3-4 (7-8 lf unit) times as much diphtheria toxoid. Children younger than 7 years of age should receive either DTaP or pediatric DT. Persons 7 years of age or older should receive the adult formulation (adult Td), even if they have not completed a series of DTaP or pediatric DT [29].

***lf unit**: Is a unit used to express the concentration of tetanus or diphtheria toxoid. Flocculation test used for the quantitative estimation of toxoids in Lf, the concentration of unknown diphtheria or tetanus toxoid preparation is estimated in relative to reference preparations of tetanus and diphtheria antitoxins, respectively (31).

Each 0.5 ml dose of adsorbed DPT vaccine consists of a mixture in isotonic saline of diphtheria and tetanus toxoid with a potency of the diphtheria component not less than 30 IU, and that of tetanus components not less than 40 IU, and that of whooping cough is not less than 4 IU (which is nearly equal to 20.000 million organisms), these components are preserved by 0.01 thiomersal [30].

2.3.3. The nature of immunity to diphtheria and tetanus

Immunity against diphtheria is antibody-mediated. Because the lethality of diphtheria is almost entirely due to diphtheria toxin, immunity to diphtheria depends primarily on antibody against the toxin. This antibody, called antitoxin, is primarily of the IgG type. Diphtheria antitoxin may be induced by diphtheria toxin produced by *C. diphtheriae* during the disease or the carrier state, or by diphtheria toxoid following immunization. These antibodies are identical and cannot be distinguished by any existing techniques [4].
Immunity to tetanus toxin is induced only by immunization; recovery from clinical tetanus does not result in protection against further attacks [29].

2.4. Techniques for measuring antibody response

2.4.1. Diphtheria

2.4.1.1. Schick test

In early studies the Schick test was the standard procedure. To perform the Schick test, 0.1 ml of diphtheria toxin (about l/50 of the minimal lethal dose for a guinea pig) is injected intradermally on the volar surface of the forearm of the person being tested. If the person has circulating diphtheria antitoxin at a

level of 0.01 to 0.03 IU/ml, the injected toxin will be neutralized and no reaction will occur. A positive reaction signifies lack of antitoxin and is characterized by inflammation appearing after 24 to 36 hours and persisting for 4 days or longer. A control test is always performed on the opposite arm using toxin inactivated by heating to 60°C for 15 minutes. A positive reaction to inactivated toxin and a positive reaction to toxin indicates an allergic response to toxin [4].

2.4.1.2. Neutralization test on animals

The *in vivo* neutralization test is usually performed on the depilated skin of rabbits or guinea pigs. Different dilutions of serum mixed with fixed amounts of diphtheria toxin are injected into the depilated skin of the animal and the antitoxin concentration is estimated based on the presence or absence of an inflammatory reaction [4].

2.4.1.3. Neutralization test on microcell cultures

The neutralization test on microcell cultures is based on the observation that the survival of mammalian cells in culture is inhibited by diphtheria toxin. This effect is neutralized when diphtheria antitoxin is present in serum supposed to contain samples. The titration of the antitoxin in the serum samples is done in plastic microtissue culture plates, in which dilutions of test sera are mixed with challenge toxin. After a short incubation, Vero (green monkey renal epithelium) cell or HeLa cell suspension in a special culture medium is added. After incubation for 3 or 4 days, results are read as a change in the color of the reagents in the microtiter plate wells. The color change is due to the metabolic formation of acid, which changes the pH. Vero cells are more sensitive to diphtheria toxin since they have large numbers of binding sites (receptors) and they take up the toxin in a highly specific, time- and temperature- dependent

manner [4]. When a serum dilution contains antitoxin in excess, the cells continue to grow, and the color of the medium changes from red to yellow. Recent improvements in the microcell neutralization test include spectrophotometric determination of the equivalence point between toxin and antitoxin and computer analysis of absorption values [32].

2.4.1.4. Passive hemagglutination

The passive hemagglutination (HA) test is frequently used to test for diphtheria antibody [33]. In the HA test, sheep, turkey, horse, or human red cells (previously treated with tannic acid or diazotized benzidine and sensitized with diphtheria toxoid) are agglutinated by diphtheria antibody. In the absence of antibody hemagglutination does not take place "Passive hemagglutination". The HA test is inexpensive and can be performed in a modestly equipped laboratory. The HA test is rapid (results available in one hour), reproducible, and sensitive. Results of the HA test for diphtheria correlate well with results of the neutralization test [34].

2.4.1.5. ELISA

The enzyme-linked immunosorbent assay (ELISA) involves the binding of antigen to polystyrene tubes, antibody such as bacterial anti-toxoid in the test solution is allowed to react and form a complex with the antigen. An enzyme-labeled antibody against the bound antibody (usually anti-IgG) is then attached to the antigen-antibody complex. The amount of enzyme bound, which indicates the amount of antibody in the test serum, can be measured by the change of suitable enzyme substrate concentration. Usually, the substrate is chosen so that when it reacts with enzyme there is a color change which can be assessed visually or photometrically [29].

2.4.2 Tetanus

2.4.2.1 Neutralization test in vivo

The *in vivo* neutralization test directly measures the biological activity of tetanus antitoxin by demonstrating the toxin-neutralizing property of serum in laboratory animals, usually mice. The neutralization test is expensive, time consuming, requires well trained personnel, a large number of animals, and a relatively large amount of serum. The neutralization test is a sensitive test which can detect an antitoxin level of one-thousandth IU/ml. This test is primarily a measure of serum IgG antitoxin [29].

2.4.2.2. Passive hemagglutination

The design of the passive hemagglutination (HA) test is simple: carrier red cells sensitized with tetanus toxoid agglutinate in a specific way in the presence of tetanus antibodies. The HA test has been widely used to assess the immune status of various age, sex, socioeconomic, and professional groups [35], to evaluate the duration of immunity post-immunization [36], and to compare the effectiveness of different vaccines and immunization schedules [29]. The HA test has also been used for screening serum or plasma donated for production of human anti-tetanus immunoglobulin [37] and for detecting tetanus antibody in the serum of injured persons [29].

2.4.2.3. ELISA

ELISA test is commonly used to assess tetanus antibody titers [38], the principle of the test have been previously described in section 2.4.1.5.

2.4.2.4 Other tests

Radioimmunoassay (RIA) tests have been used to titrate tetanus antibodies. There are several possible modifications of the RIA test; tetanus toxoid can be coupled with an insoluble sorbent, such as cellulose or agarose [29], or adsorbed passively onto a plastic surface as in the ELISA test. The specific antibodies bind to the antigen immunosorbent and are quantified by measuring the incorporation of isotope-labeled human antiglobulin attached to the antigen-antibody complex. The sensitivity of the RIA test is high and the results correlate well with values obtained by the HA test [39] and the ELISA test [40].

2.5. Protective level of antibodies

2.5.1. Diphtheria

It is believed that a circulating diphtheria antitoxin level of 0.01 IU/ml, as determined by the neutralization test in animals or in cell culture, provides clinical immunity against disease. This diphtheria antitoxin level corresponds to a negative Schick test. There is good correlation between clinical protection and the presence of serum antitoxin, whether this results from disease or immunization. In the 1984 diphtheria epidemic in Sweden, all seven patients who died or showed neurological complications had antitoxin titers < 0.01 IU/ml, whereas 92% of symptom- free diphtheria carriers showed high antitoxin titers, above 0.16 IU/ml [41]. However, it has also been shown that there is no sharply defined level of antitoxin that gives complete protection from diphtheria [4]. A certain range of variation must be accepted; the same degree of antitoxin may give an unequal degree of protection in different persons. Other factors may influence the vulnerability to diphtheria including the dose and virulence of the diphtheria bacilli and the general immune status of the person infected [42]. Thus, an antibody concentration between 0.01 and 0.09 IU/ml may be regarded

as giving basic immunity, whereas a higher titer may be needed for full protection. In most studies that used *in vitro* techniques, a level of 0.1 IU/ml was considered protective [33, 43].

2.5.2. Tetanus

The amount of circulating antitoxin needed to ensure complete immunity against tetanus is not known for certain. Establishment of a fixed level of tetanus antitoxin does not take into consideration the variable conditions of production and adsorption of tetanus toxin in the anaerobic area of a wound or a necrotic umbilical stump. A given serum level could be overwhelmed by a sufficiently large dose of toxin [29]. Therefore, there is no absolute protective level of antitoxin and protection results when there is sufficient toxin-neutralizing antibody in relation to the toxin load [44].

Immunological memory and the ability to respond quickly to booster doses of tetanus toxoid may be as important as the level of circulating antibody in determining the outcome of infection with tetanus spores [29].

Therefore, although the protective role of tetanus antitoxin is well documented, the establishment of a protective level has been somewhat arbitrary. Antitoxin activity is expressed in international units (IU) and a tetanus antitoxin level of 0.01 IU/ml serum is considered the minimum protective level. This ‘‘protective’’ level is based on animal studies that correlate antitoxin levels with symptoms or death. Experimental human data are limited and direct observations on ‘‘protective’’ levels of antibody are rare [29].

Wolters and Dehmel injected themselves with a dose of tetanus toxin equivalent to 2 or 3 human doses (calculated by weight, based on guinea pig experiments). Their postvaccination levels of serum antitoxin were 0.004 to 0.005 IU/ml and they did not suffer from tetanus after intramuscular administration of tetanus toxin [45]. The interpretation of this extraordinary

experiment is limited by ignorance about what is a real ‘‘human dose’’ of tetanus toxin [29].

Passen et al. (1986) described a case of severe, generalized tetanus in a person who had been fully immunized in childhood and who had received booster injections eight and four years before the disease. The antitoxin level was 0.16 IU/ml at the onset of the disease. The prognosis in this patient was considered poor because of the short incubation period, rapid progression from the initial symptoms to generalized spasms, and severe disease manifestation at admission. His survival and rapid recovery may have been the consequence of partial protection from pre-existing neutralizing antibody, good antibody response to toxoid doses given during the acute illness, and his young age and good general health [44].

2.6. Vaccination evaluation studies

Weiss et al [46] studied tetanus and diphtheria immunity in an elderly population in Los Angeles, this was by taking two groups of random samples from peoples > 65 years age one of them was from the attending and another from the convalescent hospital then diphtheria and tetanus antitoxin levels were determined by the hemagglutination assay. The protective level was considered 0.01 unit/ ml, 45% of the senior Citizen center, and 29% of the convalescent hospital individuals were protected against tetanus by having antitoxin level > 0.01 unit/ ml, 48.5% of the senior Citizen center, and 55.9% of the convalescent hospital were protected against diphtheria, age specific immunity was lower for male than female, and generally decreased with age.

Pichichero et al [47] studied the immune response to the first booster following the diphtheria and tetanus toxoid vaccines primary series which was given to children at 2, 4 and 6 months of age, all these children developed protective antitoxin antibody levels to diphtheria and tetanus antigens following

vaccination. By follow-up, the study showed that the antitoxin antibody levels were decreased at 18 months of age and dropped below the protective level in 4 children, a booster DT dose was given to 23 children and 38 children received DPT, following the 18-month booster dose of DT and DTP vaccine, all of the children had protective titers to diphtheria and tetanus toxin. These results suggest that the adjuvant effects of pertussis vaccine are not required to achieve adequate immunization to diphtheria and tetanus.

Schou et al [48] determined tetanus and diphtheria antitoxin content in dried samples of capillary blood. Capillary blood was drawn from 51 randomly selected healthy infants 2 years of age. Concentration of antitoxin to tetanus and diphtheria was assessed with ELISA and in vitro toxin neutralization assay respectively. Mean diphtheria antitoxin concentration was 0.53 IU/ml, and mean tetanus antitoxin concentration was 4.1 IU/ml.

Koblin et al [49] studied the immunity to diphtheria and tetanus in Inner-City women of childbearing age. Diphtheria and tetanus antitoxin titers were measured by the indirect hemagglutination assay, and 0.01 unit/ml was considered as protective level, 18.5% of the women had levels of diphtheria antitoxin below the protective level, whereas 4.3% had insufficient levels of tetanus antitoxin. The percentage of women susceptibility increased with age; non were susceptible under the age of 20, 33% and 25% of women over the age of 30 years were susceptible to diphtheria and tetanus, respectively. In every age group the percentage of women susceptible was greater to diphtheria than tetanus.

