



Diphtheria - Correlation of Clinical Disease with Lab Diagnosis and Vaccination Status

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ABSTRACT

Diphtheria is an acute infectious disease of upper respiratory tract caused by toxigenic strains of Corynebacterium Diphtheriae (CD) and Corynebacterium other than diphtheria (COD). The organism is locally invasive and causes exotoxin - mediated illness and can lead to complications like stridor, respiratory obstruction, myocarditis, nerve palsy, renal insufficiency and death in severe cases. The objective of the study was to isolate, identify and confirm the organisms in the laboratory from the throat swabs of clinical diphtheria cases, correlate clinical disease and immunization status of the individual and compare serum antibody titers in immunized & non-immunized patients with clinical disease. One hundred fifty patients presenting with fever, sore throat and membrane in the throat were included in the study and throat swabs were collected for direct microscopic examination for KLB and culture. In vitro Toxigenicity, testing by Elek's Gel precipitation test was done for all CD isolates. Serum samples were tested for circulating anti-diphtheria IgG antibody levels by ELISA. Results Showed Out of one hundred fifty clinical cases, fifty were culture positive of which thirteen were both culture and KLB positive. Elek's gel precipitation test was positive for two CD positive cases. ELISA showed a rise in antibody titer in twenty-two patients. CD was isolated in six immunized and ten non immunized individuals COD was isolated in fourteen immunized and twenty non immunized individuals. The present study concludes that diphtheria due to CD is severe and associated with high morbidity and mortality. It affects none immunized and those with low protective circulating IgG antibodies. The disease can occur in milder form in immunized individuals.

Keywords: *Diphtheria; Corynebacterium Diphtheriae; Corynebacterium other than Diphtheriae ; Immunization; Klebs-Loeffler's bacillus; Elek's Gel Precipitation Test; ELISA.*

INTRODUCTION

Diphtheria is an acute, toxin-mediated, contagious and febrile illness caused by bacterium *Corynebacterium diphtheriae*. The name diphtheria is derived from Greek word 'diphtherite' meaning leather hide. This disease was described in the 5th century B.C by Hippocrates and epidemics were described in the 6th century by Aetius.[1-7] *Corynebacterium diphtheriae* is anaerobic, gram-positive, pleomorphic, nonsporing, non-capsulated, nonacid fast, a non-motile rod with irregularly stained segments and granules. They frequently show club-shaped swellings and hence the name corynebacteria (from coryne meaning club). The most important member of the genus is *Corynebacterium diphtheriae*. [8]

It causes severe upper respiratory tract infection characterized by fever, malaise, difficulty in swallowing and grey pseudo membranous patch in the throat. [8] The the organism produces an exotoxin which occurs only when the bacillus is itself infected i.e. lysogenized by a bacteriophage carrying the genetic information for the toxin (tox gene). Only toxigenic strains can cause severe disease. Clinical disease associated with non-toxin-producing strains is generally milder. [1-7]

The toxin inhibits cellular protein synthesis and is responsible for local tissue necrosis, 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. [9]

The toxin has more affinity for myocardium, adrenals and responsible for the major complications i.e. myocarditis and neuritis and can also cause low platelet counts (thrombocytopenia) and protein in the urine (proteinuria). [8] The disease spreads from person to

person by airborne droplets, coughing, sneezing, skin lesions or articles soiled with discharge from lesions i.e. fomites. In temperate areas, diphtheria occurs during winter and spring seasons.[1-7]Incubation the period is 2 to 5 days. Presenting features include throat pain, fever, dysphagia, malaise, a pseudomembranous patch which bleeds when trying to remove it with complications like adenitis (Bull neck), airway obstruction, myocarditis, paralysis, depending on the biotype causing the infection.[8]

Despite the success of immunization in many countries, diphtheria continues to play a major role as a potentially lethal infectious disease. It remains a serious health problem in Eastern Europe, South East Asia, South America, & Indian Subcontinents. Early and accurate diagnosis is imperative since delay results in death [1-7]. Case fatality rate for diphtheria has changed very little during the last 50 years. Diphtheria was the major cause of morbidity and mortality in children in temperate and tropical areas. Overall case fatality rate for diphtheria is 5 to 10% with higher death rates among persons younger than 5 years and older than 40 years of age.[1-7]

