MICROBIOLOGICAL CHARACTERIZATION AND MOLECULAR DIFFERENTIATION OF *Corynebacterium* spp. ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN

THESIS

Submitted To

All India Institute of Medical Sciences, Jodhpur

In partial fulfilment of the requirement for the Degree of

DOCTOR OF MEDICINE (MD)

(MICROBIOLOGY)

JULY, 2020 **DR. DEBALEENA PAUL**

AIIMS, JODHPUR

MICROBIOLOGICAL CHARACTERIZATION AND MOLECULAR DIFFERENTIATION OF *Corynebacterium* spp. ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN

THESIS

Submitted To

All India Institute of Medical Sciences, Jodhpur

In partial fulfilment of the requirement for the Degree of

DOCTOR OF MEDICINE (MD)

(MICROBIOLOGY)

JULY, 2020 **DR. DEBALEENA PAUL**

AIIMS, JODHPUR

ACKNOWLEDGEMENT

I would like to express my deep and sincere gratitude to my Guide, Dr. Sarika P Kombade, M.D, Microbiology, Associate Professor, Department of Microbiology, All India Institute of Medical Sciences, Jodhpur, for giving me the opportunity to do research and providing invaluable guidance throughout this research. Her dynamism, vision, sincerity and motivation have deeply inspired me. She has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was a great privilege and honor to work and study under her guidance. I am extremely grateful for what she has offered me. I would also like to thank her for her patience during the discussion I had with her on research work and thesis preparation.

Besides my Guide, I would like to thank the rest of my thesis committee: Dr. Vidhi Jain Associate Professor, Department of Microbiology and Dr. Siya Ram Didel, Associate Professor Department of Paediatrics for their insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives.

I would like to extend my thanks to Dr. Ashwini Agarwal, former Additional Professor and head, Department of Microbiology, AIIMS, Jodhpur for providing his insightful comments in my thesis work. I am grateful to my teachers, Dr. Ravisekhar Gadepalli, Additional Professor, Dr Vibhor Tak, Additional Professor, Dr Kumar S Abhishek, Assistant Professor, and Dr Kuntal Sinha, Assistant Professor, Department of Microbiology for their kind encouragement.

I would like also like to extend my thanks to Senior Residents of Department of Microbiology, especially Dr. Neetha T. Ramanukutty, Dr. Navneet Kaur, Dr. Subhashree Samantray, Dr. Saumya Srivastava, Dr. Alisha Aggarwal, Dr. Twishi Shrimali, Dr Sunandini Kapoor for taking interest in my work and providing guidance for the same.

I am grateful to the staff of Department of Microbiology and various other departments for their co-operation in sample collection and obtaining relevant clinical information of the patients enrolled in the thesis work.

I would like to say thanks to my Seniors, Dr. Kirtika Sharma, Dr. Malika Grover, Dr. Naila Beg, Dr. Aditya Kundu, Dr Zeeshan Noore Azim, Dr. Tejashree Nare, Dr. Kirti Vishwakarma for their constant encouragement and support.

I am extending my thanks to all my Juniors especially Dr. Venkat Goutham Nag, Dr. Nikhil John S, Dr. Nithya S Roy, Dr. Shikhir Malhotra, Dr Sukanya Mehotra and Dr S Haripriya for their constant support during my research work.

I am grateful to the WHO VPD Surveillance team in Rajasthan for extending their help and support us with the funds. I also thank CMC Vellore for providing SOP, primers and probes for RT-PCR, training and clearing all the doubts in the processing and diagnosis of Diphtheria.

I also thank all the staff of Research section of AIIMS, Jodhpur for their kindness. I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future.

Finally, I could not have completed this thesis without the support of my 2 seniors, Dr. Aditya Kundu and Dr. Zeeshan Noore Azim who provided stimulating discussions as well as happy distractions to rest my mind outside of my research.

Dr. Debaleena Paul

DECLARATION

I, hereby declare that the thesis entitled **"MICROBIOLOGICAL CHARACTERIZATION AND MOLECULAR DIFFERENTIATION OF *Corynebacterium* spp. ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN"** embodies the original work carried out by me in the Department of Microbiology at All India Institute of Medical Sciences, Jodhpur.

I further state that no part of thesis has been submitted either in part or in full for any other degree of All India Institute of Medical Sciences or any other Institute/University.

Debaleena Paul

Dr. Debaleena Paul

Department of Microbiology

All India Institute of Medical Sciences, Jodhpur

CERTIFICATE

This is to certify that the thesis entitled **"MICROBIOLOGICAL CHARACTERIZATION AND MOLECULAR DIFFERENTIATION OF *Corynebacterium* spp. ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN"** is bonafide work of **DR. DEBALEENA PAUL** carried out under our guidance and supervision in the Department of Microbiology, All India Institute of Medical Sciences, Jodhpur.

Guide:

Dr. Sarika P Kombade
Associate Professor
Department of Microbiology
AIIMS, Jodhpur

Co-Guides:

Dr. Vidhi Jain
Associate Professor
Department of Microbiology
AIIMS, Jodhpur

Dr. Siya Ram Didel
Associate Professor
Department of Pediatrics
AIIMS, Jodhpur

CERTIFICATE

This is to certify that the thesis entitled **"MICROBIOLOGICAL CHARACTERIZATION AND MOLECULAR DIFFERENTIATION OF *Corynebacterium* spp. ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN"** is bonafide work of **DR. DEBALEENA PAUL** carried out under our guidance and supervision in the Department of Microbiology, All India Institute of Medical Sciences, Jodhpur.

It is further certified that the candidate has fulfilled the pre-requisites necessary for the submission of this thesis work.

Dr. Sneha Ambwani
Professor
Officiating Head
Department of Microbiology
AIIMS, Jodhpur

INDEX

LIST OF ABBREVIATIONS

NOTATION		EXPANDED FORM
AST	:	Antimicrobial Susceptibility Testing
CHC	:	Community Health Centers
CAMHB-LHB	:	Cation-adjusted Muller-Hinton broth supplemented with lysed horse blood
CLSI	:	Clinical Laboratory Standards Institute
DNA	:	Deoxyribonucleic acid
DPT	:	Diphtheria Pertussis Tetanus
ADS	:	Anti-Diphtheritic Serum
GPB	:	Gram-positive bacilli
IPD	:	In Patient Department
IM	:	Intra-muscular
IDSP	:	Integrated Disease Surveillance Program
JRF	:	Joint Reporting Form
KLB	:	Klebs-Loffler bacillus
LSS	:	Loeffler's serum slope
Lf	:	Limit of flocculation
MALDI TOF-MS	:	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MIC	:	Minimum inhibitory concentration
MacF	:	MacFarland
NCR	:	National Capital Region
NRHM	:	National Rural Health Mission

OPD	:	Out Patient Department
PHC	:	Primary Health Centers
RCH	:	Reproductive and Child Health
RT-PCR	:	Real-time reverse transcriptase polymerase chain reaction
SBA	:	Sheep blood agar
TT	:	Tetanus Toxoid
VPD	:	Vaccine Preventable disease
WHO		World Health Organization

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXURES

SUMMARY

Diphtheria is a vaccine preventable disease caused by toxigenic strains of *Corynebacterium* spp. It is an acute infectious disease ranging from mild infection to acute airway obstruction, toxemia and sudden death. Despite the success of mass immunization in many countries worldwide, diphtheria continues to play a major role as a lethal resurgent infectious disease. An accurate microbiological diagnosis of diphtheria is crucial and is always regarded as being complementary to clinical diagnosis. The rapid identification of the three potentially toxigenic *Corynebacterium* spp. is not only essential for diagnosis and treatment of diphtheria and diphtheria-like diseases with respect to the single patient, but is also important for public health reasons, since a suspected diphtheria case prompts a variety of public health actions including contact tracing and WHO notification. Thus, this study was formulated to determine the Microbiological characterization and molecular differentiation of *Corynebacterium* spp. from the clinicals specimens in a tertiary care hospital in Western Rajasthan.

AIM AND OBJECTIVES:

Aim: To study the Microbiological characterization and molecular differentiation of *Corynebacterium* spp. from the clinical specimens in a tertiary care hospital.

Objectives were to isolate and identify *Corynebacterium* spp. from clinical specimens followed by AST and Toxin detection of the isolated *Corynebacterium* spp. by conventional method. Simultaneously identification and toxin detection by molecular method.

MATERIAL AND METHODS:

It was a descriptive study (time-based study) conducted in the Department of Microbiology of a tertiary care hospital of Jodhpur from January 2021 – December 2022. Two Samples (throat swab) of suspected cases of Diphtheria fulfilling the WHO case definition were collected and the first throat swab was processed for isolation and identification of *Corynebacterium* spp. which was followed by AST and Toxigenicity testing of the isolated *Corynebacterium* spp. by conventional method. The second

throat swab was used directly for identification and Toxin detection by molecular technique Multiplex Real Time Polymerase Chain Reaction (RT-PCR).

RESULT:

A total 204 suspected cases of Diphtheria were included during the study period from January 2021- December 2022. Among the 204 cases, 27 (13.2%) cases showed GPB on direct Gram's staining and 26 (12.7%) cases showed KLB. All the 26 positive KLB were also positive for GPB.

Among the 204 cases, *Corynebacterium* spp. was isolated and identified in 25 (12.25%) cases & all these 25 cases were also smear positive. Among them *Corynebacterium diphtheriae* was speciated 9 (4.41%) cases and *Corynebacterium ulcerans* in 16 (7.84%) cases. None of these 25 isolates showed any resistance to Penicillin, Ceftriaxone, Erythromycin, Azithromycin and Amoxicillin. Modified Elek's gel precipitation test performed on 25 isolates of *Corynebacterium* spp. showed toxin production in 9 (4.41%) cases. All the toxin producing strains were from *C. diphtheriae.*

Among the 204 cases, RT-PCR detected *Corynebacterium* spp. in 55 (26.96 %) cases, in which *C. diphtheriae* was detected in 9 (4.41%) cases and *C. ulcerans* was detected in 46 (22.54%) cases.

Simultaneously out of 204 cases, *Toxin A* was detected in 10 (4.9%) cases. Among the 10 (4.9%) *Toxin A* positive cases, 9 (4.41%) was from *C. diphtheriae* and 1 (0.49%) was from *C. ulcerans.*

CONCLUSION:

This study gives an insight about the rise of Diphtheria cases in the district of Jodhpur, Jaipur, Ajmer and Bikaner since 2021 which will help Public Health Department to strengthen the full DPT vaccination coverage in this region to prevent upsurge of diphtheria cases in the future. Toxigenic strain of *C. ulcerans* and nontoxigenic variants of *C. ulcerans* are markedly found raised in this study which indicates re-emergence of diphtheria due to non-*Corynebacterium diphtheriae* strains which needs to be addressed seriously apart from *C. diphtheriae* strains. Microbiological diagnosis is complementary to the clinical diagnosis and basically supports the clinical decision-making process by proving the suspected diagnosis.

INTRODUCTION

Diphtheria is caused by toxin-producing strains of *Corynebacterium diphtheriae*, and more rarely *Corynebacterium ulcerans*, or *Corynebacterium pseudotuberculosis*. [1]. *Corynebacterium diphtheriae* was first described in 1884 by Friedrich Löffler, who also showed that this bacterium is the etiological agent of diphtheria [2–4].

The most common form of this disease is respiratory diphtheria [5], which is characterized by mild fever and an exudative pharyngitis at the beginning of infection. During progression of the infection, a greyish white pseudo-membrane may be formed on the tonsils, pharynx, and larynx, composed of fibrin and secreted by the damaged nasopharyngeal epithelia, destroyed host cells, and colonizing bacteria. This pseudo-membrane may get detached while coughing and may cause bleeding of the epithelial tissue, and subsequently, decaying erythrocytes may stain the pseudo-membrane a dirty brownish color. The extension of inflammation into the nasal cavity and larynx may cause an obstruction of the airways, resulting in dyspnea up to suffocation and death [1].

Klebs isolated *C. diphtheriae* from a pseudomembrane in 1884, and Loeffler proved it to be the etiologic agent of diphtheria. Hence known as Klebs Loeffler bacillus or KLB [6].

Classical diphtheria of the upper respiratory tract is spread from person to person via respiratory droplets. Additionally, other secretions and contaminated materials can also be a source of infection, especially in the case of cutaneous diphtheria, where wounds or insect bites are the typical entry sites [7].

Before introduction of mass vaccination, children were the main victims of diphtheria, which indicates that *C. diphtheriae* was widely disseminated among the population, leading to early contact with the pathogen. With the beginning of industrialization and urbanization, diphtheria became more prevalent and became a leading cause of infant mortality. Up to four fifths of children infected with diphtheria died [8].

As the harmful effects of diphtheria toxin are the primary contributor and sometimes having fatal outcome, it was a prime target to combat respiratory diphtheria [9].

In 1888 Roux and Yersin discovered the exotoxin and described its clinical effects. The treatment of diphtheria advanced significantly in 1890 when von Behring and Kitasato

developed antitoxin in guinea pigs, demonstrating the concept of passive immunity. For this discovery von Behring was awarded the Nobel Prize for Physiology and Medicine in 1901 [6].

The first toxoid vaccine was produced by Ramon in 1923 by formalin treatment of diphtheria toxin, and it was the basis of subsequent mass vaccination starting in industrialized countries in the 1920s [10].

After implementation of the World Health Organization's Expanded Programme on Immunization (EPI) in 1974, only relatively small and local outbreaks occurred until the 1990s [11]. This changed with the breakdown of the former Union of Socialist Soviet Republics, when a large-scale outbreak leading to a diphtheria pandemic between 1990 and 1998 occurred, with more than 157,000 reported cases and over 5000 deaths [12–15].

This pandemic was finally stopped by mass immunization, especially of adults with waning antibody levels. Despite continuing global efforts and stable vaccination coverage, diphtheria is not eradicated today. Between 2015 and 2019, diphtheria outbreaks occurred, for example, in Bangladesh, Haiti, South Africa, Venezuela, and Yemen [16–20], and, moreover, the worldwide number of reported cases of diphtheria has increased within the last few years [21,22].

C. diphtheriae is a re-emerging pathogen at the global level [23–25]. Its increasing importance can be attributed to several reasons. Firstly, it can be expected that the global SARS-CoV-2 pandemic, which overwhelmed public health systems in many countries and also recent military conflicts such as in Yemen, Ethiopia, or Ukraine may interfere with vaccination and further increase in cases of diphtheria. Secondly antibiotic-resistant strains of *C. diphtheriae* are increasingly observed [28,29]. Thirdly, interaction of *C. diphtheriae* with host cells turned out to be much more complex than initially expected when *C. diphtheriae* was considered as extracellular pathogen [30]. Therefore, not only surveillance of cases, but also continuing research focusing on the re-emerging pathogen is crucial and hence, the study was carried out.

REVIEW OF LITERATURE

Diphtheria is a potentially fatal disease caused by toxigenic strains of the Genus *Corynebacterium.*

Diphtheria has varied presentation ranging from mild infection to acute airway obstruction, toxemia and sudden death [31]. Diphtheria remains a serious health problem within many regions of the world including India. The disease can have a fatality rate as high as 30.8% as reported in a previous study from Assam [32].

Diphtheria is often confused with other conditions, such as severe streptococcal sore throat, Vincent's angina, or glandular fever [32]. Therefore, microbiological diagnosis and appropriate clinical management of patients is very crucial in management of case of Diphtheria.

In many cases of classical respiratory diphtheria, medical and public health actions are mainly based on clinical diagnosis prompting immediate action, that is isolation of the index patient, antitoxin administration, sampling of diagnostic material for microbiological diagnosis, supportive antibiotic treatment and contact or source tracing. Therefore, in unambiguous cases clinical diagnosis usually precedes microbiological confirmation thus allowing straight forward management of the patient. However, rapid diphtheria diagnosis might be hampered for several reasons. For instance, in low incidence countries clinical awareness might be low and proper diagnosis delayed. Moreover, in patients presenting with milder symptoms, example pharyngitis

without “pathognomonic” pseudo membranes or with cutaneous diphtheria, establishment of the definite diagnosis might only be achieved by the detection of a toxigenic *Corynebacterium* species. Basically, the main role of the laboratory is to provide simple, rapid and reliable methods to assist clinicians in confirming a clinical diagnosis. In these cases, microbiological diagnosis is complementary to the clinical diagnosis and basically supports the clinical decision-making process by proving the suspected diagnosis. However, the rapid identification of the three potentially toxigenic *Corynebacterium* species is not only essential for diagnosis and treatment of diphtheria and diphtheria-like diseases with respect to the single patient, but is also important for

public health reasons, since a suspected diphtheria case prompts a variety of public health actions including contact tracing and WHO notification.

During the 5th or 4th century BC Vivid depictions of diphtheria were documented during the Renaissance: Bartholin, in his descriptions of the illness, used the terms "angina puerorum" and "morbus strangulatorius," highlighting how it frequently causes excessive morbidity in young children [26]. During the 1600s, there were occasional outbreaks in Spain, and from 1735 to 1740, children in New England experienced a major epidemic [27].

During an 1818–20 epidemic in Tours, Bretonneau described diphtheria's salient clinical findings and differentiated it from other causes of "throat distemper." He named the disease "diphtheria" (from the Greek "diphthera," meaning "leather hide"), aptly depicting its characteristic pseudomembrane. He may have also been the first clinician to perform a tracheostomy successfully for pharyngeal diphtheria in 1825 [6].

Klebs isolated *C. diphtheriae* from a pseudomembrane in 1884, and Loeffler proved it to be the etiologic agent of diphtheria. In 1888 Roux and Yersin discovered the exotoxin and described its clinical effects [25]. In 1890 von Behring and Kitasato developed antitoxin in guinea pigs, demonstrating the concept of passive immunity, for this discovery von Behring was awarded the inaugural Nobel Prize for Physiology and Medicine in 1901[28]. Ramon developed a safe and immunogenic heat- and formalin-inactivated toxoid vaccine in 1923. Aluminum salt adjuvants were incorporated into the toxoid vaccine to increase its immunogenicity, and by the 1940s an effective vaccine had been developed [29,30].

The genus *Corynebacterium* is grouped within the order Actinomycetales and consists of more than 80 species, several of which are medically important. *C. diphtheriae* (from "Korune" and "diphthera," Greek for "club" and "leather," respectively) is named for its characteristic clubbed shape appearance on Gram stain and its propensity to form a leather-like pseudomembrane [33].

C. diphtheriae, the classical diphtheria agent, and the two species *C. ulcerans* and *C. pseudotuberculosis* are facultative anaerobic, non-motile, non-sporulating, unencapsulated, pleomorphic bacteria. They are Gram positive but tend to get decolourised easily [1].

The bacillus is a slender rod with a tendency to clubbing at one or both ends, measuring approximately 3-6 µm × 0.6-0.8 µm. The granules are often situated at the poles of the bacilli and are called polar bodies. They are more strongly Gram positive then the rest of the bacterial cell. Stained with Loeffler's methylene blue, the granules take up bluish purple colour and hence are called metachromatic granules. They are also called volutin or Babes-Ernest granules. These granules are composed of polymetaphosphate and serve as storage granules.

Special stains, like Albert's, Neisser's and Ponder's have been devised for demonstrating the granules clearly [34,35].

The bacilli are arranged in a characteristic fashion in smears. They are usually seen in pairs, palisades (resembling the stake of a fence) or small groups, the bacilli being at various angles to each other, resembling the letter V or L. This has been called the Chinese letter or Cuneiform arrangement. This is due to incomplete separation of the daughter cells after binary fission. Growth of *C. diphtheriae* is scanty on ordinary media. Enrichment with blood, serum or egg is necessary for good growth. The optimum temperature for growth is 37^0 C (range 15-40^0 C) and optimum pH is 7.2. Loeffler's serum slope (LSS) is an enriched medium containing horse serum. Diphtheria bacilli grow on LSS very rapidly and colonies can be seen in 6-8 hours. Colonies are small circular opaque discs and a distinct yellow tint. Potassium tellurite blood agar is a selective medium for *Corynebacterium* spp. Several modifications of tellurite blood agar have been utilized such as McLeod's and Hoyle's media. The properties of tellurite blood agar are, firstly tellurite (0.04%) inhibits the growth of most of other bacteria, acting as a selective agent and secondly Diphtheria bacilli reduce tellurite to metallic tellurium, which is incorporated in the colonies, giving them a grey or black colour. And the growth on this media may take 48 hours to appear. Based on colony morphology on the tellurite medium and other properties, Mc Leod classified diphtheria bacilli into 3 types: *gravis, intermedius, mitis* [36,37]. The names were originally proposed to relate to the clinical severity of the disease produced by the three types- *gravis*, causing the most serious, *mitis* the mildest variety, with intermedius being responsible for disease of intermediate severity. However, the association is not constant [35].

Typical colony morphology features of *Corynebacterium* spp. on different agar (Table 1) [37-39].

Species and/or biovar	**Blood agar**	**Hoyle's tellurite agar**	**Tinsdale agar**
C. diphtheriae biovar *gravis*	Non-hemolytic	Dull, grey/black, opaque, 1.5–2 mm in diameter, matt surface, friable, moveable or tending to break into small segments when touched with a loop	Black with brownishblack halo
C. diphtheriae biovar *mitis*	Small zone of β-hemolysis	Grey/black, opaque, 1.5–2 mm in diameter, entire edge and glossy smooth surface, variable in size	Black with brownishblack halo
C. diphtheriae biovar *intermedius*	Small zone of β-hemolysis	Grey/black, opaque, small, 0.5-1 mm in diameter, shiny surface, discrete, translucent, partly with black center	Black with brownishblack halo
C. diphtheriae biovar *belfanti*	Small zone of β-hemolysis	Grey/black, opaque,	Black with brownishblack halo

		1.5–2 mm in diameter, entire edge and glossy smouth surface, variable in size	
C. ulcerans	Small zone of β-hemolysis, grey/ white, dry, waxy consistency, circular, slightly convex with an entire margin	Grey/black, opaque, very dry	Black with brownishblack halo
C. pseudotuberculosis	Small zone of β-hemolysis, cream to orange coloured, concentrally ringed	Grey/black, opaque, very dry	Black with brownishblack halo
C. striatum	Non-hemolytic, white, moist, smooth	Grey/black	Black without halo
C. jeikeium	Non-hemolytic, grey/ white, low convex	Grey/black	Black without halo

Table 1: Colony morphology features of *Corynebacterium* spp. on different agar

In the late 19th century Loeffler discovered the presence of avirulent, nontoxigenic strains of *C. diphtheriae* in healthy carriers and noted that these strains are morphologically indistinguishable from toxigenic strains. Corynebacteriophages carry *tox*, the gene for exotoxin production, and convert strains of *C. diphtheriae* from

nontoxigenic to toxigenic via a lysogenic cycle. Expression of *tox* is regulated by DtxR, an iron-activated repressor that is derepressed in low iron states [33].

Along with the 3 important species that is *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*, other species of *Corynebacterium* along with their habitat, mode of transmission, Virulence factor, and spectrum of disease is summarized below (Table 2) [40].

Organism	**Habitat (Reservoir)**	**Mode of Transmission**	**Virulence Factors**	**Spectrum of Diseases and Infections**
Corynebacterium diphtheriae	Colonizer: Human nasopharynx but only in carrier state; not considered part of normal flora Isolation from healthy humans is not common.	Direct contact: Person to person by exposure to contaminated respiratory droplets Contact with exudate from cutaneous lesions, Exposure to contaminated objects.	Diphtheria toxin: A potent exotoxin that destroys host cells by inhibiting protein synthesis.	Respiratory diphtheria is a pharyngitis characterized by the development of an exudative membrane that covers the tonsils, uvula, palate, and pharyngeal wall; if untreated, life-threatening cardiac toxicity, neurologic toxicity, & other complications occur. Respiratory obstruction develops and release of toxin into the blood can damage various organs, including the heart.
Corynebacterium diphtheriae			Nontoxigenic strains: Uncertain	Cutaneous diphtheria is characterized by nonhealing ulcers and membrane formation. Immunocompromised patients, drug addicts, and alcoholics. Invasive endocarditis, mycotic aneurysms, osteomyelitis, and septic arthritis
Corynebacterium jeikeium	Colonizer: Skin flora of hospitalized patients, most commonly in the inguinal, axillary, and rectal sites.	Uncertain Direct contact: May be person to person Endogenous strain:	Unknown: Multiple antibiotic resistance allows survival in hospital setting	Systemic: Septicemia Skin infections: Wounds, rashes and nodules Immunocompromised:

		Selection during antimicrobial therapy Introduction during placement or improper care of intravenous catheters		Malignancies, neutropenia, AIDS patients. Associated with indwelling devices such as catheters, prosthetic valves, and CSF shunts
Corynebacterium ulcerans	Normal flora: Humans and cattle	Uncertain Zoonoses: Close animal contact, especially during summer	Unknown	Zoonoses: Bovine mastitis Has been associated with diphtheria-like sore throat, indistinguishable from *C. diphtheriae* Skin infections Pneumonia
Corynebacterium pseudotuberculosis	Normal flora: Animals such as sheep, goats, and horses	Uncertain Zoonoses: Close animal contact, but infections in humans are rare	Unknown	Zoonoses: Suppurative granulomatous lymphadenitis
Corynebacterium pseudodiphtheriticum	Normal flora: Human pharyngeal and occasionally skin Flora	Uncertain Endogenous strain: Access to normally sterile site	Unknown Some stains have been identified that are resistant to macrolides	Systemic: Septicemia Endocarditis Pneumonia and lung abscesses; primarily in immunocompromised
Corynebacterium urealyticum	Normal flora: Human skin	Uncertain Endogenous strain: Access to normally sterile site	Unknown Multiple antibiotic resistance allows survival in hospital setting	Immunocompromised and elderly: Urinary tract infections Wound infections Rarely: endocarditis, septicemia, osteomyelitis, and tissue infections
Corynebacterium xerosis	Normal flora: Human conjunctiva Skin Nasopharynx	Uncertain Endogenous strain: Access to normally sterile site	Unknown	Immunocompromised: Endocarditis Septicemia
Corynebacterium striatum	Normal flora: Skin	Uncertain Endogenous strain: Access to normally sterile site	Unknown	Immunocompromised: Bacteremia Pneumonia and lung abscesses Osteomyelitis Meningitis

Table 2: Pathogenesis and spectrum of diseases of different species of *Corynebacterium*

Pathogenesis

Apart from *C. diphtheriae, C. pseudotuberculosis* and *C. ulcerans* also elaborate the diphtheria toxin; both species may be differentiated from *C. diphtheriae* by means of biochemical testing.

The diphtheria exotoxin, containing 535 amino acids containing single protein. Diphtheria toxin binds to its receptor on the cell surface via R-domain and is internalized by clathrin mediated endocytosis. Low pH-induced conformational change of T-domain initiates membrane translocation steps. Catalytic fragment transfer occurs from early endosome to cytosol under regulation of cytosolic translocation factors. C-domain becomes active following disulfide bond reduction and chaperone-dependent refolding. Toxic C-domain ADP-ribosylates eEF2 in the presence of NAD+ and induces depolymerisation of F-actin. As a result of which there is inhibition of protein synthesis leading to cell death (Fig 1) [41].

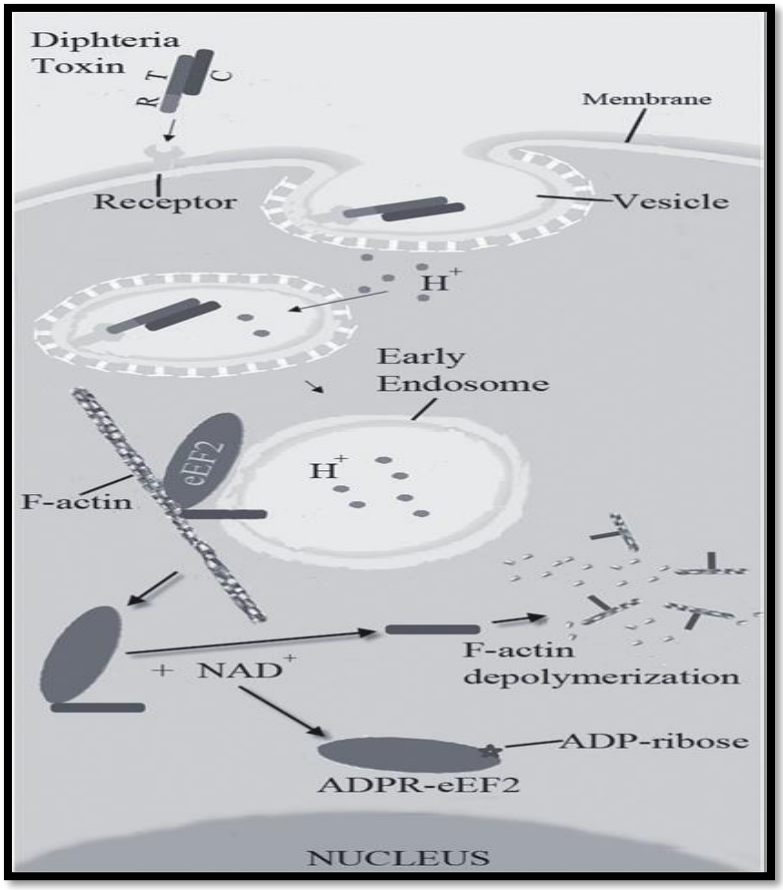

Fig 1: Schematic illustration of diphtheria toxin delivery pathway

One molecule of the exotoxin is sufficient to kill a cell; the lethal dose in humans may be as little as 100 ng/kg [42,43].