Mark et al [50] studied the level of immunity to diphtheria and the effect of vaccination with different doses of diphtheria toxoid. Blood samples were collected from children, 6, 10 and 16 years of age. Infants received routine primary vaccination with three doses of diphtheria-tetanus-toxoid or diphtheria-

tetanus-pertussis vaccine, and the 16 year-olds also had received a booster dose of tetanus with a small dose of diphtheria at the age of ten. Prior to the study booster, 15 % of the 6-year-olds had antitoxin levels against diphtheria <0.01 IU/ml; the given minimum level for protection. Of the 10-year-olds, 48 % had titers <0.01 IU/ml, while the corresponding figure for the 16-year-olds was 24 %. After a booster injection of 0.1, 0.25 or 0.5 ml of diphtheria-tetanus vaccine, more than 97 % of the children showed titer levels ≥0.1 IU/ml. In another group of 5-years-olds given diphtheria-tetanus primary vaccinations over wider intervals, only 1.4 % had antitoxin titers <0.01 IU/ml. The results show a need for serologic monitoring of vaccination programs.

Lagergard et al [51] determined the neutralizing antibodies and specific immunoglobulin isotype levels in a group of Swedish infants after vaccination against diphtheria. Serum samples were obtained from 44 infants vaccinated against diphtheria at the ages of 3, 5 and 12 months with an aluminium-adsorbed diphtheria-tetanus toxoid vaccine. Toxin-neutralizing antibodies (antitoxiod) were measured by the Vero cell assay and IgG, IgM and IgA antibodies against diphtheria toxoid by ELISA. A neutralizing antibody titer of 0.01 IU/ml is considered necessary for short-term protection. All children between 6 and 30 months of age had titers greater than or equal to 0.01 IU/ml. At 30 months only 48% had titers of greater than or equal to 0.1 IU/ml, the level considered necessary for long-term protection. Geometric mean of IgG antibody levels at 3, 5, 6, 12, 13 and 30 month were 13, 36, 216, 64, 649 and 57 IU/ml, respectively. IgM antibodies were only detected after the third vaccination. IgA antibodies were not detected in any serum sample from the ten infants tested.

Gupta et al [52] studied Diphtheria antitoxin levels in US blood and plasma donors. Plasma samples from 500 blood donors were titrated for diphtheria antitoxin by the toxin neutralization test. Only 1.6% of donors had <0.01 IU/mL diphtheria antitoxoid, the minimum protective level against

diphtheria; 15% had levels between 0.01 and <0.1 IU/mL, indicating basic protection, and 83.4% had levels ≥0.1 IU, indicating full protection.

Pasetti et al [53] studied serum antibodies to diphtheria-tetanus-pertussis vaccine components in Argentine children. The Argentine vaccination schedule against diphtheria, tetanus and pertussis (DPT) recommends three doses of DPT vaccine at 2, 4 and 6 months of age, two boosters at 18 months and 6 years, and a booster dose of tetanus vaccine every 10 years and two doses during pregnancy. To evaluate the effect of this schedule, antibodies against pertussis toxin and filamentous hemagglutinin and against tetanus and diphtheria toxoids were determined by ELISA in serum samples from children (1 month to 6 years) who received different doses of DPT vaccine: 0 dose, 1 dose, 2 doses, 3 doses, first and second booster; a group of pregnant women and their offspring, and another group of adults. High antibody levels against pertussis antigens and full protection against tetanus (titers >0.1 IU/ml) were observed in the group of adults 0.37 IU/ml, in mothers 4.4 IU/ml and their newborn offspring 5.5 IU/ml, and in children after receiving the second dose of DPT vaccine 1.86 IU/ml. The immune status for diphtheria was far lower, as most of the groups lacked adequate protection. After the third dose of DPT vaccine, only 78% of the children had antibody titers above the protective level (0.1 IU/ml). Since antibody levels are considered to provide full protection, it was only achieved after the first booster dose of DPT vaccine; the primary three-dose schedule seems to be insufficient to confer adequate immunity in all vaccinees. Because of the high proportion of non-protected adults, a booster dose of Td vaccine should be considered for this group.

Khalil et al [54] aimed to compare the immunogenicity of Vaccines produced in accordance with WHO formulas, and those used in United States according to FDA formulas which differ in concentrations. Infants from Saudi Arabia who were 6 weeks old were randomly put into 3 groups to receive 3

doses of vaccines at 6 weeks, 3 months and 5 months of age. The first group received the WHO-recommended formula of DPT and OPV with Hib vaccine. The second group received the FDA recommended formula of DPT and OPV with Hib vaccine. The third group received only the WHO recommended formula of DPT and OPV without Hib. Antibody levels for polyribosylribitol phosphate (PRP), tetanus, diphtheria and poliovirus were measured 1 month after the third dose of vaccines. Antibodies against tetanus and diphtheria were measured using ELISA and the results were expressed in international units (IU/mL). A level of 0.01 IU/mL of antitoxin was regarded as the protective level for tetanus and diphtheria. Although diphtheria and tetanus antigens in the FDA formula are half the concentration of the WHO formula, anti-tetanus and anti-diphtheria antibodies were significantly higher for the FDA. This indicates that the immunogenic response did not correlate with the concentration of the antigen in the case of DPT.

Aboud et al [55] determined the serological response in children (aged 1–15 years) immunized with diphtheria-pertussis-tetanus vaccine (DPT) alone or with a tetanus toxoid (TT) booster dose under the Expanded Programme on Immunization (EPI) in Dar es Salaam and Bagamoyo, Tanzania. Using an ELISA technique, serum levels of anti-TT antibody and anti-TT IgG subclasses were determined in a group of apparently healthy children, 94.7% and 98% of children aged 1–5 years in Dar es Salaam and Bagamoyo, respectively, had anti-TT antibody levels above that considered protective (≥ 0.1 IU/ml). Among 6–15 year old children, 53.3% in Dar es Salaam and 55% in Bagamoyo had anti-TT antibody levels ≥ 0.1 IU/ml. The predominant anti-TT IgG subclasses were IgG1 and IgG3.

Björkholm et al [56] studied the booster effect of low doses of tetanus toxoid in elderly vaccines. The recommended booster dose of diphtheria/tetanus vaccine for adults in Sweden was changed in 1986 from 0.5 ml of tetanus vaccine with a small diphtheria dose to 0.25 ml of a diphtheria/tetanus vaccine

containing 7.5 Lf tetanus toxoid and 30 Lf diphtheria toxoid/ml. This change resulted in an increase in the dose of diphtheria toxoid from 0.5 Lf to 7.5 Lf, but a decrease in the recommended booster dose of tetanus toxoid from 3.75 Lf to 1.9 Lf. Two hundred adults (median age 76 years, range 60-92 years) with no history of tetanus vaccination during the past 10 years volunteered for the study. One hundred two vaccinees were inoculated with 1.9 Lf tetanus toxoid (0.25 ml) and 98 with 3.75 Lf tetanus toxoid (0.5 ml). Paired serum samples were analyzed by the toxin-binding inhibition (ToBI) assay. Side effects were few and mild and without significant differences between the groups. Response rates were similar, with the 3.75 Lf dose eliciting a marginally higher antitoxin response. The prevaccination geometric mean titer was the same for both groups: 0.03 IU/ml. Postvaccination geometric mean titers were 1.18 IU/ml for the 3.75 Lf group and 1.93 IU/ml for the 7.5 Lf group, respectively (difference not significant). Among the vaccinees 47% had a prevaccination titer of 0.01 IU/ml. After vaccination, 85% had a titer >0.01 IU/ml. Booster vaccination with tetanus vaccine containing only 1.9 Lf of tetanus toxoid was thus found to induce an excellent immune response in elderly people, with few side effects resulting.

Fordymacka et al [57] studied the immunity against diphtheria and tetanus in various age groups and compared it with the results of seroepidemiological studies conducted during previous 40 years. The level of diphtheria and tetanus antibody was determined by means of ELISA tests in sera obtained from persons aged 1-81 years. Groups with the lowest levels of diphtheria antibody include persons aged 30-60 years (62% protected). Tetanus immunity was highest during the first three decades of life (above 90% protected), and then declined with increasing age. The comparison with results of several serologic surveys performed earlier showed gradually decreasing immunity level against tetanus in all age groups**.**

Jackson et al [58] determined diphtheria antitoxin level among children primed with diphtheria and tetanus toxoids and acellular Pertussis vaccine lot

with a subpotent diphtheria toxoid component. Diphtheria antitoxin levels were assessed before and after the fourth dose of fully potent (DTaP) vaccine. Of the 105 children evaluated, 84% had prevaccination level less than 0.1 IU/ml, after the booster dose the mean titer level was raised to 92-fold, 100% of children had level > 0.1 IU/ml, and 69% had level >1.0 IU/ml.

Kirmani et al [59] assessed the immune response of 7-year-old former extremely preterm (PT) infants to routine childhood immunizations, this was accomplished by taking blood samples from sixteen PT (<29 weeks and <1000 g) infants, followed since their primary immunizations, and 16 age-matched full-term (FT) control subjects were evaluated at 7 years of age. Antibodies to *Haemophilus influenzae* type b polyribosylribitol phosphate (Hib-PRP), tetanus, pertussis, diphtheria, polio, and hepatitis B (HBsAb) were measured. At this age, PT children had lower antibody titers to any vaccine antigens than FT children. However, most PT children maintained antibody titers in the protective range. The FT group had higher antidiphtheria geometric mean titers (GMT) than the PT group (1.07 vs 0.36 IU/mL). All FT and 13 of 16 PT had protective diphtheria antibody titers (>0.1 IU/mL). The tetanus GMT was 4.22 IU/mL (FT) and 1.99 IU/mL (PT). All children had protective tetanus titers (>0.01 IU/mL). (Both tetanus and diphtheria were measured by ELISA).

Slusarczyk et al [60] studied Immunity of children aged 6-8 years against pertussis, tetanus and diphtheria in Poland. Protective antibody levels were detected in 70%, 58%, and 45% children aged 6, 7, and 8 years, respectively.

McQuillan et al [61] studied the serologic immunity to diphtheria and tetanus in the United State to evaluate the success of immunization programs by taking random samples of persons 6 years of age or older, serum samples were tested for diphtheria by a neutralization assay in Vero monkey kidney cells while tetanus antitoxin was measured by using a solid phase enzyme immunoassay,

60.5% of Americans 6 years of age or older had fully protective levels of diphtheria antibody (>0.10 IU/mL) and 72.3% had protective levels of tetanus antibody (>0.15 IU/mL), 91% of Americans 6 to 11 years of age had protective levels of both diphtheria and tetanus antibody; this proportion decreased to approximately 30% among persons 70 years of age (29.5% for diphtheria and 31.0% for tetanus), 17% and 7% more men than women had protective levels of antibody to tetanus and diphtheria, respectively. Adult Mexican- Americans were slightly less likely to have protective levels of antibody to both toxins. Only 47% of persons 20 years of age or older had levels that were protective against both diseases, and only 63% of adults who were protected against tetanus were also protected against diphtheria.

Carlsson et al [62] studied antibody persistence in two groups of 5.5 year-old Swedish children who during infancy completed a vaccine trial of a combined diphtheria toxoid, tetanus toxoid, acellular pertussis, inactivated polio and *Haemophilus influenzae* type b conjugate vaccine. Three priming doses at ages 2-4-6 months induced higher geometric mean concentrations of antibodies for all antigens than did two doses at 3-5 months, but there were no differences in proportions with protective antibody concentrations. After the booster dose administered at 13 or 12 months of age, respectively, there were no differences in concentrations or proportions between the groups. After 4.5 years later they were tested again for antibodies, the two groups did not differ significantly in antibody concentrations or proportions with antibodies above protective or other defined levels, with the exception of poliovirus type 3 (P < or = 0.01). In all, 89% had ≥ 0.01 IU/ml antibodies against diphtheria by ELISA and 76% by the Vero cell neutralization test, 93% had > or = 0.01 IU/ml antibodies against tetanus, 96% to 99% had detectable antibodies against the polioviruses and 97% had > or = 0.15 microg/ml *H. influenzae* type b antibodies. As for pertussis only 44% had detectable antibodies against pertussis toxoid by ELISA but 99% by

Chinese hamster ovary cell neutralization test, and 94% had detectable antibodies against filamentous hemagglutinin.