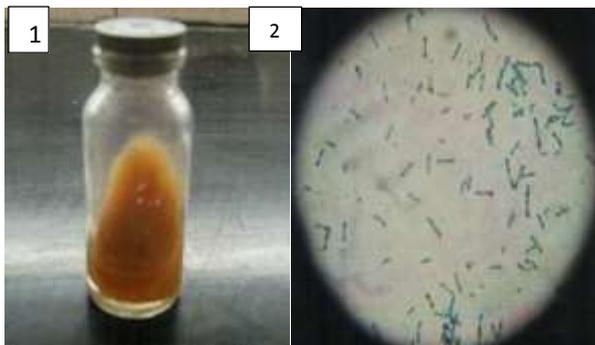


Figure 1&2 Showing Growth of Corynebacterium Diphtheriae On Loefflers Serum Slope And Alberts Stain Showing Green Bacilli With Meta Chromasia Respectively

In developing countries, after inclusion of DPT in immunization schedules, prophylaxis with active and passive immunization, anti-diphtheritic serum and antibiotics have led to decline in cases. But it is still persisting in developing and underdeveloped countries due to various factors like incomplete immunization, low social economics poverty, low immunization coverage among children and delayed reporting to hospital after acquiring of clinical symptoms [9].

In India, the reported incidence of Diphtheria during 1980 was about 39231.Immunization started in 1978 and Universal Immunization Program (UIP) started in 1985. After the introduction of UIP, total admissions for diphtheria cases decreased sharply [10]In the year 2000, there was once again a sudden increase in number of cases to 5125, which increased to 8465 in 2004, and 5826 in 2005 subsequently followed by drop to 2834 in 2006, 3812 in 2007, 3977 in 2008, 3529 in 2009, 3434 in 2010, 4233 in 2011, 2525 in 2012.[9-15]

In this backdrop, the present study was taken up at Sir Ronald Ross Institute of Tropical & Communicable Diseases, Nallakunta, Hyderabad (SRRIT&CD), an infectious disease hospital included in network of Integrated Disease Surveillance Project (IDSP), which has an isolation ward for diphtheria with the following aims and objectives –

- 1) Isolation of Corynebacterium diphtheriae by culture from suspected cases of clinical diphtheria.
- 2) Identification based on biochemical properties.
- 3) Perform in vitro toxigenicity test - Elek's gel precipitation test.
- 4) Detect serum anti-diphtheria (IgG) antitoxin titer & compare antitoxin levels in immunized & non-immunized individuals in relation to disease – ELISA

METHODOLOGY

This study was done at Sir Ronald Ross Institute of Tropical and Communicable Diseases, Hyderabad, India from April 2013 to October 2013.Total numbers of patients admitted during this period were 5801 out of these, 150 cases of Clinical diphtheria were admitted in isolation ward of the hospital.

INCLUSION CRITERIA (AS PER CDC CRITERIA [56, 57]) Patients of all ages presenting with fever and sore throat with the grayish white membrane.

EXCLUSION CRITERIA

- 1) Patients who died before any therapeutic measure could be undertaken.
- 2) Patients who left the hospital against medical advice.

On admission, the data including age, sex, IP number, address, and vaccination status of the patient were noted.Age wise, patients were categorized as <5 yrs, 5 – 10 yrs, 11 – 20 yrs, 20 -30 yrs, 30 -40 yrs, >40 yrs.

Documentation Regarding immunization status was done as per the information was given by parents or attendees or individuals. Accordingly, those who received 3 primary doses at 4 – 6 weeks intervals starting from one month of age followed by a booster

dose at 18 months and 5 years were taken as immunized. Those who did not receive any dose were considered nonimmunized. Partially immunized patients were those who did not receive either all primary doses or booster doses. Categorization of patients was done by male and female to assess gender predisposition.

Clinical data was noted down for each patient which included additional features like lymphadenopathy or bull neck, rhinitis, clinical features suggestive of Pneumonia, myocarditis.

