

A study on detection and characterization of *Corynebacterium diphtheriae* isolated from throat swabs by using manual methods and molecular techniques at a tertiary care hospital

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Abstract- *Corynebacterium diphtheriae* is an exotoxin producing Gram positive bacillus which causes an upper respiratory tract infection known as Diphtheria. It is a vaccine preventable disease. Even with the successful implementation of universal immunization, the pathogen is re-emerging in India due to waning of immunity. The diagnosis of Diphtheria is mainly based on clinical presentation, but the microbiological identification of the pathogen and detection of toxin production is as important to prevent the complications of Diphtheria. The current study is conducted at Sir Ronald Ross Institute of Tropical and Communicable diseases, Hyderabad for a period of 2 years from January 2022 to December 2023. Throat swabs were collected from the Patients presenting with clinical features of Diphtheria and processed by microscopy, culture. Biochemical reactions were performed for further identification of the pathogen. Antibiotic sensitivity testing was performed for all the isolates. All the *Corynebacterium* species were subjected to PCR for identification of toxin production and confirmation of toxin production was done by Modified Elek test. Total of 1894 throat swab samples were collected during the study period out of which 50 were positive for *Streptococcus pyogenes*, 37 were positive for *Staphylococcus aureus*, 50 were positive for *Corynebacterium diphtheriae* [CD] and 30 were positive for *Corynebacterium* species other than diphtheriae [COD]. 100% Antibiotic susceptibility was noted for all the isolates. Recent studies have shown that the non-toxigenic toxin bearing strains of *Corynebacterium* species have evolved and they can cause milder disease which has shown the importance of booster doses in adolescents and adults.

Index Terms: *Corynebacterium diphtheriae*, Pharyngitis, Diphtheria, Elek test, Toxigenic and Nontoxigenic strains.

I. INTRODUCTION

Diphtheria is a vaccine preventable disease of upper respiratory tract caused by *Corynebacterium diphtheriae*. The three strains of *Corynebacterium diphtheriae* are toxigenic, non-toxigenic toxin bearing and non-toxigenic, the latter two strains cause milder disease [1]. The most common form of Diphtheria is respiratory diphtheria transmitted by droplets. Respiratory diphtheria has a gradual onset and is characterized by Mild fever, Sore throat, Difficulty swallowing, Malaise, Loss of appetite, Hoarseness (if the larynx is involved). The hallmark of respiratory diphtheria is a pseudo membrane formed in the oropharynx that appears within 2–3 days of illness [2].

Implementation of Universal immunization has led to a major decline in diphtheria cases but because of waning of immunity, poor nutritional status, unimmunized adults, and low socio-economic status, out breaks are continued to occur [3]. In 2018 India contributed to 52.8% of total cases reported globally [4] from different states like Maharashtra, Haryana, Karnataka, Kerala, and Telangana.

Diagnosis of Diphtheria is by microscopy, culture, and molecular methods. As microscopy is not confirmatory, other methods are used for early detection, which helps to decrease the mortality and morbidity. PCR is being used to detect structural gene for toxin and the results are rapid and easy to interpret. As PCR alone cannot provide definitive results and many laboratories may not be equipped, the phenotypic method i.e. Elek test is being performed for confirmation of toxin production [5]. A lateral flow immunoassay (LFIA) is a qualitative and immunochromatographic strip method developed for toxin detection, which is not yet standardized and approved, so not in use for routine clinical settings [6].

II. AIM

To study the characterization of *Corynebacterium diphtheriae* at a tertiary care center.

III. OBJECTIVES

1. To identify *Corynebacterium* species by manual methods like culture and biochemical reactions
2. To compare the manual methods and molecular techniques for Toxin detection
3. To evaluate the Antibiotic sensitivity pattern of isolates

IV. MATERIALS AND METHODS

The current study was conducted at Sir Ronald Ross Institute of Tropical and Communicable Diseases, Fever Hospital, Hyderabad for a period of two years from January 2022 to December 2023. Complete demographic details along with clinical symptoms like fever, date of onset of illness, number of days of illness, bilateral or unilateral patch over tonsils and pharynx and immunization history were collected from all the patients admitted in diphtheria ward with complaints of upper respiratory tract infection. Two throat swabs were collected from each patient. One throat swab was used for Gram staining and Albert staining. The second swab is used for culture. For bacteriological culture blood agar, potassium tellurite agar, and Loeffler's serum slope were used. Loeffler's serum slope is mainly used for demonstration of granules. Growth suspected of *Corynebacterium* species on potassium tellurite agar was subjected to biochemical tests for speciation. Antibiotic sensitivity test was carried out by Kirby Bauer disc diffusion method to evaluate the antibiotic sensitivity pattern of the isolates. PCR and Modified Elek's gel precipitation test was done to detect Tox A gene and toxin production respectively. Nucleic acid extraction is carried out by Qiagen DNA extraction kit. Cut-off value $Ct > 35$ is negative for all targets.



