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ORIGINAL RESEARCH ARTICLE

A STUDY ON ETIOLOGICAL AGENTS OF ACUTE MEMBRANOUS PHARYNGITIS WITH SPECIAL REFERENCE TO CORYNEBACTERIUM DIPHTHERIAE

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ABSTRACT

BACKGROUND

Acute Bacterial Pharyngitis is the most common clinical condition presenting as a health care visit. Pharyngitis is inflammation of Pharynx. It may be caused by Bacteria, viruses, sometimes fungi. Most common are Staphylococcus, Group A Streptococcus, Corynebacterium diphtheriae. The name Diphtheria is derived from a greek word Diphtherite meaning Leather hide. Diphtheria being an acute infectious disease affecting the upper respiratory tract which is caused by toxigenic strains of Corynebacterium diphtheriae (CD) and Corynebacterium other than diphtheria (COD)- Corynebacterium ulcerans and Corynebacterium pseudotuberculosis.. 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). The toxin has greater affinity for myocardium, adrenals and causes major complications like myocarditis, neuritis. The toxin which is produced at the site is distributed to several organs of the body. Diphtheria toxin is easily absorbed in the surrounding tissues of the patient's throat, where it induces local inflammatory reaction in the nasopharynx and larynx. There is a serocellular exudate which forms a grayish membrane across the larynx, causing severe acute respiratory obstruction Incubation period is 2-5 days. Severe complications like stridor, respiratory obstruction, myocarditis, nerve palsy, renal insufficiency and death. This study was aimed to isolate, identify and confirm the organism in the laboratory from the throat swabs of clinically suspected cases, correlate clinical disease and immunization status of the individual.

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MATERIALS AND METHODS

The identification of Diphtheria in particular can be done by conventional or molecular methods. Conventional methods require expertise and it is time consuming but it is gold standard. Molecular methods yield results in less time. Two throat swabs collected in Amies Transport medium were processed. One was used for molecular testing. The other swab was used for culture and microscopy. It was inoculated on Blood agar, Potassium Tellurite agar, Tinsdale agar. Microscopy was done with Gram stain and Albert stain. Biochemical testing was done. Antibiotic Susceptibility testing was carried out on Blood Agar or Muller Hinton Agar with selective antibiotics.

RESULTS

Out of 50 samples processed, 5 came out to be positive for Corynebacterium diphtheriae, 5 were due to Staphylococcus aureus, 3 found to be Streptococcus pyogenes, 3 were found to be Candida albicans. Molecular testing was also done for Corynebacterium diphtheriae which is positive in 5 samples.

CONCLUSION

Diphtheria being a vaccine preventable disease it is posing a health threat. It has a case fatality rate of 10%. There is a resurgence of cases of Diphtheria due to circulation of toxigenic strains now a days. It may be due to wanning of immunity or not completing vaccination schedule. The disease is severe in unvaccinated where as it is mild in vaccinated individuals. Prompt diagnosis along with laboratory confirmation is very much essential. Availability of antiserum is another factor that plays an important role in saving life of an individual by decreasing mortality.

KEYWORDS

Diphtheria, Immunity, Antiserum, vaccination

INTRODUCTION

Sorethroat is primary symptom of Pharyngitis. Pharyngitis is inflammation of Pharynx. It is used interchangeably with Pharyngotonsillitis. Pharyngitis presents with definitive evidence of exudates, ulceration and erythema. Etiology may be Bacterial, viral or Allergic. Most common viruses were Coronavirus, Rhino virus, Adenovirus, Parainfluenza virus. Bacterial causes being Group A Streptococci, Corynebacterium diphtheriae, Staphylococci, H.influenza.[1]

Diphtheria is caused by a Gram positive bacilli named Corynebacterium diphtheria. It appears in Chinese letter arrangement with volutin granules at the ends. It produces an exotoxin which is very dreadful. French physician Pierre Bretonneau called it ‘diphtherite’.[2] Respiratory Diphtheria is the most common form and it may affect Pharynx**,** Tonsil, Larynx. These are the only form reportable to World Health Organisation. Median time from infection to prodromal onset of symptoms being 1.4 days. Children being more affected compared to adults.[3]

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Inspite of national Immunisation programs like Mission Indra Dhanush there is resurgence of diphtheria in India. Patients often present with fever, sore throat, membrane, bull neck and Respiratory distress sometimes. This is a pseudomembrane which bleeds on touch. Lack of awareness among treating Doctors, delay in clinical suspicion, lack of appropriate diagnostic facilities are the main factors that play an important role in progression of disease. Lack of timely availability of Diphtheria Anti Toxin even on clinical suspicion is another key factor for the mortality of the cases.[4]

The study was started after approval from Institutional ethical committee (GMC/IEC/011/2023).