C. diphtheriae infection leads to mucosal edema with subsequent necrosis and ulceration. A fibrinous exudate overlying the desquamated mucosae forms the adherent pseudomembrane. On histopathology the pseudomembrane consists of fibrin and denuded epithelial cells with an associated neutrophilic infiltrate and clusters of *C. diphtheriae* organisms. The pseudomembrane may extend to form a cast of the upper airways. Forcible removal of the pseudomembrane

may cause bleeding; dislodgement may lead to aspiration and asphyxiation [44]. Edematous cervical, parabronchial and mediastinal lymph nodes often hemorrhage or necrose [45].

Spread from person to person, usually through respiratory droplets, like from coughing or sneezing. People can also get sick from touching infected open sores or ulcers. Those at increased risk of getting sick include: People in the same household, People with a history of frequent, close contact with the patient, People directly exposed to secretions from the suspected infection site (e.g., mouth, skin) of the patient (fig 2) [7].

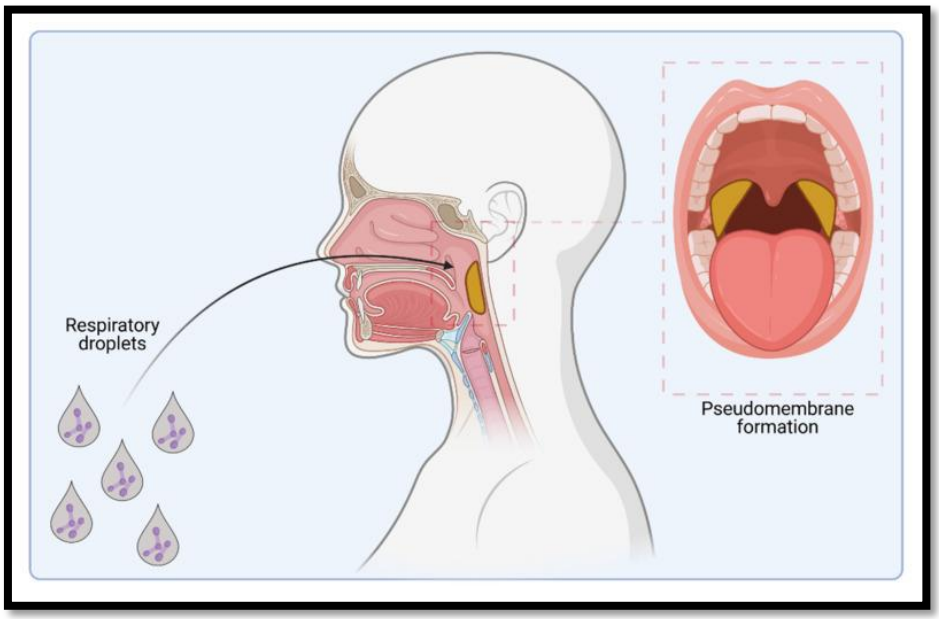

Fig 2: Transmission of pathogen

Clinical manifestations:

C. diphtheriae usually causes upper respiratory tract or cutaneous disease. Cardiac and neurologic complications are the most frequent toxin mediated manifestations. Both toxigenic and nontoxigenic strains may rarely disseminate to distant sites.

a) Respiratory Tract Diphtheria

Clinical signs and symptoms of respiratory tract disease become apparent after an incubation period of 2 to 5 days. The fauces are most commonly involved; however, disease may also occur at other sites, including the anterior nares, larynx, and tracheobronchial tree [34].

b) Anterior Nasal

Anterior nasal diphtheria is characterized by discharge which is mucopurulent that may be slightly bloody. In more severe cases a white membrane develops on the anterior nasal mucosae and septum. Very rarely, the membrane can erode through the nares and upper lip [21]. Systemic toxicity in anterior nasal diphtheria is uncommon, even in the presence of a pseudomembrane [30].

c) Faucial

Early symptoms of infection of the tonsillar pillars and pharynx include sore throat, malaise, and low-grade fever (usually less than 39°C). Approximately 3 days later a pseudomembrane forms on the tonsils or proximal pharynx in 50% to 80% of individuals (fig: 3) [46,47]. The membrane is initially white, then becomes grayish-green or black and may extend to the soft palate, nasopharynx, laryngopharynx, or bronchi. Forceful removal of the membrane causes bleeding of the underlying mucosae. Approximately one-third of affected individuals develop a “bull neck” appearance as a result of cervical lymph node enlargement and submandibular edema [48]. Local complications of pharyngeal diphtheria include stridor, airway obstruction, and subsequent respiratory failure. The case fatality rate of pharyngeal diphtheria is approximately 10%. In the absence of a pseudomembrane, disease is less severe and associated with improved outcomes [46,47].

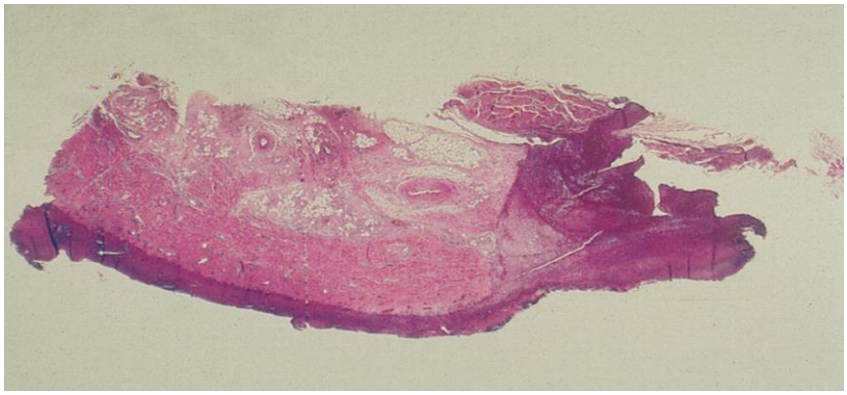

Fig 3: Hematoxylin & eosin staining of pharyngeal pseudomembrane X 2.5 magnification

d) Laryngeal and Tracheobronchial

Although primary infection of the larynx, trachea, and bronchial tree may occur, these sites are more often secondarily involved as a result of pseudomembranous extension from the pharynx. Prominent symptoms include stridor, hoarseness, and a barking cough. Airway edema or membrane dislodgement leads to eventual respiratory embarrassment and asphyxiation [30, 44].

Anatomy of upper and lower respiratory tract: The respiratory tract divided into following critical areas: the URT contains structure over the larynx, and the LRT under the windpipe to the bronchi and bronchioles, then, into the alveolar spaces, which has been shown by following fig 4.

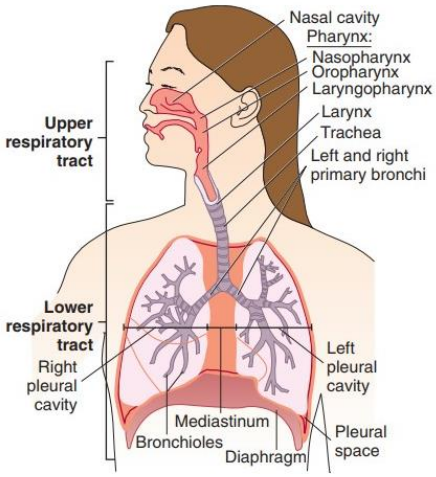

Fig 4: Anatomy of upper and lower respiratory tract

e) **Cardiac Toxicity**

Individuals with pharyngeal diphtheria may develop cardiac toxicity. Fever, tonsillar pseudomembrane, and "bull neck" appearance is predictive of cardiac involvement, which may occur acutely or approximately 10 days after the initial onset of symptoms. ST-segment and T-wave abnormalities, as well as QT interval prolongation, are electrocardiographic abnormalities of diphtheritic cardiomyopathy. Severe complications of cardiac involvement include cardiac dilatation, arrhythmias, and heart block. Approximately one-third of patients with diphtheritic cardiomyopathy suffer a fatal outcome; Third degree atrioventricular block and ST-segment depressions/T-wave inversions were associated with worse outcomes. Resolution of electrocardiographic abnormalities occurred in all survivors [49].

f) Neurologic Toxicity

A local motor neuropathy, manifesting as paralysis of the soft palate and posterior pharyngeal wall, occurs initially. Afterward, bulbar and oculomotor neuropathies may develop, leading to further paralysis of the pharynx and involvement of the extraocular and ciliary muscles. Peripheral neuritis, occurring early in the disease or up to 3 months after respiratory symptoms have abated, is characterized by a descending motor neuropathy involving the diaphragm and limbs [50-52]. Cerebrospinal fluid analysis usually reveals a cytoalbuminologic dissociation, similar to Guillain-Barré syndrome; the latter may be distinguished from diphtheritic polyneuropathy by its characteristic ascending paralysis. Sensory involvement occurs in a stocking-and-glove distribution. Signs of autonomic dysfunction, such as hypotension and urinary retention, may also develop. Cranial nerve neuropathies tend to improve at around the same time during the disease course as peripheral nerve function worsens [52]. After several weeks of neurologic involvement, complete recovery of peripheral motor and sensory nerve function is the norm.

g) Other Complications

Acute kidney injury, due to direct activity of the exotoxin, may occur. The exotoxin has been shown to induce necrosis of the kidneys, liver, and adrenal glands in animal models [53,54].

Cutaneous Diphtheria

Although widespread vaccination has led to a decline in the incidence of respiratory tract disease, cutaneous diphtheria has become increasingly recognized. Cutaneous infection due to toxigenic *C. diphtheriae* may occur in unimmunized individuals, but the majority of cases are caused by nontoxigenic strains. Cutaneous transmission may be more efficient than the respiratory route and may lead to contamination of fomites, thereby facilitating reinfections during outbreaks. Cutaneous diphtheria classically manifests as an ulcerative lesion that may be associated with a pseudomembrane Skin involvement may present uncharacteristically, however, as a scaly, impetiginous, or erythematous lesion [55].

Epidemiology

Incidence in industrialized countries decreased rapidly with diphtheria–tetanus–pertussis (DTP) vaccine introduction after World War II. Incidence in less developed countries also decreased after the launch of the World Health Organization (WHO) Expanded Programme on Immunization in 1974, which recommended that all infants receive a 3-dose series of DTP vaccine by 6 months of age [56].

A spike in incidence in the newly independent states of the former Soviet Union occurred in the 1990s (Fig 5), resulting in >157,000 cases and 5,000 deaths. This spike demonstrated the potential for severe outbreaks of diphtheria in communities that have a large population of nonimmune adults and poor vaccination coverage for children [57].

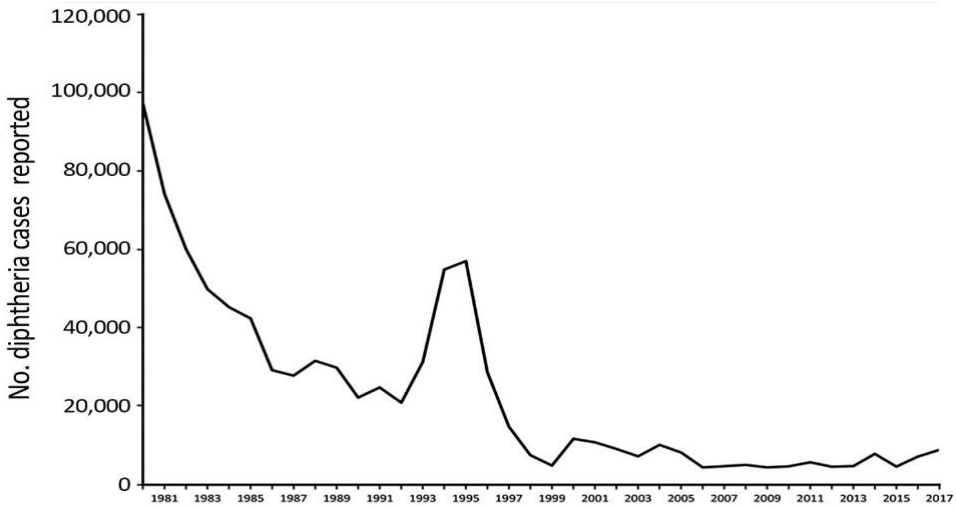

Fig 5: Cases of diphtheria as reported to the World Health Organization and the United Nations Children's Fund, through the Joint Reporting Form, worldwide, 1980−2017.

Global epidemiology:

Since 2000, the number of reported diphtheria cases worldwide in JRF (Joint Reporting Form) data initially decreased, then leveled at 4,300–5,700 reported cases/year during 2006–2013. Subsequently, the annual number of reported cases became more variable; 8,819 cases were reported in 2017, the most cases in a single year since 2004 (Fig 6). The average number of annual cases reported worldwide over the most recently reported 5-year period (2013–2017) was 6,582, an increase of 37% compared with the previous 5-year average of 4,809 cases during 2008–2012 [58-60].

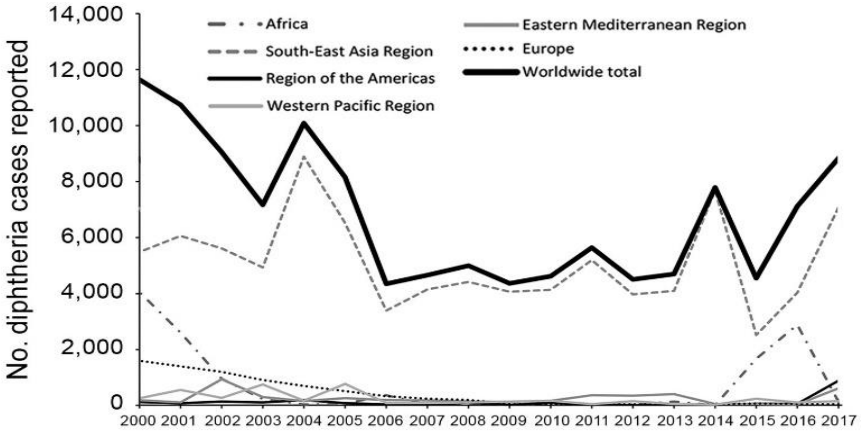

Fig 6: Reported cases of diphtheria per Joint Reporting Form, by World Health Organization region and worldwide, 2000−2017

Since 2000, the WHO South-East Asia region has reported most of the global diphtheria incidence each year. India has reported the largest proportion of diphtheria cases in aggregate JRF data since 2000 (64%); similarly, in data compiled from the literature review, >50% of cases with age and vaccination status were from India in the full dataset (8,720 [57%]). Collectively, India, Nepal, and Indonesia have reported 96%–99% of the cases in the South-East Asia region since 2000 [58-60].

According to IDSP, (Updated till 2022) Various districts of India has reported outbreak of Diphtheria. The red-coloured regions depicts the diphtheria cases as shown in fig: 7 [61].

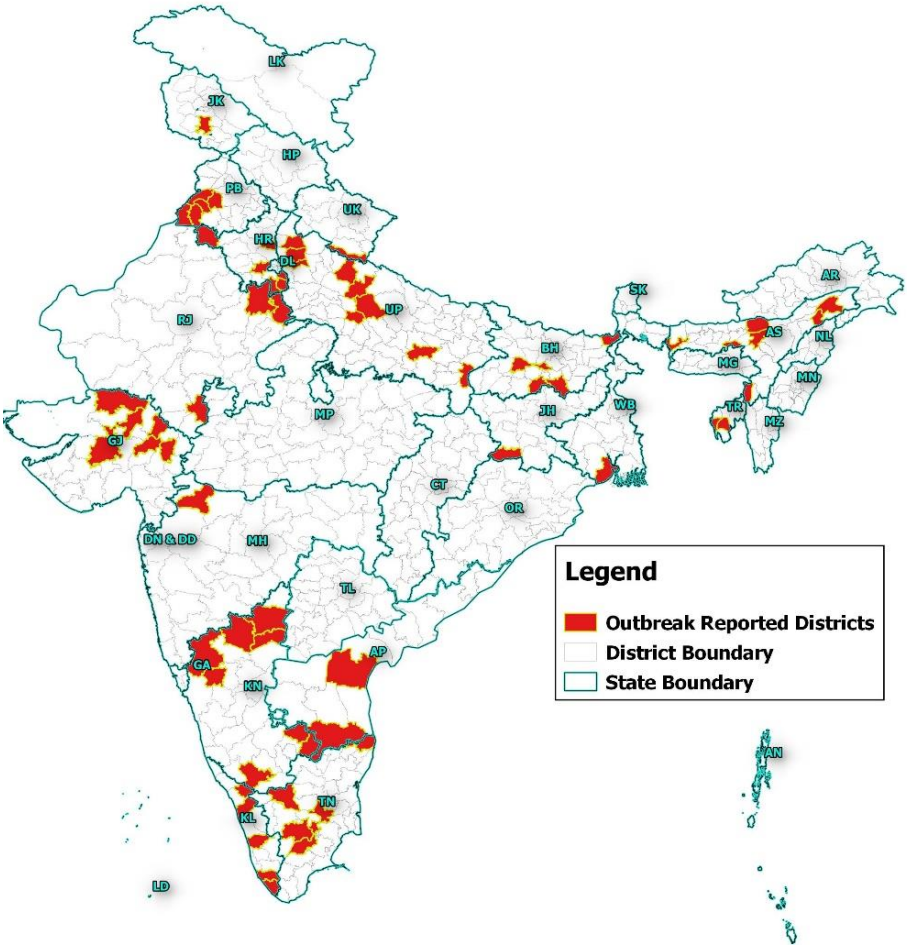

Fig 7: Map of India showing outbreak of Diphtheria in various districts

The yearly reported diphtheria cases and incidence rate globally and in India as follows (table 3) [62].

YEAR	GLOBAL		INDIA	
	Reported cases	Incidence rate (per 1,000,000 total population)	Reported cases	Incidence rate (per 1,000,000 total population)
2010	4,603	0.7	3,434	2.8
2011	5,626	0.9	4,233	3.4
2012	4,490	0.7	2,525	2
2013	4,680	0.7	3,133	2.4
2014	7,774	1.6	6,094	4.7
2015	4,535	0.7	2,365	1.8
2016	7,102	1.1	3,380	2.5
2017	8,819	1.3	5,293	3.9
2018	16,911	2.4	8,788	6.4
2019	22,986	3.4	9,622	7
2020	10,137	1.5	3,485	2.5
2021	8,638	1.3	1,768	1.3

Table: 3 Reported cases and incidence rate of Diphtheria

Although diphtheria is currently controlled by mass vaccination, still India accounts for 53.1% (8788) of cases globally in 2018 and 41.86% (9622) of cases globally in 2019 making country the highest contributor globally followed by Ethiopia 31.2%, Nigeria 9.9%, Madagascar 7.8%, Indonesia 2.1% and Yemen 1.2%in 2019 [63].

According to the study, Persistence of Corynebacterium diphtheriae in Delhi & National Capital Region (NCR) by S. Bhagat *et al* the percentage positivity of diphtheria cases in 2012, 2013 and 2014 were as 26.1, 30.6 and 17 per cent, respectively. The highest numbers of cases were obtained from Haryana (35%) followed by Uttar Pradesh (30%), Delhi (17%), Rajasthan (15%) and others (3%) during the study period. Majority of the samples were obtained from the rural areas of the respective States [64].

According to the study, Diphtheria in Andhra Pradesh–a clinical-epidemiological study by Meera M *et al*, of 61 950 admissions from January 2008 to December 2012, 2925 (4.7%) had clinical diphtheria; Culture-positive immunized patients were positive for

Corynebacterium other than diphtheriae (COD; n = 104) or Corynebacterium diphtheriae (CD; n = 23) [65].

Study conducted by Daiji Gogoi Mohan et al showed Out of the total of 99 cases, 40 cases were detected/diagnosed in 2013, 28 in 2014 and 31 in 2015 from Assam districts [66].

Outbreaks and sporadic cases of diphtheria have been reported occasionally from Assam (Table 4)

Author and reference	Year of study	District	Setting	Total cases
Nandi R *et al.*, [67]	1997-2002	Silchar	Hospital	101
Nath B and Mahanta TG [68]	2009	Dibrugarh	Outbreak	60
Saikia L et al., [32]	2010	Dibrugarh	Outbreak	13
Das PP et al., [69]	2015-2016	Dibrugarh	Outbreak	33
Devi U et al., [70]	2017	Dibrugarh	Surveillance	164
Choudhury G et al., [71]	2019-2020	Dibrugarh, Jorhat	Outbreak	3

Table 4: Diphtheria cases reported from various parts of Assam

Resurgence of Diphtheria in North Kerala, India, 2016: Laboratory Supported Case-Based Surveillance Outcomes Published in: Frontiers in Public Health, 30 August 2017 Result: A total of 533 cases were identified in 11 districts of Kerala in 2016. Almost 79% cases occurred in >10 years age group. In <18 years age group, 62% were male while in ≥18 years, 69% were females. In <10 years age group, 31% children had received three doses of diphtheria vaccine, whereas in ≥10 years, 3% cases had received all doses [72].

According to the study published in Trends of Communicable Diseases & IDSP reporting in State of Rajasthan, in 2015, district wise burden of diphtheria in Rajasthan, total 223 cases were found in which highest number of cases was in Jodhpur (104), second highest was in Bikaner (58), followed by Udaipur (44), Ajmer (5), and Alwar (2) [73].

Prevention [35, 74]

Incorporation of the diphtheria toxoid vaccine into routine immunization schedules has resulted in a dramatic decrease in the global disease incidence. In adequately immunized individuals the vaccine has a clinical efficacy of approximately 97% in preventing the development of toxigenic disease. Active immunization is done with diphtheria toxoid as it induces antitoxin production in the body. A protective titer of more than 0.01 Unit/mL of antitoxin can prevent all forms of diphtheria. However, vaccine is not effective for: Prevention of cutaneous diphtheria and elimination of carrier stage

Types of Vaccine

a) Single vaccine**:** Diphtheria toxoid (alum or formal precipitated)

b) Combined vaccine: DPT: Contains DT (diphtheria toxoid), Pertussis (whole cell) and TT (tetanus toxoid), DaPT: Contains DT, TT and acellular pertussis (aP), DT: Contains DT and TT, dT: Contains TI and adult dose diphtheria toxoid (d)

DPT Vaccine

Among the vaccine preparations available, DPT is the preparation of choice for vaccinating infants, because Infants can be immunized simultaneously against three important childhood diseases- diphtheria, tetanus and pertussis by single injection. Pertussis component acts as adjuvant and increases immunogenicity of DT and TT.

Diphtheria toxoid is prepared by two methods:

1. Plain formol toxoid (or fluid toxoid): Toxoid is prepared by incubating toxin with formalin.

2. Adsorbed (alum adsorbed): Formol 1oxoid is adsorbed on 10 alums. Alum (Aluminum phosphate, to less extent Aluminum hydroxide) acts as adjuvant and increases the immunogenicity of toxoid.

Administration of DPT

Schedule: DPT is scheduled under National Immunization schedule of India.

Total five doses are given, three doses at 6, 10 and 14 weeks of birth followed by two boosters

doses at 16-24 months and 5 years.

Site: DPT is given deep intramuscularly (IM) at anterolateral aspect of thigh, (gluteal region is not preferred as fat may inhibit DPT absorption).

Thiomersal (0.01%) is used as preservative.

Storage: DPT should be kept at 2-8^0 C, if accidentally frozen then it has to be discarded.

Dose: One dose (O.5 ml) of vaccine contains:

Glaxo: 25 Lf (DT), 5 Lf (TT), 20,000 million (pertussis killed bacilli).

Kasauli: 30 Lf (DT), 10 Lf (TT), 32,000 million (pertussis killed bacilli).

Protective titer: Following vaccination, an antitoxin titer of~ 0.01 unit/mL is said to be protective.

Adverse Reactions following DPT Administration

Mild: Fever and local reaction (swelling and indurations) are observed commonly.

Severe: Whole cell killed vaccine of *B. pertussis* is encephalitogenic. It is associated with neurological complications. Hence, DPT is not recommended after 6 years of age.

Absolute contraindication to DPT: Hypersensitivity to previous dose and progressive neurological disorder. Acellular pertussis (aP) vaccine: This form of pertussis vaccine is devoid of neurological complication and is given safely to older children (DaPT).

India introduced pentavalent vaccine from the Serum Institute of India in the states of Tamil Nadu and Kerala in December 2011. This was followed by expansion of vaccine usage in the states of Goa, Pondicherry, Karnataka, Haryana, Jammu and Kashmir, Gujarat and Delhi during the second half of 2012 through the first quarter of 2013.

Pentavalent Vaccine

About-Pentavalent vaccine is a combined vaccine to protect children from five diseases Diphtheria, Tetanus, Pertussis, Hemophilus influenza type b infection and Hepatitis B. Three doses are given at 6, 10 and 14 weeks of age (can be given till one year of age). Route and site-Pentavalent vaccine is given intramuscularly on anterolateral side of mid-thigh

Tetanus and adult diphtheria (Td) vaccine:

TT vaccine has been replaced with Td vaccine in UIP to limit the waning immunity against diphtheria in older age groups. Td vaccine is administered to adolescents at 10 and 16 years of age and to pregnant women. Pregnant women- Td-1 is given early in pregnancy as first dose and 4 weeks after Td1, second dose of Td as Td-2 is given. Td-

Booster is given, if pregnant woman has received 2 TT/Td doses in a pregnancy within the last 3 years. Intra-muscular Upper Arm

Immunization has a major role in prevention of Diphtheria. The immunization coverage Globally and in India is summarized in table 5 [62].

Year	**Global**		**India**	
	DTP-containing vaccine, 1st dose	DTP-containing vaccine, 3rd dose	DTP-containing vaccine, 1st dose	DTP-containing vaccine, 3rd dose
2010	89%	83%	86%	79%
2011	90%	84%	89%	82%
2012	90%	84%	89%	82%
2013	89%	84%	90%	83%
2014	90%	85%	90%	85%
2015	89%	85%	90%	87%
2016	90%	86%	91%	88%
2017	90%	86%	92%	89%
2018	90%	86%	93%	90%
2019	90%	86%	94%	91%
2020	87%	83%	87%	85%
2021	86%	81%	88%	85%

Table 5: Immunization coverage of DPT vaccine

Concomitant efforts should now focus on improving and monitoring primary immunization and booster coverages across all age groups.

AIMS AND OBJECTIVES

AIM:

Microbiological characterization and molecular differentiation of *Corynebacterium* species isolated from clinical specimens in a tertiary care hospital in Western Rajasthan.

OBJECTIVES:

- Isolation and identification of *Corynebacterium* spp. from clinical samples received in Microbiology laboratory by conventional method.
- Drug susceptibility testing of identified *Corynebacterium* spp. by conventional method.
- Detection of toxin production by modified Elek's gel precipitation test in the isolates identified as *Corynebacterium* spp.
- Identification of *Corynebacterium* spp. by molecular technique Multiplex Real Time Polymerase Chain Reaction (RT-PCR).
- Detection of tox gene by Multiplex Real Time Polymerase Chain Reaction (RT-PCR).

MATERIAL AND METHODS

STUDY DESIGN:

It is a Descriptive study (Time based study).

PLACE OF STUDY:

The study was carried out in the Department of Microbiology, All India Institute of Medical Sciences, Jodhpur.

STUDY DURATION:

The study was carried out from January 2021 to Dec 2022.

PATIENT SELECTION

The **case definition** of a suspected case of diphtheria [75]

An illness of the upper respiratory tract characterized by the following: pharyngitis, nasopharyngitis, tonsillitis

or

laryngitis AND adherent pseudomembrane of the pharynx, tonsils, larynx and/or nose.

Note: A diphtheria pseudomembrane is an exudate that is greyish, thick, firmly adherent and patchy to confluent. Dislodging the pseudomembrane is likely to cause profuse bleeding.

INCLUSION CRITERIA:

Minimum 2 samples (throat swab) of suspected cases of Diphtheria fulfilling the case definition were received in Microbiology laboratory, AIIMS Jodhpur.

EXCLSUION CRITERIA:

- All those who are not having clinical suspicion of Diphtheria.
- Sample is unlabeled or unmarked and there is mismatch of the identity.
- Submission in an improper and non-sterile container and leaking container.
- Only one sample received.
- Patients not willing to give consent.