Serum was collected from all patients to measure the level of antitoxin titers by IgG ELISA.

Urine examination for ketone bodies and ECG are done to find systemic complications like renal insufficiency and Myocarditis.

On day 1 two throat swabs were collected from each patient. The first swab was inoculated on Loeffler's Serum slope for culture followed by incubation in 0.5 ml Hartley's Broth for enrichment.

The second swab was inoculated on Potassium Tellurite agar, Tinsdale agar or Diphtheria virulence agar, Blood agar and incubated at 37° C. The same swab was used for smear preparation for direct microscopic examination for Klebs - Loeffler's bacillus (KLB).

Throat swab was repeated for the same patient on the 5th and 7th day of admission to assess the prognosis of the disease. Inoculated Loeffler's serum slope was examined for growth at 2-hour intervals up to 6 hrs beyond which it was refrigerated to prevent the growth of contaminants. Before refrigeration, any growth observed was processed. The smear was made from the water of condensation and stained by Albert method and examined for greenish bacilli with black Metachromatic granules.

On day 2 growth on Potassium Tellurite agar, Tinsdale agar, blood agar was noted. Smears made from the colonies and Albert staining was done.

Colony characters on Loeffler's serum slope: Colonies are at first small, circular, white opaque discs but enlarge on continued incubation and may acquire a distinct yellow tint.

Colony characters on Potassium Tellurite agar: Black dome-shaped opaque colonies.

Colony character on Tinsdale Agar: Grey black opaque dome-shaped colonies.

If no appreciable growth was noted on Potassium Tellurite agar and Tinsdale agar, then the plates were further incubated for another 24 hrs before considering as no growth.

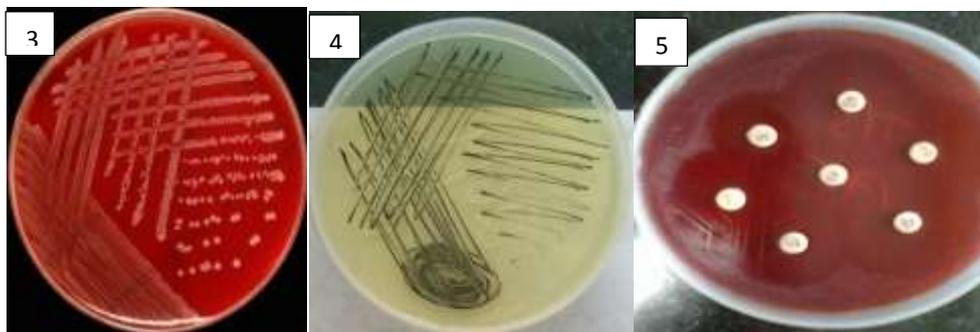


Figure 3,4, 5 Showing Growth On Blood Agar, Tinsdale Agar And Antibiotic Sensitivity

Growth from PT subculture in Hartley's Broth and biochemical identification was done using 25% Hiss serum sugars for Glucose, Maltose, Sucrose, and Trehalose. Starch fermentation and Urease production by Christensen's urea medium was noted for each isolate. All isolates which showed Glucose, Maltose, Starch, Trehalose fermentation were identified as CD and those which gave reactions differently from CD were grouped as COD.

Antibiotic sensitivity testing was done using 0.5McFarland standard inoculum. Antibiotics tested were Penicillin, Azithromycin, Erythromycin, Ciprofloxacin, Ceftriaxone, and Cefotaxime.

The isolates were maintained on blood agar slants with regular weekly subcultures.

INVITRO TOXIGENICITY TESTING: Two methods were adopted

- 1) Original Elek's gel precipitation test using 20% Horse serum agar.
- 2) Modified Elek's test using nonserum based enrichment.

ELEKS GEL PRECIPITATION BY 20% HORSE SERUM AGAR: This is the traditional original method used by ELEK In this method, a sterile Whatman filter paper soaked in 1000 IU of anti-diphtheritic serum is kept in 20% horse serum agar medium

and positive control, negative control and test strains are streaked at right angles to strip, incubated at 37^o C and examined for “MOUSTACHE LIKE LINES OF PRECIPITATION “after 24 - 48hrs.