Fernandes et al [63] studied tetanus Immunity in Long-Term Care Facilities. A simple random sample of nursing home residents was obtained and tetanus antitoxoid level measured with a solid-phase enzyme immunoassay. Protective level was considered > 0.15 IU/mL, 76.7% had adequate tetanus titers. There were significant associations between immunity and prior history of military service. There were no significant associations between immunity and past history of immunization, education, socioeconomic status, or sex.

Swartz et al [64] studied the immune response to diphtheria and tetanus toxoid components of a combined diphtheria tetanus whole-cell pertussis/enhanced inactivated poliovirus (DTwP/eIPV) vaccine, administered in a three-dose schedule to Israelian infants at 2, 3.5 and 10 months of age and followed by a booster dose at the age of 8 years, this was compared with the immune profile of a group of children at the same age given the customary DTwP vaccine schedule at 2, 4, 6, and 12 months of age and a booster at the age of 8 (control group). Diphtheria- and tetanus-antitoxoid titers were measured in parallel by ELISA and RIA. After the reinforcing dose given at 10 months of age, diphtheria antitoxoid concentrations of ≥ 0.01 IU/ml were found in 100% of infants in the study group, 91.7% of whom reached a titer of ≥ 0.1 IU/ml and a geometric mean titer (GMT) of 0.40 and 0.93 IU/ml in ELISA and RIA, respectively. At 3 and 6 years of age, diphtheria antitoxoid values of ≥ 0.01 IU/ml were detected in 100% and 94% of children with GMT of 0.043 and 0.024 IU/ml, respectively. Seropositivity and GMT values indicative of protection were measured by both ELISA and RIA after the booster dose at the age of 8 years. Similar results were found in the control group, although the GMT tended to be higher. A good correlation between results obtained by ELISA vs. RIA was evident throughout. Hence Priming at 2 and 3.5 months with diphtheria and

tetanus antitoxoid, as a component of a DTwP program, and reinforcing 6 months later induced an immune response indicative of protection against these diseases, which persisted up to the age of the booster recommended at school entry.

Symeonidis et al [65] performed a survey of immunity to tetanus in adult population of Northern Halkidiki, Greece. The study was performed on 405 adult more than 21 years old, tetanus antitoxoid titer was measured by ELISA, 64.4% of the studied population was found protected (tetanus antitoxoid levels > 0.1 IU/ ml). The percentage of protected people decreased as age increased from 83.3% in the 21- 30 to 51.2% in the > 60 age group. There was a significant difference in protection between male and female (82.1% versus 52.6% respectively).

Viviani et al [66] evaluated EPI vaccines-induced antibody prevalence to EPI vaccines in 8–9 year-olds in The Gambia. This included measles, polio 1 and 3, and tetanus toxoid antibodies. The results of the survey were compared with a previous survey performed with the same objectives and same methodology but in different children at 3–4 years of age. Blood samples were collected from children who had received BCG, DPT, OPV, measles and yellow fever immunization. The measles haemoagglutination inhibition test (HAI) was used to detect measles antibody. Antibodies to polioviruses 1 and 3 were tested using the standard polio neutralization assay. ELISA was used to measure tetanus toxoid antibodies. A high proportion of children were fully vaccinated in both age groups. A significant overall lower proportion of 8–9 year-old children had detectable tetanus toxoid antibodies compared to 3–4 year-old children (87% vs. 95%), as well as those who received four doses of DPT (90% vs. 97%).This study indicated that there is a high vaccine coverage achieved in the Gambia with EPI.

Tejedor et al [67] studied the immunogenicity and safety of 3 doses of the combined (DTPa-HBV-IPV/Hib) vaccine (Infanrix hexa) when coadministered with a conjugated meningococcal C vaccine (Meningitec) **.**

The immunogenicity analysis included collection of blood samples from 452 healthy infants before the first dose of vaccine and one month after the third dose, 228 in the coadministration group (receive DTPa-HBV-IPV/Hib and meningococcal C conjugate vaccines at 2, 4, and 6 months of age) and 224 in the separate administration group (received the DTPa-HBV-IPV/Hib vaccine at 2, 4 and 6 months of age and the meningococcal C conjugate vaccine at 3, 5 and 7 months of age). Antibodies against diphtheria and tetanus toxoids, the 3 pertussis antigens and hepatitis B were determined with ELISA. The assay cutoff values were 0.1 IU/mL for the diphtheria and tetanus toxoid antibodies, 5 ELISA units (EL.U)/mL for the 3 pertussis antibodies, 0.15 µg/mL for anti-PRP, 10 mIU/mL for anti-HBs, 99.1% of subjects in both groups achieved anti-polyribosylribitol phosphate antibody concentrations =0.15 µg/mL. The vaccine response against pertussis antigens was at least 99.1% in both groups. For all other DTPa-HBV-IPV/Hib vaccine antigens, at least 97.8% of all subjects from both groups were seroprotected. In addition, 99.5% of all subjects had protective meningococcal C bactericidal antibody titers. Coadministration of both vaccines did not result in an increased local or general reactogenicity compared with separate administration. These results indicated that coadministration of the combined DTPa-HBV-IPV/Hib vaccine and the meningococcal C conjugate vaccine during the same vaccination visit was immunogenic and safe.

Pichichero et al [68] studied the immunogenicity and reactogenicity of a tetanus-diphtheria 5-component (pertussis toxoid, filamentous hemagglutinin, pertactin, and fimbriae types 2 and 3) acellular pertussis vaccine (Tdap) in adolescents and adults. Randomized samples were collected from adolescents aged (11-17) and adults aged (18- 64) years. Before and 28 days after the administration of a single 0.5-mL intramuscular dose of either (Tdap) or tetanus-

diphtheria vaccine (Td), and then antibody titers to diphtheria and tetanus toxoids and pertussis antigens were measured in sera by ELISA method. For both Tdap and Td, more than 94% and nearly 100% of vaccinees had protective antibody concentrations of at least 0.1 IU/mL for diphtheria and tetanus, respectively. Geometric mean antibody titers to pertussis toxoid, and antigens exceeded (by 2.1 to 5.4 times) levels in infants following immunization at 2, 4, and 6 months with DTaP (they were studied in a previous efficacy trial).

3.1. Study design and selection of subjects

This was an open random study conducted from the end of May to the end of July 2005.

Samples were obtained from apparently healthy children who had received the 3 primary doses of DPT vaccine and the fourth booster dose. The percent of DPT vaccine coverage in 2002 was 100% for the 3 primary doses and 99.3% for the booster dose according to the annual report of the Palestinian Ministry of Health (14). Subjects were excluded if they had a major congenital defect or serious chronic illness, any confirmed or suspected immunosuppressive or immunodeficient condition, receipt of immunoglobulin or blood product therapy.

A total of 180 samples were collected. Eligible subjects were grouped according to age (1:1:1) ratio into 3 study groups, (2-4) year olds were chosen from the reception department of Al Naser children hospital, (7-8) years and (11-12) years were chosen from four primary schools in Gaza. The last two groups had received another DT booster dose at the school entry age; 6 or 7 years.

3.2. Ethical considerations

This study was conducted according to the good clinical practice guidelines and the declaration of Helsinki Health Research Committee and Ministry of Education. Sample collection was conducted under the supervision of School Health Directorate and AlNaser hospital nursing staff. Before study enrollment, the purpose of the study was explained to the child's parents, and then, written informed consent was obtained from the parent of every child.

3.3. Materials

3.3.1. Vaccine

DPT and DT vaccines used in Palestinian vaccination centers are manufactured by Aventis Pastuer and they are approved by WHO. Each 0.5 mL dose is formulated to contain 6.7 Lf of diphtheria toxoid, 5 Lf of tetanus toxoid, and that of whooping cough not less than 4 IU (which is nearly equal to 20.000 million organism), these components are preserved by 0.01 thiomersal, the same components are available in DT vaccine, except that for Pertussis.

3.3.2. Reagents

There are two reagents used in the study:
1- ELISA kit for diphtheria anti toxoid (Adaltis Italia S.p.A.)
2- ELISA kit for tetanus anti toxoid (Adaltis Italia S.p.A.)
Two kits were used in the study for each.

3.3.3. Instruments for reading

ELISA reader (TC 89+) Teco Dignostics.

3.4. Serum samples

A volume of 3-4 ml of blood was drawn by veinpuncture under aseptic conditions and serum was separated by centrifugation, each sample was distributed into 3 tubes and then stored at – 80^{o}C until the time of serological assay.

3.5. Assessment of tetanus and diphtheria antitoxoid titer

Before test performance a pilot study was made by taking random samples from each age group and examined together with controls to adjust the technique, this study indicated that the sera should be diluted with higher dilution in the second age group (7-8) years.

3.5.1. Determination of diphtheria antitoxoid level

Diphtheria antitoxoid titer was estimated in all serum samples by ELISA technique. The EIAgen *C. diphtheriae* toxin kit used is an enzyme immunoassay for the quantitative determination of IgG class anti-diphtheria toxioid antibodies (diphtheria antitoxoid) in human serum. This allows the determination of the immune status of the subjects after vaccination.

The analytical sensitivity of the method – defined as the apparent concentration of the analyte that can be distinguished from the zero calibrator – is 0.01 IU/ml. **(Adaltis Italia S.p.A.)**

3.5.1.1. Principle of the assay

The quantitative immunoenzymatic determination of IgG-class antibodies against *C. diphtheriae* toxin is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique.

Microtiterstrip wells were precoated with inactivated specific *C. diphtheriae* toxin (toxoid) antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material, horseradish peroxidase (HRP) labelled anti-human IgG conjugate was added. This conjugate binds to the captured *C. diphtheriae* toxoid-specific antibodies. The immune complex formed by the bound conjugate was visualized by adding

Tetramethylbenzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of *C. diphtheriae* toxin-specific IgG antibodies in the specimen. Sulphuric acid was added to stop the reaction. This produces a yellow endpoint color. The optical density (OD) at 450 nm was read using an ELISA microwell plate reader.

3.5.1.2. Content of the kit

1- Microplate

One microplate of 12 strips of 8 breakable wells. The wells are coated with *C. diphtheriae* toxin antigens. Ready to use.

2- Conjugate

One vial of 20ml of solution containing anti-human-IgG conjugated to HRP in buffer, stabilizers, 0.2% Bronidox L and an inert red dye.

3- Calibrators

Four vials, labeled with Calibrator 0, 1, 2 and 3, each containing 2ml calibrator solution. With the following concentrations 0,000 IU/mL, 0,015 IU/mL, 0.075 IU/mL, 0.150 IU/mL. Each contain 0.1 % Kathon. Ready to use.

4- Sample Diluent:

One vial containing 100 ml of phosphate buffer pH 7.2 ± 0.2., stabilizers, 0.1% Kathon and an inert yellow dye. It is used for the dilution of the patient specimen. Ready to use.

5- Washing Buffer 20X

One vial containing 50 ml of a 20-fold concentrated buffer, detergents and preservatives for washing the wells. pH 7.2 ± 0.2. Contains 0.01 % Kathon after dilution. (need dilution with distilled water)

6- Substrate (TMB)

One vial containing 15ml 3, 3', 5, 5'tetra-methylbenzidine (TMB)/hydrogen peroxide system. Ready to use

7- Stop Solution

One vial containing 15 ml of 0.2 mol/l H_2SO_4 stop solution. Ready to use

3.5.1.3. Assay procedure

Samples and controls were brought to room temperature (20…25°C) before starting the test run.