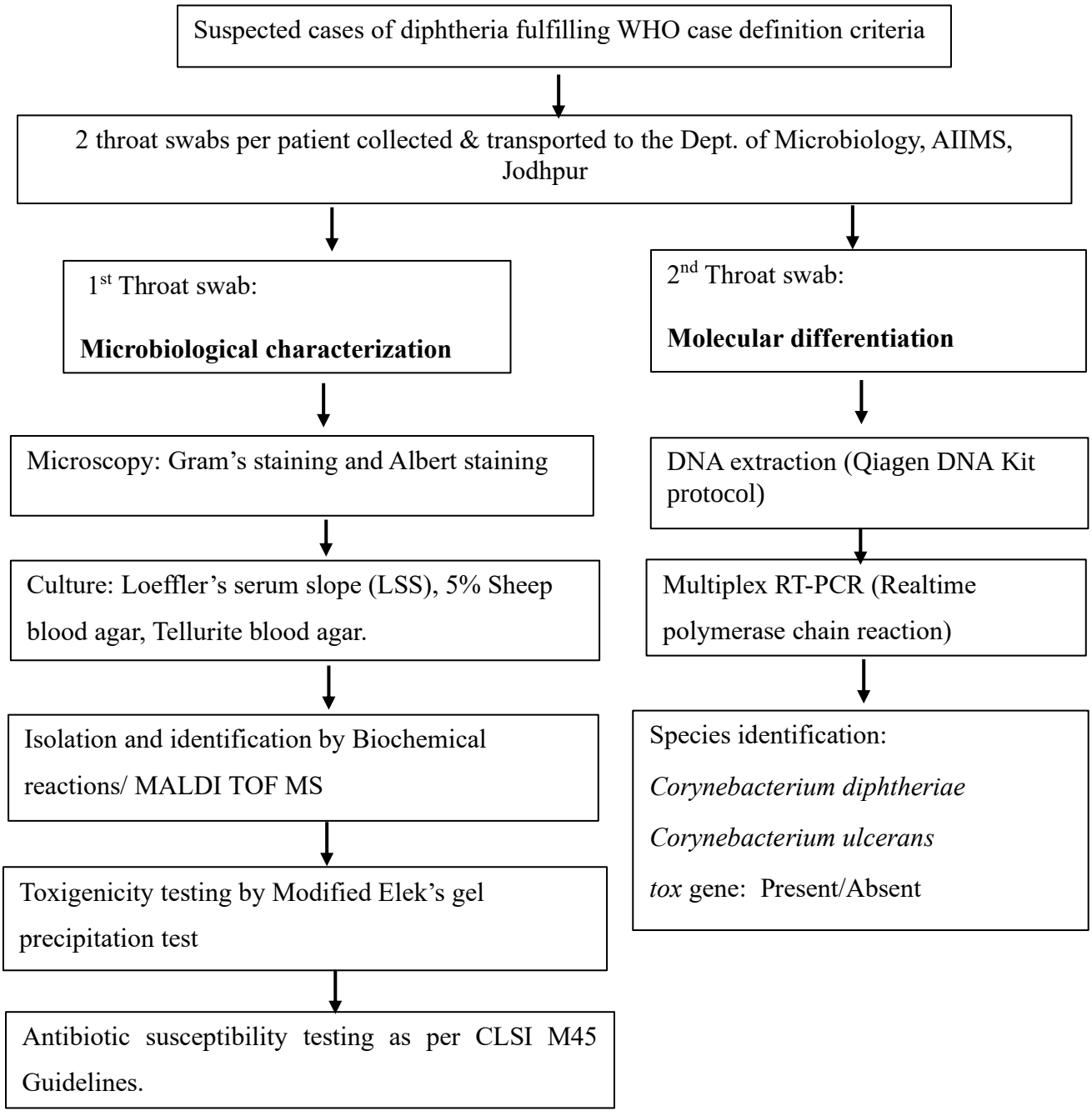

Fig 8: Flowchart of the method followed in this study

SAMPLE SIZE: 204 samples were included during the study period.

This study was conducted from January 2021 to December 2022.

Patients attending Primary health centre (PHC) or Community health centre (CHC) of various districts of Rajasthan, with any of the following clinical features like sore throat, greyish membrane over tonsils, hoarseness of voice, fever, difficulty in breathing, difficulty in swallowing and bull neck, was enrolled as study participants.

Two throat swab specimens from all such patients were collected on the same day by the attending medical officer. However, samples of the patients attending AIIMS OPD and IPD were collected at the Department of Microbiology AIIMS, Jodhpur. Samples from other districts were then transported in Amies transport medium by health care workers to the Department of Microbiology AIIMS Jodhpur within 1-2 days.

Samples received were processed in the Department of Microbiology, AIIMS, Jodhpur. Among the two throat swabs from each 204 patients, the first throat swab was used for conventional method of processing of *Corynebacterium* spp. by microscopy and culture in which final identification was made by Biochemical reactions and further confirmation done by MALDI-TOF MS (Biomerieux Pvt ltd). Which was followed by antimicrobial susceptibility testing by conventional method and toxin detection by Modified Elek's gel precipitation test of the identified *Corynebacterium* spp.

Simultaneously, multiplex PCR was used for molecular diagnosis of diphtheria from the clinical samples. DNA extraction was performed from the second throat swab and subjected to multiplex RT-PCR-based detection of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Toxin A.*

COLLECTION AND TRANSPORT OF SPECIMENS:

Sample was collected by the following procedure (fig 9)

1. Patient's face was turned against the light and slightly tilted their head backwards, and asked them to open their mouth and phonate an "ah".
2. Patient's tongue was gently depressed using a wooden tongue depressor so that the throat was well exposed

3. A sterile swab was guided over the tongue without touching the side of the mouth to the posterior pharynx and tonsillar arches where pseudomembrane was present.
4. Then the swab was rubbed gently over the tonsils and pseudomembrane care was taken not to remove the pseudomembrane to avoid bleeding.
5. Then the swab was taken out carefully without touching the lips, cheeks and tongue and was then placed in a sterile container.

The samples were transported by health care workers to the department of Microbiology AIIMS Jodhpur within 1-2 days cold chambers (fig 10).

I) IDENTIFICATION, ISOLATION OF *Corynebacterium* spp. FOLLOWED BY ANTIMICROBIAL SUSCEPTIBILITY TESTING AND TOXIN DETECTION BY CONVENTIONAL METHOD [76, 77].

National Collection of Type Cultures (NCTC) strains, *C. ulcerans* NCTC 12077, *C. diphtheriae* NCTC 10648, *C. diphtheriae* NCTC 3984, provided by CMC Vellore was used as controls.

A. MICROSCOPY

1. Gram Staining:

After receiving the sample first Gram stain was done. As it gives a preliminary idea of the organism causing the pathogenesis. It was done according to the standard bacteriological procedure.

Principle of Gram-Stain:

1. Gram-positive bacteria have a thick peptidoglycan layer and these cells have more acidic protoplasm. So, they will retain the primary dye and appear blue in colour.

2. Gram negative bacteria contain lipid layers and these lipid layers make the primary dye to permeable and will take the counterstain. These will appear pink in colour.

Procedure:

Part 1: The slide was prepared by making it grease free dust free oil free by rubbing with a dry tissue paper or passing through flame. After cleaning, the slides were allowed for air drying until further use.

Part 2: Next step labelling of the slides were done. A circle on the slide was made using a glassware marking pen to clearly designate area for the smear.

Part 3: Preparation of the smear

From sample: The swab was taken and a smear was made on the slide and allowed to air dry. The sample was spread by means of circular motion 1 cm in diameter and allowed to dry. Bacterial plate cultures: A drop of saline was put on the slide. The isolated colony was picked up by sterilized loop and a smear of 1cm x 1cm was made.

Part 4: Heat Fixing: Slide was fixed by heat fixation by passing over flame 2-3 times.

Gram Stain Procedure:

1. Placed slide with heat fixed smear on staining tray.
2. Gently flooded smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorization was done using acetone for 4-5 seconds. Then the slide was rinsed with water.
7. Immediately rinses with water.
8. Saffranine was added for counterstaining of slide and kept for 1 minute.
9. Then slide was rinsed with tap water or distilled water using a wash bottle.
10. After air drying slide was focused under oil immersion lens of microscope.

Interpretation: Gram positive bacilli (GPB) typically arranged in Chinese letter or cuneiform arrangement (V-or-L shaped) (fig 11).

2. <u>Stain for volutin granules:</u>

The presence of metachromatic granules in *C. diphtheriae* is shown using Albert's metachromatic stain. Because of the metachromasia property, which causes the storage granules in this organism to appear in a colour other than the staining colour, they are known as metachromatic granules. The granules appear violet when stained with polychrome methylene blue, while the rest of the bacillus appears blue. The

polymetaphosphate granules go by a number of different names, including Babe-Ernst granules, volutin bodies, and polar bodies. When the bacteria are cultured on nutrient-rich media like Loeffler's serum slope, it forms the granules in large quantities. The cytoplasm is counterstained pale green, while the granules stain purple-black. This makes diphtheria bacillus easier to recognize from the majority of the short non-pathogenic diphtheroid.

Albert staining method:

Composition of Albert stain:

Albert I: Comprises of toluidine blue, malachite green, glacial acetic acid-alcohol (95% ethanol) and distilled water.

Albert's II: Contains iodine in potassium iodide.

Principle of Albert's staining:

Albert's solution I acts as a staining solution. Toluidine blue and malachite green are basic dyes that have a high affinity for acidic tissue components like cytoplasm. Albert's stain pH is adjusted to 2.8 by using acetic acid which becomes basic for volutin granules (as their pH is highly acidic) and acidic for cytoplasm (as their pH is neutral).

When toluidine blue is applied to the smear, it stains the volutin granules as it is the most acidic part of the cell. Malachite green stains the cytoplasm green. Due to the metachromatic property, volutin granules appear red in color.

Albert's solution II contains iodine as a mordant which doesn't let the metachromasia show and thus, granules appear blue-black in color. This also allows the dye to bind and hold the chemical dye stuck on micro-organisms.

Procedure of Albert's staining:

1.Placed slide with heat fixed smear on staining tray.

2. Smear is covered with Albert I stain for 5 minutes

3. After 5 minutes, drain out the excess stain

4. Albert's II (Iodine solution) is added for 1 minute

Slide is washed with water, blotted dry and examined under oil immersion lens of microscope. Interpretation: Green-colored, rod-shaped bacilli that are arranged at

angles to each other resembling the English letter 'V', 'L', or Chinese letter pattern. Bluish black metachromatic granules in the polar region (fig 12).

B. CULTURE [76, 77]

The specimen was first inoculated to Loeffler's serum slope (LSS), 5% Sheep blood agar plate, selective media like Potassium/Cystine-tellurite blood agar and which was incubated at 37^0 C.

After 4-6 hours Loeffler's serum slope (LSS) was taken out and examined for any growth. And also, from the water of condensation of LSS a subculture was made on 5% Sheep blood agar plate, selective media like Potassium/Cystine-tellurite blood agar and a smear for Albert's staining.

The plates were examined in 18 to 24 hours for 5% sheep blood agar and Potassium/Cystine-tellurite blood plates were examined after 36 to 48 hours of incubation.

1. Loeffler's serum slope (LSS):

LSS is an enriched medium containing glucose and Horse serum in nutrient broth at pH 7.6. colonies of *Corynebacterium* spp. on LSS appear as small, circular, glistening and white with a yellowish tinge (fig 13).

Advantage: a) Growth is detected as early as 6-8 hours

b) Best medium for metachromatic granules.

Disadvantage: Being an enriched medium, if incubated beyond 6-8 hours, it supports growth of other throat commensals also.

2. Blood agar:

5% Sheep Blood agar (SBA) is widely used in medical bacteriology. In addition to being an enriched medium, it is also an indicator medium showing the hemolytic properties of bacteria. The media is prepared by adding blood to sterile nutrient agar that has been melted and cooled to 50^0 C. The concentration of blood may be varied from 5-50%.

Corynebacterium diphtheriae form grey-white, smooth, nonhemolytic colonies on 5% SBA and *Corynebacterium ulcerans* on blood agar form white colour and a dry to waxy appearance with a small zone of beta-hemolysis around the colony (fig 14, 15).

3. Potassium tellurite blood agar:

Potassium tellurite blood agar is a selective medium, it inhibits normal flora and are best for isolation of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and also *Corynebacterium pseudotuberculosis*, all of them produces black coloured colonies after 48 hours of incubation.

These organisms reduce potassium tellurite to tellurium and thereby produces grey-black coloured colonies (fig 16, 17).

4. Tinsdale's medium:

Hydrogen sulphide (H_2S) production from Cystine is observed in the formation of a brown colonies on Tinsdale medium. Both *C. diphtheriae* and *C. ulcerans* produce H_2S from Cystine (fig 18)

C. BIOCHEMICAL REACTIONS [76, 77]

1. Catalase test

Principle: Catalase decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Catalase converts hydrogen peroxide into oxygen and water $2H_2O_2 \rightarrow 2H_2O + O_2$ (gas bubbles)

Procedure:

1. With an inoculating needle or a wooden applicator stick, transferred growth from the center of a colony to the surface of a glass slide.

2. Added one drop of 3% hydrogen peroxide and observe for bubble formation.

Interpretation: The rapid and sustained appearance of bubbles or effervescence constitutes a positive test.

Positive control: *Staphylococcus aureus*

Negative control: *Streptococcus species*

Corynebacterium diphtheriae and *Corynebacterium ulcerans* both are catalase positive.

2. Oxidase test

Principle: The cytochromes are iron-containing hemoproteins that act as the last link in the chain of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome oxidase test uses certain reagent dyes, such as p-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptors. In the reduced state, the dye is colourless; in the presence of cytochrome oxidase and atmospheric oxygen, p-phenylenediamine is oxidized, forming indophenol blue.

Procedure: Commercial disk impregnated with dried p-phenylenediamine dihydrochloride are used. Suspected colony is smeared into the Disc.

Interpretation: Bacterial colonies having cytochrome oxidase activity develop a deep blue colour at the inoculation site within 10 seconds.

Positive control: *Pseudomonas aeruginosa*

Negative control: *Escherichia coli*

Corynebacterium diphtheriae and *Corynebacterium ulcerans* both are oxidase negative.

3. Urease Test

Principle: Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea, forming ammonia and carbon dioxide. Presence of ammonia increases the pH (>8.1) of media, thus converting colourless phenolphthalein to pink red coloured phenolphthalein

Procedure:

1.The surface of the agar slant is streaked with the test organism.

2.Incubated at 35°C for 18–24 hours.

Interpretation: Organisms that hydrolyze urea rapidly may produce positive reactions that is conversion of the colour of media to red within 1 or 2 hours.

Positive control: *Proteus species*

Positive control (weak): *Klebsiella species*

Negative control: *Escherichia coli*

C. diphtheriae does not produce urease, so this test may be used to distinguish this organism from *C. ulcerans* and *C. pseudotuberculosis* (both urease positive).

4. Nitrate Reduction Test

Principle:

The capability of an organism to reduce nitrates to nitrites. $NO_3^- + 2e^- + 2H \rightarrow NO_2 + H_2O$

Procedure

1. Inoculate the nitrate medium with a loopful of the test organism isolated in pure culture on agar medium, and incubate at 35°C for 18–24 hours.
2. After incubation, add 1 mL each of reagents to the test medium.

Interpretation: The development of a red colour within 30 seconds after adding the test reagents indicates a positive reaction for nitrate reduction

Positive control: *Escherichia coli*

Negative control: *Acinetobacter baumanni*

Nitrate test can differentiate *C. diphtheriae* and *C. ulcerans.*

Corynebacterium diphtheriae reduce nitrates to nitrites whereas *Corynebacterium ulcerans* do not reduce nitrates to nitrites.

5. Methyl Red test

Principle: Methyl red is a pH indicator, with a range between 6.0 (yellow) and 4.4 (red). Test organism producing large quantities of acid from the carbohydrate substrate changes the pH.

Procedure:

1. Inoculate the glucose phosphate broth with a pure culture of the test organism. Incubate the broth at 35°C for 48–72 hours.

2. Add 5 drops of the methyl red reagent directly to the broth.

Interpretation:

The development of a stable red colour in the surface of the medium indicates a positive test

Positive control: *Escherichia coli*

Negative control: *Enterobacter aerogenes*

6. Voges-Proskauer Test

Principle:

Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose, is metabolized through various metabolic pathways. One such pathway results in the production of acetoin (acetyl methyl carbinol). In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and α-naphthol serves as a catalyst to bring out a red complex.

Procedure:

1. Inoculate a tube of glucose phosphate broth with a pure culture of the test organism.
2. Incubate for 24 hours at 35°C. Add 0.6 mL of 5% α-naphthol followed by 0.2 mL of 40% KOH. 3.Shake the tube gently to expose the medium to atmospheric oxygen.

Interpretation:

A positive test is represented by the development of a red colour 15 minutes or more after addition of the reagents.

Positive control: *Enterobacter aerogenes*

Negative control: *Escherichia coli*

7. Citrate Utilization Test

Principle:

Certain bacteria can obtain energy by using citrate as the sole source of carbon. The utilization of citrate is detected in citrate medium by the production of alkaline by-products. Sodium Citrate→ alkaline metabolic products and ↑pH bromothymols blue (Green pH: 6.9) →bromothymol blue (Blue pH: 7.6)

Procedure:

1. A well-isolated colony is picked and inoculated as a single streak on the slant surface of the citrate agar tube.

2. The tube is incubated at 35°C for 24–48 hours.

Interpretation: A positive test is represented by the development of a deep blue color within 24–48 hours.

Positive control: *Enterobacter aerogenes*

Negative control: *Escherichia coli*

8. Carbohydrate Fermentation Test:

Hiss's serum sugar media is used to test the fermentation reactions. The medium is inoculated with the test organism; one tube with serum Control (as adding serum lowers the pH) and is incubated over night at 37°C.

The biochemical properties of *C. diphtheriae* and *C. ulcerans* are enlisted in table 6 (fig 19, 20)

S. No.	Tests	*C. diphtheriae*	*C. ulcerans*
1	Catalase	Positive	Positive
2	Oxidase	Negative	Negative
3	Nitrate	Positive	Negative
4	Urease	Negative	Positive
5	Methyl red	Positive	Positive
6	Voges-Proskauer	Negative	Negative
7	Citrate	Negative	Negative
8	Gelatin liquification test	Negative at 22°C	Positive at 22°C
9	Cystinase	Positive	Positive
Carbohydrate fermentation test			
10	Glucose	Positive	Positive
11	Maltose	Positive	Positive
12	Sucrose	Negative	Negative
13	Trehalose	Negative	Positive*

Table 6: Biochemical properties of *Corynebacterium* spp.

**C. ulcerans* may take up to 14 days to ferment Trehalose.

D. <u>AUTOMATED METHOD: MALDI-TOF MS:</u> [78] (fig 21)

MALDI stands for matrix which assists in desorption and ionization of highly abundant bacterial and fungal proteins through energy from a laser. The matrix (e.g., α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid) isolates bacterial or fungal molecules from one another, protecting them from fragmentation and enabling their desorption by laser energy; the majority of the laser energy is absorbed by the matrix, converting it to an ionized state. 30 As a result of random collision in the gas phase, charge is transferred from matrix to microbial molecules. Ionized microbial molecules are then accelerated through a positively charged electrostatic field into a time of flight, or TOF, tube. Inside the tube, which is under vacuum, the ions travel toward an ion detector, with small analytes traveling the fastest, followed by increasingly larger analytes; a mass spectrum is produced, representing the number of ions of a given mass impacting the detector over time. It is highly abundant (predominantly ribosomal) proteins which generate the mass spectrum. Computer software compares the generated mass spectrum to a database of reference spectra, generating a list of the most closely related organisms with numeric rankings (fig 22, 23). Turnaround time for MALDI-TOF MS is 3 minutes or very less as per standard methods.

E. ANTIBIOTIC SUSCEPTIBILITY TESTING:

After the organism is isolated and identified the antimicrobial susceptibility of the isolate is done using both conventional methods [79].

MIC testing by E-strip:

Medium: CAMHB-LHB

Inoculum: Direct suspension, equivalent to 0.5 MacF standard.

MIC of the drug was detected by E-strip on Mueller-Hinton Agar plate and interpreted according to CLSI M45 (Table 7), (fig 24)

Antimicrobial class	Antimicrobial agents	MIC (µ/ml)		
		Interpretive criteria		
		Sensitive	Intermediate	Resistant
PENICILLINS				
	Penicillin	<=0.12	0.25-2	>=4
CEPHEMS				
	Cefepime	<=1	2	>=4
	Cefotaxime	<=1	2	>=4
	Ceftriaxone	<=1	2	>=4
CARBAPENEMS				
	Meropenem	<=0.25	0.5	>1
GLYCOPEPTIDES				
	Vancomycin	<=2	-	-
MACROLIDES				
	Erythromycin	<=0.5	1	>=2
FLUROQUINOLONES				
	Ciprofloxacin	<=1	2	>=4
TETRACYCLINES				
	Doxycycline	<=4	8	>=16
	Tetracycline	<=4	8	>=16
LINCOSAMIDES				
	Clindamycin	<=0.5	1-2	>=4
FOLATE PATHWAY INHIBITORS				
	Trimethoprim-sulfamethoxazole	<2/38	-	>=4/76

Table 7: Breakpoints of the antibiotics used for *Corynebacterium* spp.

F. TOXIN DEMONSTRATION:

Elek's gel precipitation test [35]

This is a type of immunodiffusion in gel described by Elek (1949).

A rectangular strip of filter paper soaked in diphtheria antitoxin (1000 units per mL) is placed on the surface of a 20% horse (or sheep or rabbit) serum agar plate before the

medium solidifies. When the agar solidifies, the test main is streaked at right angle to the filter paper strip. The plate was incubated at 37^0C for 24-48 hours.

Precipitation band: If the strain is toxigenic, the toxin diffuses in the agar, meets with the antitoxin and produces arrow-shaped precipitation band.

Nontoxigenic stains will not produce any precipitation line.

This test can also be used to know the relatedness between the strains isolated during an outbreak.

The precipitate lines would fuse with each other if the toxins produced by these strains are identical.

Modified Elek's gel precipitation test [80]

Elek Media preparation:

1. Agar 1.5gm/100ml
2. Sodium chloride 0.25 gm
3. Proteose peptone (Difco) 2gm
4. 100ml distilled water

After dissolving the above contents and adjust the pH 7.8 and autoclave for 15 minutes for 115^0C.

After that cool the media to 50^0C and add 20 ml Horse serum and add 5ml of 1% Potassium tellurite and then pour 10-12 ml in 90mm Petri dish.

Steps:

1. Using pre-flamed forceps, place a filter paper disc of 6mm diameter in the centre of the Elek's media plate.
2. In a biosafety cabinet and wearing gloves, with a 1 μl loop, inoculate the plate with the two test strains and the three control strains (*C. ulcerans* NCTC 12077, *C. diphtheriae* NCTC 10648, *C. diphtheriae* NCTC 3984) at a distance of 9 mm from the filter paper disc.
3. Place 10ul of diphtheria antitoxin in previously placed 6mm filter paper disc.
4. Incubate the plate in the 37 ^{0}C room for 12-24 hours only.

Interpretation:

Test may be read on the open bench wearing gloves after 12 hours of incubation.

1. Using a suitable light source and wearing gloves, examine the plate carefully after overnight incubation looking for precipitin lines of identity between the test strains and the strong and weak positive control strains.
2. The negative control strain should not demonstrate any precipitin lines (fig 25).

II) IDENTIFICATION AND TOXIN GENE DETECTION BY MOLECULAR METHOD

A. DNA extraction:

DNA Extraction is done using QiAamp DNA mini-Kit (Qiagen, Germany). Extraction is done asper Qiagen DNA Kit protocol.

a) Prepare 1X TE buffer from 10X TE buffer with pH 8:
   1X TE buffer = 1ml 10X TE buffer + 9ml distilled water.
   Reconstitute 80mg of Lysozyme powder and add in 1ml of 1X TE buffer.

b) STEPS:

1.Throat swab resuspended in 400 µl of 0.85% saline and vortex.

2. Take new microcentrifuge tube and add 200 µl of the above saline solution (sample) and add 100 µl of lysozyme and vortex.

3. Incubate 60 minutes at 37^0C in heat block

4. Add 20 µl of proteinase K and vortex

5. Incubate 30 minutes at 56^0C in heat block

6. Add 200 µl of AL buffer

7. Incubate at room temperature for 10 minutes

8. Add 250 µl of chilled absolute alcohol and vortex

9. Take new spin column and add 800 µl of the above mixture into it

10. Centrifuge at 8000 rpm for 1 minute

11. Keep the column in another collection tube and discard the supernatant.

12. Add 500 µl of AW1

13. Centrifuge 8000rpm for 1 minutes

14. Add 500 µl of AW2

15. Centrifuge 14000 rpm for 3 minutes

16. Keep the column in another collection tube and discard the supernatant

17. Add 40 µl of Elution buffer.
18. Incubate at room temperature for 1 minute
19. Keep the DNA in fresh autoclaved Eppendorf and store at -20^{0}C.

Extracted DNA from throat swab/culture isolate can be used as the template for detecting diphtheria toxin (*tox*) gene, species identification *Corynebacterium diphtheriae/ Corynebacterium ulcerans.*

B. Real-Time Multiplex PCR [81]

The primers and probes for the real-time PCR was standardised by following the protocol provided by the Department of Clinical Microbiology, Christian Medical College, Vellore. The primers and probes (provided by CMC, Vellore under WHO VPD project) for the detection of *C. diphtheriae*, *C. ulcerans* and *Toxin A* are listed in table 8.

Target	Primer/ Probe	Sequence 5' – 3'	Amplicon length (bp)
C. diphtheriae rpoB	dip_rpob_F dip_rpob_R dip_probe	CGTTCGCAAAGATTACGGAACCA CACTCAGGCGTACCAATCAAC HEX-AGGTTCCGGGGCTTCTCGATA TTCA-BHQ1	97
C. ulcerans rpoB	ulc_rpob_F ulc_rpob_R ulc_probe	TTCGCATGGCTCATTGGCAC TCCAGGATGTCTTCCAGT CC FAM- CCAGCAGGAGGAGCTGGGTGA A-BHQ1	98
Toxin A	toxA_F toxA_R toxA_probe	CTTTTCTTCGTACCACGGGACTAA CTATAAAACCCTTTCCAATCA TCGTC CY5- AAGGTATACAAAAGCCAAAAT CTGGTACAC- BHQ2	117

Table 8: Primers and probes for the real time detection of *C. diphtheriae, C. ulcerans* and *Toxin A*

Preparation of Primer mix: (for 25 reactions): 100 µl (Table 9)

	C. diphtheriae	***C. ulcerans***	***tox*A**	**RNase P**
Forward primer	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Reverse primer	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Probe	1 µl	1 µl	1 µl	1 µl
RNase free water	19 µl	19 µl	19 µl	19 µl
Total	25 µl	25 µl	25 µl	25 µl

Table 9: Preparation of Primer mix

Preparation of PCR reaction mix (Table 10)

	For 1 reaction	For 25 reactions
Master mix	10 µl	250µl
C. diphtheriae primer mix	1 µl	25µl
C. ulcerans primer mix	1 µl	25µl
*tox*A primer mix	1 µl	25µl
RNase P primer mix	1 µl	25µl
Molecular grade water	1 µl	25µl
Total	**15µl**	375µl
DNA template	5µl	

Table 10: Preparation of PCR reaction mix

PCR Program AB7500 (Fig 26):

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:

Data Interpretation for Real Time PCR based on Ct values is shown in table 11 (fig 24-27).

