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Original research article

A study on vaccine preventable diseases in the state of Andhra Pradesh

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Abstract

Background: Pertussis is a highly contagious, vaccine-preventable acute respiratory infection caused by Bordetella pertussis (*B. Pertussis*) and affects all age groups. Since 1947, the Global Expanded Program on Immunization (EPI) was executed, and its incidence has been effectively curtailed. The reasons for the resurgence are multifactorial including the waning of vaccine acquired immunity, the difference in vaccination strategies, the adaptation of *B. Pertussis* strains, and an increase in disease awareness due to the strengthening of diagnostic sensitivity and surveillance sensitivity. Notably, Hewlett and Edwards proposed that the shift in pertussis transmission patterns plays an important role in pertussis resurgence. Diphtheria is an acute infectious disease of upper respiratory tract caused by toxigenic strains of Corynebacterium Diphtheriae (CD) and Corynebacterium other than diphtheriae (COD). The organism is locally invasive and causes exotoxin - mediated illness and can lead to complications like stridor, respiratory obstruction, myocarditis, nerve palsy, renal insufficiency and death in severe cases. The objective of the present study is to isolate and identify the etiological agents in clinically suspected cases of Diphtheria and Pertussis.

Materials & Methods: This Prospective study has been conducted in the department of Microbiology from Feb 2022 to Aug 2024. Samples with high clinical suspicion were received and processed. Two Throat swabs were collected in patients with clinical suspicion of Diphtheria. They were inoculated on Blood agar, Potassium Tellurite agar, Tinsdale agar. Gram staining and Albert staining were done. Molecular testing also performed for *C. diphtheriae*. Two nasopharyngeal swabs were collected in patients with clinical suspicion of Pertussis. They were inoculated on Bordet Gengou medium, Charcoal Agar with Cephalexin and Blood Agar and Mac Conkey agar. Gram Staining was performed and Biochemical testing was carried out. Molecular testing was carried out for *B. Pertussis* and *B. parapertussis*.

Results: Out of 53 samples with clinical suspicion of Diphtheriae, 6 were positive for Corynebacterium diphtheria by culture. Molecular testing was also done for testing of rpo B gene and Tox A gene. 6 samples came to be positive for *C. diphtheria* rpo B gene where as one was positive for tox gene production.

Out of 20 cases with strong suspicion of Pertussis, culture was positive for *Bordetella pertussis* in 2 samples. Molecular testing came out to be positive for one sample. Testing for the antibodies by ELISA two patients were serologically positive for Pertussis. Both Culture and molecular testing was positive in one sample. Both culture and serology was positive in one sample. Only serology was positive in one sample.

Conclusion: A constant surveillance is needed for vaccine preventable diseases to predict the outbreak or resurgence of cases.

Keywords: Diphtheria, Pertussis, Vaccination, Myocarditis.

Introduction

The impact of vaccination on the health of the world's peoples is hard to exaggerate. With the exception of safe water, no other modality has had such a major effect on mortality reduction and population growth" (Plotkin and Mortimer, 1988). One of the scientific advances of the 21st century is development of safe and efficacious vaccination. Along with this, sanitation and clean drinking water as public provision as public interventions which are responsible for improved health outcomes.

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Vaccines prevented 6 million deaths annually from vaccine preventable diseases (Ehreth 2003). Vaccines are estimated to prevent almost six million deaths/year and to save 386 million life years and 96 million disability-adjusted life years (DALYs) globally (Ehreth, 2003). The traditional measures of vaccine impact include: vaccine efficacy, the direct protection offered to a vaccinated group under optimal conditions e.g., trial settings; or vaccine effectiveness, the direct and indirect effect of vaccines on the population in a real-life setting. Globally, the provision of vaccines is more challenging in many low- and middle- income countries (LMIC), as evidenced by the failure to make the EPI vaccines available to every child by 1990, irrespective of setting (Keja *et al*., 1988). The vaccine coverage is varied in low middle and high income countries because of economical and political factors as well as variable access to Gavi, the vaccine alliance. There has been a decrease in vaccine preventable pathogens giving healthier lives to many children. Global eradication of a disease is restricted to the pathogens having human reservoirs. Eradication requires good population immunity so that no ongoing transmission occurs. Surveillance systems should be in place with accurate diagnositic facilities to check for decrease in disease burden [1].