MATERIALS AND METHODS

This Prospective Cross sectional study was conducted for over a period of 18 months from January 2022 to August 2023 in the department of Microbiology, Guntur Medical College, Guntur. Throat swabs two in number were collected from patients with clinical suspicion and were sent to the laboratory in Amies Transport medium without Charcoal which is shown in fig. 1.

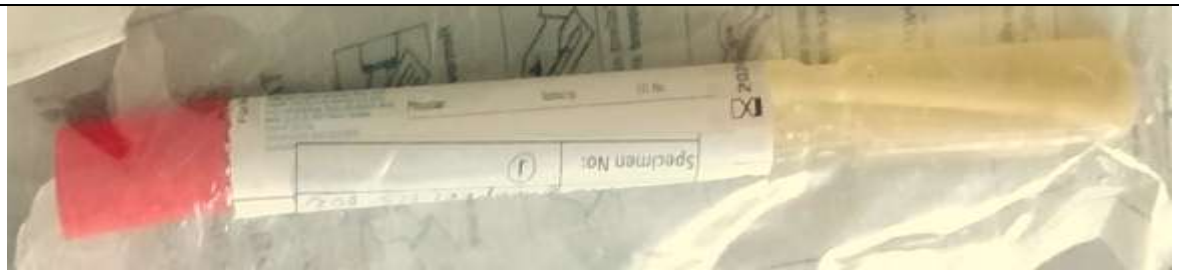

Figure 1. Amies Transport medium without charcoal

Upon receiving in the laboratory one swab was used for inoculation on Blood Agar, Potassium Tellurite Agar, Tinsdale Agar. The plates were incubated at 37^{C} and observed for 5-7 days. The same swab was used to perform Gram stain and Albert stain.

Colony characters on Potassium Tellurite agar: Black dome-shaped opaque colonies which was shown in fig. 2.

Colony characters on Tinsdale Agar: Grey black opaque dome-shaped colonies which was shown in fig. 3.

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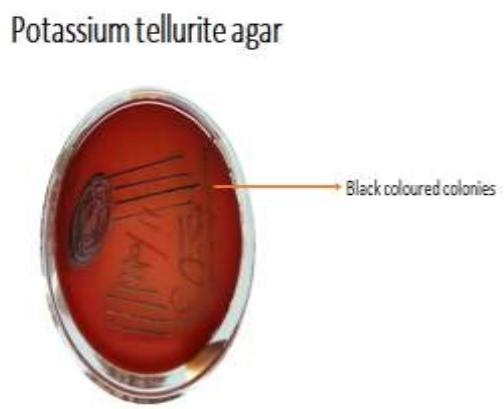

Figure 2. Growth on Potassium Tellurite Agar

Figure 3. Growth on Tinsdale agar

After careful observation of colonies on plates staining was done with Gram stain which showed Gram positive bacilli shown in fig. 4.