Ct Cut offs for positivity	***C. diphtheriae***	***C. ulcerans***	***tox*A**
Minimum	≤31.24	≤28.96	≤31.05
Maximum	34.06	31.12	35.03

Table 11: Data Interpretation for Real Time PCR based on Ct values

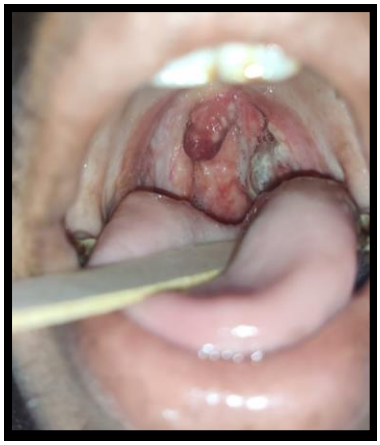

Fig 9: Collection of throat swab

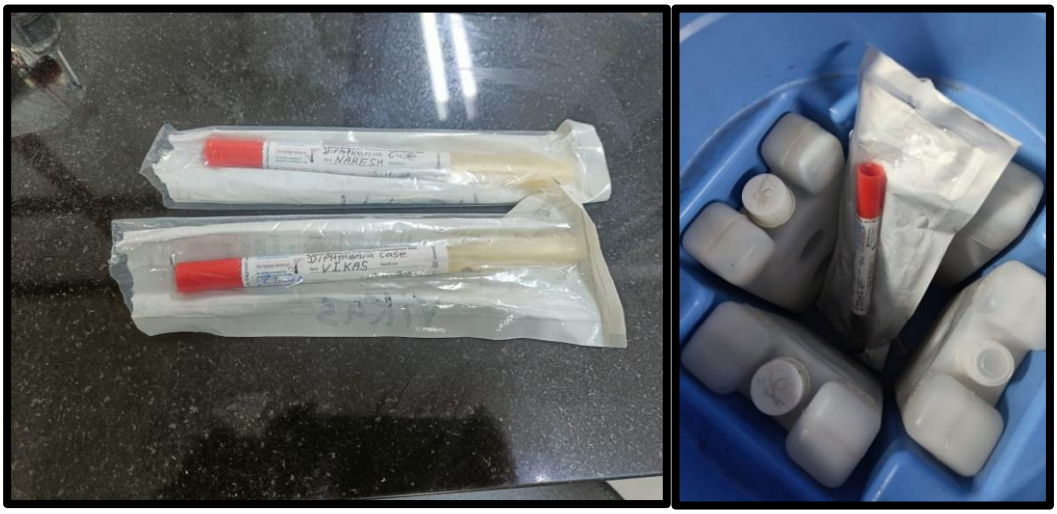

Fig 10: Samples transportation in cold chain

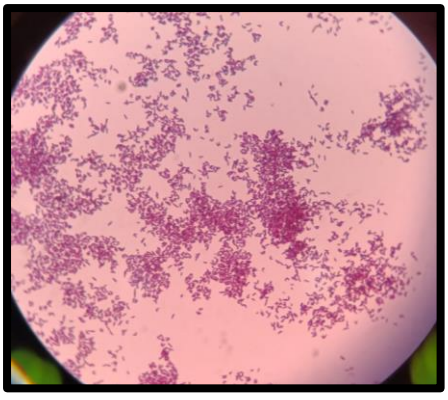

Fig 11: Gram staining- showing Gram positive bacilli (GPB) typically arranged in Chinese letter or cuneiform arrangement (V-or-L shaped)

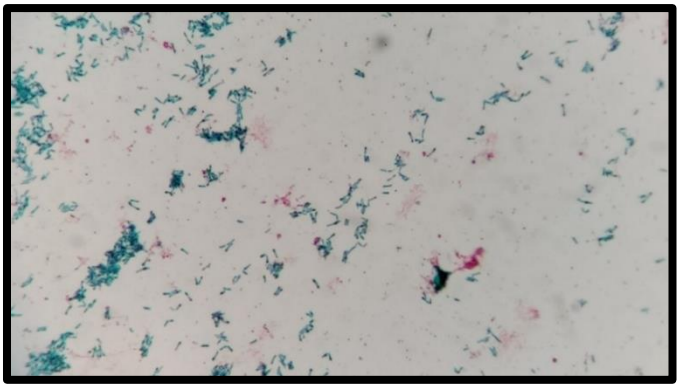

Fig 12: Albert stain- showing Green-colored, rod-shaped bacilli with Bluish black metachromatic granules in the polar region

Fig 13: Growth of *Corynebacterium* spp. on LSS

Fig 14: Blood agar showing growth of *C. diphtheriae*

Fig 15: Blood agar showing growth of *C. ulcerans*

Fig 16: Potassium tellurite blood agar showing growth of *C. diphtheriae*

Fig 17: Potassium tellurite blood agar showing growth of *C. ulcerans*

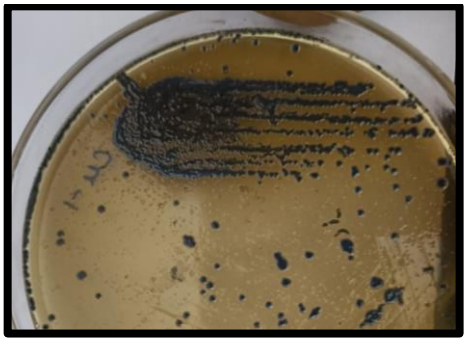

Fig 18: Tinsdale medium showing growth of *C. diphtheriae*

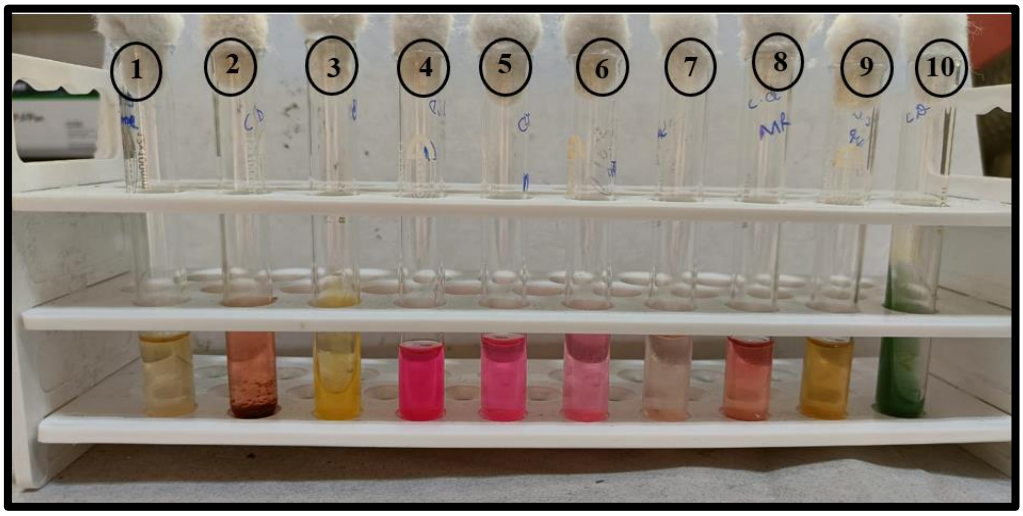

Fig 19: Various biochemical reactions and Carbohydrate fermentation properties of *C. diphtheriae*

1. Peptone water broth 2. Nitrate 3. Urease 4. Glucose 5. Ribose 6. Maltose
7. Sucrose 8. Methyl Red 9. Voges-Proskauer 10. Citrate

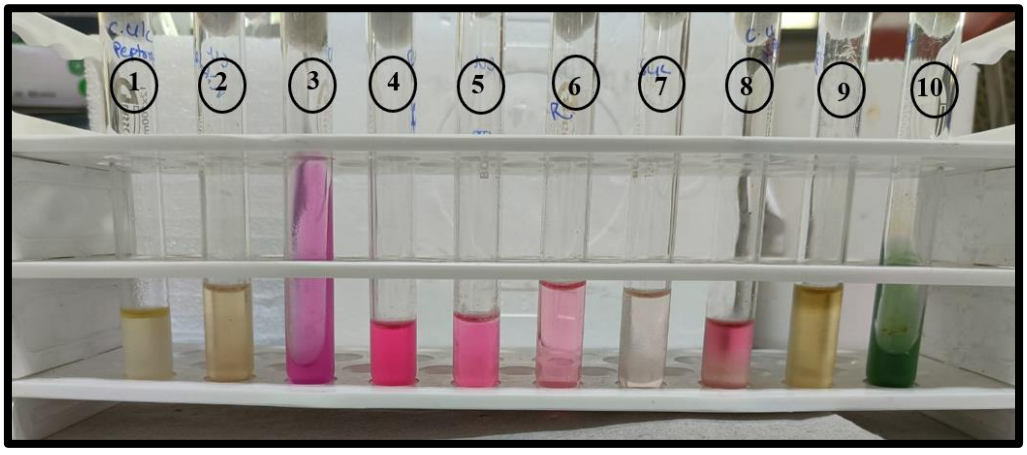

Fig 20: Various biochemical reactions and Carbohydrate fermentation properties of *C. ulcerans*

1. Peptone water broth 2. Nitrate 3. Urease 4. Glucose 5. Ribose 6. Maltose
7. Sucrose 8. Methyl Red 9. Voges-Proskauer 10. Citrate

Fig 21: MALDI TOF MS

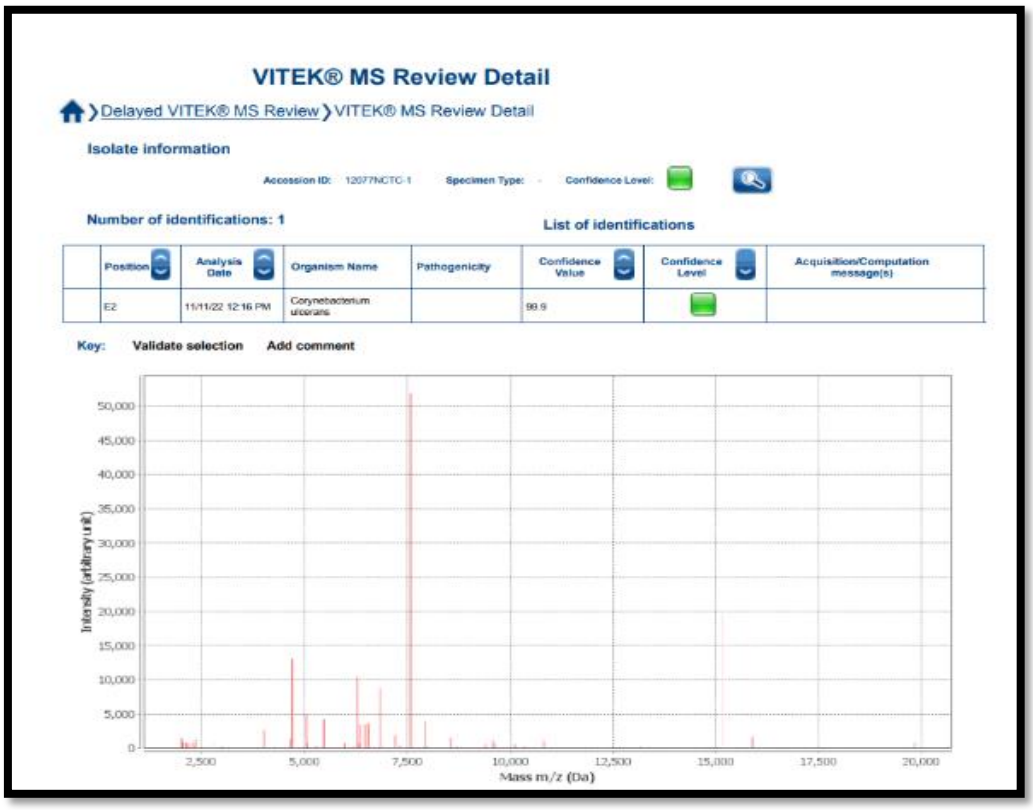

Fig 22: Identification of *C. ulcerans* by MALDI TOF MS

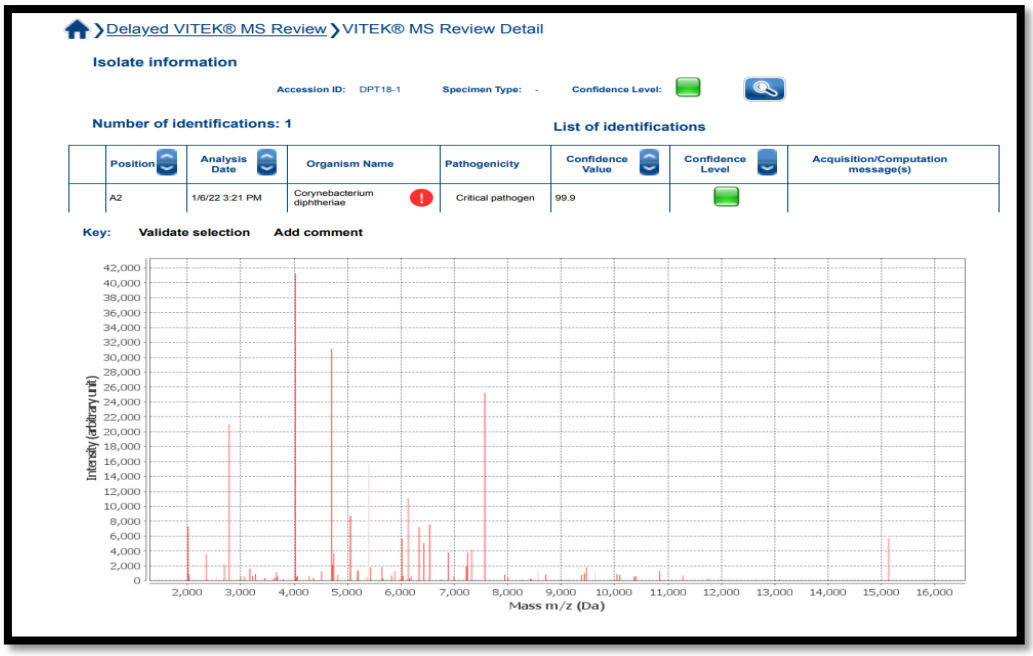

Fig 23: Identification of *C. diphtheriae* by MALDI TOF MS

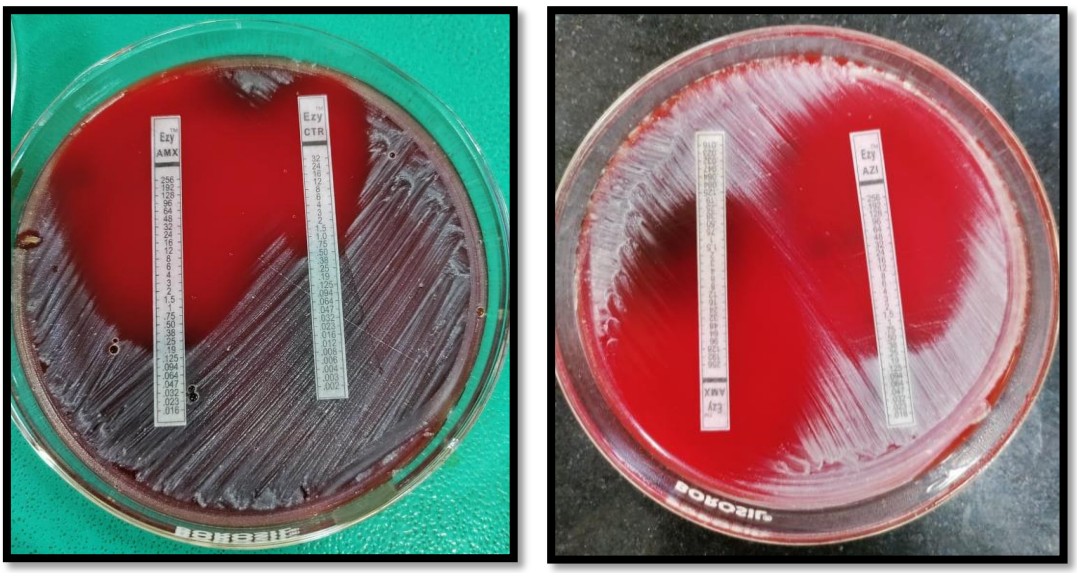

Fig 24: Antimicrobial susceptibility testing of *Corynebacterium* spp.

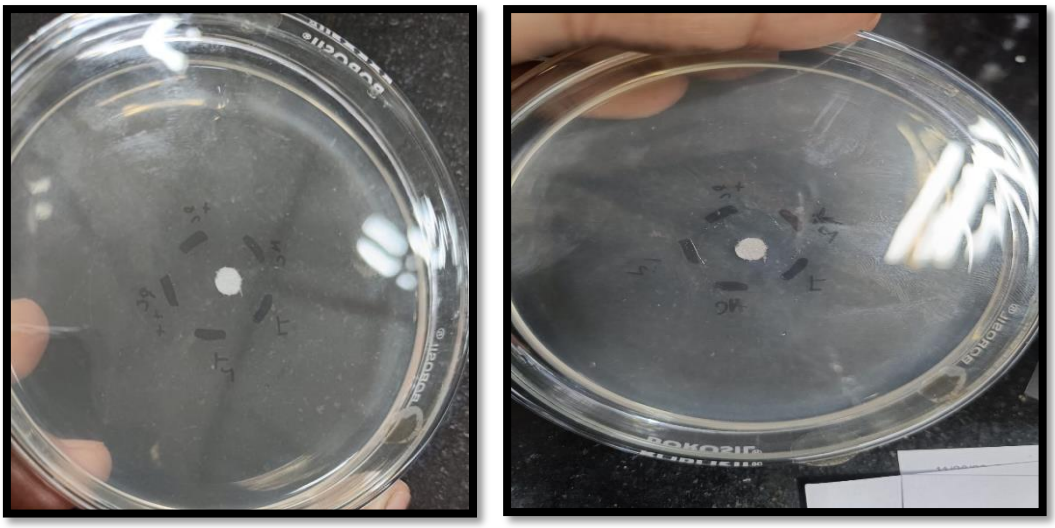

Fig 25: Modified Elek's gel precipitation test

Fig 26: BIO-RAD CFX96^{TM}Real-Time System (PCR)

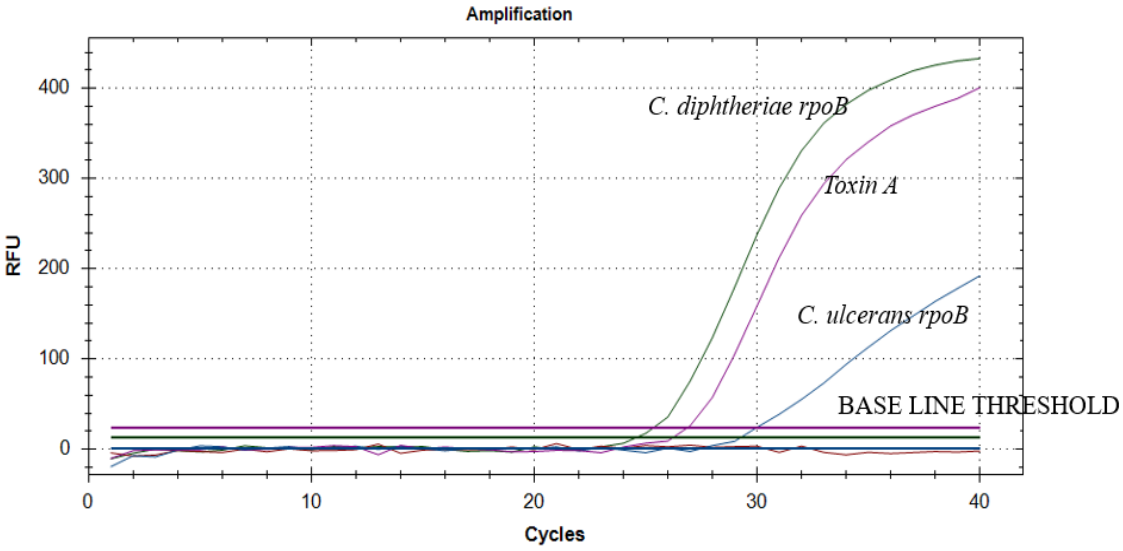

Fig 27: Amplification plot of *C. ulcerans, C. diphtheriae* & *Toxin A*

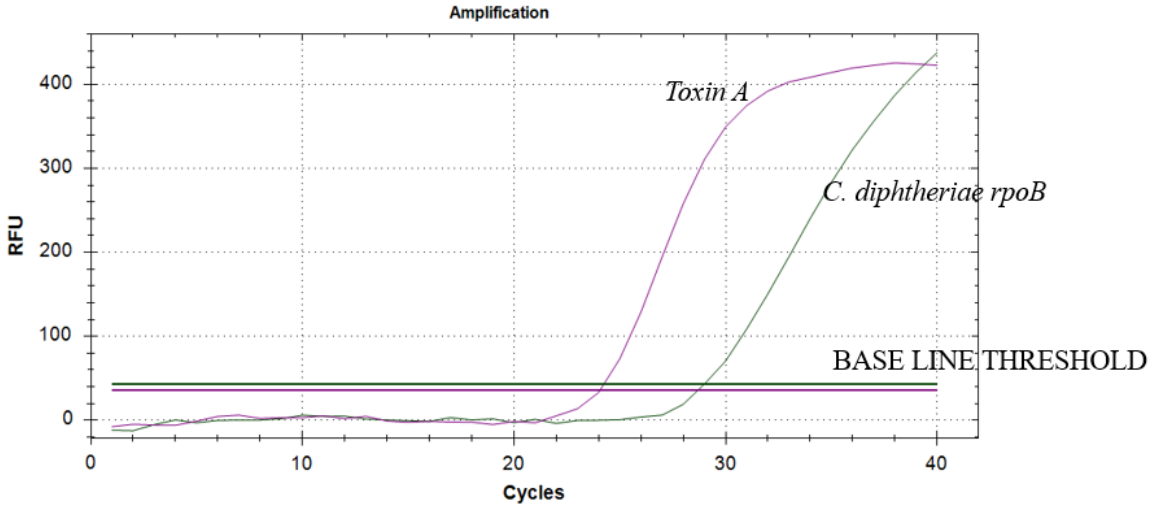

Fig 28: Amplification plot of *C. diphtheriae* & *Toxin A*

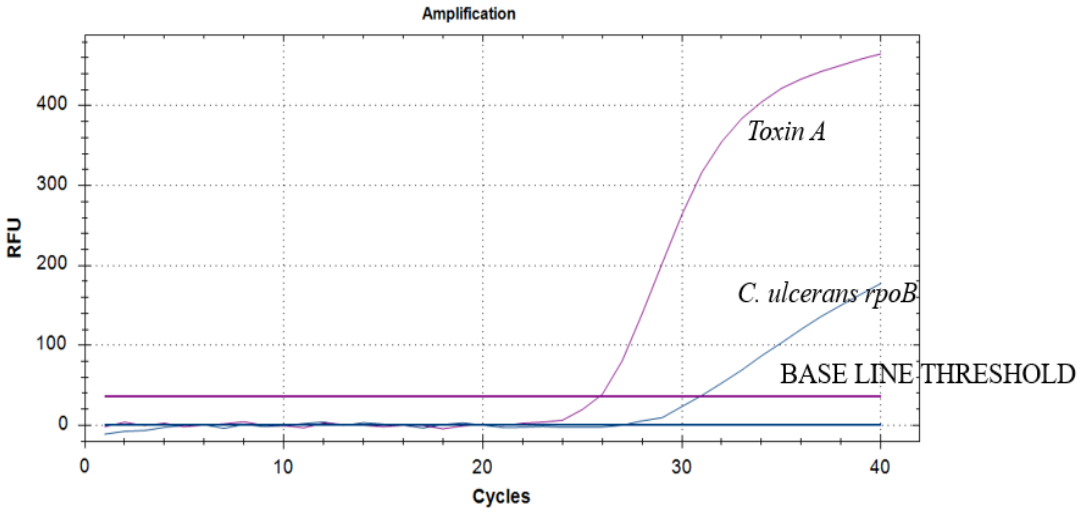

Fig 29: Amplification plot of *C. ulcerans* & *Toxin A*

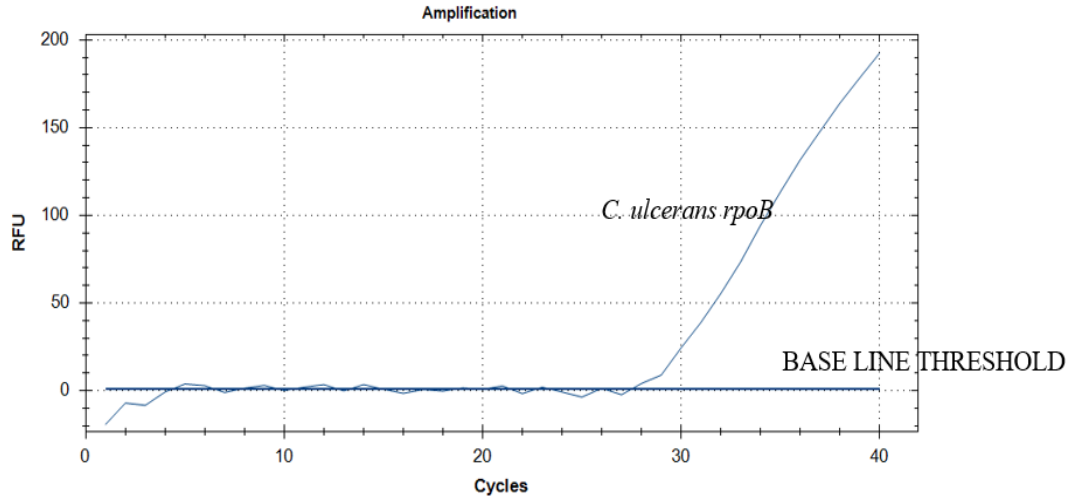

Fig 30: Amplification plot of *C. ulcerans*

RESULT

During the study period from January 2021 to December 2022, a total of 217 suspected cases of Diphtheria were enrolled in the study whose throat swab specimens were received in the Department of Microbiology, AIIMS Jodhpur. Out of them, 13 patients were excluded from the study as they did not fulfill the inclusion criteria. Therefore, a total of 204 patients were included in the study.

Fig 31 Shows the distribution of suspected cases of Diphtheria in various districts of Rajasthan.

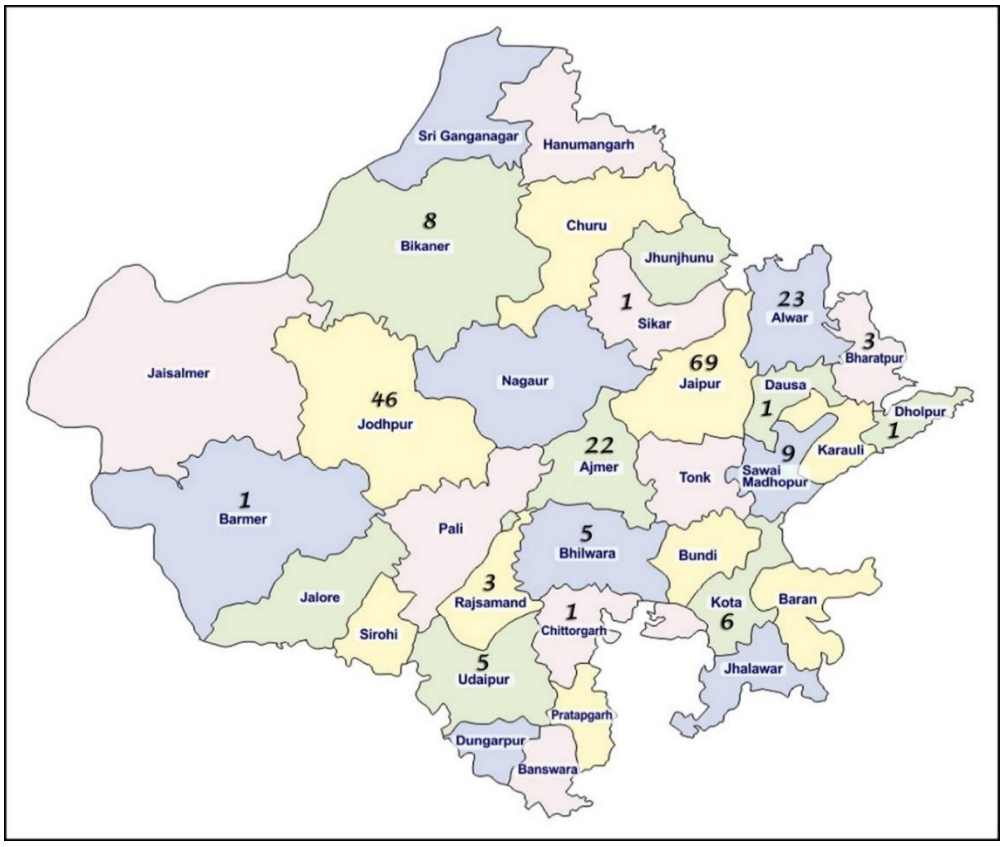

Fig 31: Map showing distribution of suspected cases of Diphtheria from various districts of Rajasthan

Among 204 suspected cases of Diphtheria, maximum number of samples were received from Jaipur 69 (34%) followed by Jodhpur 46 (23%), as depicted in Fig 32.

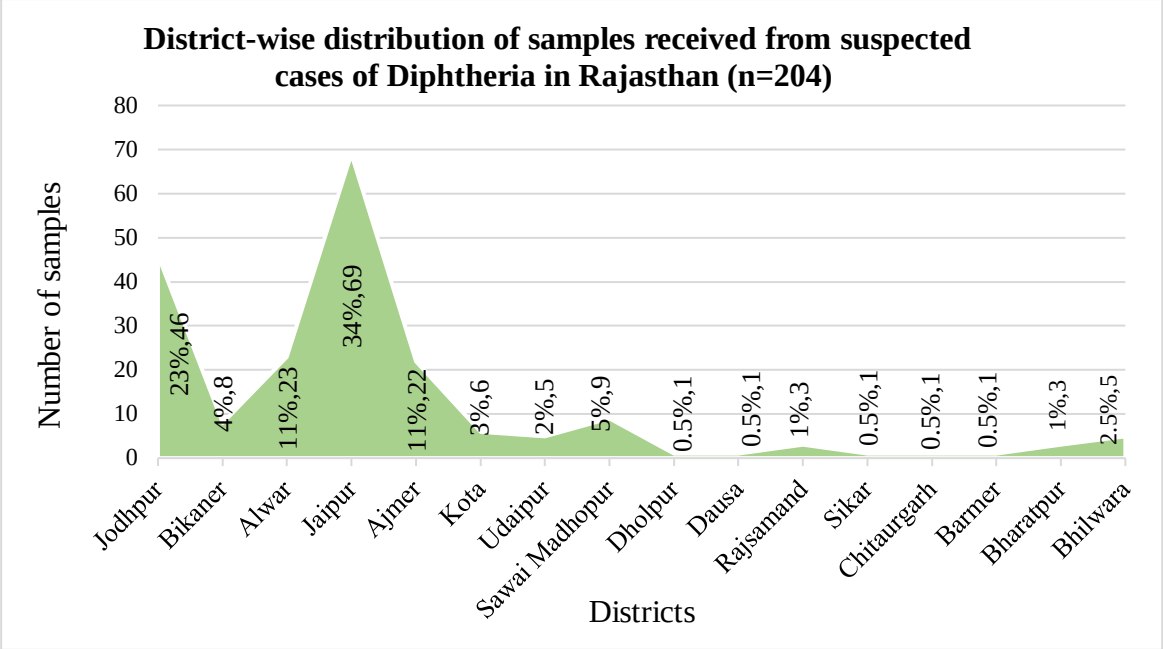

Fig 32: District-wise distribution of samples received from suspected cases of Diphtheria in Rajasthan (n=204)

As shown in fig 33, majority of the samples were received in the month of October 35 (17%) followed by September 34 (16%) on average.