2) ELEK’S GEL PRECIPITATION TEST USING NON SERUM BASED ENRICHMENTS (DIPHThERIA VIRULENCE AGAR): Herman et al used the concept of non-serum based enrichments to overcome the difficulties encountered during usage of horse serum

Procedure for media preparation, plating is followed as per manufacturer’s instructions

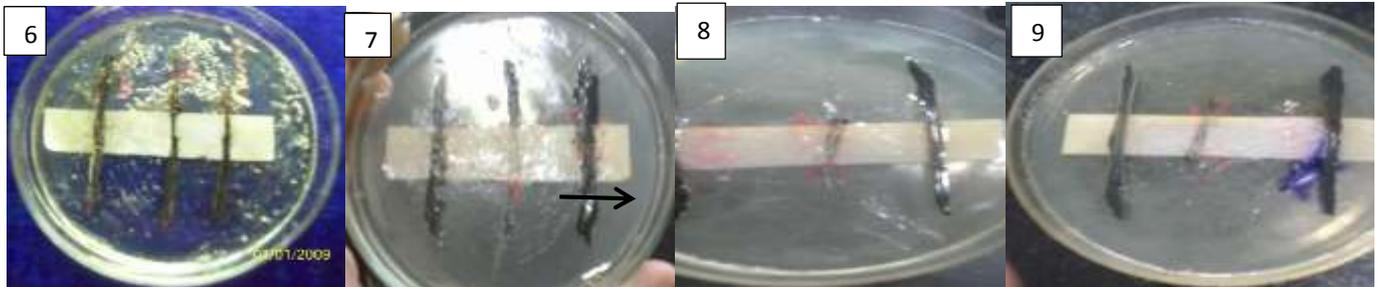


Figure 6,7,8,9 Eleks Showing Lines Of Precipitation On Diphteria Virulence Agar

Positive control used is ATCC 13812 STRAIN (KIWI STICK) obtained from **micro biologics, USA.STOCK CULTURE ATCC 13812:** This is a lyophilized preparation of standard strain of Corynebacterium diphtheria It was used as positive control for quality assessment of Elek’s gel precipitation test Product reconstitution and maintenance of stock culture is done as per guidelines are given by the manufacturer.

ANTI-DIPHThERIA IGG TOXOID ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY)

This procedure provides a quantitative in-vitro assay for human antibodies of IgG class against diphtheria toxoid in serum or plasma. It measures antitoxin levels which are directly proportional to the level of protection in the individual.

INTERPRETATION OF RESULTS:

Value	Result
<0.01 IU/ml	No protection
0.01-0.99	Uncertain Protection
>0.1IU/ml	Immunization Protection present
>1IU/ml	long-term immunization present

The graph is plotted based on OD values recorded and range of protection is noted.

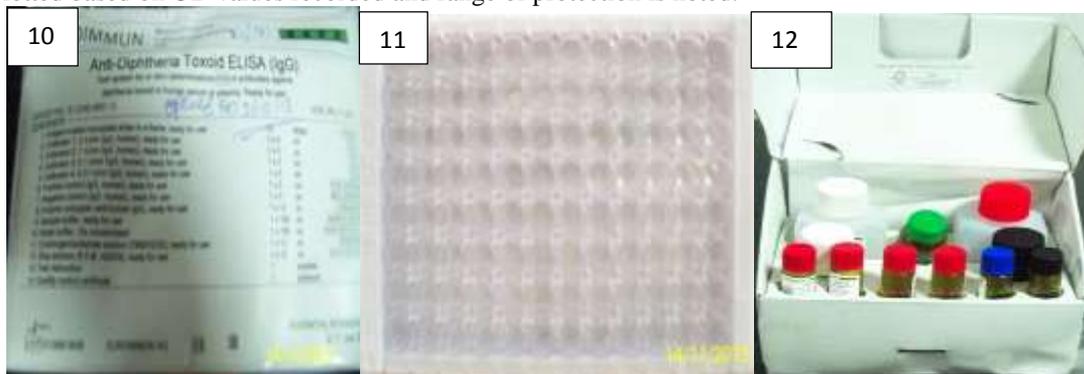


Figure 10, 11, 12 Showing Elisa Kit And Reagents

CONCLUSION

- ❖ 150 clinically diagnosed cases of diphtheria were subjected to smear and culture examination.
- ❖ 87 (58%) cases were male and 63 (42%) cases were female showing male preponderance.