3.5.1.3.1. Sample dilution

Before assaying, all samples were diluted 1:101 with sample diluent. By dispensing 10 µl sample and 1ml sample diluent into tubes to obtain a 1:101 dilution. A second 1:3 dilution of this 1:101 diluted patient sample was performed for the first (2-4 years) and third (11-12 years) group, while (1:6) dilution was performed for the (7-8 years) group. Calibrators are ready to use and were not diluted.

3.5.1.3.2. Test preparation and procedure

A. Into the respective wells, 100 µl of each Calibrator (0, 1, 2 and 3) and diluted samples were dispensed while one well was left for substrate blank.

B. Wells were covered with the foil supplied in the kit, and incubated for 1 hour ± 5 min at 37±1°C.

C. When incubation had been completed, the foil was removed, the content of the wells aspirated and each well was washed three times with 300µl of Washing Solution. At the end, the remaining fluid was carefully removed by tapping strips on tissue paper prior to the next step.

D. The conjugate was added in which 100µl *C. diphtheriae* toxin anti-IgG-HRP conjugate dispensed into all wells except for the blank well.

E. Wells were covered with the foil and the plate was incubated for 30 min at room temperature.

F. Step C repeated.

G. The substrate was added (100µl TMB Substrate Solution dispensed into all wells).

H. Wells incubated for exactly 15 min at room temperature in the dark.

I. Finally, 100µl Stop Solution was dispensed into all wells in the same order and at the same rate as for the TMB Substrate Solution.

Any blue color developed during the incubation turned into yellow.

J. The OD of the specimen measured at 450/620 nm within 30 min after addition of the Stop Solution.

3.5.1.3.3. Calculation of diphtheria antibody titers

In order to obtain quantitative results in IU/ml**,** the (mean) OD values of 4 calibrators 0, 1, 2 and 3 was plotted against their corresponding concentrations (0.0 / 0.015 / 0.075 and 0.150 IU/ml) by the use of Excel software; which is a computer program that facilitates calculations, a calibrator calibration curve (OD values on the horizontal x-axis, concentrations on the vertical y-axis) was obtained (figure 1), then results were calculated.

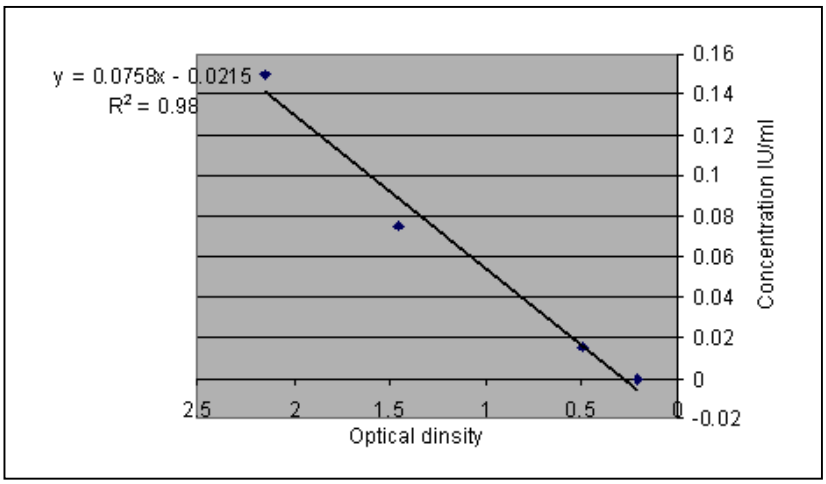

Figure 2: Calibration curve for diphtheria antibody titer.

3.5.2. Determination of tetanus antitoxoid level

Tetanus antitoxoid titer was estimated in all serum samples by ELISA technique. The EIAgen Tetanus toxin kit used which is an enzyme immunoassay for the quantitative determination of IgG class anti-Tetanus toxioid antibodies in human serum. This allows the determination of the immune status of the patients facilitating individual recommendations about the necessity of a basic immunization or booster injection.

The analytical sensitivity of the method – defined as the apparent concentration of the analyte that can be distinguished from the zero calibrator – is < 0.05 IU/ml. **(Adaltis Italia S.p.A.)**

3.5.2.1. Principle of the assay

The quantitative immunoenzymatic determination of IgG-class antibodies against *C. Tetani* toxin is based on the ELISA technique.

Microtiterstrip wells were precoated with inactivated specific Tetanus toxin (toxoid) antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgG conjugate was added. This conjugate binds to the captured *C. tetani* toxin-specific antibodies. The immune complex formed by the bound conjugate was visualized by adding TMB substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of tetanus toxoid-specific IgG antibodies in the specimen. Sulphuric acid was added to stop the reaction. This produces a yellow endpoint color. The optical density (OD) at 450 nm was read using an ELISA microwell plate reader.

3.5.2.2. Content of the kit

1- Microplate

One microplate of 12 strips of 8 breakable wells. The wells are coated with Tetanus toxin antigens. Ready to use.

2- Conjugate

One vial of 20ml of solution containing anti-human-IgG conjugated to HRP in buffer, stabilizers, 0.2% Bronidox L and an inert red dye.

3- Calibrators

Four vials, labeled with Calibrator 0, 1, 2 and 3, each containing 2ml calibrator solution. With the following concentrations 0,00 IU/mL, 0.1 IU/mL, 0.5 IU/mL, 1.0 IU/mL. Each contain 0.1 % Kathon. Ready to use.

4- Sample Diluent:

One vial containing 100 ml of phosphate buffer pH 7.2 ± 0.2., stabilizers, 0.1% Kathon and an inert yellow dye. It is used for the dilution of the patient specimen. Ready to use.

5- Washing Buffer 20X

One vial containing 50 ml of a 20-fold of a concentrated buffer, detergents and preservatives for washing the wells. pH 7.2 ± 0.2. Contains 0.01 % Kathon after dilution. (need dilution with distilled water)

6- Substrate (TMB)

One vial containing 15ml TMB/hydrogen peroxide system. Ready to use

7- Stop Solution

One vial containing 15 ml of 0.2 mol/l H_2SO_4 stop solution. Ready to use

3.5.2.3. Assay procedure

Samples and controls were brought to room temperature (20…25°C) before starting the test run.

3.5.2.3.1. Sample dilution

Before assaying, all samples were diluted 1:101 with sample diluent. By dispensing 10 µl sample and 1ml sample diluent into tubes to obtain a 1:101 dilution. A second 1:3 dilution of this 1:101 diluted patient sample was performed for the first (2-4 years) and third (11-12 years) groups, while (1:6) dilution was performed for the (7-8 years) group. Calibrators are ready to use and were not diluted.

3.5.2.3.2. Test preparation and procedure

A. Into the respective wells, 100 µl of each Calibrator (0, 1, 2 and 3) and diluted samples dispensed while one well was left for substrate blank.
B. Wells were covered with the foil supplied in the kit, and incubated for 1 hour ± 5 min at 37±1°C.
C. When incubation had been completed, the foil was removed, the content of the wells was aspirated and each well was washed three times with 300µl of washing solution. At the end, the remaining fluid was carefully removed by tapping strips on tissue paper prior to the next step.
D. The conjugate was added in which 100µl *C. tetani* toxin anti-IgG-HRP conjugate dispensed into all wells except for the blank well.
E. Wells were covered with the foil and the plate was incubated for 30 min at room temperature.
F. Step C repeated.

G. The substrate was added (100µl TMB Substrate Solution dispensed into all wells).

H. Wells incubated for exactly 15 min at room temperature in the dark.

I. Finally, 100µl Stop Solution dispensed into all wells in the same order and at the same rate as for the TMB Substrate Solution.

J. The OD of the specimen measured at 450/620 nm within 30 min after addition of the Stop Solution.

3.5.1.3.3. Calculation of tetanus antibody titers

In order to obtain quantitative results in IU/ml**,** the (mean) OD values of 4 calibrators 0, 1, 2 and 3 was plotted against their corresponding concentrations (0.0 / 0.1 / 0.5 and 1.0 IU/ml) by the use of Excel software; a calibrator calibration curve (OD values on the horizontal x-axis, concentrations on vertical y-axis) was obtained (figure 2), then results were calculated.

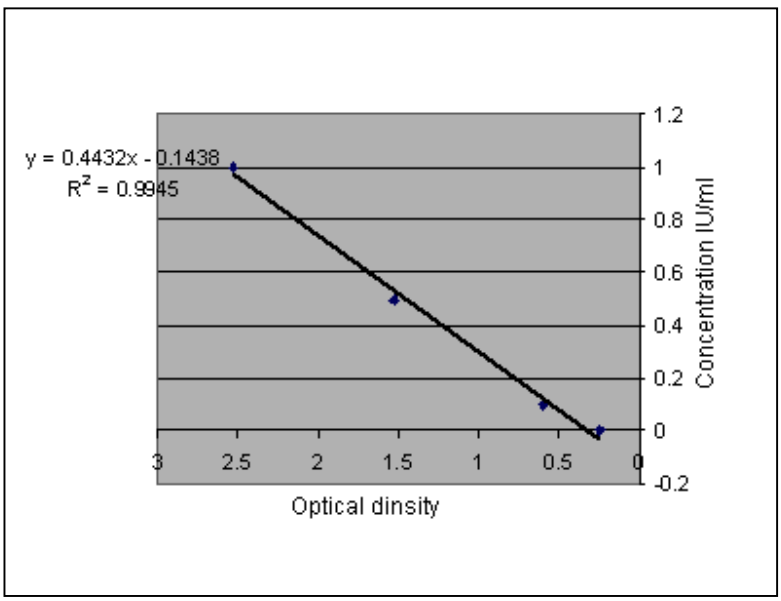

Figure 3: Calibration curve for tetanus antibody titer.

3.6. Data analysis

The present study included 180 children from age 2 to 12 years, "90 males and 90 females" (Table 4), the subjects were classified according to age into 3 age groups; 60 (2-4) years, 60 (7-8) years and 60 (11-12) years (Table 5).

Data were coded numerically and entered on Statistical Package for Social Science (SPSS) and sorted according to sex and age. The arithmetic mean of antibody titers was calculated for age groups, statistical significance was analyzed using one way analysis of variance (ANOVA). The percentage of children who had antibody titers less or more than 0.1 IU/ml was calculated, statistical significance in vaccination efficacy among age groups and between male and female were analyzed using Chi square test.

4.1. Diphtheria

Results showed that 87.8% of children below 12 years old were well immunized against diphtheria and had a protective level of diphtheria antibody (≥0.1 IU/ml) Table (6).

As shown in Table 8 and Figure 3, about 83.3% of the children of 2-4 years age group were protected against diphtheria and 16.7% were not protected, in 7-8 olds, 95% were protected and 5.0% were not, and in 11-12 years age group, 85% were protected and 15% were not. There was also a significant difference of protection against diphtheria after the DT booster dose given at 6 years age, (p =0.040), Table (9).

Results showed also that diphtheria vaccine efficacy was 83.3% in male and 92.2% in female; there was no significant difference between male and female (p=0.069), Table (12).

Table (13) and Figure (4) showed that the mean titers were 0.239 IU/ ml, 0.632 IU/ ml and 0.460 IU/ml in the three age groups respectively, with a significant difference between each two age groups, Table (15).

4.2. Tetanus

Results showed that 98.3% of the study group had protective level of tetanus antibody (≥0.1 IU/ml) Table (7).

Table 16 and Figure 5 showed that all the children in the two age groups, 2-4 and 7-8 years were protected against tetanus, and in 11-12 years age group, 95% were protected and 5.0% were not.

Results showed also that tetanus vaccine efficacy was 97.8% in male and 98.9% in female; there was no significant difference between male and female (p=0.56), Table (18).