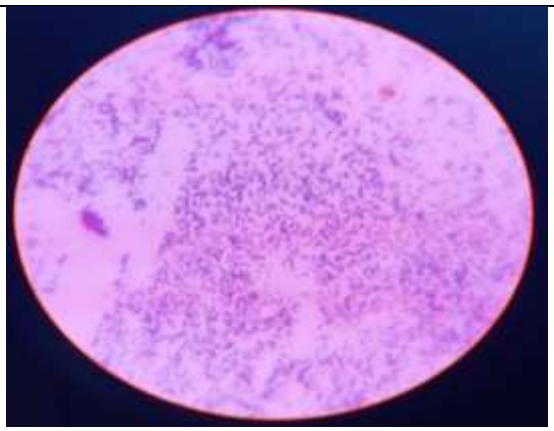

Figure 4. Gram positive bacilli on Gram stain

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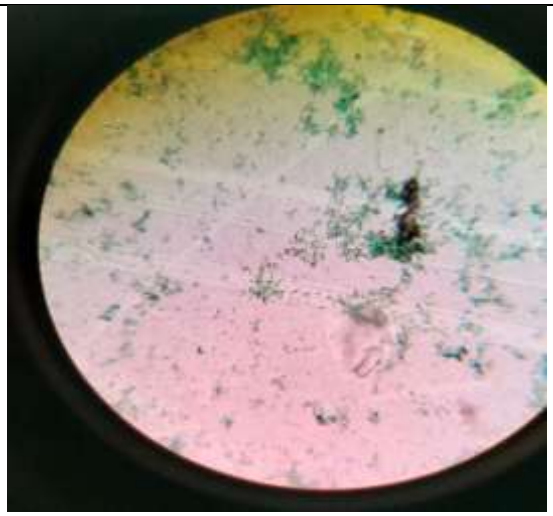

Figure 5. Green coloured bacilli on Albert stain

Albert staining was done for confirmation which showed greencoloured bacilli with metachromatic granules at the end.

Further biochemical testing was done with Hiss Serum sugars - Glucose, Maltose, Sucrose, Ribose, Christensens Urease agar, Nitrate Reduction Test.

Gram positive cocci was seen in few samples on Gram stain. Catalase test was done to differentiate Staphylococcus and Streptococcus. Further confirmation was done with Slide coagulase and Tube Coagulase test. Bacitracin disc was used for identifying Streptococcus pyogenes on Antibiotic susceptibility testing.

Gram positive budding yeast cells were seen in few samples resembling like candida species. Germ Tube Test was done for identifying Candida albicans.

Germ Tube Test: In a sterile test tube 1ml of serum is taken and 4-5 identical colonies suspicious of candida were added and incubated at 37 C for not more than 2 hours. Then a drop of suspension was added on a clean glass slide and coverslip is placed and observed under microscope for production of germ tubes.

Antibiotic susceptibility testing was carried out for selecting appropriate antibiotic. The following antibiotics were tested – Penicillin, Erythromycin, Azithromycin, Ceftriaxone, Cefotaxim, Rifampin, Cefoxitin, Bacitracin. The other swab was used for molecular testing for detection of tox gene and rpoB gene for Corynebacterium diphtheriae.

Extracted DNA from isolate can be used as the template for detecting diphtheria toxin (tox) gene using oligonucleotide primers.

Target Primer/Probe	**Primer/ probe**	**Sequence 5’ – 3’**	**Amplicon length (bp)**
C.diphtheriae rpoB	dip_rpob_F dip_rpob_R dip_probe	CGTTCGCAAAGATTACGGAACCA CACTCAGGCGTACCAATCAAC HEX-AGGTTCCGGGGCTTCTCGATATTCA-BHQ1	97
C.ulcerans rpoB	ulc_rpob_F ulc_rpob_R	TTCGCATGGCTCATTGGCAC TCCAGGATGTCTTCCAGTCC	98

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	ulc_probe	FAM-CCAGCAGGAGGAGCTGGGTGAA-BHQ1	
ToxinA	tox A_F toxA_R toxA_probe	CTTTTCTTCGTACCACGGGACTAA CTATAAAACCCTTTCCAATCATCGTCCY5- AAGGTATACAAAAGCCAAAATCTGGTACAC- BHQ2	117

Table 1 – Primers and Probes.

PCR Master Mix

Primer mix can be prepared for (rpoB for C. diphtheriae, C. ulcerans, toxA and RNaseP) separately by adding 10 µl of each primer, 4 µl of probe and make up with 76 µl of nuclease free water.

Reaction Mixture (1rxn)

1. Master mix 10 µl
2. C. diphtheriae primer mix 1 µl
3. toxA primer mix 1 µl
4. C. ulcerans primer mix 1 µl
5. RNaseP primer mix 1 µl
6. Water (PCR grade) 1 µl
7. DNA template 5 µl

Total 20 µl

PCR Program AB7500:
Thermal Cycle Conditions;

Initial Activation: 95 ^{0}C for 10 mins
Combined 45 cycles of:

Denaturation 95 ^{0}C for 15 secs
Annealing and Extension 60 ^{0}C for 30 secs.