Figure 1: Loeffler's serum slope with growth.

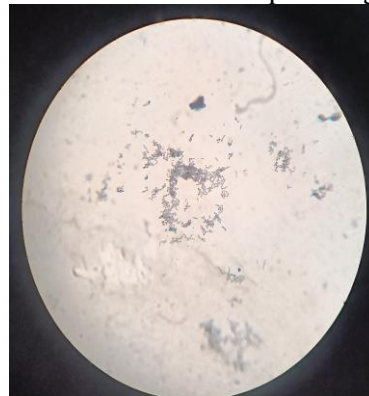


Figure 2: Kleb Loeffler bacilli on Albert staining.



Figure 3: Potassium tellurite agar showing jet black color colonies of *Corynebacterium* species.



Figure 4: Modified Elek's test.

PCR-Corynebacterium diphtheria

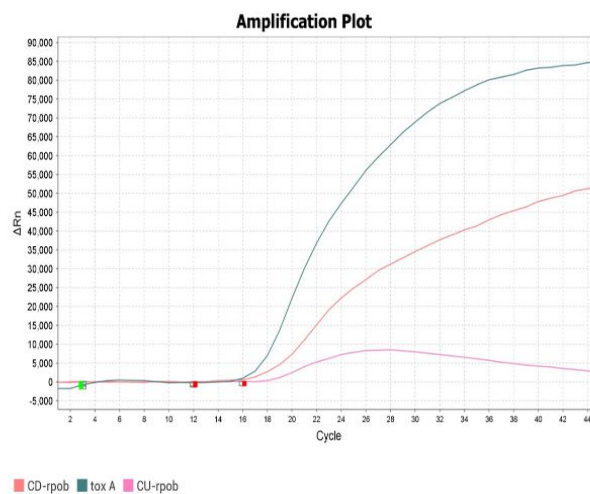


Figure 5: PCR-Amplification plot

V. RESULTS

A total of 1894 throat swab samples were collected during the study period and processed. 38/1894 (2%) samples were positive for Kleb Loffler bacilli by direct microscopy of smears stained by Albert's staining. Out of 1894 clinically suspected cases with symptoms of upper respiratory tract infection the organism isolated are *Streptococcus pyogenes* 50/1894, *Staphylococcus aureus* 37/1894 and *Corynebacterium* species 80/1894. Culture confirmation of *Corynebacterium* species was done by observing the growth on potassium tellurite agar for 80 samples and these were further processed by biochemical reactions like sugar fermentation test for speciation. By sugar fermentation tests 50 samples were positive for *Corynebacterium diphtheriae*, 7 were positive for *Corynebacterium xerosis*, 1 is positive for *Corynebacterium pseudodiphtheriticum*, 1 was positive for *Corynebacterium ulcerance* and 21 were considered as other *Corynebacterium* species. Modified Elek's gel precipitation test and PCR were performed on all the *Corynebacterium* isolates for toxin detection. 50 isolates of *Corynebacterium diphtheriae*, 1 isolate of *Corynebacterium ulcerance* and 1 isolate of *Corynebacterium xerosis* were positive for TOX A gene by PCR. 42/50 *Corynebacterium diphtheriae* isolates, 1 isolate of *Corynebacterium ulcerance* and 1 isolate of *Corynebacterium xerosis* were positive for toxin production by Elek's gel test.