Table 19 and Figure 6 showed that the mean titers were 1.005 IU/ ml, 2.625 IU/ ml and 1.202 IU/ml in the three age groups respectively, with a

significant difference between 2-4, 7-8 and 11-12 age groups, but there was no significant difference between 2-4 and 11-12 age groups. Table (21)

4.3. Descriptive tables

Table 4: Distribution of children according to sex

Sex of each child	frequency	percentage
male	90	50.0
female	90	50.0
total	180	100.0

Table 5: Distribution of children according to age

Age (years)	frequency	percentage
2-4	60	33.3
7-8	60	33.3
11-12	60	33.3
Total	180	100.0

Table 6: **Protection against diphtheria among children**

Titer (IU/ml)	frequency	percentage
< 0.1 (not protected)	22	12.2
≥ 0.1 (protected)	158	87.8
Total	180	100.0

Table 7: Protection against tetanus among children

Titer (IU/ml)	Frequency	percent
< 0.1(not protected)	3	1.7
≥ 0.1(protected)	177	98.3
Total	180	100.0

4.4. Results Tables

4.4.1. Diphtheria

Table 8: Protection against diphtheria among children with different age groups.

Age groups	Diphtheria antibody titer			
	< 0.1 (IU/ml) (Not protected)		≥ 0.1 (IU/ml) (Protected)	
	No	%	No	%
2-4 years old	10	16.75	50	83.3
7-8 years	3	5.00	57	95.0
11-12 years old	9	15.00	51	85.0
Total	22	12.20	158	87.8

(X^2)= 4.45, p= 0.108, df=2

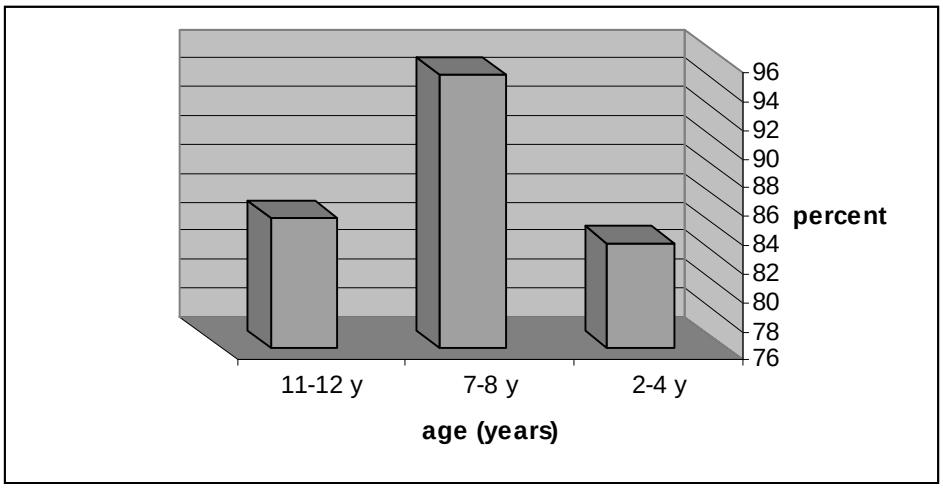

Figure 4: Protection against diphtheria among children with different age groups

Table 9: Protection against diphtheria in (2-4) and (7-8) age groups.

Age groups	Diphtheria antibody titer			
	<0.1 (IU/ml) (Not protected)		≥0.1 (IU/ml) (Protected)	
	No	%	No	%
2-4 years old	10	16.75	50	83.3
7-8 years old	3	5.00	57	95.0
Total	13	10.80	107	89.2

(X^2)= 4.227, p=0.040, df=1

Table 10: Protection against diphtheria in (7-8) and (11-12) age groups

Age groups	Diphtheria antibody titer			
	< 0.1 (IU/ml) (Not protected)		≥ 0.1 (IU/ml) (Protected)	
	No	%	No	%
7-8 years old	3	5.0	57	95.0
11-12 years old	9	15.0	51	85.0
Total	12	10.0	108	90.0

(X^2)= 3.333, p=0.068, df=1

Table 11: Protection against diphtheria in (2-4) and (11-12) age groups

Age groups	Diphtheria antibody titer			
	< 0.1 (IU/ml) (Non protected)		≥ 0.1 (IU/ml) (Protected)	
	No	%	No	%
2-4 years old	10	16.75	50	83.3
11-12 years old	9	15.00	51	85.0
Total	19	15.80	101	84.2

(X^2) = 0.063, p=0.803, df=1

Table 12: Protection against diphtheria among children according to sex

Sex of each child	Diphtheria antibody level			
	<0.1IU/ml Non protected		≥0.1IU/ml Protected	
	No	%	No	%
Male	15	16.7	75	83.3
Female	7	7.8	83	92.2
Both	22	12.2	158	87.8

(X^2) = 3.314, p =0.069, df = 1

Table 13: Mean titer of diphtheria antibody in different age groups

	Mean titer of diphtheria antibody (IU/ml)			
Age group	**2-4 years**	**7-8 years**	**11-12 years**	**All age groups**
Sex				
Male	0.2161	0.7205	0.4498	0.4621
Female	0.2632	0.5450	0.4704	0.4262
Both	0.23965	0.63275	0.4601	0.4442

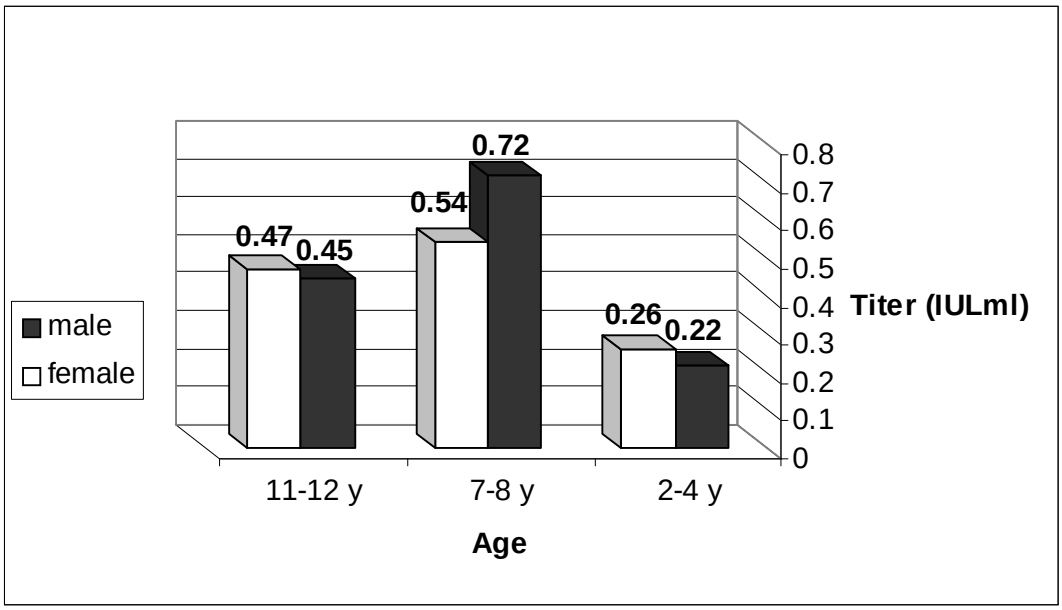

Figure 5: Mean titer of diphtheria antibody in different age groups

Table (14): One way analysis of variance (ANOVA) for mean titers among age groups in diphtheria

	Sum of squares	df	Mean squares	F	Sig
Between groups	4.781	2	2.390	30.716	0.000
Within groups	13.775	177	0.078		
Total	18.556	179			

Table (15): Pairwise Comparison among mean differences for age groups in diphtheria.

Age 1	Age 2	Mean difference Age (1-2)	Sig.
2-4 years	7-8 years	0.398500*	0.000
2-4 years	11-12 years	0.219767*	0.000
11-12 years	7-8 years	0.178733*	0.003

*. The mean difference is significant at the .05 level.

There is a significance difference between each two groups

4.4.2. Tetanus

Table 16: Protection against tetanus among children with different age groups

Age groups	Tetanus antibody titer			
	<0.1 (IU/ml) (Non protected)		≥0.1 (IU/ml) (Protected)	
	No	%	No	%
2-4 years	00	0.0	60	100.0
7-8 years	00	0.0	60	100.0
11-12 years	3	5.0	57	95.0
Total	3	1.7	177	98.3

(X2)= 6.102, P=0.04, df=2

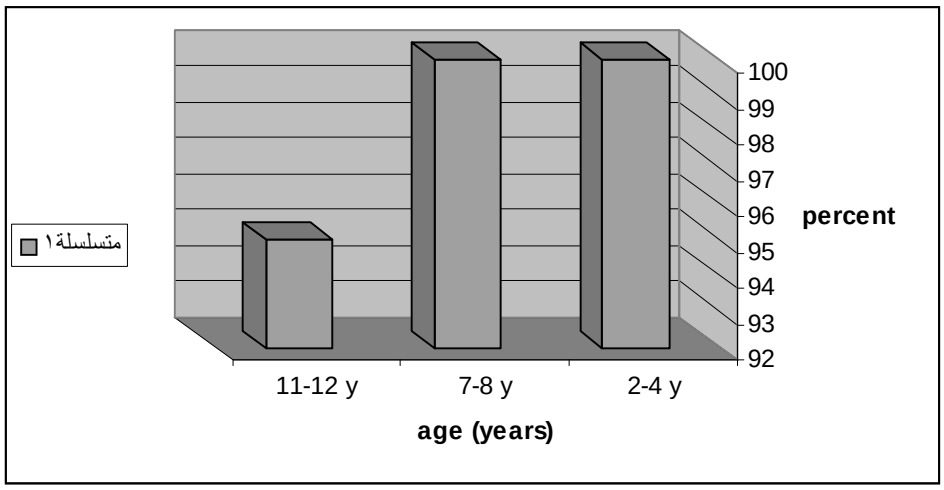

Figure 6: Protection against tetanus among children with different age groups

Table 17: Protection against tetanus in (7-8) and (11-12) age groups

Age groups	Tetanus antibody titer			
	<0.1 (IU/ml) (Not protected)		≥0.1 (IU/ml) (Protected)	
	No	%	No	%
7-8 years	00	0.0	60	100.0
11-12 years	3	5.0	57	95.0
Total	3	2.5	177	97.5

(X^2) = 3.077, p= 0.079, df =1

The same results obtained when compared tetanus efficacy in (2-4) and (11-12) age groups.

Table 18: Protection against tetanus among children according to sex

Sex of each child	Tetanus antibody level (IU/ml)			
	<0.1IU/ml Not protected		≥0.1IU/ml Protected	
	No	%	No	%
Male	2	2.2	88	97.8
Female	1	1.1	89	98.9
Both	3	1.7	177	98.3

(X^2)= 0.339, p= 0.56, df=1

Table 19: Mean titer of tetanus antibody in different age groups

	Mean titer of tetanus antibody (IU/ml)			
Age group	2-4 y	7-8 y	11-12 y	All age groups
Sex				
Male	0.8981	3.2337	1.1481	1.759
Female	1.1121	2.0174	1.2564	1.462
Both	1.0051	2.6255	1.2022	1.6105

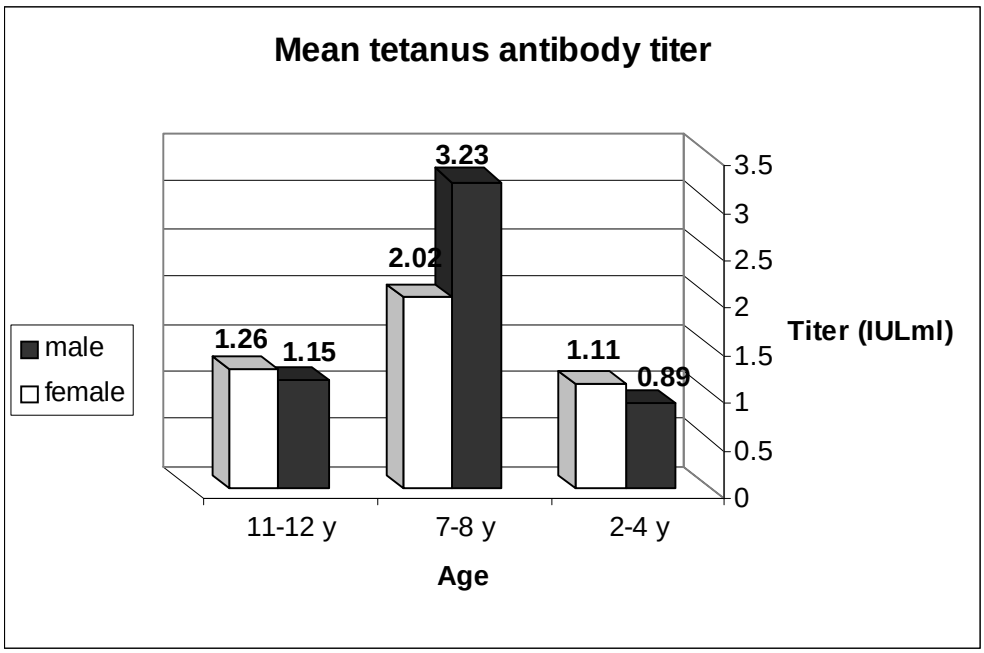

Figure 7: Mean titer of tetanus antibody in different age groups

Table (20): One way analysis of variance (ANOVA) for mean titers among age groups in tetanus

	Sum of squares	df	Mean squares	F	Sig
Between groups	93.196	2	46.598	61.013	0.000
Within groups	135.183	177	0.764		
Total	228.379	179			

Table (21): Pairwise Comparison among mean differences for age groups in tetanus.