Data Interpretation

Ct cut off for Positivity	C.diphtheriae	C. ulcerans	Tox A
Minimum	≤31.24	≤28.96	≤31.05
Maximum	34.06	31.12	35.03

Table 2: Data Interpretation for Real Time PCR based on Ct values

A duplex sigmoid curve indicated detection of *C. diphtheria* and *Tox* gene, i.e., a positive result. The detection of both bacterium and diphtheria toxin gene indicated the presence of potentially toxigenic *C.* diphtheriae.

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Results: A total of 50 patients were involved in the study. 34 were males where as 16 were females. Males are more in number than females. Male to female ratio is 2.1:1 which is shown below.

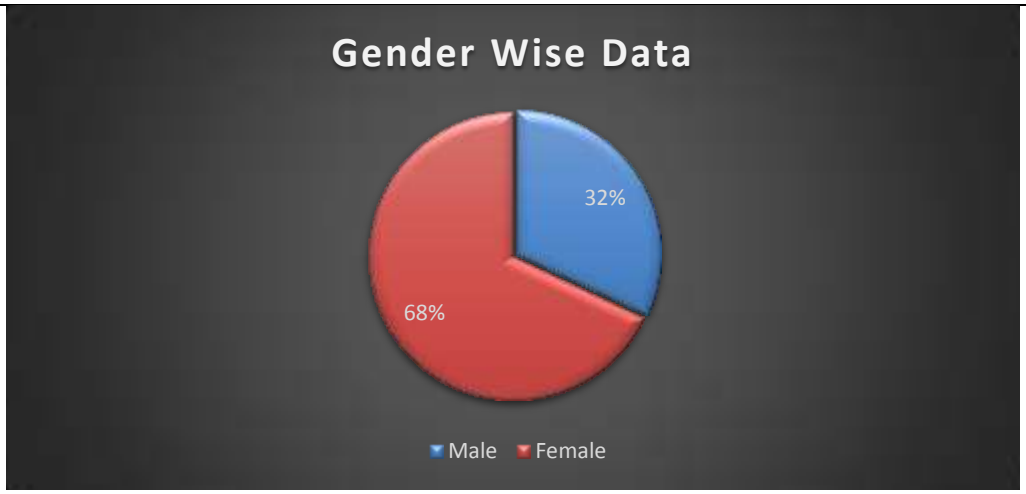

Figure 6. Gender Wise Data

The patients included in the study ranged from 1 month old baby to 15 years old child which is shown below. 5- 10 years is the predominant age group affected followed by 1-5 years.

Figure 7. Age Wise Data

Majority of the patients presented with Fever, Sorethroat, Membrane over Tonsil followed by Bull neck, Hoarseness of voice which is shown below. Majority of patients had fever followed by sore throat followed by membrane. The present study also included patients with bull neck, dysphagia, hoarseness of voice sometimes bleeding also.

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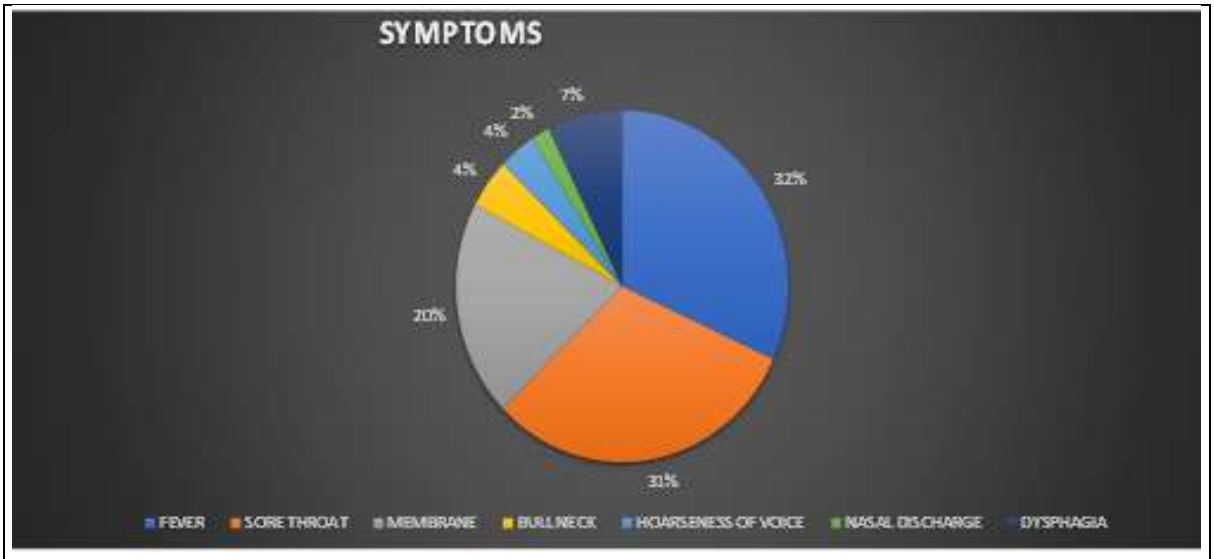