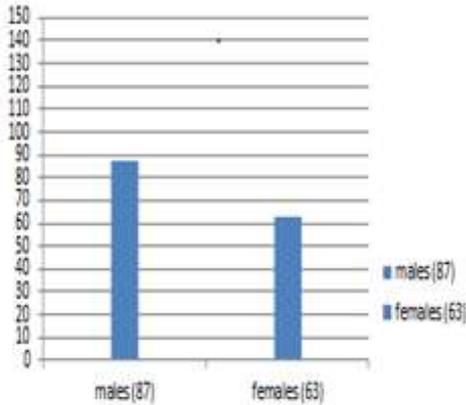


Chart 1 showing comparison of clinical

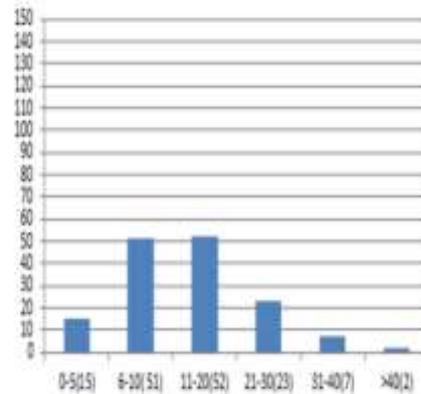


chart 2 showing comparison of clinical cases of diphtheria with age cases of diphtheria with gender

- ❖ 118 (78%) were in age group of 0 -20. Maximum incidence was seen in 6 – 20 years.
- ❖ Out of 150 cases 68 (45.33%) were immunized and 83(54.67%) unimmunized. This indicates that despite successful implementation of the universal immunization program, the clinical disease occurring in unimmunized cases was found to be high.
- ❖ Among the immunized, there were 46 males and females 26 while among unimmunized 41 were males and 37 were females showing more unimmunized females than males. Complications were found to be more in males than females.
- ❖ Number of unimmunized cases as also complications were in the age group of 6 - 20 years.

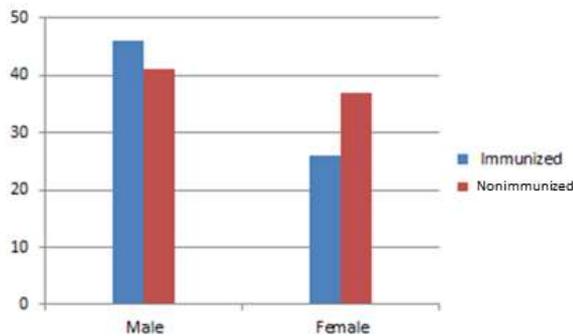


Chart 3 comparing gender and immunisation chart 4 comparing gender and complications

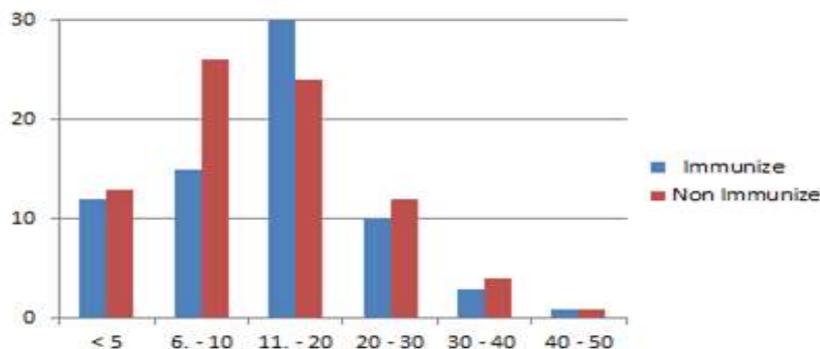
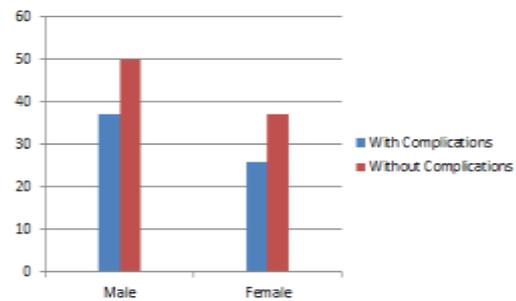