Age 1	Age 2	Mean difference Age (1-2)	Sig.
2-4 years	7-8 years	1.612017*	0.000
2-4 years	11-12 years	0.188787	0.498
11-12 years	7-8 years	1.423230*	0.000

*. The mean difference is significant at the 0.05 level.

This study focused on the humoral immune response to diphtheria and tetanus toxoids, this was accomplished by measuring antibody titers for these toxoids in the sera of children younger than 12 years old, who were classified into 3 different age groups, titers were compared with the minimum protective level recommended by the WHO which is ≥ 0.1IU/ml for in vitro measurement of both diphtheria and tetanus antitoxoids. The percentage of children who had titers more or less than the protective level was calculated for each age group.

Since the integration of immunization programs in Gaza, the vast majority of children have been vaccinated against the major vaccine preventable diseases, including BCG, DPT and measles. The trends in mortality and morbidity from these diseases have subsequently decreased dramatically. Although diphtheria and tetanus are now rare diseases in Gaza strip and since several years no cases were reported, analysis of serum specimen from children under 12 years demonstrates that considerable proportion of them lack protective levels for diphtheria antibodies. Although DPT vaccine has a high percent of coverage (100% for the 3 primary doses and 99.3% for the booster dose) [14] about 12% of the tested subjects are not protected against diphtheria, but 1.7% only are not protected against tetanus, hence there is fear in case of diphtheria outbreaks to affect many not protected peoples.

5.1. Diphtheria

5.1.1. Efficacy of diphtheria vaccination

The efficacy of diphtheria vaccination which represents the percentage of children who have antibody titer equals to or more than the protective level was calculated. About 87.8% of children in the different age groups have antitoxoid level ≥ 0.1 IU/ml and 12.2% of children are not protected, this finding is lower than the efficacy obtained by McQuillan et al [61] who studied the protective level for diphtheria and tetanus in several age groups in the United States, 91% of children from 6-11 years old were protected against diphtheria. Carlsson et al

[62] studied antibody persistence in 5.5 years old Swedish children, 89% were protected which is a relatively high percentage despite the fact that those children received only one booster dose.

The study of Mark et al [50], which was conducted on children 6, 10, and 16 years old, revealed that 15%, 48% and 24%, respectively, were not protected against diphtheria, and had antibody titers < 0.01 IU/ml. Efficacy was low compared to our study especially when we talk about 0.01 IU/ml as cut off value since there is no titer less than 0.01 IU/ml was estimated in this study. The results of our study are also higher than the efficacy obtained by Slusarczyk et al [60] who studied the immunity of children aged (6-8 years) against pertussis, tetanus and diphtheria, protective levels were detected in 70%, 58%, and 45% of children aged 6, 7 and 8 years respectively, this percentage is low due to the absence of a school entry age booster dose. Legergard et al [51] determined the neutralizing antibodies and specific immunoglobulin isotype levels in infants after vaccination against diphtheria. Between 6-30 months of age all children were protected, at 30 months only 48% remained protected, this result is much lower than the result obtained in this study.

5.1.2. Efficacy of diphtheria vaccination among age groups

The study children were classified into 3 age groups, the first was (2-4) years old, children in this group completed the three primary doses of DTaP vaccination at 2, 4, 6 months of life and received a booster dose at 12 months.

Efficacy in this group was 83.3% with a mean titer of 0.2342 IU/ml, which is relatively low titer when compared with the titer obtained by Schou et al [48] who determined antitoxoid in 2 years infant, the mean titer of diphtheria antitoxoid was 4.1 IU/ml.

The second group was 7-8 years; children in this group completed the four doses during the first year of life and had a booster dose at 6-7 years, after

one year of this booster dose vaccine efficacy was 95% which is considered a high percent after the booster dose, and a mean titer of 0.6327 IU/ml.

Regarding the efficacy and mean titer in these age groups, we found that the DT booster dose increased the efficacy significantly (p=0.04), and elevated the mean antibody titer (p=0.000). Higher efficacy obtained by Tejedor et al [67] who studied vaccine efficacy in children one month after the fourth booster dose, efficacy was 97.8%, the same study performed by Pichichero et al [47] efficacy was 100%, these results were anticipated because there was no long time lag between the booster dose and sample collection.

The third age group was (11-12) years, 85% of children in this age group had protective level for diphtheria vaccine, with a mean titer of 0.4403 IU/ml. In this age group, after 5 years from the booster dose, efficacy was decreased (p=0.068) and the mean antibody titer decreased (p=0.003). Symeonidis [65] found that the efficacy in older population (21-30y) was 83.3%, the same results were also obtained also by McQuillan et al [61] for 12-19 year olds. In comparison higher efficacy must be obtained in 11- 12 years age group of our study.

Furthermore, we noted that both efficacy and mean titer dropped to the degree at which there was no significant difference between this group and the first group in efficacy (p=0.803), this means that the booster dose may not give a long term protection.

The results obtained from previous studies show that the efficacy of diphtheria vaccine is variable from one study to another, differences in these results may be due to several reasons including the modifying effect of passively acquired maternal antibodies in young infants [69]; a level of passive antibody titer higher than 0.1 IU/ml temporarily interferes with active immunization of infants, whereas a level below 0.02 IU/ml does not [4]. Studies in the United

States suggest that passively-acquired diphtheria antibody may influence the early response to DPT vaccine. Children with a high level of diphtheria antibody in cord serum (0.24 IU/ml), showed a decline in antibody level to 0.05 IU/ml at 2 months of age, and the first dose of DPT vaccine given at two months did not change the declining trend in antibody level [70]. Other studies show that when the level of diphtheria antibody at the time of the first injection of DPT vaccine is below 0.1 IU/ml, the suppressive effect of this passively acquired antibody is less evident. Passive diphtheria antibody seems to show a transient suppression of the antibody response to the second injection of DPT vaccine, but no effect is seen on the response to the third injection of DPT vaccine [4].

Endemicity of diphtheria is another reason for explaining the variability of results among various studies; in developing countries, a high rate of skin infections caused by *C. diphtheriae* creates a primary reservoir of diphtheria organisms. This appears to be an important factor in the early development of natural immunity against the disease [4]; mothers and their infants may have high diphtheria antibody titers [71]. On the other hand, in areas where the reservoir of *C. diphtheriae* is reduced, mothers are less likely to have immunity and their babies seldom acquire passive protection. There is a logarithmic rate of loss of passively-acquired antitoxoid in babies, which averages about 14% per week [4]. Furthermore, the duration of active immunity in children not continually exposed to diphtheria may be shorter than in similar groups of children from communities where diphtheria is prevalent [4]. Socioeconomic changes especially migration from rural to other areas, and sociocultural changes, including improved hygiene and different styles of living, may change the epidemiological patterns of diphtheria. In developing countries the disease could emerge as an epidemic disease with more serious forms, including lethal laryngeal and pharyngeal diphtheria [72].

Vaccination schedule affects the magnitude of response among vaccinated children; primary immunization with three doses of DPT vaccine stimulates antibody levels that considerably exceed the minimum protective level. The antibody level starts to increase after the second dose of DPT vaccine and the level is considerably higher after the third dose. After the primary series, 94% to 100% of children have antibody levels higher than 0.01 IU/ml [47, 48], with the mean level ranging between 0.1 and 1 IU/ml [70] or more [73]. A booster dose administered at the end of the second year of life or at the age of 4 to 6 years stimulates abundant production of diphtheria antibody with the mean levels above 1 IU/ml [74] which is higher than the level obtained by our study which is 0.6327 IU/ml.

The percentage of children with diphtheria antibody above 0.01 IU/ml following two doses of DPT vaccine administered two months apart is similar to that following three doses of DPT vaccine administered with one month between doses. However, the mean antibody levels are significantly lower with a two-dose schedule [73] and it is likely that the duration of immunity after two doses is shorter than after three doses.

Studies have demonstrated that recommended ages and intervals between doses of the same antigen(s) provide optimal protection or have the best evidence of efficacy; administration of vaccine doses at intervals less than the recommended minimal intervals or earlier than the minimal ages because decreasing the interval between doses of a multidose vaccine may interfere with antibody response and protection while increasing the interval between doses of a multidose vaccine does not diminish the effectiveness of the vaccine, Table (22) shows the recommended minimal ages and minimal intervals between immunizations for DPT doses in the recommended childhood immunization schedule [75]. The 4^{th} dose in Gaza strip schedule is given at 12 month which is the minimum age recommended, increasing this age to 15 or 18 month of age will increase the interval between this dose and the previous one, and enhance better immune response.

Table 22: The recommended minimal ages and minimal intervals between immunizations for DPT doses in the recommended childhood immunization schedule (76)

Dose no	Recommended age for this dose	Minimum age for this dose	Recommended interval to next dose	Minimum interval to next dose
DTaP-1	2 months	6 weeks	2 months	4 weeks
DTaP-2	4 months	10 weeks	2 months	4 weeks
DTaP-3	6 months	14 weeks	6-12 months	6 months
DTaP-4	15-18 months	12 months	3 years	6 months

The nature of the pertussis component of DPT vaccine does not seem to affect the immune response to the diphtheria component of the vaccine. Several studies show that the diphtheria antibody response following DPT containing whole cell- or acellular pertussis components is similar (70).

Storage and distribution of vaccines may affect the validity of vaccine; all vaccines are sensitive biological substances and lose their potency, i.e. their ability to give protection against disease, with time. The rate of loss increases as vaccines are exposed to higher temperatures. In order to maintain their efficacy, vaccines must be continuously stored at the appropriate temperature which is (2-8)^{O}C for DPT, DT and Td from the time they are manufactured until the moment of use. Once potency is lost it cannot be regained or restored. Without proper care a vaccine may eventually lose its entire potency. If this occurs, the vaccine no longer provides any protection against the disease and is useless. In some cases, heat exposure leads to loss of potency [77].

Some vaccines are also sensitive to low temperature; freezing or exposure to temperatures below 0°C which can cause loss of potency and the vaccines become useless. Therefore, it is essential to protect them not only from heat but also from freezing [77].

Some vaccines are very sensitive to strong light and their exposure to ultraviolet light causes loss of potency. Consequently, they must always be protected against sunlight or fluorescent (neon) light [77].

DPT is considered the third most sensitive vaccine for high and low temperatures as well as light, while the other forms of vaccine (DT, TT and Td) are less sensitive (77).

Taking into consideration that the electricity in Gaza is disconnected frequently, this may explain in part the relatively low efficacy of diphtheria vaccine.

There are some other factors including some frequently observed program errors such as, too much or too little vaccine in one dose or immunizations given in wrong part of body and variability in vaccine synthesis and preparation [77].