Figure 8. Symptoms Wise Data

Most of the positives were in the age group of 1-5 years. 64% were vaccinated which is shown below. Vaccination was done with DPT vaccine which is given at 6,10,14 weeks followed by Booster at 18-24 months and one more booster of dT at 4-5 years.

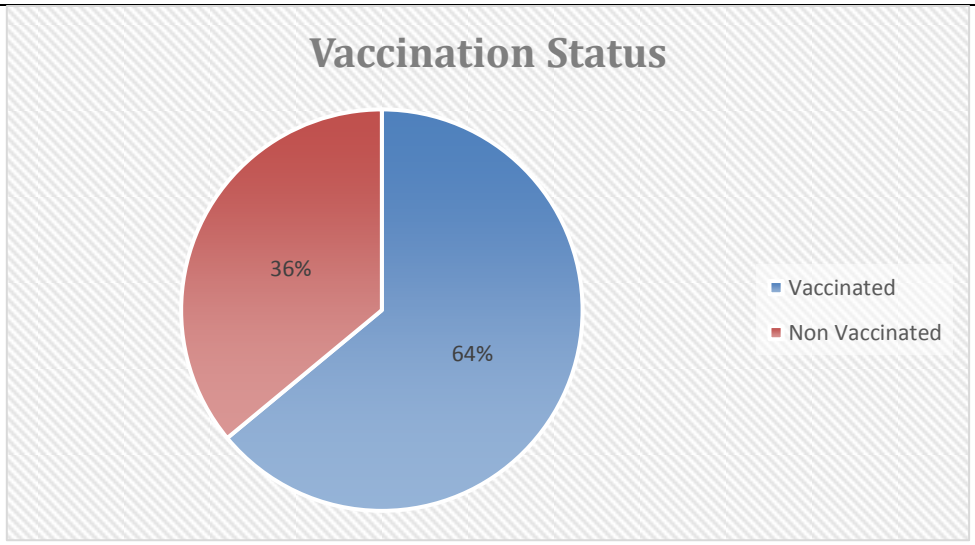

Figure 9. Vaccination Status

Out of 50 samples processed, 16 were positives. Among 16 positive isolates, 5 were positive for Staphylococcus aureus, 3 were positive for Streptococcus pyogenes, 5 were positive for Corynebacterium diphtheriae, 3 were positive for Candida albicans. Antibiotic sensitivity testing was carried out on Blood Agar/ Muller Hinton Agar with antibiotics – Penicillin, Ceftriaxone, Cefotaxim, Erythromycin, Azithromycin, Rifampin, Bacitracin. The most sensitive antibiotics were Ceftriaxone, Cefotaxime, Azithromycin which is shown below.