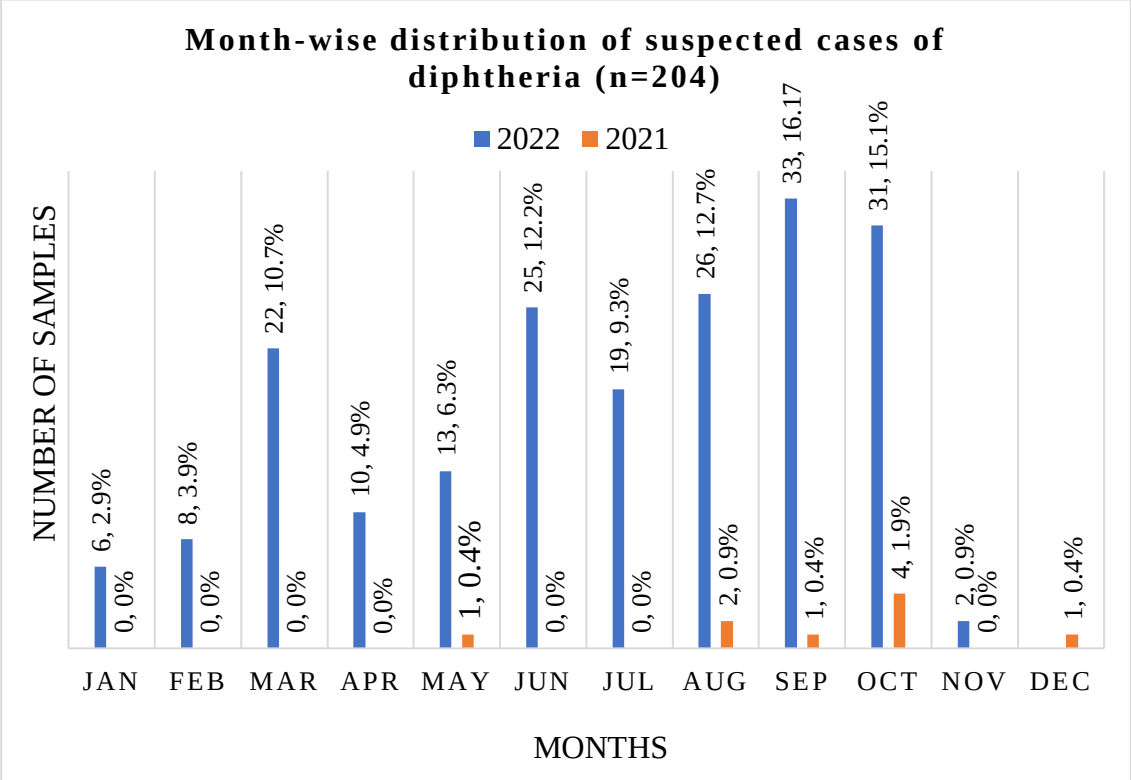

Fig 33: Month-wise distribution of suspected cases of diphtheria (n=204)

The age of patients ranged between 9 months to 50 years. Maximum participants 80 (39%) belonged to the 0-5 years age group, followed by 69 (33%) in the age group of 6-10 years, as summarized in Fig 34.

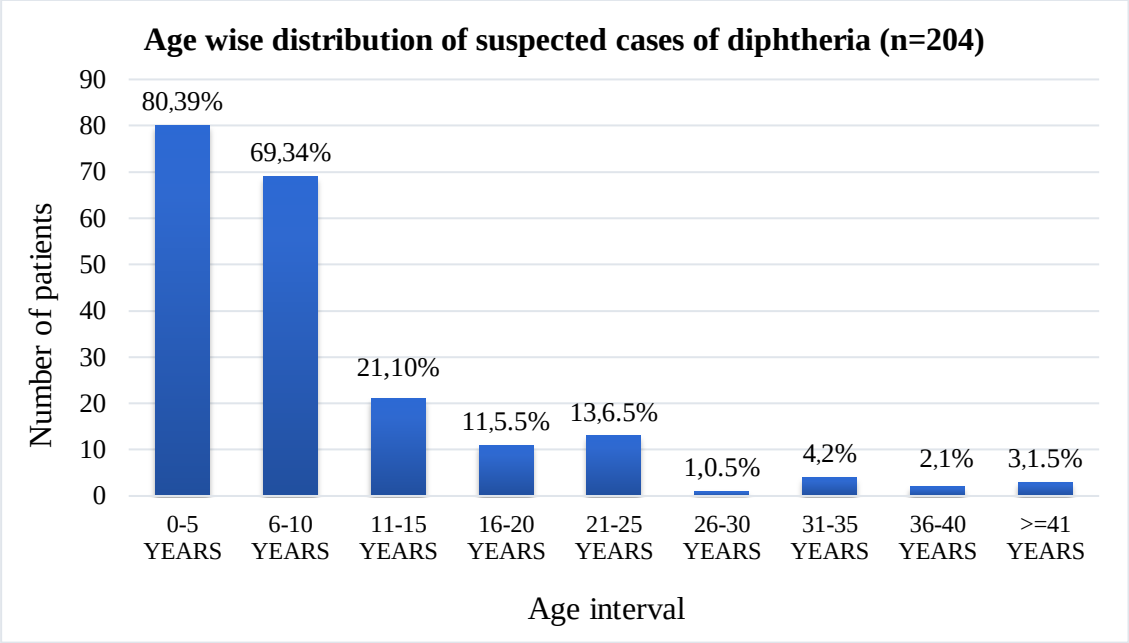

Fig 34: Age wise distribution of suspected cases of diphtheria (n=204)

Out of the total 204 suspected cases of diphtheria, males 132 (64.70%) outnumbered female patients 72 (35.29%) as shown in Fig 35.

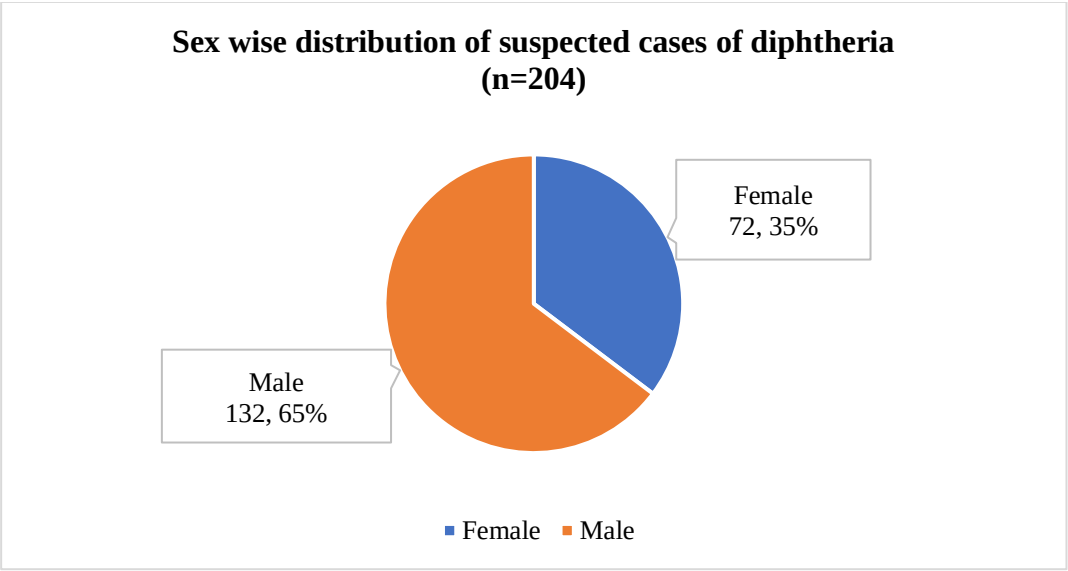

Fig 35: Sex wise distribution of suspected cases of diphtheria (n=204)

Upon taking history of all the 204 patients, 113 (55%) patients did not know their DPT vaccination details, and 92 (45%) were fully vaccinated for age (fig 36).

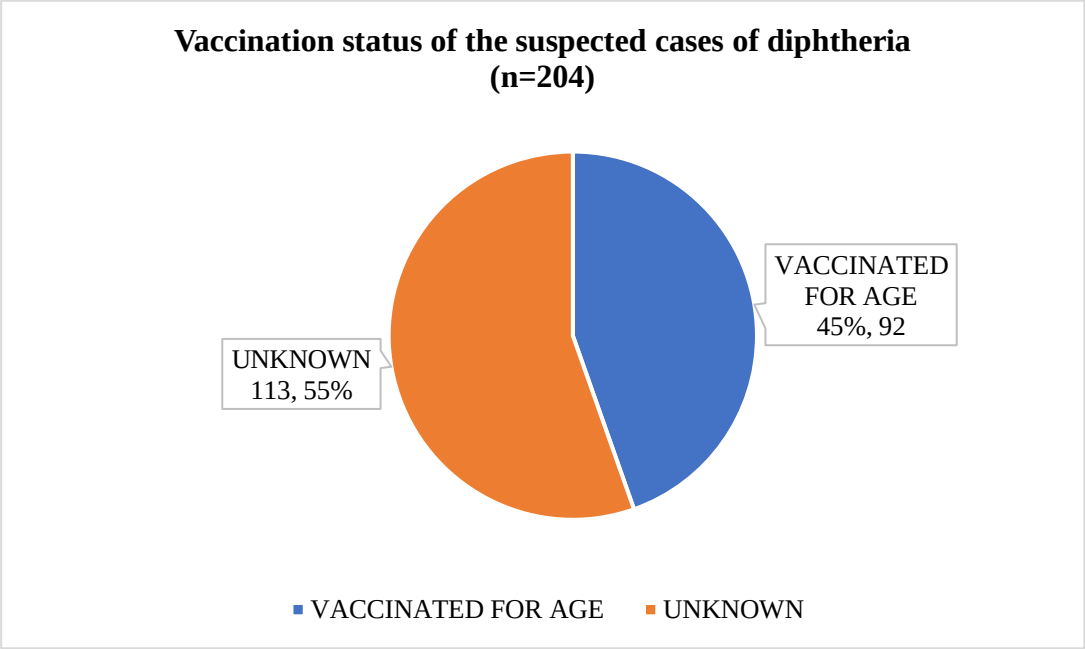

Fig 36: Vaccination detail of the suspected cases of diphtheria (n=204)

On Gram's staining, 27 (13.2%) cases showed GPB suggestive of *Corynebacterium* spp. and 26 (12.7%) cases showed KLB on Albert staining s/o *Corynebacterium* spp. (Table 12).

S No.	**Staining method**	**Interpretation**	**Result n (%)**
1.	Gram's staining	GPB seen	27 (13.2%)
		GPB not seen	177 (86.8%)
TOTAL			204
2.	Albert's staining	KLB seen	26 (12.7%)
		KLB not seen	178 (87.2%)
TOTAL			204

Table 12: Results of Gram's staining and Albert's staining

All the 26 positive KLB were also positive for GPB. Hence microscopy was positive in 26 (12.7%) cases.

After microscopy, the 204 throat swabs was subjected to conventional culture. No growth was observed in 118 (57.84%) cases, commensal flora grown in 61 (29.90%) cases and *Corynebacterium* spp. was seen in 25 (12.25%) cases.

Among the 25 *Corynebacterium* spp. grown in culture, 09 (36%) were speciated as *Corynebacterium diphtheriae* and 16 (64%) as *Corynebacterium ulcerans* by biochemical reactions as well as MALDI TOF MS. All these 25 *Corynebacterium* spp. were also microscopy positive.

Therefore, out of 204 cases, 09 (4.41%) cases were positive for *Corynebacterium diphtheriae* and 16 (7.84%) cases for *Corynebacterium ulcerans* (Table 13).

S No.	**Culture findings**		**Result n (%)**
1.	No growth		118 (57.84%)
2.	Commensal flora		61 (29.90%)
3.	*Corynebacterium* spp.	*C. diphtheriae* - 09 (4.41%)	25 (12.25%)
		C. ulcerans - 16 (7.84%)	
Total			**204**

Table 13: Findings of Conventional culture

Further, the Modified Elek's gel precipitation test was performed on all 25 *Corynebacterium* spp. Toxin was detected in 9 (36%) out of 25 *Corynebacterium* spp. isolates. All the 9 (100%) isolates of *C. diphtheriae* were toxin producing, while none of the isolates of *C. ulcerans* 0 (0%) showed any detectable toxin production (Table 14).

S No.	*Corynebacterium* spp.	Number of isolates n (%)	Number of isolates showing Toxin production n (%)
1	*Corynebacterium diphtheriae*	9 (36%)	9 (100%)
2	*Corynebacterium ulcerans*	16 (64%)	0 (0%)
TOTAL		**25 (100%)**	**9 (100%)**

TABLE 14: Result of toxin detection by Modified Elek's gel precipitation test performed on 25 isolates of *Corynebacterium* spp.

Therefore, 09 (4%) out of 204 patients grew toxigenic *Corynebacterium* spp. (*C. diphtheriae)* by conventional methods.

Antimicrobial susceptibility of all the *Corynebacterium* spp. was performed on CAMHB-LHB plates using E-strips. All the 9 isolates of *C. diphtheriae* and 16 isolates of *C. ulcerans* were susceptible to antibiotics Penicillin, Ceftriaxone, Erythromycin, Azithromycin and Amoxicillin.

Simultaneously, multiplex PCR was used for molecular diagnosis of diphtheria from the clinical samples. DNA extraction was performed from the second throat swab and subjected to multiplex RT-PCR-based detection of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Toxin A.*

Out of the 204 cases, *Corynebacterium* spp. was detected in 55 (26.96 %) cases and rest 149 (73.03%) cases were negative.

Among the 55 *Corynebacterium* spp. *C. diphtheriae* was detected in 9 (16.4%) cases and *C. ulcerans* was detected in 46 (83.6%) cases.

Therefore, out of 204 cases, *C. diphtheriae* was detected in 9 (4.41%) cases and *C. ulcerans* in 46 (22.5%) cases (Table 15).

S No.	PCR		Result n (%)
1.	Negative		149 (73.03%)
2.	*Corynebacterium* spp.	*C. diphtheriae* - 9 (4.41%)	55 (26.96 %)
		C. ulcerans - 46 (22.54%)	
Total			**204**

TABLE 15: PCR Result

Simultaneously *Toxin A* was detected by PCR in 10 (18%) cases out of 55 *Corynebacterium* spp. Among the 10 *Toxin A* positive cases, 9 (90%) was from *C. diphtheriae* and 1 (10%) was from *C. ulcerans* (Table 16).

S No.	***Corynebacterium* spp.**	Number of samples	Number of samples showing ***Toxin A***
1	*Corynebacterium diphtheriae rpoB*	9 (16.4%)	9 (90%)
2	*Corynebacterium ulcerans rpoB*	46 (83.6%)	1 (10%)
TOTAL		55 (100%)	10 (100%)

TABLE 16: Result of molecular detection *Toxin A*

Therefore, 10 (5%) out of 204 patients had toxigenic *Corynebacterium* spp. by molecular method. Out of the 10, 1 case was due to toxin producing *C. ulcerans* and 9 cases was due to toxin producing *C. diphtheriae*.

All the culture positive cases were also positive by PCR. And all the 9 toxin producing isolates by Modified Elek's gel precipitation test were also positive by PCR.

Among the 10 cases with laboratory confirmed toxigenic *Corynebacterium* spp. All 10 (100%) cases had fever greyish adherent membrane over tonsils, 8 (80%) cases had difficulty in breathing, 7 (70%) cases had sore throat, hoarsness of voice was present in 6 (60%) cases, 3 (30%) cases had Bull neck and 3 (30%) cases had difficulty in swallowing (Fig 37).

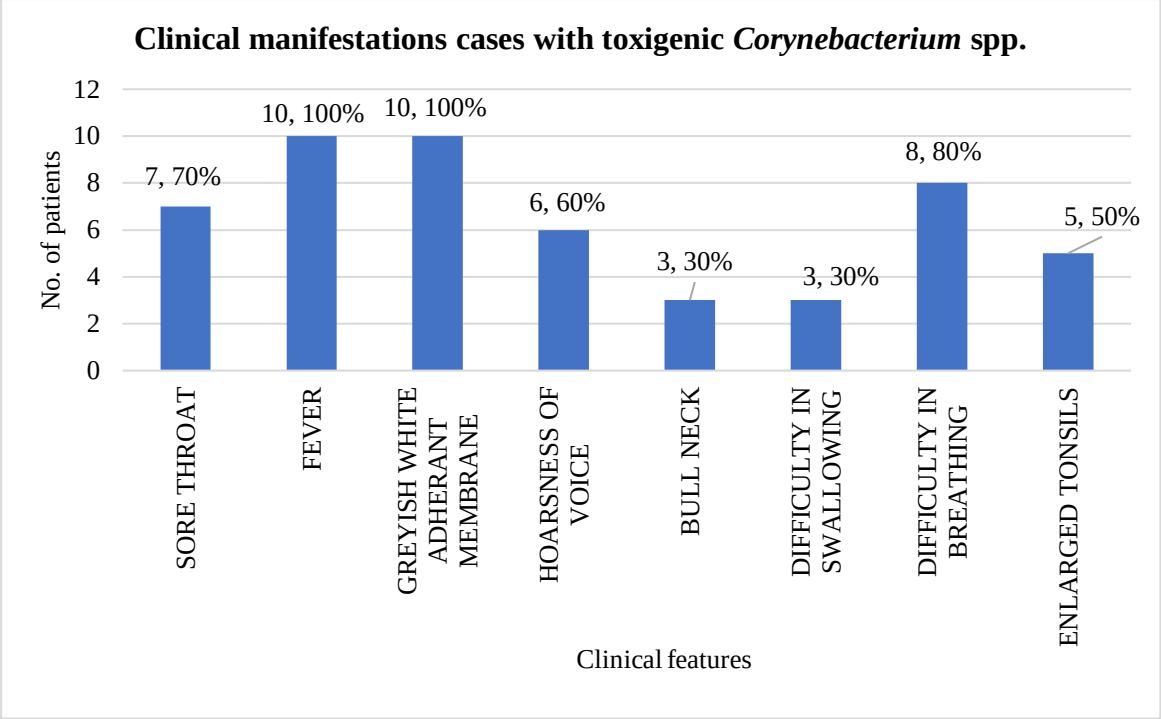

Fig 37: Clinical manifestations of cases with toxigenic *Corynebacterium* spp. (n=10)

All the 10 cases with toxigenic *Corynebacterium* spp. vaccination details were unknown.

Out of all 10 cases with laboratory confirmed toxigenic *Corynebacterium* spp. majority of the cases 7 (70%) were between the age group 6-10 years followed by 1 (10%) case in each age group 0-5 years, 11-5 years and 21-25 years as shown in fig 38.

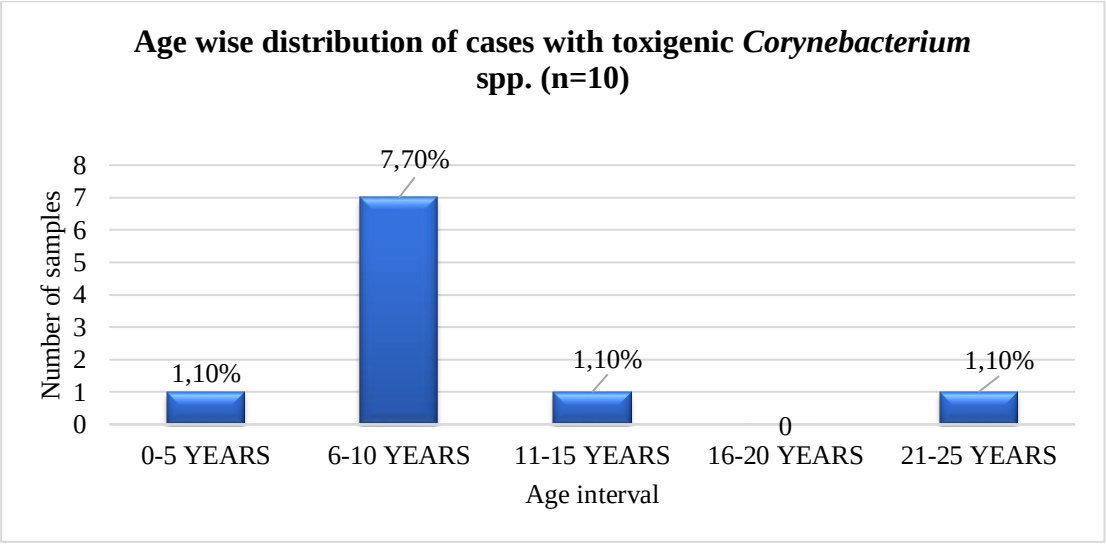

Fig 38: Age wise distribution of cases with toxigenic *Corynebacterium* spp. (n=10)

6 (60%) cases with the toxin producing strains of *Corynebacterium* spp. were female and 4 (40%) were male patient as shown in Fig 39.

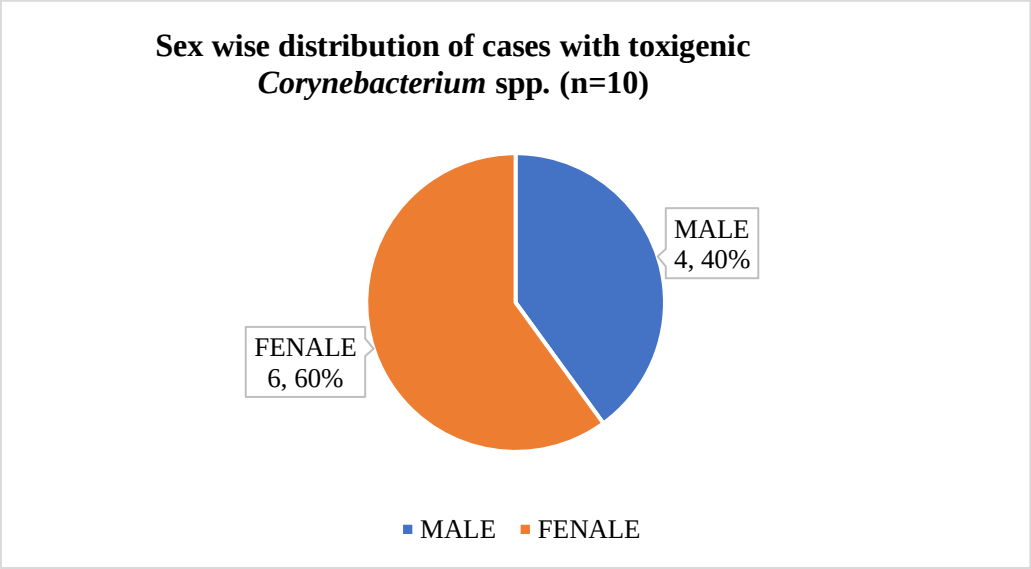

Fig 39: Sex wise distribution of cases with toxin producing strains of *Corynebacterium* spp. (n=10)

Cases of toxin producing strains of *Corynebacterium* spp. was seen more in the month of October 3 (30%) cases followed by November and July 2 (20%) cases each as shown in Fig 40.

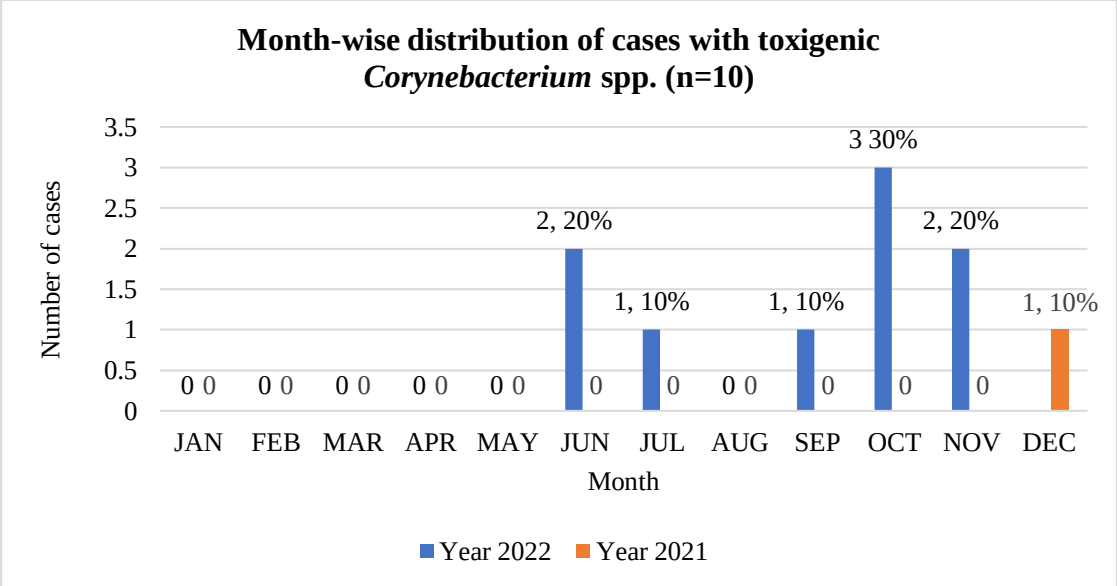

Fig 40: Month-wise distribution of cases with toxigenic *Corynebacterium* spp. (n=10)

Majority of the cases of toxigenic *Corynebacterium* spp. was from Jodhpur 60%(n=6) followed by Jaipur 20%(n=2) as shown in Fig 41.

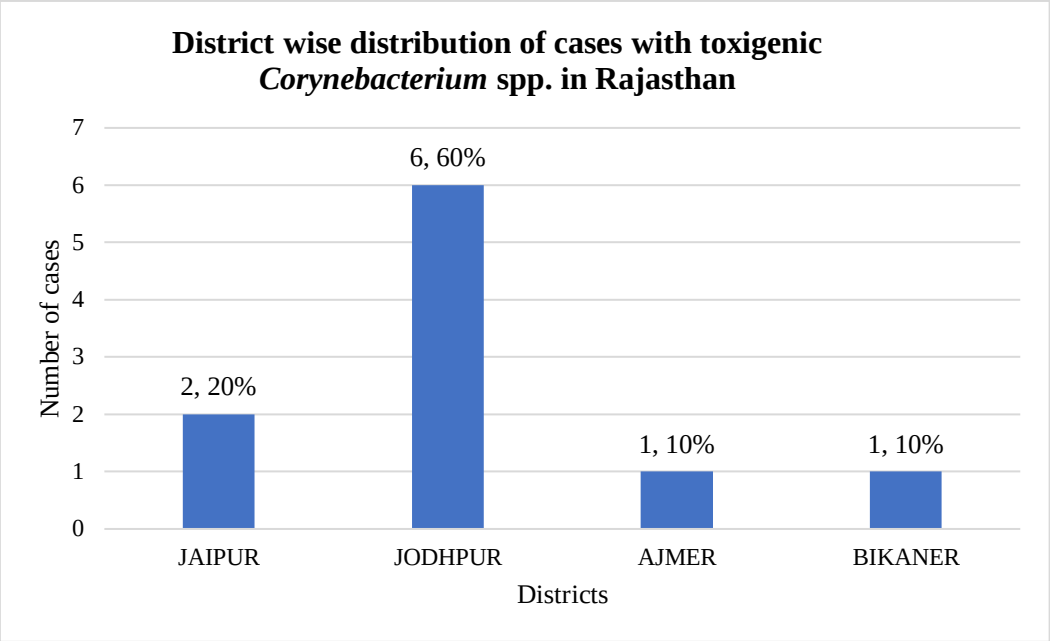

Fig 41: District wise distribution of cases with toxigenic *Corynebacterium* spp. in Rajasthan

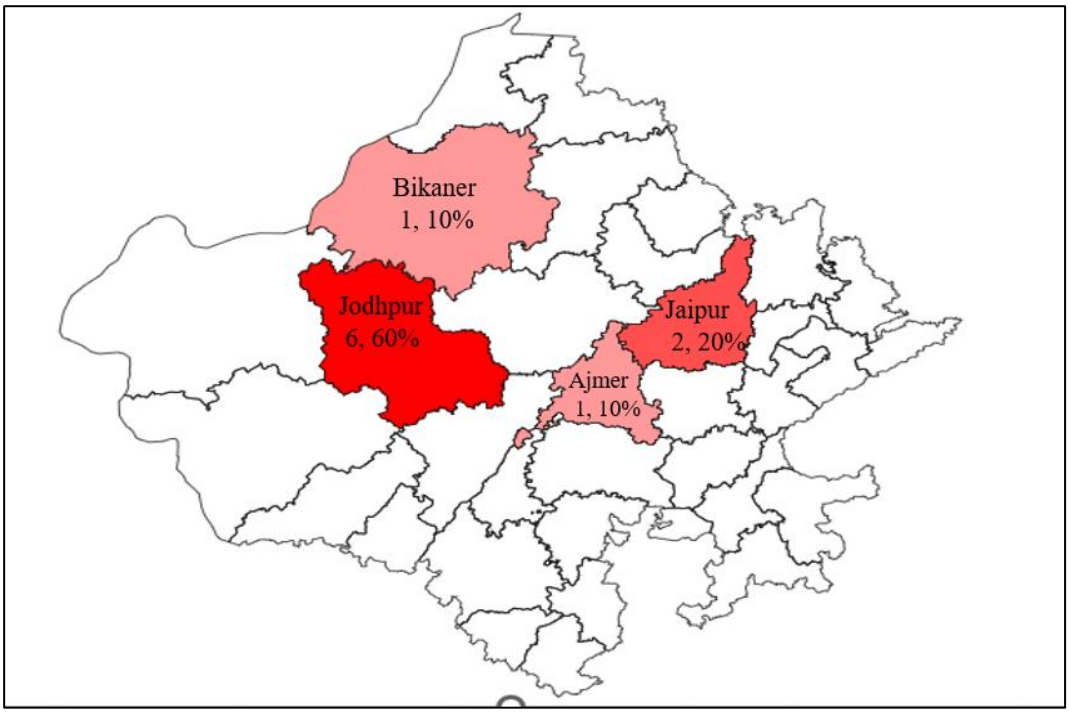

Fig 42: Map of Rajasthan showing distribution of cases with toxigenic *Corynebacterium* spp.