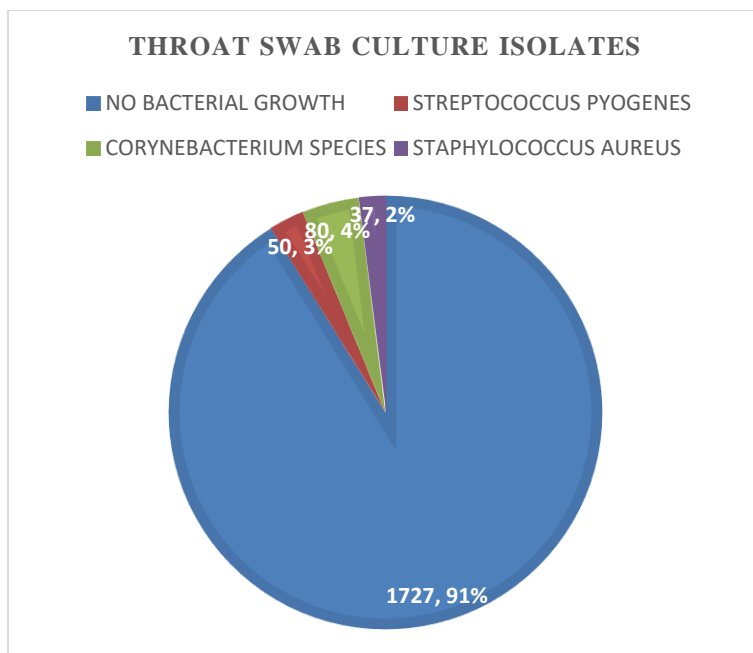


Figure 6:Throat swab culture results.

In the current study most of the patients affected with Corynebacterium species were presented with fever, difficulty in swallowing, throat pain and patch over tonsils. The most common age group affected with Corynebacterium diphtheriae is 11 to 15 years followed by 6-10 years and majority were males. For Corynebacterium species other than diphtheriae (COD) the most affected age group is above 20 years and majority were females. The least common age group for both CD and COD is 0-5years.

AGE GROUP	CD	COD
0-5 years	3	1
6-10 years	12	11
11-15 years	16	4
16-20 years	9	2
>20years	10	12

Table 1: Age distribution for CD and COD (In years):

GENDER	CD	COD
MALE	33	14
FEMALE	17	16

Table 2: Gender distribution for CD and COD:

Clinical symptoms	Percentage % (n=80)
Fever	80 (100%)
Throat pain	80 (100%)
Membrane in oral cavity	80 (100%)
Difficulty in swallowing	48(60%)
Bull neck	44 (55%)

Table 3: clinical symptoms in patients affected with Corynebacterium species.

Antibiotic sensitivity testing was performed by Kirby-Bauer disc diffusion method for all the isolates. Corynebacterium and Streptococcus isolates were sensitive to all the antibiotics and Staphylococcus aureus isolates were sensitive to gentamycin, amikacin, ciprofloxacin, ceftioxin and resistant to azithromycin. The highest number of culture positive Diphtheria cases were reported from the month of September 2022 and October 2023. We observed rise in admissions and number of positive cases from the month of July to November 2022 and January to March 2023.