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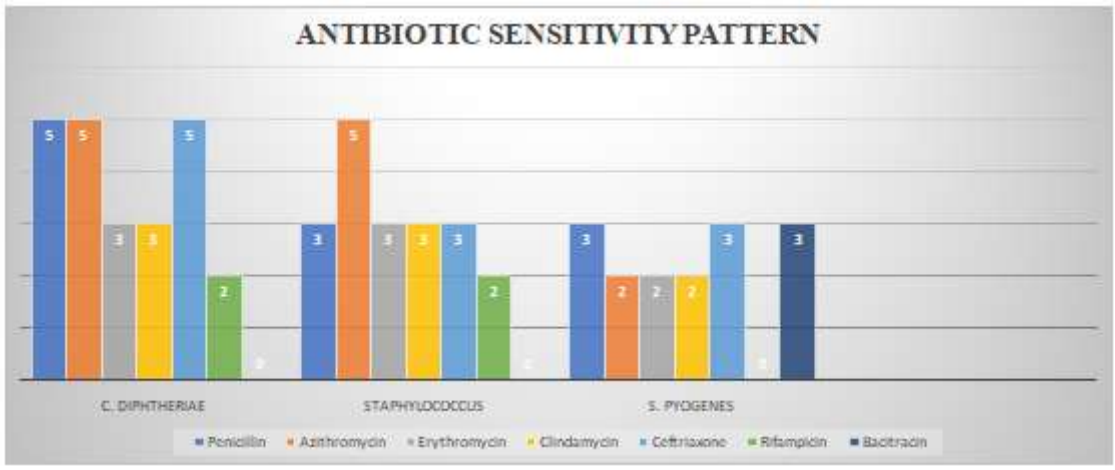

Figure 10. Antibiotic Sensitivity Pattern

Molecular testing was also done for testing of rpo B gene and Tox A gene. 5 samples came to be positive for C. diphtheriae rpo B gene where as one was positive for tox gene production.

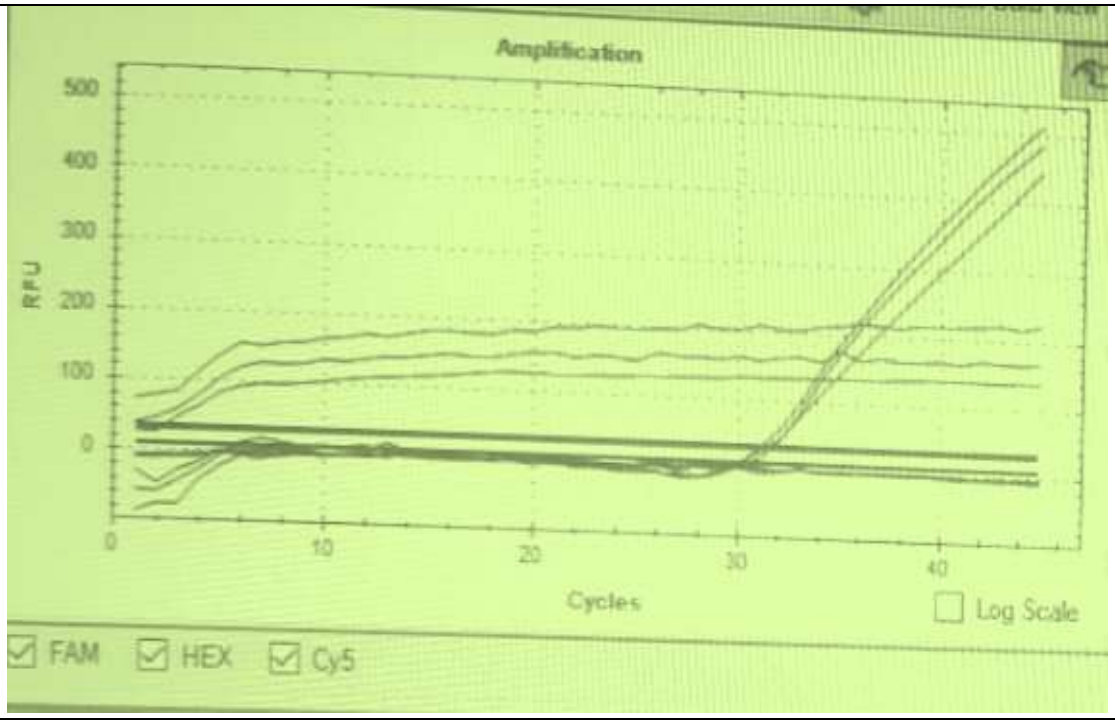

Figure 11. Molecular Testing for C. Diphtheriae

DISCUSSION

Diphtheria is a re-emerging infectious disease with a capacity for larger epidemics. Transmission occurs from person to person by respiratory droplets, sometimes by cutaneous lesions. Diphtheria being a leading cause of childhood mortality, the global burden of diphtheria has fallen dramatically, from more than a million cases a year in the mid-1900s to

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7097 cases reported in 2016.[3] In India and many other countries, cases of diphtheria have shown comeback because of waning of immunity.[5]