Mortality was seen in 1 patient out of 10 laboratory confirmed cases of toxigenic *Corynebacterium* spp. The cause of death was due to Respiratory failure. Laboratory diagnosed that this patient was infected with toxin producing strain of *Corynebacterium ulcerans* by molecular method.

DISCUSSION

Diphtheria is a highly contagious bacterial disease, if not diagnosed and treated promptly, can lead to significant mortality and morbidity because of its severe critical complications such as airway obstruction, myocarditis, polyneuritis, cranial nerve palsies, and secondary pneumonia [41].

Among members of the genus *Corynebacterium, C. diphtheriae* is well known as the causative pathogen of diphtheria. *C. ulcerans* is a known zoonotic pathogen of cats and dogs. Certain strains of *C. ulcerans* have an ability to produce the diphtheria toxin and can cause a serious condition in the respiratory tract similar to clinical diphtheria [82,1].

In the present study, a total of 204 suspected human clinical cases of diphtheria were enrolled. An active search for suspected cases of Diphtheria was routinely being carried out in Rajasthan by the WHO VPD surveillance team during the COVID-19 pandemic. Out of 33 districts, samples for suspected cases of diphtheria were received from 16 districts. More cases were reported from the eastern part of the state i.e., from Jaipur (34%), Alwar (11%), Sawai Madhopur (5%), Bharatpur (1%) and Dholpur (0.5%), as compared to other districts like Jodhpur (22.5%) and Barmer (0.5%). The higher rate of suspected cases of diphtheria from eastern part of the state can be due to a higher population density in the eastern part, as compared to other areas [73].

In the present study, a seasonal variation was seen in the suspected cases of diphtheria. Cases were seen more in the month of October 17.1% followed by September 16.6% on average. This can be due to better survival of the pathogen in autumn season i.e., during autumn season. Similar finding was observed in study conducted by S. Bhagat, S.S. Grover *et al* [64] and Meera M. *et al* [65].

In the present study, suspected cases were seen more in the age group 0-5years (39%) followed by 6-10 (34%) years. The disease is rare in the first year of life due to the passive immunity obtained from the mother, reaches a peak between 2 and 5 years, falls slowly between 5 and 10 years, and rapidly between 10 and 15 years with only very low incidence afterwards because of active immunity acquired by repeated subclinical infections [35]. Similar finding was observed in the study conducted by Parande MV. *et al* [83].

The present study shows a male predominance in the suspected cases of diphtheria compared to female patients, whereas, Meera M. *et al* has shown that in their study females (60%) outnumbered male (40%) cases [65].

In the present study of 204 suspected cases of diphtheria, Microscopy positive cases were 12.7% both by Gram's staining and Albert staining. Although, staining gives an immediate preliminary report but interpretation of staining also depends on the quality of staining and both quality and quantity of sample. Study conducted by Gogoi Mohan *et al* has shown microscopy positivity rate of 26.6% [66]. Another study conducted by Meera M. *et al* showed smear positivity (3.1%) [65].

Present study has shown culture positive cases of *Corynebacterium* spp. to be 12.25%. Study conducted by S. Bhagat *et al* [64] showed 23.2% culture positive cases and another study conducted by Parande MV *et al* [83] has 8% culture positive cases. Comparable finding was also found in the study conducted by Daiji Gogoi Mohan *et al* [66] as shown in table 17.

Studies	**S. Bhagat *et al* [64]**	**Parande MV *et al* [83]**	**Gogoi Mohan *et al* [66]**	**Meera M. *et al* [65]**	**Present study**
Culture positivity rate	23.2%	8.79%	26.26%	18.8%	12.25%

Table 17: Comparison of the culture positivity rate of various studies among suspected cases of diphtheria in India

In the present study, the 55 *Corynebacterium* spp. were speciated by biochemical reactions and MALDI-TOF MS, in which, 09 (36%) were speciated as *Corynebacterium diphtheriae* and 16 (64%) as *Corynebacterium ulcerans.* The Accuracy of the MALDI-TOF system for the identification of *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans* is very high (97–100%) [84].

All the microscopy positive cases were also culture positive. There was only 01 (0.5%) case in which the microscopy was positive but the culture was negative (Table 18).

S No.	Correlation between microscopy and culture	Number of samples n (%)
1	Microscopy positive & culture positive	25 (12%)
2	Microscopy positive & culture negative	1 (0.5%)
3	Microscopy negative & culture positive	0 (0%)
4	Microscopy negative & culture negative	178 (87.5%)
Total		**204**

Table 18: Correlation between microscopy and culture among the suspected cases of diphtheria in the current study

In the present study, 100% isolates of *C. diphtheriae* were toxin producing, while none of the isolates of *C. ulcerans* 0% showed any detectable toxin production by the conventional method.

In the present study, all the 09 isolates of *C. diphtheriae* and 16 isolates of *C. ulcerans* were susceptible to the antibiotic panel: Penicillin, Ceftriaxone, Erythromycin, Azithromycin and Amoxicillin. Penicillin resistance was seen in 33 (86.84%) of 38 isolates by study conducted by Parande MV *et al* [83], which was not seen among our cases. The same may be attributed to regional variation in the epidemiology of *C. diphtheriae.*

In the present study, out of 204 cases, *C. diphtheriae* was detected in 09 (4.41%) cases and *C. ulcerans* in 46 (22.5%) cases by multiplex RT-PCR. All the 9 cases of *C. diphtheriae* were *Toxin A* positive and one *C. ulcerans* case was also found to be *toxin A* positive. This toxin producing *C. ulcerans* had a fatal outcome for the patient. Study conducted by Williams MM. *et al* showed (33) 31.4% *C. diphtheriae* and 0% *C. ulcerans* out of 105 samples by PCR. All the 33 *C. diphtheriae* were *tox* positive. Study conducted by Otsuji K Fukuda K Endo T *et al* reported a fatal case of a Japanese patient with respiratory failure due to pseudomembrane formation in the central airways caused by *C. ulcerans* probably transfected from pet cats [86].

Real-time PCR (RT-PCR) is a fast, reliable and sensitive tool for the identification of toxigenic *Corynebacterium* spp.

In present study, RT-PCR identified 55 *Corynebacterium* spp. compared to conventional method which identified only 25 *Corynebacterium* spp. In the present study, all the culture positive cases were also positive by PCR (Table 19).

S. No.	*Corynebacterium* spp.	Culture n (%)	PCR n (%)
1	*C. diphtheriae*	9 (36%)	9 (16%)
2	*C. ulcerans*	16 (64%)	46 (84%)
Total		**25**	**55**

Table 19: Correlation between detection of *Corynebacterium* spp. by conventional and molecular method in the present study

In our study, a total of 10 toxigenic strains were identified, of which 09 were *C. diphtheriae*. The single toxigenic strain of C. ulcerans was missed on conventional Modified Elek's gel precipitation test but was found to harbour the *tox A* gene on RT-PCR testing. Among *C. diphtheriae* isolates, there was 100% correlation between conventional and molecular method of toxin detection (Table 20). Since toxigenicity is the primary basis of differentiating true pathogen from commensal diphtheria, RT-PCR is a useful tool for diagnosing true diphtheria cases in modern era.

Study conducted by Williams MM. *et al* shows RT-PCR targets demonstrated 100% sensitivity for isolates, compared to the Elek test for toxigenicity determination [85].

S No.	Interpretation	Toxin detection by Modified Elek's gel precipitation test	*Tox A* detection by PCR
1.	Positive	9 (4%)	10 (5%)
2.	Negative	195 (96%)	194 (95%)
Total		204	204

Table 20: Correlation between conventional method and molecular method of toxin detection in the present study

In standard laboratories procedures, suspected colonies are tested for toxin production using the Elek's test, which takes 24–48 hours and 12-16 hours for Modified Elek's test before any positive reaction can be observed. The preparation of Elek's media and the procedure is time consuming and sometimes need to be repeated because of plate

contamination or inconclusive results. Also, Elek's test is prone to misinterpretation especially in microbiological laboratories that rarely performed this. PCR amplification and visualization of PCR product would only take approximately 4 hours. In some rare cases, the presence of toxin gene in the isolates of *C. diphtheriae* does not necessarily express a biologically active protein (Zakikhany, Neal and Efstratiou, 2014) [87].

In the present study, 10 laboratory confirmed cases of diphtheria were diagnosed. All 10 (100%) cases had fever greyish adherent pseudo-membrane over tonsils. A total of 8 (80%) cases had difficulty in breathing, 7 (70%) had sore throat, hoarsness of voice was present in 6 (60%) cases, 3 (30%) cases had Bull neck and 3 (30%) cases had difficulty in swallowing. Similar clinical features were reported in the study conducted by Meera M. *et al* in which all patients (N = 2925) presented with fever, sore throat, and a pseudomembrane [65].

Vaccination history was unavailable for all the 10 cases with toxigenic Corynebacterium spp. in our study, similar thing was observed in study conducted by Revati K Phalkey et al. and the disease was more severe due to incomplete immunization [88, 89].

The question whether Immunized or not- does it really matter? While the Government of India recommends DPT immunization at 6, 10, 14 weeks and followed by booster dose at 16-24 months and 5-6 years, the same does not guarantee 100% protection against diphtheria. Study conducted by Patel UV *et al* and another study by Dravid MN *et al* have report invasive disease in a child with documented primary immunization and death in a partially immunized child. Disease despite primary immunization has been reported earlier from Rajkot, Gujarat and from Malegaon and Dhule district [90, 91]. Also, study conducted by Daiji Gogoi Mohan. *et al* [66] shows 31.58% of the fully immunized patients were also culture positive. Immunization by itself is reported to confer only 95% protection, not 100% and Study conducted by Meera M. *et al* [65] has also shown that Patients who were completely immunized against diphtheria suffered from milder disease, and most of them recovered uneventfully.

The Present study shows, out of all 10 cases with laboratory confirmed toxigenic *Corynebacterium* spp. majority of the cases 07 (70%) were between the age group 6-

10 years. Diphtheria mainly affects children aged between 1 to 5 years, however, due to good vaccine coverage worldwide, a shift in age incidence has been observed from preschool to school age (5-15 years) with more and more cases now being reported in adults [64]. Study conducted by Sunarno S. *et al* shows most of the diphtheria-confirmed cases were < 15- years-old (67.5%), with the age range of 6 - 10 years being the most prevalent one (27.5%) [92].

It was found in the present study that 06 (60%) cases with the toxin producing strains of *Corynebacterium* spp. were female and 4 (40%) were male patient. Study conducted by Sangal L, Joshi S, Anandan S. *et al* showed overall sex distribution of diphtheria cases is almost proportionate in males and females [72]. Another study conducted by Parande MV, Roy S, Mantur BG. *et al* showed the confirmed cases in males were three times higher than in females [83]. Another study conducted by Meera M. *et al* had similar finding that is Sixty percent of the affected were females [65].

In the present study, cases of toxin producing strains of *Corynebacterium* spp. was seen more in the month of October 3 (30%). Study conducted by Meera M. *et al* has shown similar seasonality pattern, they observed peak of disease among the vaccinated cases occurred in the month of October (33 cases on average) and the peak among the unvaccinated occurred in September (45 cases on average) [65].

Majority of the cases of toxigenic *Corynebacterium* spp. was from Jodhpur 6 (60%) followed by Jaipur 2 (20%) The higher number in this area could be due to the existence of pockets of low immunization coverage or compared to other districts.

Diphtheria cases are seen in almost every part of India. Table 21 shows diphtheria cases reported in various studies from different parts of India.

S. No.	Author and References	Location	Study period	Setting	Sample size	No. of Diphtheria cases
1.	Bhatnagar R. *et al* [93]	Uttar Pradesh	May 2016 to September 2018 (1.5 yrs.)	Hospital	53	53 cases only clinically confirmed
2.	Raghupati N. *et al* [94]	Multicentric India	2015 to 2020 (6yrs.)	Surveillance	218	Culture positive: 32 PCR positive: 43 (*C. diphtheriae*)
3.	Parande MV. *Et al* [83]	Karnataka	2012-15 (4 yrs.)	Outbreak	432	Culture positive: 38 (*C. diphtheriae)*
4.	Sharma N *et al* [95]	Gujarat	2019–2020	Outbreak	188	Culture positive: 21 (*C. diphtheriae*)
5.	Dash N *et al* [96]	Chandigarh	2008- 2015 (8yrs)	Hospital	99	Albert positive: 21 Culture positive: 28 (*C. diphtheriae*)
6.	Choudhury G et al. [70]	Dibrugarh, Jorhat, Assam	2019-2020 (2 yrs.)	Outbreak	3 isolates obtained from 3 different cluster of cases	3 (Multi-Locus Sequence Typing)
6.	Present study	Rajasthan	Jan 2021-Dec 2022 (2 yrs.)	Surveillance	204	Albert positive:26 Culture positive: 25 RT-PCR Positive: 55 Toxin producing strain: 10 (9 *C. diphtheriae* and 1 *C. ulcerans*)

Table 21: Number of cases of Diphtheria reported in various studies from different parts of India.

The number of *C. ulcerans* strains outnumbered *C. diphtheriae* in the present study. The emergence *C. ulcerans* and its possible correlation with domestic animals and the significance of non-toxigenic strains causing systemic disease have underlined the need for further screening and confirmation of toxigenicity.

Since diphtheria has been seen continuously since the past one year, there is a likelihood of the disease becoming once again endemic to the region and therefore needs to be controlled quickly. Also, global SARS-CoV-2 pandemic, has overwhelmed public health systems including immunization coverage so it is likely that diphtheria will re-emerge globally.

Challenges faced during the study:

The present study relied on throat swab collected from patients located in different districts of Rajasthan, by WHO health care workers, who transported the same to AIIMS Jodhpur. Since there was no direct contact with the patients, we had to rely on the clinical data available in the case record forms submitted aby the health care workers and on the WHO VLIFA software. The patient consent forms were already filled but often the guardians could not recall vaccination history of their child and left many categories blank. Later telephonic follow-up was done on the phone number provided by the guardians.

The healthcare workers reported that they faced difficulties in proper sample collection because of patient's inability to open their mouth wide enough for a proper throat swab collection due to lymphadenopathy and edema/bull neck. Also, majority of the patients were children and sample collection from pediatric age group was more challenging.

Attempts made by the health care workers to obtain vaccination history through registers at the PHC, vaccination diaries of subcenter and PHC staff and the National Rural Health Mission (NRHM) Reproductive and Child Health II (RCH II) databases were in vain due to incomplete documentation. Secondly, parental recall and vaccination cards were sought during visits and also via telephonic communication but were also unsuccessful.

Follow up of the patients was difficult. Follow up was done either through telephonic communication with the patient/ Guardian/ the health care worker.

Although, immunization is advised for all as per NIS. More importance regarding follow- up of the patients with laboratory confirmed non-toxigenic *Corynebacterium* spp. was given to complete their vaccination.

Significance of this study:

Our study highlights some important epidemiological features of diphtheria in Rajasthan. Classically considered a vaccine preventable illness, in the under 5 age group, 10% cases contracted the disease due to low primary immunization coverage. Circulating strains of diphtheria probably went on to cause clinical infections among 6-10 years age group in 70% cases. Maximum cases seen in our study belonged to this

age group highlighting the importance of booster dose and adolescent vaccination. A single case of adult diphtheria is an alarming pointer towards weaning adult immunity in the region. The study was able to correctly identify and characterize toxigenic diphtheria among all age groups. A notable finding among these is the emergence of *C. ulcerans* confirmed by both RT-PCR and culture. This is a Nobel finding and no other studies from India has reported the isolation of *C. ulcerans* in currently published literature.

A single case of toxigenic *C. ulcerans* highlights the importance of changing epidemiology of diphtheria in Rajasthan which may be distinct from other parts of India and warrants further study.

A recommendation of our study is to further investigate the role of *C. ulcerans* as a causative agent of Clinical diphtheria in our region.

While strains tested negative to *Tox A* gene, there may be presence of other toxins like *Tox B* which can be incorporated in the methodology of future studies from Western Rajasthan.

CONCLUSION

The current study identified ten cases of active clinical diphtheria among 204 suspected cases in Rajasthan during the ongoing COVID-19 pandemic of 2020-2022. A combination of conventional and molecular techniques helped diagnose *Corynebacterium diphtheriae* as the causative agent in nine cases. A single strain of *Corynebacterium ulcerans* was established as true pathogen in a child who eventually succumbed to the illness. Molecular techniques like RT-PCR have contributed greatly towards the understanding the current epidemiology of this re-emerging disease. Luckily all strains were susceptible to penicillin and other antibiotics tested in the panel.

The COVID-19 Pandemic induced lockdowns and consequent challenges faced by the health care infrastructure in India have caused a set-back for the National Immunization programs in 2020-2022. There is an urgent need to identify pockets of poor immunization and encourage catch-up vaccination of such children through targeted public health initiatives such as door to door vaccination, fixed vaccination posts and administration of vaccine in schools.

Active detection and proper treatment of diphtheria is needed to interrupt its transmission in the community. Also, there is an urgent need of making Anti-Diphtheritic Serum (ADS) available at all big hospitals to decrease mortality.

The present study, even with its limitations, was the first attempt by the Department of Microbiology, AIIMS, Jodhpur, in diagnosing and reporting active cases of diphtheria in Rajasthan. Our prompt reporting resulted in correct treatment and recovery among most of these children, which is a humble contribution of this study towards our fight against vaccine preventable diseases in India.

BIBLIOGRAPHY

1) Burkovski, A. Diphtheria and its etiological agents. In Corynebacterium diphtheriae and Related Toxigenic Species; Burkovski, A., Ed.; Springer: Dordrecht, The Netherlands, 2014; pp. 1–14

2) Löffler, F. Studies on the importance of microorganisms for the development of diphtheria in humans, pigeons and calves. Mitt. The quay. Healthy. 1884, 2, 421–499.

3) Sangal, V.; Hoskisson, P.A. Evolution, epidemiology and diversity of Corynebacterium diphtheriae: new perspectives on an old foe. Infect. Genet. Evol. 2016, 43, 364–370.

4) Hoskisson, P.A. Microbe Profile: Corynebacterium diphtheriae—An old foe always ready to seize opportunity. Microbiology 2018 164, 865–867.

5) Sharma, N.C.; Efstratiou, A.; Mokrousov, I.; Mutreja, A.; Das, B.; Ramamurthy, T. Diphtheria. Nat. Rev. Dis. Primers 2019, 5, 81

6) English PC. Diphtheria and theories of infectious disease: centennial appreciation of the critical role of diphtheria in the history of medicine. Pediatrics. 1985; 76:1–9.

7) Galazka AM, Roberson SE. Diphtheria: changing patterns in the developing world and the industrialized world. *Eur J Epidemiol*. 1995; 11:107–117.

8) Behring, E. Ueber ein neues Diphtherieschutzmittel. Dtsch. Med. Wochenschr. 1913, 19, 873–876

9) Mandell, Douglas and Bennett's principles and practice of infectious diseases. 8th ed.

10) Relyveld, E.H. A history of toxoids. In A History of Vaccine Development; Plotkin, S.A., Ed.; Springer: New York, USA, 2011; pp. 57–64v

11) Tiwari, T.S.P.; Wharton, M. Diphtheria toxoid. In Plotkin´s Vaccines, 7th ed.; Plotkin, S.A., Offit, P.A., Orenstein, W.A., Edwards, K.M., Eds.; Elsevier: Philadelphia, PA, USA, 2012; pp. 261–275

12) Vitek, C.R.; Wharton, M. Diphtheria in the former Soviet Union: Reemergence of a pandemic disease. Emerg. Infect. Dis. 1998, 4, 539–550.

13) Dittmann, S.; Wharton, M.; Vitek, C.; Ciotti, M.; Galazka, A.; Guichard, S.; Hardy, I., Kartoglu, U.; Koyama, S.; Kreysler, J.; et al. Successful control of epidemic diphtheria in the states of the former Union of Soviet Socialist Republics: Lessons learned. J. Infect. Dis. 2000, 181, S10–S22

14) Markina, S.S.; Maksimova, N.M.; Vitek, C.R.; Bogatyreva, E.Y.; Monisov, A.A. Diphtheria in the Russian Federation in the 1990s. J. Infect. Dis. 2000, 181, S27–S34.

15) Clarke, K.E.N.; MacNeil, A.; Hadler, S.; Scott, C.; Tiwari, T.S.P.; Cherian, T. Global epidemiology of diphtheria, 2000–2017. Emerg. Infect. Dis. 2019, 25, 1834–1842.

16) Matsuyama, R.; Akhmetzhanov, A.R.; Endo, A.; Lee, H.; Yamaguchi, T.; Tsuzuki, S.; Nishiura, H. Uncertainty and sensitivity analysis of the basic reproduction number of diphtheria: A case study of a Rohingya refugee camp in Bangladesh, NovemberDecember 2017. Peer J. 2018, 6, e4583.

17) Exavier, M.M.; Hanna, M.P.; Muscadin, E.; Freishstat, R.J.; Brisma, J.-P.; Canarie, M.F. Diphtheria in children in Northern Haiti J. Trop. Pediatr. 2019, 65, 183–187.

18) Mahomed, S.; Archary, M.; Mutevedzi, P.; Mahabeer, Y.; Govender, P.; Ntshoe, G.; Kuhn, W.; Thomas, J.; Olowolagba, A.; Blumberg, L.; et al. An isolated outbreak of diphtheria in South Africa, 2015. Epidemiol. Infect. 2017, 145, 2100–2108.

19) Strauss, R.A.; Herrera-Leon, L.; Guillén, A.C.; Castro, J.S.; Lorenz, E.; Carvajal, A.; Hernandez, E.; Navas, T.; Vielma, S.; Lopez, N.; et al. Molecular and epidemiologic characterization of the diphtheria outbreak in Venezuela. Sci. Rep. 2021, 11, 6378.

20) Dureab, F.; Al-Sakkaf, M.; Ismail, O.; Kuunibe, N.; Krisam, J.; Müller, O.; Jahn, A. Diphtheria outbreak in Yemen: The impact of conflict on a fragile health system. Confl. Health. 2019, 13, 19

21) World Health Organization. Diphtheria Reported Cases. Available online: https://apps.who.int/immunization_monitoring/globalsummary/timeseries/tsincidencediphtheria.html (accessed on 22 February 2022).

22) World Health Organization. Third Dose of Diphtheria Toxoid, Tetanus Toxoid and Pertussis Vaccine—Reported Estimates of DTP3 Coverage. Available online: https://apps.who.int/immunization_monitoring/globalsummary/timeseries/tscoveragedtp3.html (accessed on 22 February 2022).

23) Burkovski, A. Pathogenesis of Corynebacterium diphtheriae and Corynebacterium ulcerans. In Human Emerging and Re-Emerging Infections; Singh, S.K., Ed.; John Wiley & Sons: Hoboken, NJ, USA, 2016; Volume 2, pp. 697–708.

24) Lai, Y.; Purnima, P.; Ho, M.; Ang, M.; Deepak, R.N.; Chew, K.L.; Vasoo, S.; Capulong, D.F.; Lee, V. Fatal case of diphtheria and risk for reemergence, Singapore. Emerg. Infect. Dis. 2018, 24, 2084–2086.

25) Scheifer, C.; Rolland-Debord, C.; Badell, E.; Reibel, F.; Aubry, A.; Perignon, A.; Patey, O.; Brisse, S.; Caumes, E. Re-emergence of Corynebacterium diphtheriae. Med. Mal. Infect. 2019, 49, 463–466.

26) Shulman ST. The history of pediatric infectious diseases. *Pediatr Res*. 2004; 55:163–176.

27) Caulfield E. A history of the terrible epidemic, vulgarly called the throat distemper, as it occurred in his majesty's New England colonies between 1735 and 1740. *Yale J Biol Med*. 1939; 11:219–272.

28) Hajj Hussein I, Chams N, Chams S, et al. Vaccines through centuries: major cornerstones of global health. *Front Public Health*. 2015; 3:1–16.

29) Roush SW, Murphy TV, Vaccine-Preventable Disease Table Working Group. Historical comparisons of morbidity and mortality for vaccine-preventable disease in the United States. *JAMA*. 2007;298: 2155–2163.

30) Centers for Disease Control and Prevention. Diphtheria. In: Hamborsky J, Kroger A, Wolfe S, eds. *Epidemiology and Prevention of Vaccine-Preventable Diseases*. 13th ed. Washington D.C.: Public Health Foundation; 2015: 107–118.

31) Magill AJ, Ryan ET, Solomon T, Hill DR. Hunter's Tropical Medicine and Emerging Infectious Disease: Ninth Edition. Hunter's Tropical Medicine and Emerging Infectious Disease: Ninth Edition. 2012. 1-1190 p.

32) Saikia L, Nath R, Saikia NJ, Choudhury G, Sarkar M. A diphtheria outbreak in Assam, India. Southeast Asian J Trop Med Public Health. 2010;41(3):647-52.

33) Murphy JR. *Corynebacterium diphtheriae*. In: Baron S, ed. *Medical Microbiology*. 4th ed. Galveston, TX: University of Texas Medical Branch at Galveston; 1996:413–422.

34) Whitley OR, Damon SR. A transparent dextrose serum tellurite plating medium; its use as an adjunct to microscopic examination of smears made from Loeffler slants in routine diphtheria diagnosis. *Public Health Rep*.1949; 64:201–212.

35) R. Ananthanarayan. Ananthanarayan and Paniker's Textbook of Microbiology.8th ed. Universities Press (India), 2009

36) Efstratiou A, George RC (1996) Microbiology and epidemiology of diphtheria. Rev Med Microbiol 7(1):31–42

37) Dorella FA, Pacheco LG, Oliveira SC, Miyoshi A, Azevedo V (2006) *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Vet Res 37(2):201–218

38) Riegel P, Ruimy R, de Briel D, Prévost G, Jehl F, Christen R, Monteil H (1995) Taxonomy of *Corynebacterium diphtheriae* and related taxa, with recognition of *Corynebacterium ulcerans* sp. nov. nom. rev. FEMS Microbiol Lett 126(3):271–276

39) Health Protection Agency (2008) National Standard Method: Identification of *Corynebacterium* species, BSOP ID2i3. www.evaluations-standards.org.uk.

40) Patricia M. Tille Bailey & Scott's Diagnostics Microbiology, 13th Edition.

41) Varol B, Bektaş M, Nurten R, Bermek E (2012) The cytotoxic effect of diphtheria toxin on actin cytoskeleton. Cell Mol Biol Lett 17(1):49–61.

42) Yamaizumi M, Mekada E, Uchida T, et al. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell*. 1978; 15:245–250.

43) Gill DM. Bacterial toxins: a table of lethal amounts. *Microbiol Rev*. 1982; 46:86–94.

44) Hadfield TL, McEvoy P, Polotsky Y, et al. The pathology of diphtheria. *J Infect Dis*. 2000;181(suppl 1): S116–S120.

45) Byard RW. Diphtheria "the strangling angel" of children. *J Forensic Leg Med*. 2013; 20:65–68.