5.1.3. Booster dose

As shown in table (8) efficacy for diphtheria vaccination was 87.8%, which represents the efficacy in all age groups, while efficacy in the last age group was 85% which is lower than the efficacy recommended by WHO (> 87%) [78]. On the other hand, these results were obtained by ELISA and may be lower when performed by other reference methods such as *in vivo* neutralization technique. Moreover, efficacy gradually declines with age. Hence, there is a need to Td booster dose at the age of 13-14 year.

5.2. Tetanus

5.2.1 Efficacy of tetanus vaccination

In our study 98.3% of children in the different age groups had antitoxoid level ≥ 0.1 IU/ml and 1.7% of children are not protected, this finding is in agreement with the study of Tejedor et al [67] who investigated vaccine efficacy in children one month after the fourth booster dose, efficacy was 97.8%, the same study was performed by Pichichero et al [47], efficacy was 100%. These results were anticipated because there is no long time elapsed between the booster dose and sample collection.

Similar results were obtained by McQuillan et al [61] who studied the protective level for diphtheria and tetanus in several age groups in the United States, 91% of children from 6-11 years old were protected against tetanus, and Aboud et al [55] who determined the serological response in children aged 1-15 years immunized with DPT vaccine alone or with a tetanus toxoid (TT) booster dose in Dar es Salaam and Bagamoyo. Percentage of protected children were 94.7% and 98% of children aged (1-5 years) and 53.3% and 55% aged (6-15 years) in Dar es Salaam and Bagamoyo, respectively, and Carlsson et al [62] who studied antibody persistence in 5.5 year olds Swedish children, in which 93% were protected. Lower results obtained by Viviani et al [66] who evaluated EPI vaccine induced antibody prevalence in 8-9 year-olds in the Gambia, 87% have protective antibody levels versus 95% protection in 3-4 year-olds. And Slusarczyk et al [60] who studied immunity of children aged (6-8 year olds) against pertussis, tetanus and diphtheria, protective tetanus levels were detected in 70%, 58%, and 45% of children aged 6, 7,and 8 years, respectively.

The variability among various studies supports the notion that our study should not be a copy of other previous studies; however, it is more or less similar to the majority of similar studies.

5.2.2. Efficacy versus age groups

In the first and the second age groups, all children had protective antibody titers ≥ 0.1 IU/ml, with a mean titer of 1.0135 IU/ml for the first and 2.625 IU/ml for the second, there was a significant difference (p=0.000) between the mean titer in the two groups due to the booster dose effect.

In the third group 95.0% were protected with a mean titer of 1.202 IU/ml, this titer is significantly different from the second group (p=0.000), but there is no significant difference between the third and the first groups, this indicates that the titer returns to its level before the booster dose, and may continue to drop to decrease the protective level, this correlates with results obtained by diphtheria which indicates that vaccine does not provide individuals with a long term protection.

In Schou et al study [48] the mean titer was higher 4.1 IU/ml. In the study of Björkhom et al [56], the booster dose elevates the mean titer from 0.03 IU/ml to 1.18 IU/ml, and another from 0.03 IU/ml to 1.93 IU/ml, and in the study of Aboud [55] the mean titer for tetanus antitoxoid was in 1-5 years 0.82 IU/ml, in 6-10 years 0.13 IU/ml and in 11-15 years after a booster dose given at 10 years was 0.45 IU/ml, the study classified participants according to the time after vaccination, in those who took the last dose 4 years or less, titer was 0.87 IU/ml and those of 4-9 years titer was 0.29 IU/ml.

Differences in the efficacy and mean antibody titer of our study and other studies may be due to several factors, the most important factor is natural immunity; it has been proposed that ‘‘natural immunity’’ against tetanus can be induced by a sublethal dose of tetanus toxin or by fragments of tetanus toxin released from tetanus bacilli located in the digestive tract, as a result of ingesting tetanus spores [79]. Some authors reported finding tetanus antitoxin in the sera of persons who were not immunized [29] or who claim not to have been immunized with tetanus toxoid [79]. Tetanus toxin can be adsorbed from the

gastrointestinal tract. The rate of adsorption depends on the concentration of toxin, the species, and the age and condition of the mucosal lining [29].

There are many unanswered questions about ‘‘natural immunity’’ against tetanus and much of the evidence is open to criticism. Tetanus organisms are widely distributed in nature. Proponents of the natural immunity hypothesis believe that ‘‘natural immunity’’ to tetanus occurs in developing countries due to the presence of tetanus bacilli in the intestinal tract. Furthermore, the carrier state (transient or established) does not protect animals from tetanus infection, nor does it cause the appearance of detectable quantities of antitoxin in the serum [29]. On the other hand, studies in African schoolchildren, Indian military recruits, persons taking care of horses, pregnant women in New Guinea and healthy persons in Upper Volta have demonstrated that populations in developing countries with a high level of exposure to tetanus spores usually lack tetanus neutralizing antitoxins [29].

The implications of ‘‘natural immunity’’ are understood quite differently. One group believes that naturally immunized people are sensitized by contact with tetanus toxin and will respond as primed persons when tetanus toxoid is administered parenterally [29]. Another group speculates about a tolerant state to tetanus toxoid resulting from chronic clostridial contamination of the small intestine [79]. Neither speculation is confirmed by experimental data; in most studies in developing countries the response to primary immunization has not been significantly different from that in industrialized countries [29].

Immunity to tetanus toxin is induced only by immunization; recovery from clinical tetanus does not result in protection against further attacks. A small amount of tetanus toxin, although enough to cause the disease is insufficient to stimulate antibody production. To reach the protective level 2-3 fatal doses are needed [80]. Therefore, all patients with clinical tetanus should be immunized with tetanus toxoid, either at the time of diagnosis or during convalescence [29].

Furthermore recovery from clinical tetanus is achieved by immunoglobulin doses which prevent the immune response to tetanus by neutralizing bacterial toxins.

Another factor makes variability of results among various studies is the interference between passive antibodies and development of active immunity; IgG antibodies produced by the immunized mother are transferred across the placenta to the fetus and provide transient, passive protection of the newborn against tetanus. Some authors suggest "transplacental immunization" as a different mechanism of neonatal protection. According to this concept, the fetus is actively immunized with tetanus toxoid transported transplacentally [29].

After one month, about 80% of antitoxin transferred from the mother is still present in the circulation of the newborn, with an increasing proportion of women immunized with tetanus toxoid, more and more infants will have high levels of passively acquired tetanus antitoxin. Such passive immunity could suppress the development of active immunity following early administration of DPT vaccine. The interference was accentuated in infants who had cord serum titers above 0.1 IU/ml. Data from Thailand on infants immunized at 3, 4, and 6 months of age show a suppressive effect of passive immunity after the first dose of DPT vaccine, but not following the two subsequent doses [29].

In developed countries, the majority of women of childbearing age are immune against tetanus. In the USA, the mean tetanus antibody level in cord serum is high, exceeding 10 IU/ml, when measured by the hemagglutination test [70]. With a half-life of about one month, the antibody level determined by the neutralization test declines in the range of 0.3 to 0.5 IU/ml by the age of two months, when the first dose of DPT vaccine is administered [81], this level of passive immunity interferes with the first dose(s) of DPT, but the third dose of DPT exerts a strong antigenic stimulus [29].

In addition to the previous factors, although immunization with tetanus toxoid is one of the most effective prophylactic procedures, as proved by this

study and other studies, several apparently real failures to achieve protection following tetanus toxoid immunization have been reported. Some of these reports are inadequately documented, some cases follow incomplete immunization, several occurred years after primary or basic immunization, and only a few had received basic immunization plus a booster injection [29].

Vaccination schedule affect the degree of response among vaccinated children; the degree and duration of immunity increases with the number of tetanus toxoid doses given; one dose of tetanus toxoid ensures little, if any, protection. Two to four weeks after the second dose the mean level of tetanus antitoxoid usually exceeds the minimum ‘‘protective’’ level of 0.01 IU/ml, although the percentage of poorly protected persons (“bad responders’’) can still be up to 10%. Immunity declines with time. After one year the percentage of poorly protected persons may increase to 20% and the mean titer may fall to the threshold level. A third dose of tetanus toxoid induces plentiful antitoxoid production, with mean levels between 1 and 10 IU/ml. The level of immunity induced by a course of three injections is high and durable. One month following the third dose the percentage of bad responders is negligible and the protective level lasts for at least 5 years. After the third dose, each additional dose given with at least a one year interval increases the tetanus antitoxoid level and prolongs the duration of immunity. Immunity will last for 10 years after the fourth dose and for at least 20 years after the fifth dose [81].

Immunization of infants with 3 doses of DPT vaccine will provide tetanus immunity for one to three years. Usually, three doses of tetanus toxoid received as an infant are counted as two doses received as an adult. Reinforcing the infant immunization with a fourth dose given somewhere between the 15th and 24 month of life will prolong tetanus immunity for another five years, e.g. until 6 or 7 years of age. A fifth dose of tetanus toxoid (given as Td or DT vaccine) at school entry will provide immunity for another 10 years, e.g. until 17 or 18 years

of age. An additional dose at school leaving or during military service will assure sufficient immunity for at least two more decades [82].

There are other factors including, administration of vaccine doses at intervals less than the recommended minimal intervals or earlier than the minimal ages, storage and distribution of vaccines, some frequently observed program errors and variability in vaccine synthesis and preparation had been discussed previously.

5.2.3. Booster dose

As shown in Table (16) efficacy for tetanus vaccination was 98.3%, which represents the efficacy in all age groups, while efficacy in the last age group was 95% which is in agreement with the efficacy recommended by WHO (> 95%) [78]. As noted, efficacy gradually declines with age. Hence, there is a need to Td booster dose at the age of (13-14 year).

5.3. Efficacy and sex

As shown in Table (12) among all children 87.8% of samples have protective level for diphtheria antitoxoid, the percent of protected female was 92.2% which is 11% higher than protected male, which was 83.3% but the difference between male and female is not significant (p=0.069). And in Table (18) the disparity between male and female with protective levels of antibody was less clear in tetanus, 97.8% male versus 98.9% female, p value=0.56 indicates that there is no significant difference, actually it is difficult to determine the significance because only 3 cases have antibody titers less than the protective level. Hence, there is no significant difference in efficacy between male and female, because they receive the same vaccine and the same dose,

and there is no difference between male and female in the immune response. This is in agreement of the study of Fernandes et al [63] and most other studies.

Some studies such as McQuillan et al [61] study in the USA, and Symeonidis et al [65] study in Greece maleS were with protective antibodies level increased significantly than female in old ages. This may be due to the fact that the majority of men were vaccinated during the military service.

5.4. Techniques to measure antibody response

5.4.1. Diphtheria

ELISA is considered now the most widely used method to measure antitoxoid level for diphtheria. Pichichero et al [68], Tejedor et al [67], Carlsson et al [62], Kirmaniet al [59], Fordymacka et al [57], Khalil et al [54], Pasetti et al [53], and Lagergard et al [51], all of those used ELISA for the measurement of diphtheria antitoxoid. Results of the direct ELISA test are highly reproducible [83] when the antibody level is above 0.1 IU/ml, the results of the ELISA test correlate well with results of the *in vivo* neutralization test in guinea pigs [84] and the results of the neutralization test in tissue culture [83]. Levels of 0.1 IU/ml are considered to be protective in ELISA which corresponds 0.01 IU/ml with *in vivo* techniques. When the antibody titer is low, the results of the ELISA test correlate poorly with results of the neutralization test. Titers of 0.001 IU/ml with the neutralization test can be 10 to 100 times higher (0.01 to 0.1 IU/ml) with the direct ELISA test [83, 84]. Others such as Weiss et al [46], and Koblin et al [49] used the hemagglutination assay. Level of 0.01 IU/ml was considered as a protective level. Results of the HA test for diphtheria correlate well with results of the neutralization test, although the HA test tends to underestimate low concentrations of diphtheria antibody [34]. This is in contrast to the HA for tetanus, which tends to overestimate antibody titers. The results of the HA test for diphtheria can be distorted by non-specific agglutinins in the sera directed against the antigens on the surface of the red cell. These effects can be

minimized by heating the sera at 56^{o}C, pre-treating sera with 2-mercaptoethanol, or absorbing the sera with unsensitized erythrocytes [4].