In the present study males were more compared to females. This is in correlation with a study by Hingolekar Ambika et al.[6] The maximum number of patients were in the age group of 5-10 years which is in correlation with Boghani et al.[7] The most common clinical features observed in our study were throat pain, fever, dysphagia, and bull neck which is in correlation with Mallesh Gampa et al.[8] In a study conducted by Meshram and Patil, similar patterns of clinical features were also observed, where the most common feature is throat pain followed by bull neck and respiratory difficulty.[9]

Common features in diphtheria are mostly due to local effects of the toxin. In this study 64% were vaccinated which is correlating with Hingolekar et al. The reason for low rate of vaccination may be myths and misconceptions on immunisation, literacy aspects of parents, migration from one area to other area. Mortality from diphtheria varies from 8% to 46% in various studies.[10]

PCR has contributed significantly to the development of modern molecular approaches in the laboratory diagnosis of diphtheria because it allows for preliminary presumptive results on toxigenicity within a few hours of specimen collection. A valid negative *tox* PCR test on suspected isolates will exclude the diagnosis and prevent further control measures. In addition to the speed of the test, interpretation of results is simple, and PCR facilities are becoming increasingly available in many laboratories throughout the world.[11]

Two samples were positive by culture where as 5 found to be positive by PCR. Antibiotics may alter the culture result by killing bacteria where as PCR is less affected. There are two fatal cases which are positive both by culture and PCR. This is in correlation to a study by Rosana et al.[12] Out of two cases one case had mortality inspite of administering Diphtheria antitoxin. Diphtheria anti-toxin decreased mortality in 76% of patients, but anti-toxin only neutralised circulating toxins, and its effectiveness was correlated with timely administration.[12]

Worldwide, in 2016, according to figures from the World Health Organization, there were 7,097 reported cases of diphtheria, mainly from India and Madagascar There is a higher fatality rate in young children. From the literature it showed that vaccinations does not provide life long immunity, booster doses are needed. Regular doses are needed for every ten years to provide protection. It is much needed for those visiting endemic areas. For Diphtheria it took 25 years to cause fatality.[13]

Vaccination does not provide protection against non toxigenic strains of C.diphtheriae. Toxigenic and non toxigenic strains are morphologically indistinguishable. Disease by these non toxigenic strains is more common in vulnerable population with pre existing conditions. Non toxigenic strains can be converted to toxigenic via bacteriophage mediated lysogenesis.[14]

Most of the outbreaks affect vulnerable populations living in or fleeing conflict zones with failing health care systems. The common features of these systems are: 1) critical shortage of clinicians knowledgeable in managing patients with diphtheria; 2) lack of laboratory capacity to make the diagnosis; and 3) limited basic medical equipment, supplies

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and medicines to provide early treatment, adequate monitoring and advanced airway management.[15]

To control an outbreak effectively, availability of resources and laboratory diagnosis is of utmost importance along with effectiveness of vaccines. There should be a functional laboratory surveillance system. It is recommended that laboratories should be strengthened for diagnosis of Diphtheria. Full vaccination provides more protection compared to partial vaccination. In addition timely administration of Diphtheria antitoxin is of life saving in suspected or confirmed Diphtheria.[16]

In 2010, India shared 3123 (77.1%) of 4053 diphtheria cases contributed by 24 countries, reported to WHO, which may possibly be a gross underestimate due to lack of a good surveillance system & facility for microbiologic diagnosis.[17] As per the CBHI (Central Bureau of Health Intelligence) data, during 2005–2014, India reported 41,672 cases (average: 4,167 per year) with 897 deaths (case fatality ratio: 2.2%). Ten Indian states (Andhra Pradesh, Assam, Delhi, Gujarat, Haryana, Karnataka, Nagaland, Maharashtra, Rajasthan, and West Bengal) accounted for 84% cases reported.[18]

CONCLUSION

Diphtheria is a communicable disease affecting all age groups. Nasopharyngeal Diphtheria being the most common.The disease is milder in vaccinated where as severe complications occur in unvaccinated individuals. Re emergence of Diphtheria ia a point of discussion since a past decade. A familiar proverb Prevention is better than cure applies here too. Its being a vaccine preventable disease primary immunisation with booster doses at regular intervals will decrease morbidity and mortality. Diphtheria toxoid vaccine, antibiotics, isolation plays a critical role in interrupting transmission. Availability of diphtheria antitoxin is critical in reducing mortality and this necessitates an immediate action to resolve the global shortage of Diphtheria antitoxin.