Chart 5 comparing age with immunisation status

- ❖ Out of 150 cases, 13(8.7%) were smeared (KLB) positive and 137(91.3%) were smear (KLB) negative. Culture positive for either CD/COD was 50 (33.3%) showing less microbiological confirmation rate.

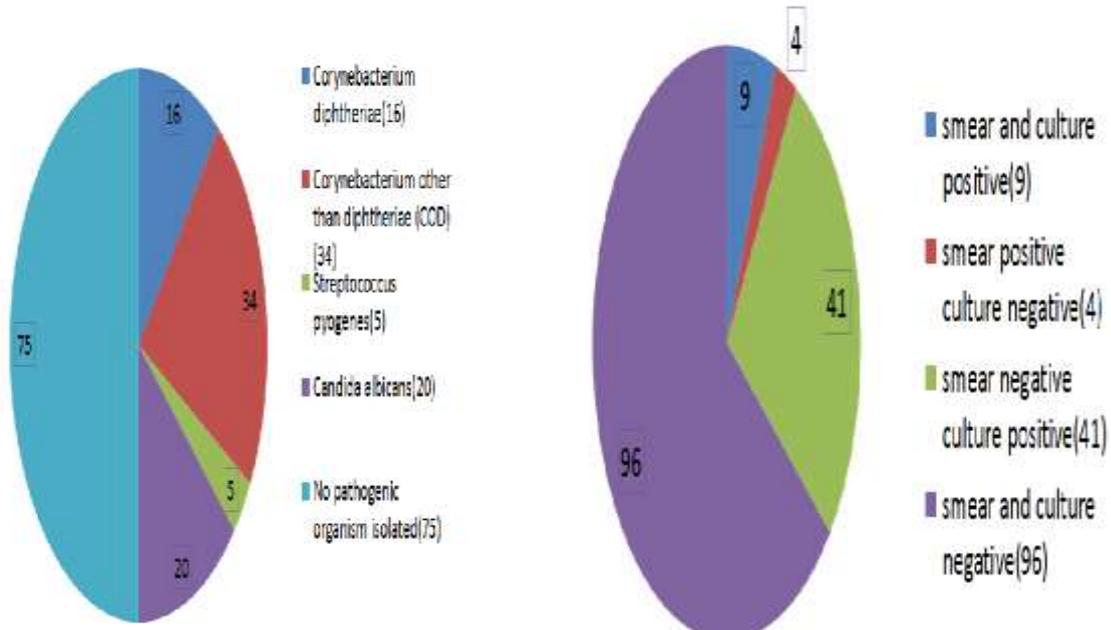


Chart 6 showing various organisms isolated from chart Clinical diphtheria cases

7 showing smear and culture positive and negative

- ❖ Corynebacterium diphtheriae isolated were 16 (10.67%), Corynebacterium other than diphtheriae in 34 (22.6%) cases, biotype gravis was the most common isolate with complicated diphtheria and Arcanobacterium pyogenes among COD.
- ❖ Myocarditis (9.33%), renal insufficiency (12.66%) and lymphadenopathy (12.66%) were the most common complications observed in unimmunized cases showing low level of serum IgG anti titer protection.
- ❖ Elek’s gel precipitation test showed “Moustache like lines of precipitation” in two cases belonging to Corynebacterium diphtheriae biotype mitis.
- ❖ Elisa results showed rise in serum antitoxin titer in cases who recovered, at the time of discharge