46) Dobie RA, Tobey DN. Clinical features of diphtheria in the respiratory tract. *JAMA*. 1979;242: 2197–2201.

47) Quick ML, Sutter RW, Kobaidze K, et al. Epidemic diphtheria in the Republic of Georgia, 1993-1996; risk factors for fatal outcome among hospitalized patients. *J Infect Dis*. 2000;181(suppl 1): S130–S137

48) Khadirova R, Kartoglu HU, Strebel PM. Clinical characteristics and management of 676 hospitalized diphtheria cases, Kyrgyz Republic, 1995. *J Infect Dis*. 2000;181(suppl 1): S110–S115.

49) Kneen R, Nguyn MD, Solomon T, et al. Clinical features and predictors of diphtheritic cardiomyopathy in Vietnamese children. *Clin Infect Dis*. 2004; 39:1591–1598.

50) Manikyamba D, Satyavani A, Deepa P. Diphtheritic polyneuropathy in the wake of resurgence of diphtheria. *J Pediatr Neurosci*. 2015; 10:331–334.

51) Alesen LA. Postdiphtheritic paralysis of the diaphragm. *JAMA*. 1925;84: 730–731

52) Piradov MA, Pirogov VN, Popova LM, et al. Diphtheritic polyneuropathy: clinical analysis of severe forms. *Arch Neurol*. 2001;58: 1438–1442.

53) Jayashree M, Shruthi N, Singhi S. Predictors of outcome in patients with diphtheria receiving intensive care. *Indian Pediatr*. 2006; 43:155–160.

54) Holmes RK. Biology and molecular epidemiology of diphtheria toxin and the *tox* gene. *J Infect Dis*. 2000;181(suppl 1): S156–S167.

55) Belsey MA, Sinclair M, Roder MR, et al. *Corynebacterium diphtheriae* skin infections in Alabama and Louisiana: a factor in the epidemiology of diphtheria. *N Engl J Med*. 1969; 280:135–141.

56) Van Panhuis WG, Grefenstette J, Jung SY, et al. Contagious diseases in the United States from 1888 to the present. N Engl J Med. 2013; 369:2152–2158.

57) World Health Organization. Diphtheria Vaccine: WHO Position Paper: August 2017. Diphtheria Vaccine: WHO Position Paper: August 2017. Wkly Epidemiol Rec. 2017; 92:417–435. http://www.who.int/immunization/

58) Clarke K, MacNeil A, Hadler S, Scott C, Tiwari T, Cherian T. Global Epidemiology of Diphtheria, 2000–2017. Emerg Infect Dis. 2019;25(10):1834-1842. https://doi.org/10.3201/eid2510.190271.

59) Clarke K, MacNeil A, Hadler S, et al. Global Epidemiology of Diphtheria, 2000–2017. Emerging Infectious Diseases. 2019;25(10):1834-1842. doi:10.3201/eid2510.190271.

60) Clarke, K., MacNeil, A., Hadler, S., Scott, C., Tiwari, T., & Cherian, T. (2019). Global Epidemiology of Diphtheria, 2000–2017. *Emerging Infectious Diseases*, *25*(10), 1834-1842. https://doi.org/10.3201/eid2510.190271.

61) www.idsp.mohfw.gov.in/outbreak_d/Home.html

62) https://www.who.int

63) https://www.who.int/southeastasia/our-work/vaccine-preventable-disease

64) Bhagat S, Grover SS, Gupta N, Roy RD, Khare S. Persistence of Corynebacterium diphtheriae in Delhi & National Capital Region (NCR). Indian J Med Res. 2015 Oct;142(4):459-61. doi: 10.4103/0971-5916.169212. PMID: 26609038; PMCID: PMC4683831.

65) M M, M R. Diphtheria in Andhra Pradesh-a clinical-epidemiological study. Int J Infect Dis. 2014 Feb;19: 74-8. doi: 10.1016/j.ijid.2013.10.017. Epub 2013 Dec 1. PMID: 24295558.

66) Gogoi Mohan, Daiji & Gogoi, Mayuri & Hazarika, N & Sharma, Ajanta. (2018). Sporadic outbreaks of Diphtheria: A three-year study from a tertiary care centre

of Northeast India. IOSR Journal of Dental and Medical Sciences. 17. 42-45. 10.9790/0853-1707104245.

67) Nandi R, Mriganka De, Simon Browning. Diphtheria: The patch remains. J Laryngol Otol. 2003;117(10):807-10.

68) Nath B, Mahanta TG. Investigation of an outbreak of diphtheria in Borborooah block of Dibrugarh district, Assam. Indian J Community Med. 2010;35(3):436-38

69) Das PP, Patgiri SJ, Saikia L, Paul D. Recent outbreaks of diphtheria in Dibrugarh District, Assam, India. J Clin Diagnostic Res. 2016;10(7): DR01-03.

70) Devi U, Baruah PJ, Borah PK, Mahanta J, Dutta P. Report of diphtheria cases & surveillance among contacts in Dibrugarh, Assam, India. Indian Journal of Medical Research. Wolters Kluwer-Medknow Publications. 2017;145(6):847-48.

71) Gargi Choudhury, Navonil Gogoi, Reema Nath, Pallabi Sargiary, Partha Pratim Das, Binita Bhuyan, Uttara Borkotoki. Molecular Characterisation of Corynebacterium diphtheriae Isolates of Faucial Diphtheria Cases from Assam: A Cross-sectional Study. Journal of Clinical and Diagnostic Research. 10.7860/JCDR/2022/52934.16379.

72) Sangal L, Joshi S, Anandan S, Balaji V, Johnson J, Satapathy A, Haldar P, Rayru R, Ramamurthy S, Raghavan A, Bhatnagar P. Resurgence of Diphtheria in North Kerala, India, 2016: Laboratory Supported Case-Based Surveillance Outcomes. Front Public Health. 2017 Aug 30;5 :218. doi: 10.3389/fpubh.2017.00218. PMID: 28913330; PMCID: PMC5582196.

73) Rathore Monika, Trends of Communicable Diseases & IDSP reporting in State of Rajasthan, Dept. Of Community Medicine S.M.S. Medical College, Jaipur.

74) https://nhm.gov.in/New_Updates_2018/NHM_Components/Immunization 2018

75) https://www.who.int/.../diphtheria-outbreak-toolbox

76) Procop GW, Church DL, Hall GS, Janda WM, Koneman EW, Schreckenberger PC, et al. Koneman's Colour Atlas and Textbook of Diagnostic Microbiology. 7th ed. Philadelphia: Wotter's Kluwer Health; 2017.

77) Mackie TJ, McCartney JE. Handbook of practical bacteriology: a guide to bacteriological laboratory work. E. & S. Livingstone; 1953.

78) Procop GW, Church DL, Hall GS, Janda WM, Koneman EW, Schreckenberger PC, et al. Koneman's Colour Atlas and Textbook of Diagnostic Microbiology. 7th ed. Philadelphia: Wotter's Kluwer Health; 2017.

79) Wayne, PA: Clinical and Laboratory Standards Institute; 2015.Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. 3rd ed. CLSI guideline M45.

80) Engler KH, Glushkevich TG, Mazurova IK, George RC, Efstratiou A. A modified Elek test for the detection of toxigenic corynebacteria. J Clin Microbiol 1997;35: 495–8

81) Anandan Shalini et al. Christian Medical College Vellore, India. Hands-on Training on Laboratory Diagnosis & Molecular Characterization of Pertussis and Diphtheria.

82) Yasuda I., Matsuyama H., Ishifuji T., Yamashita Y., Takaki M., Morimoto K., et. al.: Severe pneumonia caused by toxigenic Corynebacterium ulcerans infection, Japan. Emerg Infect Dis 2018; 24: pp. 588-591.

83) Parande MV, Roy S, Mantur BG, Parande AM, Shinde RS. Resurgence of diphtheria in rural areas of North Karnataka, India. Indian J Med Microbiol. 2017 Apr-Jun;35(2):247-251. doi: 10.4103/ijmm.IJMM_17_48. PMID: 28681814.

84) Konrad, R. et al. Matrix-assisted laser desorption/ ionisation time-of-flight (MALDI-TOF) mass spectrometry as a tool for rapid diagnosis of potentially toxigenic Corynebacterium species in the laboratory management of diphtheria-associated bacteria. Eurosurveillance 15, 19699 (2010).

85) Williams MM, Waller JL, Aneke JS, Weigand MR, Diaz MH, Bowden KE, Simon AK, Peng Y, Xiaoli L, Cassiday PK, Winchell J, Tondella ML. 2020. Detection and characterization of diphtheria toxin genebearing Corynebacterium species through a new real-time PCR assay. J Clin Microbiol 58: e00639-20. https://doi.org/10.1128/JCM.00639 -20

86) Otsuji K., Fukuda K., Endo T., Shimizu S., Harayama N., Ogawa M., et. al.: The first fatal case of Corynebacterium ulcerans infection in Japan. JMM Case Rep 2017.

87) Zakikhany, Katherina et al. "Emergence and molecular characterisation of non-toxigenic tox gene-bearing Corynebacterium diphtheriae biovar mitis in the United Kingdom, 2003-2012." *Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 19 22 (2014).

88) Singh J, Harit AK, Jain DC, Panda RC, Tewari KN, Bhatia R, Sokhey J: Diphtheria is declining but continues to kill many children: analysis of data from a sentinel centre in Delhi, 1997. Epidemiol Infect 1999, 123(2):209–215.

89) Singhal T, Lodha R, Kapil A, Jain Y, Kabra SK: Diphtheria-down but not out. Indian Pediatr 2000, 37(7):728–738

90) Patel UV, Patel BH, Bhavsar BS, Dabhi HM, Doshi SK: A Retrospective study of diphtheria cases. Rajkot: Gujarat. Indian Journal of Community Medicine; 2004.

91) Dravid MN, Joshi SA: Resurgence of diphtheria in Malegaon & Dhule regions of north Maharashtra. Indian J Med Res 2008, 127(6):616–617.

92) Sunarno S, Puspandari N, Sariadji K, Febriyana D, Febrianti T, et al. Microbiological and Clinical Aspects of Diphtheria-Confirmed Cases from Capital City of Indonesia, Jakarta, and Surrounding Areas in 2017. Jundishapur J Microbiol. 2021;14(8): e118751. Doi: 10.5812/jjm,118751.

93) Bhatnagar R, Sahai L. Resurgence of diphtheria and its outcome among children in western Uttar Pradesh: a battle to conquer. Int J Contemp Pediatr 2020; 7:149-53.

94) Devanga Ragupathi NK, Muthuirulandi Sethuvel DP, Murugan D, Ranjan R, Gautam V, Gupta P, Johnson J, Sharma NC, Mutreja A, Haldar P, Kumar A, Bhatnagar P, Sangal L, Veeraraghavan B. Divergent evolution of Corynebacterium diphtheriae in India: An update from National Diphtheria

Surveillance network. PLoS One. 2021 Dec 15;16(12): e0261435. doi: 10.1371/journal.pone.0261435. PMID: 34910778; PMCID: PMC8673651.

95) Sharma N, Desai HR, Chaudhary A, Shrivastava AK, Hasan AA. Resurgence of diphtheria in Northern Gujarat: Aretrospective study done in Banas Medical College & Research Centre, Palanpur, Gujarat. J Family Med Prim Care 2022; 11:7163-7

96) Dash N, Verma S, Jayashree M, Kumar R, Vaidya PC, Singh M. Clinico-epidemiological profile and predictors of outcome in children with diphtheria: a study from northern India. Trop Doct. 2019 Apr;49(2):96-101. doi: 10.1177/0049475518823657. Epub 2019 Jan 12. PMID: 30636517.

ANNEXURE-1

Institutional Ethical Committee certificate

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2021/3475 **Date: 12/03/2021**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2021/3310**

Project title: **"Microbiological Characterization and molecular differentiation of *Corynebacterium Spp* Isolated from clinical specimens in a tertiary care hospital in western Rajasthan"**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Debaleena Paul**
Guide:	**Dr. Ashwini Agarwal**
Co-Guide:	**Dr. Vidhi Jain & Dr. Siya Ram Didel**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member Secretary
Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com

ANNEXURE-2

All India Institute of Medical Sciences, Jodhpur
अनुसंधान अनुभाग
Research Section

No.: AIIMS/RES/2021/6637 Dated: 23/10/21

To
Dr. Ashwini Agarwal
Additional Professor & Head,
Department of Microbiology,
AIIMS, Jodhpur.

Subject: Change of PG Guide for MD Microbiology Junior Residents: Reg.

Dear Dr. Agarwal,

This is in reference to your letter no. AIIMS/Micro/2021/1498 dated 08/10/2021. I am directed to inform you that Dean (Research) accorded his permission to change the guide & appointment of co-guide for following students as per your request, if they are eligible for guideship or co-guideship as per institutional guidelines. Details as follows:

Sr. No.	Name of Student	Session	Thesis Title	New Guide	New Co-Guide
1.	Dr. Jeeshan Noore Azim	July-2019	To study the incidence and etiology of ventilator associated events in cases admitted in adult ICU in a tertiary care Centre in western Rajasthan	No Change	Dr. Vibhor Tak
2.	Dr. Tejashree Nare	Jan-2020	Antifungal Susceptibility Profile for Terbinafine and Iitraconazole among Dermatophytes at a Tertiary Care Hospital in Western Rajasthan	Dr. Ravishekhar Gadepalli	Dr. Vidhi Jain
3.	Dr. Debaleena Paul	Jul-2020	Microbiological Characterization and molecular differentiation of Corynebacterium Species Isolated from Clinical Specimens in a Tertiary Care Hospital in Western Rajasthan	Dr. Sarkila Kombade	-
4.	Dr. Nikhil John	Jan - 2021	Clinical relevance of nontuberculous mycobacteria isolated In a tertiary care hospital in western Rajasthan	Dr. Vibhor Tak	-

Dr. Jaykaran Charan
Sub Dean (Research)
Sub Dean (Research)
All India Institute of Medical Sciences
Jodhpur (Raj.)-342005 India

Copy for Information to: -

1. Dr. Ravishekhar Gadepalli, Additional Professor, Dept. of Microbiology, AIIMS, Jodhpur
2. Dr. Vibhor Tak, Associate Professor, Dept. of Microbiology, AIIMS, Jodhpur
3. Dr. Sarkila Kombade, Associate Professor, Dept. of Microbiology, AIIMS, Jodhpur
4. Dr. Vidhi Jain, Assistant Professor, Dept. of Microbiology, AIIMS, Jodhpur
5. Concerned PG Student
6. Member Secretary, IEC, AIIMS. Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005, **Website:** www.aiimsjodhpur.edu.in, **Phone:** 0291-2740741 Extn. 3109
Email: deanresearch@aiimsjodhpur.edu.in, reserachcell@aiimsjodhpur.edu.in

ANNEXURE - 3

All India Institute of Medical Sciences, Jodhpur
Informed Consent Form

Title of the project: **"MICROBIOLOGICAL CHARACTERIZATION AND MOLECULAR DIFFERENTIATION OF *Corynebacterium* spp. ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY CARE HOSPITAL IN WESTERN RAJASTHAN"**

Name of the Principal Investigator: Dr Debaleena Paul

Tel. No. (Mobile): - 7005841535/9774693679
Patient ID No: _____

I, _____S/o or D/o_____
R/o _____give my full, free, voluntary consent to be a part of the study: Microbiological characterization and molecular differentiation of *Corynebacterium* spp. isolated from clinical specimens in a tertiary care hospital in western Rajasthan, the procedure and nature of which has been explained to me in my own language to my full satisfaction. I confirm that I have had the opportunity to ask questions.

I understand that my participation is voluntary and I am aware of my right to opt out of the study at any time without giving any reason.
I understand that the information collected about me and any of my medical records may be looked at by responsible individual from AIIMS Jodhpur or from regulatory authorities. I give permission for these individuals to have access to my records.

Date: _____ _____
Place: _____ Signature/Left thumb impression
(Patient/Caregiver)

This to certify that the above consent has been obtained in my presence.

Date: _____ _____
Place: _____ Signature of Principal Investigator

Witness1 Witness2
_____ _____
_____ _____
Signature Signature

ANNEXURE - 4

अखिल भारतीय चिकित्सा विज्ञान संस्थान

सूचित सहमति पत्र

परियोजना का शीर्षक: "पश्चिमी राजस्थान में एक तृत्तीयक देखभाल अस्पताल में नैदानिक नमूनों से पृथक *Corynebacterium* प्रजातियों के सूक्ष्म जीव विज्ञानी और मोलकुलर भेदभाव"

अन्वेषक का नाम: डॉ देबलेना पौल मोबाइलन. 7005841535/9774693679

रोगी आईडी नं._____

मैं. _____ एस /ओयाडी / ओ._____अध्ययन का हिस्सा बनने के लिए मेरी पूर्ण,स्वतंत्र,स्वैच्छिक सहमति है।"पश्चिमी राजस्थान में एक तृत्तीयक देख भाल अस्पताल में दानिक नमूनों से पृथक Corynebacterium प्रजातियों के माइक्रोबायोलॉजिकल निस्रपण और मोलकुलर भेदभाव",को मैंने अपनी भाषा में अपनी पूर्ण संतुष्टि के लिए मुझे समझाया है।मैं पुष्टि करता हूं कि मुझे सवाल पूछनेका अवसर मिला है।

मैं समझता हूं कि मेरी भागीदारी स्वैच्छिक है और मुझे बिना कोई कारण बताए कि सीभी समय अध्ययनसे बाहर निकलने के मेरे अधिकार के बारे में पता है।

मैं समझता हूं कि मेरे सीभी मेडिकल रिकॉर्ड के बारे में एक त्रित जानकारी को एम्स, जोधपुर के जिम्मेदार व्यक्ति या नियामक अधिकारि यों से देखा जा सकता है।

दिनांक. _____

_____

स्थान. _____ प्रिंसिपल जांचकर्ता के हस्ताक्षर

1.साक्षी. 2.साक्षी

_____ _____

ANNEXURE 5

All India Institute of Medical Sciences, Jodhpur

PATIENT INFORMATION SHEET

Changes in the epidemiology of diphtheria have been reported worldwide. The prevalence of toxigenic *Corynebacterium* spp. Highlights the need for proper clinical and epidemiological investigations to quick identify and treat affected individuals, along with public health measures to prevent and contain the spread of this disease.

PURPOSE OF STUDY: "Microbiological characterization and molecular differentiation of *Corynebacterium* spp. isolated from clinical specimens in a tertiary care hospital in Western Rajasthan"

METHODS INVOLVED: Relevant sample will be collected from patient with clinical suspicion of diphtheria and will transport for proper bacteriological profiling and AST.

BENEFIT OF STUDY TO THE PATIENT: It will be helpful in the proper diagnosis and treatment to the patient and will helpful in selecting antimicrobial drugs.

RISK INVOLED TO THE PATIENT: There is no risk of any kind to the patient in this study. No drug or vaccines are being tested in the study.

CONFIDENTIALITY OF RECORDS: The patient's records/reports/ shall be kept confidential.

ANNEXURE 6

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर

रोगी कीसूचना पत्र

डिप्थीरिया की महामारी विज्ञान में परिवर्तन दुनिया भरमें सूचित किया गया है।टोक्सीजेनिक Corynebacterium प्रजाति यों का प्रचलन। इस बीमारी के प्रसार को रोकने के लिए सार्वजनिक स्वास्थ्य उपायोंके साथ-साथप्रभावित व्यक्तियों की त्वरित पहचान और उपचार के लिए उचित नैदानिक और महामारी विज्ञान संबं धीजांच की आवश्यकता पर प्रकाश डाला गया।

अध्ययन का उद्देश्य- "पश्चिमी राजस्थान में एक तृत्तीयक देखभाल अस्पताल में नैदानिक नमूनों से पृथक Corynebacterium प्रजातियों के सूक्ष्म जीव विज्ञानी और मोलकुलर भेदभाव ।"

विधिइनवॉ ल्ड: प्रासंगिक नमूना रोगी से डिप्थीरिया के नैदानिक संदेह के साथ एक त्रकिया जाएगा और उचित बैक्टीरियलॉजिकल प्रोफाइलिंग और एएसटी के लिए परिवहन करेगा।

रोगी को अध्ययन कालाभ: यह रोगी को उचित निदान और उपचार में मददगार होगा और रोगाणुरोधीद वाओं का चयनकरने में सहायक होगा।

रोगी के लिए आमंत्रितजोखिम: इस अध्ययन में रोगी को किसीभी प्रकार का कोई खतरा नहीं है।अध्ययन में किसीभी दवा या टीके का परीक्षण नहीं किया जारहा है।

रिकॉर्ड कीमान्यता: रोगी के रिकॉर्ड / रिपोर्ट / गोपनीय रखे जाएंगे।

ANNEXURE- 7

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

DEPARTMENT OF MICROBIOLOGY

Case record form

Patient's Name: Father's Name:

Age/sex: Address:

HOSPITALIZATION: YES/NO

If Yes; Name of the Hospital :

Date of admission: Date of Discharge: Date of Death:

CLINICAL SYMPTOPMS OF DIPHTHERIA:

☐ SORE THROAT ☐ FEVER ☐ GREYISH WHITE MEMBRANE IN THROAT

☐ REDNESS OF TONSILS ☐HOARSNESS OF VOICE ☐BULL NECK

☐ DIFFICULTY IN SWALLOWING ☐ DIFFICULTY IN BREATHING

VACCINATION STATUS

☐ DPT1 ☐ DPT2 ☐ DPT3 ☐DPT Booster16-24months

DPT Booster 5years ☐ Td 10years ☐ Td 16 years

Source of Vaccination Status **Date of the last dose**

TREATMENT HISTORY

ANTIBIOTIC GIVEN : YES/NO

☐ Penicillin ☐Cotrimoxazole ☐ Doxycycline ☐ Augmentin

☐ Azithromycin ☐ Clarithromycin ☐ Amoxicillin ☐Cefixime

☐ Erythromycin ☐ Tetracycline ☐ Ampicillin ☐Unknown

☐ Others Diphtheria Antitoxin

COMPLICATIONS

☐ Myocarditis ☐ Bulbar palsy (palatal, pharyngeal, facial, oculomotor)

☐Peripheral Neuropathy ☐ Pneumonia ☐Otitis Media ☐Respiratory Insufficiency

ANNEXURE-8

Abstract presented in MICROCON 2021

Laboratory detection of *Corynebacterium* spp. from suspected cases of diphtheria from Western Rajasthan

Dr Debaleena Paul[1], Dr Sarika Prabhakar Kombade[1], Dr Siya Ram Didel[2], Dr Vidhi Jain[1],

Dr Ashwini Aggarwal[1]

1. Department of Microbiology.
2. Department of Pediatrics.

Institute: All India Institute of Medical Sciences, Jodhpur (AIIMS Jodhpur)

BACKGROUND

Diphtheria is a vaccine preventable disease of childhood caused by bacterium *Corynebacterium diphtheriae*. Due to COVID 19 Pandemic, the coverage of National Immunization Program has been insufficient. Therefore, early detection and timely treatment for this life-threatening disease of childhood is the need of an hour. Our study focused on detection of *Corynebacterium* spp. from suspected cases of diphtheria, by microscopy, culture and RT-PCR, as part of the WHO vaccine preventable disease surveillance program.

METHODS:

Throat swabs received from suspected cases of diphtheria were subjected to Albert's staining and conventional culture. Further confirmation was done by RT-PCR for *Corynebacterium* spp. and *tox* gene detection.

RESULTS:

During the 17-month study period from 1stJanuary 2021 to 1stMay 2022, the laboratory received throat swabs from 74 clinically suspected cases of diphtheria. The mean age of cases were 4.6 years and Male Female ratio was 2:1. Of these, 10(13.5%) tested positive on Albert's staining. Culture positivity was seen in 7(9.45%) cases.

RT-PCR was put for only 19 samples, of which 12 tested positive. The most common species isolated was *Corynebacterium ulcerans* (11 cases) followed by *Corynebacterium diphtheria* (1 case). The *tox* gene was detected in one case only.

CONCLUSIONS:

In this short period of study, 12 active cases of clinical diphtheria were diagnosed, pointing to a prevalence of 16% in Western Rajasthan. Albert's staining remains the quickest and cheapest diagnostic tool, while RT-PCR adds further speciation advantage. *Corynebacterium ulcerans* was the most common species detected, an unusual finding in our area. We hope to increase clinician awareness regarding the re-emergence of clinical diphtheria in India.

ANNEXURE-9

Abstract presented in ID-CON 2021

Resurgence of Diphtheria in western Rajasthan: A case report

Authors: Dr Debaleena Paul, Dr Sarika P Kombade, Dr Vidhi Jain, Dr Ashwini Agarwal, Dr Aditya Kundu, Dr Zeeshan Noore Azim

AIIMS Jodhpur

Introduction:

Diphtheria is a vaccine preventable disease caused by bacterium Corynebacterium diphtheriae that make a toxin. Due to COVID 19 pandemic many places of India have failed to provide proper vaccination and there is increasing trend in the number of cases of diphtheria. Hereby we are reported a laboratory confirmed case of a 1year old boy with unknown vaccination status presented with the chief complaints of fever and difficulty in swallowing, and on clinical examination pseudomembrane was present. 2 throat swabs were sent for further bacteriological profile.

AIM: To study the identification of isolates from throat swabs received in bacteriology laboratory, AIIMS Jodhpur.

Materials and methods:

2 throat swabs received in bacteriology laboratory from Bikaner, direct Gram stain and Albert stain was done then the swabs were inoculated in Loeffler's serum slope (LSS). The Microscopic findings were documented and after 6 hours, subculture was done from LSS to LSS, Blood agar, Chocolate agar, MacConkey agar, Potassium tellurite blood agar (KTBA), along with smears (Albert stain) were made.

Results:

On Gram stain, gram positive club-shaped bacilli were seen and on Albert stains, Green coloured bacilli with metachromatic granules were present arranged in V and L form resembling Klebs–Löffler bacillus. And on culture there was growth of greyish black colony on KTBA. Confirmation of toxin producing Corynebacterium diphtheria was made using MALDI-TOF followed by Modified Elek's gel test for toxin detection and also PCR was performed for the same.

Conclusion:

Apart from other complications, pseudomembrane causes blockage of your air way which is life threatening so early diagnosis and early treatment with antitoxin is of utmost importance and Vaccination can prevent diphtheria altogether.