Others measured the antitoxoid by two methods such as Swartz et al [64], who measured the antibody titer by both ELISA and RIA; a good correlation between results obtained by ELISA vs. RIA was evident.

And Carlsson et al [62], measured titers by both ELISA and by the Vero cell neutralization test. In all, 89% ≥ 0.01 IU/ml antibodies against diphtheria by ELISA and 76% by the Vero cell neutralization test.

5.4.2. Tetanus

ELISA is considered now the most widely used method to measure antitoxoid level for tetanus as well. Pichichero et al [68], Tejedor et al [67], Viviani et al [66], Symeonidis et al [65], Kirmani et al [59], Fordymacka et al [57], Aboud et al [55], Pasetti et al [53] and Schou et al [48] measured tetanus antitoxoid by ELISA, which is simple, sensitive, rapid, and inexpensive, but they are generally less specific than the *in vivo* neutralization method and tends to show falsely increased titers in the range of “protective” titers.

Koblin et al [49] and Weiss et al [46], measured the antitoxoid level by the HA assay. Although the correlation between the HA test and the neutralization test is generally high, differences of up to tenfold or more between the results of the two techniques have been reported in individual serum samples. These differences were especially noted at low levels of antibodies [29].

Björkholm et al [41] measured antitoxoid level by the toxin-binding inhibition (ToBI) test which is another modification of the ELISA test. Tetanus toxin is preincubated with serum dilutions and the mixture is exposed to antitoxin-coated plates [68]. The ToBI test is based on detection of unbound toxin in a toxin-antitoxin mixture and therefore is similar to the neutralization test. The difference between the ToBI test and the neutralization test is the way in which free toxin is detected: in the ToBI test, toxin is detected by the enzyme-labeled antitoxin; while in the neutralization test, direct toxic effects are observed in mice. [29]

Better correlation with *in vivo* tests has been obtained with the ToBI test than with the standard ELISA test. However, experience with the ToBI test is limited and further data on the relationship between results of this test and the neutralization test are needed, especially for sera with low antibody content [29].

Generally the results of *in vitro* techniques should be interpreted carefully and verified against the *in vivo* neutralization method which is more sensitive but require staff with special skills in tissue culture techniques and a laboratory with special equipment and are expensive tests.

6.1. Conclusion

Finally we conclude that the efficacy of tetanus vaccination among children below 12 years in Gaza is very high (98.3%), while that of diphtheria was little lower (87.8%). Although, efficacy for diphtheria is lower but it's valuable, this indicates that vaccination against diphtheria and tetanus has an important role in protection against both diseases.

On the other hand, we can recognize that vaccination efficacy and antibody levels in older children is lower than the younger ones, this could be noted clearly form the significant difference between the last two age groups, furthermore, relatively low antibody titers were elicited by primary immunization, which means that vaccine may not give a long term protection; hence, children at the age of 13 years need a Td booster dose.

Moreover, DT booster dose which is given at the school entry age is highly immunogenic and raises both vaccine efficacy and antibody titers. The significant difference in both efficacy and antibody titers in both diphtheria and tetanus before and after the reinforcing dose, emphasizes the need for such booster.

In addition, our study shows that there is no significant difference between male and female in efficacy. This means that the effect of diphtheria and tetanus vaccination in both sexes is the same.

6.2. Recommendations

In fact, I wish that this study will be followed by other studies in the field of immunization. In my opinion, I think that this subject deserves more studies since it is a very important subject, but unfortunately they are few in Palestine. I would like to end by the following recommendations:

- It's necessary to give a Td booster dose at the age of 13-14 years.

- It's advisable to give the first booster dose at 15 months instead of 12 months in order to increase the response to the booster dose and may prolong its duration.

- Regular booster doses with the combined tetanus-diphtheria toxoid should be routinely given at mid-decade ages and whenever tetanus toxoid is indicated.

- There is a need for serologic monitoring of the whole vaccination program in Gaza Strip due to the instability of sociopolitical conditions which affect the validity of vaccines.

- Further studies should be conducted to cover other age groups to determine where we stand from herd immunity.

- Continuously monitor the cold chain preservation.

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Appendix A

The study sample titers

Serial no.	Age (Years)	Sex	Diphtheria Antitoxoid Titer (IU/ml)	Tetanus Antitoxoid Titer (IU/ml)
1	8	male	0.76	3.03
2	8	male	0.54	1.32
3	8	male	0.02	2.14
4	8	male	0.9	1.13
5	8	male	0.8	3.13
6	8	male	0.72	2.5
7	8	male	0.34	3.27
8	8	male	0.85	0.9
9	7	male	0.69	4.93
10	7	male	0.92	3.43
11	7	male	0.78	2.7
12	8	male	0.90	3.51
13	8	male	0.57	3.4
14	8	male	0.97	6.14
15	7	male	0.28	1.5
16	8	male	0.72	4.83
17	8	male	0.72	6.18
18	8	male	0.95	1.3
19	8	male	0.99	4.18
20	7	male	0.92	4.17
21	7	male	0.41	6.08
22	7	male	0.98	1.45
23	7	male	0.68	3.14
24	7	male	1.22	4.6
25	7	male	0.57	4
26	7	male	0.67	3.6
27	7	male	1.03	2.65
28	7	male	0.87	4.25
29	7	male	0.06	1.25
30	7	male	0.78	2.3
31	7	female	0.98	1.91
32	8	female	0.23	2.2

33	8	female	0.95	1.27
34	8	female	0.86	2.07
35	8	female	0.02	1.38
36	8	female	0.73	2.27
37	8	female	0.78	2.25
38	7	female	0.75	2.15
39	8	female	0.29	2.08
40	7	female	0.23	2.04
41	8	female	1.01	1.62
42	8	female	0.25	1.98
43	8	female	0.32	2.38
44	7	female	1.00	2.19
45	7	female	0.84	2.4
46	7	female	0.31	2.19
47	8	female	0.3	2.00
48	7	female	0.32	2.34
49	8	female	0.31	1.99
50	8	female	0.39	1.62
51	7	female	0.81	2.06
52	7	female	0.31	1.91
53	8	female	0.28	1.31
54	8	female	0.21	1.16
55	7	female	1.15	2.38
56	8	female	0.50	2.28
57	7	female	0.68	2.34
58	7	female	0.78	2.27
59	7	female	0.32	2.15
60	7	female	0.44	2.33
61	12	male	0.39	1.62
62	12	male	0.02	0.05
63	12	male	0.41	2.44
64	12	male	0.02	0.93
65	12	male	0.37	0.68
66	12	male	0.10	0.31
67	12	male	0.34	0.39
68	12	male	0.37	2.43
69	12	male	0.31	0.04
70	12	male	0.16	1.90
71	12	male	0.09	2.38
72	12	male	0.82	2.09

73	12	male	0.37	1.19
74	12	male	0.72	1.68
75	12	male	0.2	1.06
76	11	male	0.14	0.71
77	11	male	0.32	0.97
78	11	male	0.39	1.25
79	11	male	0.25	1.67
80	11	male	0.4	2.41
81	11	male	0.76	1.6
82	12	male	0.08	0.21
83	11	male	0.88	1.34
84	12	male	1	0.93
85	12	male	0.08	0.2
86	12	male	1.03	0.68
87	11	male	0.39	0.92
88	11	male	0.65	0.5
89	11	male	0.91	0.7
90	12	male	1.17	1.16
91	11	female	0.22	0.74
92	11	female	0.45	0.65
93	11	female	0.89	1.61
94	11	female	0.37	0.88
95	11	female	0.4	2.43
96	12	female	0.35	0.78
97	12	female	0.94	1.61
98	12	female	0.24	1.08
99	12	female	0.34	1.11
100	12	female	0.76	1.92
101	12	female	0.27	1.62
102	12	female	0.80	1.72
103	12	female	0.10	1.39
104	12	female	0.28	1.56
105	12	female	0.39	1.13
106	12	female	0.07	0.63
107	12	female	0.13	0.08
108	12	female	0.29	2.09
109	12	female	0.33	1.16
110	12	female	0.67	1.44
111	12	female	0.39	2.23
112	12	female	0.36	1.61

113	12	female	0.39	1.01
114	12	female	0.17	0.73
115	12	female	0.33	1.72
116	11	female	0.07	0.74
117	11	female	0.37	0.91
118	11	female	0.91	0.83
119	11	female	1.16	1.19
120	11	female	1.68	1.10
121	3	male	0.4	0.73
122	3	male	0.09	1.62
123	2	male	0.37	0.82
124	3	male	0.18	1.06
125	2	male	0.18	0.97
126	3	male	0.11	0.33
127	2	male	0.26	0.89
128	4	male	0.29	0.53
129	4	male	0.05	0.96
130	3	male	0.12	0.52
131	2	male	0.37	1.34
132	4	male	0.42	2.19
133	2	male	0.13	0.53
134	3	male	0.14	0.67
135	3	male	0.11	0.25
136	2	male	0.06	0.77
137	3	male	0.26	1.45
138	3	male	0.18	0.29
139	4	male	0.2	1.38
140	3	male	0.05	0.71
141	3	male	0.40	1.42
142	2	male	0.01	0.79
143	3	male	0.09	0.49
144	3	male	0.11	1.24
145	4	male	0.36	0.38
146	4	male	0.29	1.25
147	3	male	0.02	0.74
148	3	male	0.13	1.01
149	2	male	0.5	1.04
150	2	male	0.59	0.61
151	4	female	0.12	0.15
152	2	female	0.11	0.84

153	4	female	0.28	2.05
154	4	female	0.15	0.74
155	4	female	0.21	1.41
156	3	female	0.13	0.94
157	3	female	0.21	1.83
158	2	female	0.11	1.25
159	3	female	0.21	0.3
160	4	female	0.33	0.27
161	4	female	0.21	1.06
162	3	female	0.21	0.76
163	3	female	0.08	1.24
164	3	female	0.21	1.21
165	4	female	0.06	0.67
166	4	female	0.06	0.90
167	2	female	0.33	2.20
168	2	female	0.18	1.82
169	4	female	0.17	0.56
170	2	female	0.15	0.91
171	3	female	0.39	1.99
172	2	female	0.41	1.6
173	3	female	0.35	1.63
174	4	female	0.23	0.72
175	3	female	0.59	1.23
176	3	female	0.46	0.65
177	2	female	0.50	2.12
178	4	female	0.21	0.16
179	2	female	0.46	2.19
180	3	female	0.46	0.48

APPENDIX B

السيد ولي أمر الطفل :.....
والمولود بتاريخ :..... بعد التحية.
تقوم باحثة بالجامعة الإسلامية تحت إشراف الطاقم الطبي لمستشفى النصر بعمل دراسة حول كفاءة التطعيم المعطى للأطفال ضد بعض الأمراض الخطيرة وقد قامت بجمع عينات دم عشوائية لهذا الغرض لذلك نرجو التكرم من سيادتكم بالموافقة على سحب عينة دم من طفلكم من (3-4) مل لهذا الغرض. علما بأنه في حالة الرفض أو القبول لن تتأثر الخدمة الطبية المقدمة إلى طفلكم. مع جزيل الشكر.

توقيع ولي أمر الطفل:.....

الباحثة بالجامعة الإسلامية

السيد ولي أمر الطفل :.....
والمولود بتاريخ :..... بعد التحية.
تقوم باحثة بالجامعة الإسلامية تحت إشراف دائرة الصحة المدرسية بعمل دراسة حول كفاءة التطعيم المعطى للأطفال ضد بعض الأمراض الخطيرة وقد قامت بجمع عينات دم عشوائية لهذا الغرض لذلك نرجو التكرم من سيادتكم بالموافقة على سحب عينة دم من طفلكم من (3-4) مل لهذا الغرض. مع جزيل الشكر.

توقيع ولي أمر الطفل:.....

الباحثة بالجامعة الإسلامية

APPENDIX C

APPENDIX D

APPENDIX E