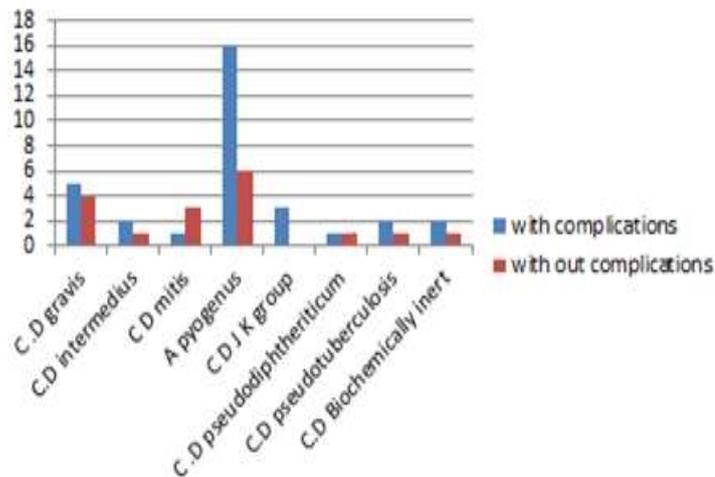


CHART 8 SHOWING CORYNEBACTERIUM SPECIES ISOLATED IN COMPLICATED AND UNCOMPLICATED CLINICAL DIPHTHERIA CASES

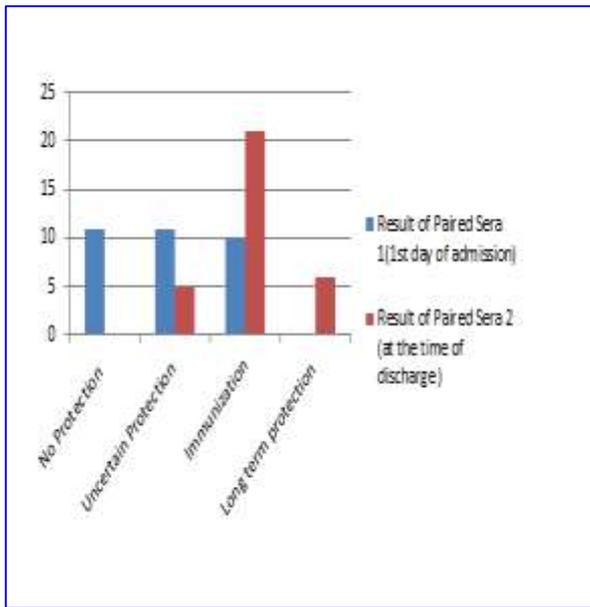


Chart 9 showing results of 32 paired sera Samples

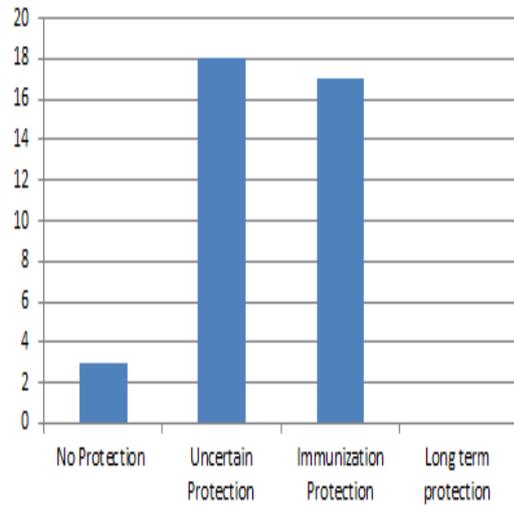


chart 10 showing result of 38 single sera Samples

SUMMARY

Diphtheria is a communicable disease affecting all age groups in spite of measures to immunize children and adults; it is still persisting worldwide, especially in developing countries. The commonest clinical type was nasopharyngeal diphtheria. Myocarditis was observed to be the most common complication. Patients who were completely immunized against diphtheria suffered from milder disease and most of them recovered uneventfully. Clinical illness in those patients whose throat swab culture yielded *Corynebacterium diphtheriae* was severe when compared to disease due to *Corynebacterium* other than diphtheria. Deaths occurred in younger and unimmunized age group. Recovery was faster in older age group and in those who are immunized completely. Since Diphtheria is a vaccine-preventable disease, complete primary immunization with boosters at regular intervals in both children and adults is the only way to prevent the disease and measuring the level of serum antibodies against the disease in affected helps in assessing the individual patient’s immune status in relation to the disease.

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