Lab ID	Name	EP ID Code	Date of Receiving	No. Of Swabs	Age	Sex	G/S	A/S	Culture	PCR	FINAL RESULT (TOX)	VACCINATION	SORE THROAT	FEVER	GREYISH WHITE ADHERANT MEMBRANE	HOARSNESS OF VOICE	BULL NECK	DIFFICULTY IN SWALLOWING	DIFFICULTY IN BREATHING	ENLARGED RED TONSILS
DPT 1	Saurobh	DTHJDP20002	3/10/2021	2	5	M	GPC, GNB	NEGATIVE	No growth	negative	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	Y
DPT 2	HARUN	DTHJDP20003	6/10/2021	2	2	M	GPC, GNB	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 3	POOJA	DTHJDP20004	13/10/2021	2	8	F	GPB	POSITIVE	Corynebacterium ulcerans	FAM 28.09 C.ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 4	DIVYANSHU	DTHBKN21008	11/5/2021	2	1	M	GPB	POSITIVE	Corynebacterium ulcerans	FAM 30.01 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 5	SAINA	DTHJDP21025	6/8/2021	2	2	F	GPC, GPB	POSITIVE	Corynebacterium ulcerans	FAM 29.01 C. ULCERANS	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 6	GAURAV	DTHJDP21028	31/8/2021	2	5	M	GPC,GPB,BYC	POSITIVE	Corynebacterium ulcerans	FAM 29.11 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 7	SAKIB	DTHJDP21029	27/9/2021	2	11	M	GPC, GPB	POSITIVE	Corynebacterium ulcerans	FAM 30.12 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	Y
DPT 8	AAHIL	DTHJDP21031	4/10/2021	2	7	M	GPC,BYC,GNB	POSITIVE	Corynebacterium ulcerans	FAM 29.89 C. ULCERANS	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	Y
DPT 9	SAROJ	DTHJDP21045	28/12/2021	2	10	F	GPC ,GPB	POSITIVE	Corynebacterium diphtheriae Elek +	TOX-A CY5 24.12 HEX 28	POSITIVE	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 10	LOKESH JAT	DTHALW21001	10/1/2022	2	16	M	GPC,GNB	NEGATIVE	COMMENSAL FLORA	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 11	SONU	DTHJPR22003	31/1/2022	2	22	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 12	SUNIL KUMAR	DTHJPR22002	31/1/2022	2	24	M	NIL	NEGATIVE	No growth	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	Y
DPT 13	ADITYA	DTHJPR22004	31/1/2022	2	3	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	N	Y	Y	N	N	Y	Y
DPT 14	AAKASH	DTHAJM22001	30/1/2022	2	10	M	GPC	NEGATIVE	COMMENSAL FLORA(S. mitis/oralis)	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	Y
DPT 15	JITENDER	DTHAJM22002	16/2/2022	2	5	M	GPC	NEGATIVE	COMMENSAL FLORA (S. parasanguinis)	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	N
DPT 16	AKSHI SHARMA	DTHRJJPR22006	15/2/2022	2	19	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	N	Y	Y	Y	N	N	Y	N
DPT 17	ARMAN	DTHRJJPR22005	15/2/2022	2	8	M	GPC	NEGATIVE	COMMENSAL FLORA (S. parasanguinis)	negative	negative	VACCINATED TILL AGE	N	Y	Y	N	N	Y	N	N
DPT 18	PRINCE	DTHRJJPR22007	15/2/2022	2	8	M	GPC	NEGATIVE	COMMENSAL FLORA(Neisseria flava/S. mitis/oralis)	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 19	NEERANDRA	DTHRJJPR22008	23/02/2022	2	58	M	GPC	NEGATIVE	COMMENSAL FLORA(S. parasanguinis)	C.ulcerans -FAM 30.75	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 20	SAMEER	DTHRJALW22002	24/2/2022	2	11	M	GPC	NEGATIVE	COMMENSAL FLORA(S. vestibularis)	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	N
DPT 21	RIMJHIM	DTHKTA22002	26/2/2022	2	12months	F	GPB,GNB	POSITIVE	Corynebacterium ulcerans	C.ulcerans-FAM 27.34	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	Y
DPT 22	VIRAT MAHIWAL	DTHALW22003	24/02/2022	2	10	M	GNCB, GPC	NEGATIVE	COMMENSAL FLORA(S. salivarius)	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	Y
DPT 23	SUBHASH	DTHRJJDP22022	7/3/2022	2	7	M	GPC	NEGATIVE	COMMENSAL FLORA(S.salivarius, Granulicatella adi	C.ulcerans-FAM 27.49	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 24	HIMANSHI	DTHRJALW22004	3/3/2022	2	3	F	GPC	NEGATIVE	COMMENSAL FLORA(S.aureus, Granulicatella adiace	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	N	N	Y
DPT 25	NISHANT RATHORE	DTHKTA22003	9/3/2022	2	6	M	GPC	NEGATIVE	COMMENSAL FLORA(S.oralis/mitis)	C.ulcerans-FAM 27.25	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 26	MONISA	DTHAJM22004	12/3/2022	2	21	F	GPC	NEGATIVE	COMMENSAL FLORA(S.parasanguinis)	C.ulcerans-FAM 28.80	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	Y
DPT 27	KUSHBOO	DTHAJM22003	12/3/2022	2	35	F	GPC	NEGATIVE	COMMENSAL FLORA(S.parasanguinis)	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	Y
DPT 28	BHUMIKA	DTHJPR22009	14/3/2022	2	6	F	GPC	NEGATIVE	S. pneumoniae	C.ulcerans-FAM 28.80	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	Y
DPT 29	RAMHARI	DTHALW22005	14/3/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 30	RADHIKA	DTHRJJPR22001	20/1/2022	2	8	F	NIL	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	N	N	N	N	N	Y
DPT 31	SHAHRUKH	DTHUDP22001	28/1/2022	2	2	M	NIL	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	Y
DPT 32	PURVANSHI	DTHJDP22003	17/3/2022	2	3	F	GPC	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	Y
DPT 33	JAISHARI	DTHRJJPR22010	16/3/2022	2	5	F	GPC	NEGATIVE	No growth	negative	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	N
DPT 34	KASHNI	DTHRJJPR22011	16/3/2022	2	16	F	NIL	NEGATIVE	COMMENSAL FLORA(Granulicatella adiacens)	negative	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	N
DPT 35	SHIVA	DTHUDP22002	16/3/2022	2	7	M	NIL	NEGATIVE	Commensal flora(S. mitis/oralis)	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	N
DPT 36	MONIKA	DTHSAW22001	21/3/2022	2	18	F	GPC	NEGATIVE	COMMENSAL FLORA(S.mitis/oralis, N. flava)	negative	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	N
DPT 37	GORANSH	DTHJPR22012	23/3/2022	2	3.5	M	NIL	NEGATIVE	No growth	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	N
DPT 38	TAKSH	DTHSAW22002	23/3/2022	2	7.6	M	GPC, GPB	POSITIVE	C.ulcerans	C.ulcerans -FAM26.36	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	N
DPT 39	GUNJAN	DTHBKN22001	23/3/2022	2	4	F	GPC,BYC	NEGATIVE	COMMENSAL FLORA (S. sanguinis)	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	N
DPT 40	HIMANSHU	DTHDLP22001	23/3/2022	2	3	M	GPC,GPB	POSITIVE	Granulicatella adiacens	C.ULCERANS-FAM28.93	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	N
DPT 41	DEVESH SAINI	DTHRJJPR22013	25/3/2022	2	7	M	GPB	POSITIVE	C. ulcerans	C.ULCERANS-FAM28.35	negative	UNKNOWN	Y	Y	Y	N	N	N	N	Y
DPT 42	KHUSHVEER	DTHRJDSA22001	26/3/2022	2	1.6	M	GPC	NEGATIVE	COMMENSAL FLORA(S.mitis/oralis)	C.ULCERANS-FAM27.67	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	Y
DPT 43	DIVYANSHI	DTHRJJPR22014	28/3/2022	2	9	F	GPB	POSITIVE	C.ulcerans	C. ULCERANS FAM 29.03	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 44	BHANU	DTHRJJPR22015	28/3/2022	2	3	M	NIL	NEGATIVE	COMMENSAL FLORA(S.mitis/oralis)	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	N	Y
DPT 45	RAHAN KHAN	DTHRJJPR22016	28/3/2022	2	7	M	NIL	NEGATIVE	COMMENSAL FLORA(S.mitis/oralis)	negative	negative	UNKNOWN	Y	Y	N	N	N	N	N	Y
DPT 46	KHUSHI	DTHAJM22005	30/3/2022	2	1MONTH	F	NIL	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 47	MONISH	ATHRJALW22006	1/4/2022	2	10	M	GPC	NEGATIVE	COMMENSAL FLORA (N.flava, Aeromonas)	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 48	SEEMA	DTHJPR22017	1/4/2022	2	9	F	BYC	NEGATIVE	S. hemolyticus	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 49	HARSH	DTHJPR22018	1/4/2022	2	4MONTH	M	GNB,BYC,GPC	NEGATIVE	S.aureus, E. coli	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 50	BHAVESH	DTHUDP22003	5/4/2022	2	10	M	BYC,GPC	NEGATIVE	Enterococcus faecium	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 51	SUNITA	DTHUDP22004	5/4/2022	2	14	M	GPC,BYC	NEGATIVE	Bacilis pumilus	negative	negative	VACCINATED TILL AGE	Y	N	Y	Y	N	Y	N	Y
DPT 52	SOORAJ	DTHALW22007	5/4/2022	2	11.4	M	GNB	NEGATIVE	Psseudomonas aerugina	negative	negative	UNKNOWN	Y	N	Y	N	N	Y	Y	N
DPT 53	MAHIMA	DTHRJSAW22003	14/4/2022	2	7	F	GPC	NEGATIVE	CPMMENSAL FLORA (S. mitis/oralis)	negative	negative	UNKNOWN	Y	N	Y	Y	N	N	Y	N
DPT 54	GIRIJA	DTHBLW22001	19/4/2022	2	1.6	F	GPC,GNB	NEGATIVE	Pseudomonas alcaligens	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 55	RUHANUDDIN	DTHJPR22019	21/4/2022	2	3	M	NIL	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	N
DPT 56	HASNEN	DTHJPR22021	27/4/2022	2	1	M	GPC	NEGATIVE	No growth	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	N

DPT 57	CHIRAJ	DTHJPR22002	3/5/2022	2	6	M	NIL	NEGATIVE	No growth	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	N	N
DPT 58	DEVYANSH	DTHJPR22023	6/5/2022	2	1MONTH	M	GNB	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	N	Y	N	N	N	Y	N
DPT 59	JAYESH	DTHRJJPR22025	11/5/2022	2	5	M	GPC (FEW)	NEGATIVE	No growth	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	N
DPT 60	ANUP SINGH	DTHRJJPR22024	11/5/2022	2	12	M	NIL	NEGATIVE	No growth	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	N
DPT 61	RAMLILA	DTHRJSAW22004	11/5/2022	2	35	F	GPC IN PAIRS(Few), BYC	NEGATIVE	CPOMMENSAL FLORA	negative	negative	UNKNOWN	Y	N	Y	N	N	Y	Y	N
DPT 62	MOHD TABIS	DTHRJAJM22007	13/5/2022	2	3	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	N
DPT 63	ARABHU KAZI	DTHRJJPR22026	16/5/2022	2	12	M	NIL	NEGATIVE	NO GROWTH	FAM 28.17 C ULCERANS	negative	UNKNOWN	Y	N	Y	N	N	Y	N	N
DPT 64	JAVI KHANDELWAL	DTHRJJPR22027	18/5/2022	2	11	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	N	N	N	N	N	Y	N
DPT 65	HAPPY	DTHRJAJM22008	18/5/2022	2	4	M	GPC(FEW)	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	N
DPT 66	ISHTA	DTHRJBLW22002	18/5/2022	2	16	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	N
DPT 67	KEVYAN	DTHRJBLW22003	18/5/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	Y
DPT 68	NAVEEN	DTHRJSAW22005	18/5/2022	2	11	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	N	N	N	Y	Y	Y
DPT 69	JAIJEET	DTHRJALW22008	26/5/2022	2	5	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	Y
DPT 70	RAVI	DTHRJJDP22004	1/6/2022	2	12	M	GNCB	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 71	CHESTHA	DTHRJAJM22009	1/6/2022	2	4	F	GNCB	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	N	Y	N	N	Y	Y
DPT 72	KIRAN	DTHRJBLW22006	1/6/2022	2	36	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	N	N	N	Y	Y	Y
DPT 73	SHIVAM	DTHRJSAW22006	1/6/2022	2	5	M	GNCB	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	Y
DPT 74	MOHIT	DTHRJJPR22029	1/6/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 75	KHEVANSH	DTHJRALW22009	1/6/2022	2	6	M	GPC	NEGATIVE	No growth	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	N	Y	N	N	Y	Y
DPT 76	VANSHIKA SHARMA	DTHRJJPR22031	4/6/2022	2	8	F	GPC CHAINS	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	Y
DPT 77	MANVI	DTHRJKTA22004	7/6/2022	2	9 MONTHS	F	NIL	NEGATIVE	NO GROWTH	C.ULCERANS-FAM 24.92	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 78	YOVAN SHARMA	DTHRJJPR22030	1/6/2022	2	10	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 79	BHAVISHY	DTHRJJPR22028	10/6/2022	2	3	M	GPC PAIRS	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	N
DPT 80	AARADHYA	DTHRJBLW22005	20/6/2022	2	6	F	GPC	NEGATIVE	NO GROWTH	C.ULCERANS-FAM30.82	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	N
DPT 81	ARSHAD	DTHRJJPR22032	19/6/2022	2	8	M	BYC,GPC	NEGATIVE	NO GROWTH	C.ULCERANS-FAM30.91	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	N
DPT 82	SONU	DTHRJJPR22033	19/6/2022	2	3	M	NIL	NEGATIVE	NO GROWTH	C.ULCERANS-FAM 29.51	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	N
DPT 83	RUPESH GURJAR	DTHRJJPR22034	19/6/2022	2	9	M	NIL	NEGATIVE	No growth	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	N
DPT 84	DAKSH	DTHRJJPR22035	20/6/2022	2	3	M	GPC	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 85	VIKAS	DTHRJRSM22001	21/6/2022	2	7	M	GPB FEW	NEGATIVE	S.vestibularis	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	N
DPT 86	SIDHARTH	DTHRJUDP22005	22/6/2022	2	1.7	M	GPC	NEGATIVE	S. parasanguinis	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	Y
DPT 87	CHANCHAL BANSWA	DTHRJAJM22006	22/6/2023	2	5		NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	N	Y	N	N	Y	Y
DPT 88	AJAAN	DTHRJSKR22001	22/6/2022	2	1	M	NIL	NEGATIVE	NO GROWTH	C.ULCERANS-FAM26.71	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 89	KULDEEP MAWAI	DTHRJJPR22010	22/6/2022	2	18	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 90	AAYUSH	DTHRJALW22010	22/6/2022	2	5	M	NIL	NEGATIVE	Pseudomonas sutzeri	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 91	LAKSH BAIRWA	DTHRJJPR22038	26/6/2022	2	7	M	GPB	POSITIVE	C. diph Elek+	TOX A 20.09 Hex 27.9	POSITIVE	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 92	TANISH	DTHRJJPR22037	24/6/2022	2	2	M	GPB	POSITIVE	C.diph Elek +	TOX A 21.98 Hex 30.1	POSITIVE	UNKNOWN	N	Y	Y	N	N	N	Y	N
DPT 93	JAMNA	DTHRJJPR22037	29/6/2022	2	46	M	GPC	NEGATIVE	COMMENSAL FLORA	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 94	VIJAY JANGID	DTHRJJPR22039	8/7/2022	2	10	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 95	PARESH KUMAR	DTHRJJPR22006	6/7/2022	2	12	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 96	DALI (Expried)	DTHRJAJM22010	7/7/2022	2	8	F	GPB	POSITIVE	C. ULCERANS Elek -	C.ULCERANS FAM-28.61 TOX-A C	POSITIVE	UNKNOWN	Y	Y	Y	Y	Y	Y	Y	Y
DPT 97	FIJA	DTHRJALW22012	4/7/2022	2	9	F	GNB	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 98	MOHIT	DTHRJALW22007	9/7/2022	2	20	M	GNB,GPB	POSITIVE	S. mitis/oralis, C. ulcerans	C.ULCERANS FAM-29.34	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 99	SANCHITA	DTHRJALW22011	29/6/2022	2	4	F	GNCB	NEGATIVE	COMMENSAL FLORA	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	Y
DPT 100	DEEPESH SINGH	DTHRJAJM22011	12/7/2022	2	18	M	GPC,GPB	POSITIVE	S.parasanguinis, C. ulcerans	C.ULCERANS FAM-29.34	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 101	HARMAN	DTHRJAJM22012	12/7/2022	2	6	M	GPC,GPB	POSITIVE	S.parasanguinis, C. ulcerans	C.ULCERANS FAM-31.05	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	Y
DPT 102	VIDHIKA	DTHRJSAW22007	11/7/2022	2	10	F	GPB,GPC	POSITIVE	S.parasanguinis, C. ulcerans	C.ULCERANS FAM-30.48	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	N
DPT 103	CHIRAG SAIN	DTHRJJPR22040	11/7/2022	2	2	M	GNB	NEGATIVE	COMMENSAL FLORA	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	N
DPT 104	DHEERAJ	DTHRJALW22013	10/7/2022	2	5	M	GPC	NEGATIVE	COMMENSAL FLORA	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 105	YASH	DTHRJKTA22066	19/7/2022	2	5	M	GNB	NEGATIVE	Granulicatella adiacens	C.ULCERANS FAM-28	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	N	N
DPT 106	POORTI	DTHRJKTA22005	14/7/2022	2	2	F	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 107	MADHU KANWAR	DTHRJJDP22008	19/7/2022	2	23	F	GPC,BYC	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	N
DPT 108	JASWANT SINGH	DTHJDP22005	17/7/2022	2	12	M	NIL	NEGATIVE	COMMENSAL FLORA	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	N
DPT 109	BHAVIKA SAINI	DTHRJJPR22041	16/7/2022	2	24	F	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	N
DPT 110	YOGENDRA	DTHRJJPR22042	15/7/2022	2	11	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	N
DPT 111	PIYUSH TAK	DTHRJAJM22013	20/7/2022	2	12	M	NIL	NEGATIVE	COMMENSAL FLORA	C.ULCERANS FAM-27.77	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	N
DPT 112	DIVY	DTHRJJPR22043	21/7/2022	2	3	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	N
DPT 113	SHAHNAWAZ	DTHRJRSM22003	1/8/2022	2	19	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	N
DPT 114	DIVEYANSH	DTHRJCTG22001	1/8/2022	2	4	M	GPC	NEGATIVE	COMMENSAL FLORA	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	N	N
DPT 115	NAMAN	DTHRJAJM22014	2/8/2022	2	7	M	GPC	NEGATIVE	COMMENSAL FLORA	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 116	SORYAVEER	DTHRJJPR22045	4/8/2022	2	3	M	BYC	NEGATIVE	CANDIDA SPP	negative	negative	VACCINATED	Y	Y	Y	N	N	Y	N	Y
DPT 117	SHYAM SHARMA	DTHRJJPR22044	4/8/2022	2	28	M	BYC,GPC,GPB	POSITIVE	C. unlcerans	C.ULCERANS FAM 30.09	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 118	SARTHAK	DTHRJJPR22046	8/8/2022	2	6	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	Y
DPT 119	DEVENDRA	DTHRJBRM22001	8/8/2022	2	6	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	Y
DPT 120	NADIM	DTHRJJPR22048	10/8/2022	2	10	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 121	MOHAMMAD SHIFAN	DTHRJJPR22047	10/8/2022	2	2	M	GPC	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	Y

DPT 122	SEHRUNA	DTHRJALW22014	13/8/2022	2	23	F	GPC	NEGATIVE	S.aureus, E. coli	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 123	HARSHA	DTHRJAJM22015	12/8/2022	2	23	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 124	INDRAJEET	DTHRJALW22015	12/8/2022	2	11	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 125	VIVEK	DTHRJJDP22009	12/8/2022	2	10	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED	Y	Y	Y	N	N	N	N	Y
DPT 126	SANJEV KAUR	DTHRJJPR22049	13/8/2022	2	7	F	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED	Y	Y	Y	Y	N	N	Y	Y
DPT 127	LAXMAN RAM	DTHRJJDP22010	17/8/2022	2	6	M	GPC	NEGATIVE	COMMENSAL FLORA	negative	negative	NOT VACCINATED	Y	Y	Y	Y	N	N	Y	Y
DPT 128	PRIYANSH	DTHRJBKN22003	18/8/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 129	AMIT	DTHRJBKN22004	18/8/2022	2	5	M	GPC,BYC	NEGATIVE	CANDIDA SPP	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	Y
DPT 130	USHA DEVI	DTHRJJDP22011	22/8/2022	2	50	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 131	MANISH	DTHRJJDP22016	22/8/2022	2	31	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	Y	Y
DPT 132	CHELSI SUBLANIYA	DTHRJJPR22050	23/8/2022	2	10	F	GPC	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 133	YUVANSH	DTHRJJPR22051	23/10/2022	2	3	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	N
DPT 134	JESHMIN	DTHRJSAW22008	23/8/2022	2	40	F	BYC,GPC	NEGATIVE	CANDIDA SPP	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 135	PRINCE	DTHRJBKN22005	24/8/2022	2	1	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	N
DPT 136	NIPUN	DTHRJJPR22052	24/8/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	N
DPT 137	LOKESH GURJAR	DTHRJJPR22053	27/8/2022	2	5	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	N
DPT 138	ANJALI	DTHRJALW22016	29/8/2022	2	3	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	N
DPT 139	RAM PRATAP	DTHRJBKN22006	30/8/2022	2	2	M	GPC	NEGATIVE	ENTEROCOCCUS HIRAE	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	N
DPT 140	SAURABH GURJAR	DTHRJJPR22056	2/9/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	N
DPT 141	MAHESH KUMAWAT	DTHRJJPR22054	2/9/2022	2	8	M	GPC	NEGATIVE	STREPTOCOCCUS PARASANGUINIS	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	N
DPT 142	LIPU VASHISHT	DTHRJJPR22055	2/9/2022	2	5	F	GPC	NEGATIVE	STREPTOCOCCUS PARASANGUINIS	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	N
DPT 143	MANISHA	DTHRJAJM22917	3/9/2022	2	3	F	NIL	NEGATIVE	STREPTOCOCCUS PARASANGUINIS	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	Y
DPT 144	LALITA	DTHRJJDP22013	5/9/2022	2	11	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	Y	Y	Y	Y
DPT 145	PREM	DTHRJJDP22015	8/9/2022	2	6	F	GPC	NEGATIVE	STREPTOCOCCUS MITIS	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 146	NAJMA	DTHRJJDP22015	8/9/2022	2	5	F	NIL	NEGATIVE	ROTHIA MUCIAGINOSA	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	Y
DPT 147	APRITA	DTHRJKTA22007	8/9/2022	2	4	F	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	Y	Y
DPT 148	ADHYAN	DTHRJALW22017	8/9/2022	2	21	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 149	AHISTA	DTHRJALW22018	8/9/2022	2	11	F	GPC	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	N	N	N	N	Y
DPT 150	HARDIK	DTHRJJDP22012	8/9/2022	2	7	M	GPC	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	Y
DPT 151	ALIYA	DTHRJAJM22018	10/9/2022	2	10	F	BYC	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 152	TIRKAL	DTHRJJDP22016	12/9/2022	2	10	F	BYC	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 153	MONISH	DTHRJALW22019	12/9/2022	2	5	M	GNB	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	Y
DPT 154	NILOFAR	DTHRJSAW22009	13/9/2022	2	24	F	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 155	PUNIT	DTHRJAJM22019	15/9/2022	2	6	M	NIL	NEGATIVE	NO GROWTH	30.09 FAM C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 156	MANISHA	DTHRJJDP22017	19/9/2022	2	8	F	GPC	NEGATIVE	NO GROWTH	FAM 30.04 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 157	UMESH YOGI	DTHRJJPR22057	26/9/2022	2	9	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 158	PRIYANSHI	DTHRJJPR22058	26/9/2022	2	6	F	GNB	NEGATIVE	MICROBACTERIUM AURUM	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 159	DIVYANSH	DTHRJJPR22059	26/9/2022	2	5	M	GPC	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	N	Y
DPT 160	ADITYA SAINI	DTHRJJPR22060	26/9/2022	2	3	M	GPC	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	Y
DPT 161	LUCKY MEENA	DTHRJJPR22061	26/9/2022	2	5	M	GPC,GNB	NEGATIVE	NO GROWTH	FAM 28.40 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 162	TEJAS	DTHRJJPR22062	26/9/2022	2	21	M	GNB	NEGATIVE	COMMENSAL FLORA	28.76 FAM C. ULCERANS	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	N
DPT 163	AKASH KUMAR	DTHRJJPR22064	26/9/2022	2	5	M	GNB,BYC	NEGATIVE	COMMENSAL FLORA	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 164	SAHISTHA	DTHRJALW22021	28/9/2022	2	7	M	NIL	NEGATIVE	NO GROWTH	30.68 FAM C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	N
DPT 165	ARYAT	DTHRJALW22020	28/9/2022	2	7	M	GNB,GPC	NEGATIVE	COMMENSAL FLORA	FAM 289.84 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	N
DPT 166	AAYUSH	DTHRJJDP22018	27/9/2022	2	5	M	BYC,GPC	NEGATIVE	COMMENSAL FLORA	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 167	PRINCE	DTHRJJDP22019	27/9/2022	2	12	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 168	DEEPIKA	DTHRJJDP22020	27/9/2022	2	9	F	GPC	NEGATIVE	COMMENSAL FLORA	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 169	SURAJ	DTHRJJDP22021	27/9/2022	2	5	M	NIL	NEGATIVE	COMMENSAL FLORA	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 170	NIRMA	DTHRJJDP22029	30/9/2022	2	11	F	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 171	PARVATI	DTHRJJDP22024	30/9/2022	2	7	F	GPC,GPB	POSITIVE	C.DIPHTHERIA (SENSITIVE TO Penicillin 1, Ceftriax	TOX A 28.93 Hex 29	POSITIVE	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 172	RUPESH GURJAR	DTHRJJDP22025	30/9/2022	2	11	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 173	SAROJ	DTHRJJDP22026	4/10/2022	2	9	F	GPB	POSITIVE	C.DIPHTHERIA (SENSITIVE TO Penicillin 1.5, Ceftria	TOX A 34.29 , Hex 30.1	POSITIVE	UNKNOWN	N	Y	Y	N	Y	N	N	N
DPT 174	BHAGIRATH	DTHRJJDP22027	4/10/2022	2	8	M	NIL	NEGATIVE	NO GROWTH	FAM 26.98 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 175	RAVINDRA	DTHRJJDP22028	4/10/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	FAM 28.65 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	Y	Y	Y	Y
DPT 176	SAYAN	DTHRJALW22022	6/10/2022	2	5	M	NIL	NEGATIVE	NO GROWTH	FAM 29.37 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 177	MADINA	DTHRJJDP22029	7/10/2022	2	8	F	NIL	NEGATIVE	NO GROWTH	FAM 29.95 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 178	SURESH	DTHRJAJM22020	8/10/2022	2	4	M	GPC,GPB	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 179	MEGHA	DTHRJAJM22021	8/10/2022	2	21	F	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	Y	N	N	Y	Y
DPT 180	RASHI	DTHRJBTP22002	10/10/2022	2	9	F	NIL	NEGATIVE	NO GROWTH	FAM 30.22 C. ULCERANS	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	N	Y
DPT 181	KUSHAL	DTHRJJDP22030	11/10/2022	2	7	M	GPC,GNB	NEGATIVE	NO GROWTH	FAM 25.48 C. ULCERANS	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 182	KASHBIYA	DTHRJJDP22031	11/10/2022	2	10	F	NIL	NEGATIVE	NO GROWTH	FAM 30.14 C. ULCERANS	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 183	GOVIND PATEL	DTHRJJDP22032	11/10/2022	2	6	M	NIL	NEGATIVE	NO GROWTH	FAM 27.12 C. ULCERANS	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N
DPT 184	SANDEEP	DTHRJJPR22065	11/10/2022	2	9	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	N
DPT 185	NANSHU	DTHRJJPR22066	11/10/2022	2	2	F	NIL	NEGATIVE	NO GROWTH	FAM 29.57 C. ULCERANS	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	N	N
DPT 186	SONAM	DTHRJJPR22067	11/10/2022	2	4	F	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	N

DPT 187	DEEPAK LODHA	DTHRJJPR22068	11/10/2022	2	19	M	NIL	NEGATIVE	NO GROWTH	NEGATIVE	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	N
DPT 188	RAJVEER	DTHRJJPR22069	11/10/2022	2	5	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	N	N
DPT 189	SHIYANI	DTHRJJDP22033	11/10/2022	2	7	F	GPC,GNB	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 190	RAVINA	DTHRJJDP22034	11/10/2022	2	22	F	GPC,GNB	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 191	DUGAR RAM	DTHRJJDP22035	11/10/2022	2	32	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	Y	Y
DPT 192	LAVKUSH	DTHRJBTP22003	12/10/2022	2	7	M	GPC	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	N	Y	Y
DPT 193	KUSHAL	DTHRJRSM22004	15/10/2022	2	11	M	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	N	N	Y	N	Y
DPT 194	MAHENDRA	DTHRJAJM22022	15/10/2022	2	9	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 195	DIVESH	DTHRJAJM22023	19/10/2022	2	4	M	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	Y	Y	Y
DPT 196	GRASTHI	DTHRJBTP22004	15/10/2022	2	1	F	NIL	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	N	Y
DPT 197	SARIK	DTHRJJDP22037	17/10/2022	2	2	M	GPC,GNB	NEGATIVE	NO GROWTH	NEGATIVE	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 198	BHAVA RAM	DTHRJJDP22038	18/10/2022	2	11	M	GPB,GPC	POSITIVE	C.DIPHTHERIA Elel+	TOX A 20.79 Hex 29.1	POSITIVE	UNKNOWN	Y	Y	Y	N	N	Y	Y	Y
DPT 199	BHAGWATI	DTHRJJDP22036	18/10/2022	2	4	F	GPC,GNB	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	Y	N	Y	Y	Y
DPT 200	SEEMA	DTHRJJDP22039	19/10/2022	2	18	F	NIL	NEGATIVE	NO GROWTH	negative	negative	UNKNOWN	Y	Y	Y	Y	N	Y	N	Y
DPT 201	AALIYA	DTHRJBKN22007	19/10/2022	2	6	F	GNB,GPC	NEGATIVE	NO GROWTH	negative	negative	VACCINATED TILL AGE	Y	Y	Y	N	N	N	N	Y
DPT 202	FIRDOSH	MS/JDH/2022/10/002471 J	6/10/2022	2	23	M	GPB	POSITIVE	C.DIPHTHERIA Elek+	TOX A 21.05 , Hex 28.3	POSITIVE	UNKNOWN	Y	Y	Y	Y	Y	N	Y	N
DPT 203	SANA JAVED	DTHRJBKN22009	7/11/2022	2	10	F	GPB	POSITIVE	C. diph Elek+	TOX A 22.06, Hex 28.7	POSITIVE	UNKNOWN	Y	Y	Y	Y	N	N	N	Y
DPT 204	NISHANT RATHORE	DTHRJJDP22050	7/11/2022	2	6	F	GPB	POSITIVE	C. diph Elex+	TOX A 20.05, Hex 29.7	POSITIVE	UNKNOWN	N	Y	Y	N	N	N	Y	N