Conflicts of Interest: Nil

Funding: This study is being carried out under WHO VPD surveillance project.
This has been presented as an oral paper in 2nd ICCAM conference.

REFERENCES

[1] Shah R, Bansal A, Singhi SC. Approach to a child with sore throat. The Indian Journal of Pediatrics 2011;78:1268-72.

[2] Murthy GS, Bhimeswar R, Kumar MV, Prasad PK. Resurgence of diphtheria: Are we ready to treat. Int J Phonosurg Laryngol 2013;3(2):42-5.

[3] Truelove SA, Keegan LT, Moss WJ, Chaisson LH, Macher E, Azman AS, et al. Clinical and epidemiological aspects of diphtheria: a systematic review and pooled analysis. Clinical Infectious Diseases 2020;71(1):89-97.

[4] Gupta A, Gujral M, Singh A, Chawla K. Diphtheria resurgence in India: a case study from south Karnataka. Journal of Family Medicine and Primary Care 2020;9(11):5776-8.

[5] Prasad PL Rai PL. Prospective study of diphtheria for neurological complications. J Pediatr Neurosci 2018;13(3):313-6.

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[6] Hingolekar A, Bai MM. Diphtheria–correlation of clinical disease with lab diagnosis and vaccination status. International Journal for Advance Research and Development 2017;2(10):37-44.
[7] Boghani S, Shah HD, Fancy M, Parmar T, Bansal S, Wanjari MB, Saxena D. A study on the characteristics and outcomes of reported diphtheria patients in a Western state in India. Cureus 2023;15(3):e35769.
[8] Gampa M, Karna PN, Reddy KV, Priyadarshini T. Study of diphtheria and its complications: a retrospective study from a tertiary care hospital. Pediatric Infectious Disease 2021;3(4):140-2.
[9] Meshram RM, Patil A. Clinical profile and outcome of diphtheria in central India: a retrospective observational study. Int J Clin Pract 2018;5(4):1600-5.
[10] Mateen FJ, Bahl S, Khera A, Sutter RW. Detection of diphtheritic polyneuropathy by acute flaccid paralysis surveillance, India. Emerg Infect Dis 2013;19:1368-73.
[11] Efstratiou A, Engler KH, Mazurova IK, Glushkevich T, Vuopio-Varkila J, Popovic T. Current approaches to the laboratory diagnosis of diphtheria. J Infect Dis 2000;181(Suppl 1):S138-45.
[12] Rosana Y, Prilandari LI, Ajisman R, Hartono TS, Yasmon A. Detection of toxin-producing Corynebacterium diphtheriae from throat swabs of diphtheria patients using duplex real-time PCR. Iranian Journal of Microbiology 2020;12(6):508.
[13] Yeo LS, Quindipan ML, Liew CW, Murukuti P, Chia NC. An unexpected case of diphtheria-the need for vigilance. Journal of Emergency and Critical Care Medicine 2018;2(2).
[14] Alvarez GV, Rupert DD, Sinclair S, Miyara SJ, Guevara S, Shih P, Hoey C. Cardiac arrest associated with non-toxigenic corynebacterium diphtheria strain: a case report. Frontiers in Medicine 2023;9:1003193.
[15] Blumberg LH, Prieto MA, Diaz JV, Blanco MJ, Valle B, Pla C, et al. The preventable tragedy of diphtheria in the 21st century. International Journal of Infectious Diseases 2018;71:122-3.
[16] Medugu, N, Musa-Booth, TO. A review of the current Diphtheria outbreaks. Afr J Clin Exper Microbiol 2023;24(2):120-9.
[17] Ujwal MV, Pol RR, Vanaki R, Yelamali BC, Badakali AV. Resurgence of diphtheria in rural north Karnataka: Clinical profile and outcome. Int J Med Paediatr Oncol 2019;5(1):17-20.
[18] Murhekar M. Epidemiology of diphtheria in India, 1996–2016: implications for prevention and control. The American Journal of Tropical Medicine and Hygiene 2017;97(2):313-8.