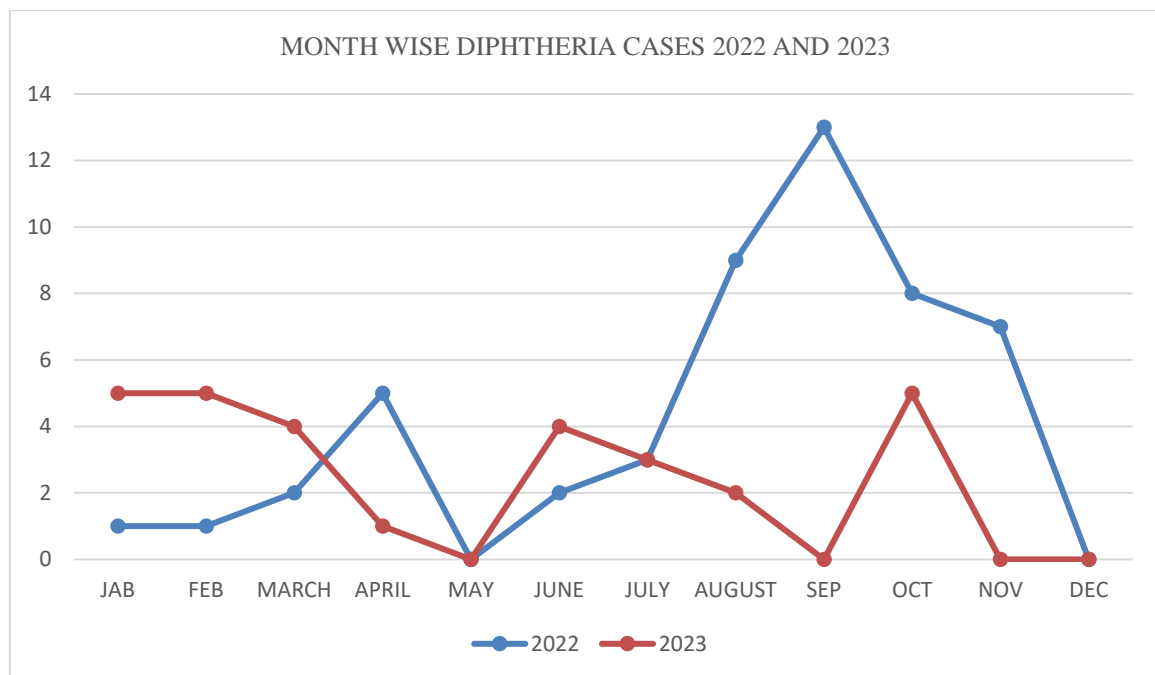


Figure 7: Monthly distribution of Diphtheria cases and comparison between 2 years (2022 and 2023)

Immunization history of all the patients was taken. Out of 80 patients 39 were completely immunized along with booster doses, 31 were partially immunized without booster doses, 5 were unimmunized and 5 patients don't know about their immunization status.

Total number of patients affected	CD N=50	COD N=30
Completely immunized	23	16
Partially immunized	21	10
Unimmunized	4	1
Status unknown	2	3

Table 4: Immunization history of patients affected with CD and COD

VI. DISCUSSION

The clinical symptoms of diphtheria include mainly high-grade fever, sore throat, fatigue or malaise, shortness of breath and signs includes swollen neck /bull neck and lymphadenopathy [7]. On examination pseudo membrane is seen in the oral cavity and over pharynx. These signs and symptoms are due to toxin production. Early diagnosis and prompt treatment of diphtheria can prevent complications such as obstruction to airway, myocarditis, polyneuritis, cranial nerve palsies and pneumonia. In the current study *C. diphtheriae* is detected by bacterial culture and biochemical reactions and PCR. Toxin production is detected by both modified Elek's test and PCR. In PCR targets used are *rpo b* gene and *Tox A* gene which represents the β subunit of RNA polymerase and A subunit of diphtheria toxin respectively [8]. In the current study the total prevalence of *Corynebacterium diphtheriae* and other *Corynebacterium* species is 2.63% and 1.5% respectively. In a study conducted by Meera M et al in 2013 [9] total prevalence of *Corynebacterium diphtheriae* is 5% in immunized patients. The most common age group affected is 6 to 20 years. This could be due to waning of vaccine induced immunity and lack of awareness about the booster doses in adolescents. In a study by Guntur sowjanya the most affected age group by diphtheria is 5 to 10 years [10]. In a study [11] conducted at kerala 7% of the patients affected with CD were below the age of 5 years and 79% were above 10 years. In the current study most common age group affected with *Corynebacterium diphtheriae* is 11 to 15 years followed by 6-10 years and majority were males. For *Corynebacterium* species other than *diphtheriae* (COD) the most affected age group is above 20 years and majority were females. As childhood immunization is widely going on in India less cases were reported below the age of 5 years. Even though the males and females were equally affected by pharyngitis due to other causes along with diphtheria during the study period, out of 50 patients who were affected with CD, 33 were males and 17 were females. In a study [12] Similar findings were noted with male preponderance. This can be because of exposure of males to community will be more, where they can come in contact with carriers. Along with toxin producing and non-toxin producing strains, currently a third category, non-toxigenic *Tox* gene bearing strains have been evolved. These strains are genotypically *tox*-positive, but they will not express diphtheria toxin [13]. In the current study 52 out of 80 *Corynebacterium* isolates were positive for *tox* gene on PCR and 44 out of those 52 were positive for toxin production on Modified Elek's test. Out of 50 patients who affected with CD, 23 were completely immunized with 3 doses along with booster doses, 21 were partially immunized, 4 were

unimmunized and 2 patients doesn't know about their immunization status. Like in many previous studies, most all the patients have been presented with fever, throat pain and membrane in the oral cavity. No deaths were reported in the patients affected with *Corynebacterium* species in the current study.

VII. CONCLUSION

The current study has concluded that Diphtheria exists in the community even after the proper implementation of universal immunization. This is because of the waning of immunity in adults and adolescents. A shift in the age group is observed in the study which showed the importance of booster doses in adults. As overcrowding, lack of education and poor economic conditions are the most important factors which plays vital role in transmission of Diphtheria, people need to be educated about the importance of booster doses along with universal immunization.

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