Infectious diseases are a leading cause of morbidity and mortality. As of 2018, the total world population of children < 5 years of age was roughly estimated at 679 million. Of these, an estimated 5.3 million children died of all causes in 2018, with an estimated 700,000 who died of vaccine-preventable infectious diseases. Pertussis is a highly communicable infectious disease caused by Bordetella pertussis. The disease presents with paroxysms of coughing followed by whoop so it is named as Whooping cough. Complications include Pneumonia and sub dural bleed [2].

The burden of infectious diseases is further exacerbated by seasonal nature of some diseases. The burden of infectious disease is thus not trivial, even in high-income countries: It has been estimated that just 4 vaccine-preventable diseases (VPDs) - influenza, pneumococcal disease, herpes zoster and pertussis cost the US $26.5 billion in direct medical and societal cost annually, with the largest part due to influenza [3].

Due to increased risk of transmission in health care settings, investigating and preventing outbreaks of vaccine preventable diseases among health care workers is crucial to ensure their safety. The World Health Organization (WHO) recommends HCWs have to be vaccinated according to their countries' vaccination schedule and vaccinate against the following VPDs: Tuberculosis (TB), Hepatitis B virus (HBV), poliomyelitis, diphtheria, measles, rubella, meningococcal disease, influenza, varicella, and pertussis. Vaccinating health care workers is a direct means of giving protection to them which is of utmost importance. Outbreaks can result in increased health care costs, morbidity and mortality. It is the way to further reduce health care associated infections due to VPD s.

Vaccination rates of HCWs are often suboptimal due to several factors, including access barriers to vaccinations, lack of vaccination-specific policy recommendations, low uptake of vaccines by HCWs, and vaccine hesitancy [4].

Vaccine preventable diseases are costly at both individual and societal levels. The largest study of VPDs in travellers was conducted by the GeoSentinel surveillance network, over a 10-year period and included 37 552 ill travellers, 508 of whom were found to have contracted a VPD. In addition to individual consequences, VPDs may have public health consequences if they are reintroduced in to susceptible population by the travelers [5].

Materials and Methods

This Prospective Cross sectional study was conducted for over a period of 18 months from February 2022 to August 2024 in the department of Microbiology, Guntur Medical College, Guntur. Throat swabs were collected in patients with clinical suspicion of Diphtheria. Two in number were collected from patients with clinical suspicion and were sent to the laboratory in Amies Transport medium without Charcoal which is shown in Fig. 1.

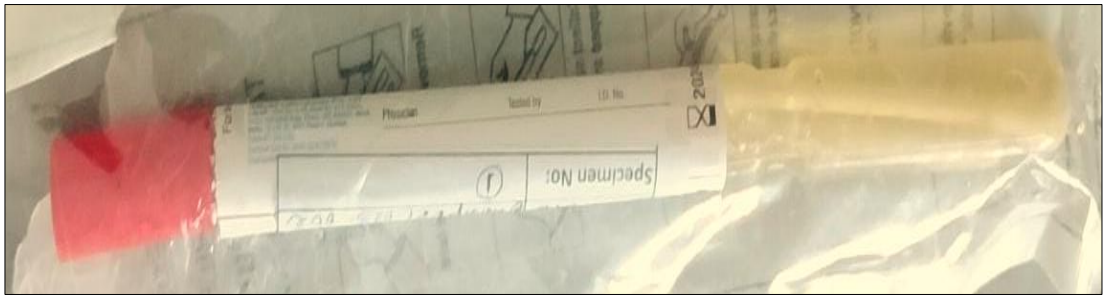

Fig 1: Amies transport medium without charcoal

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

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

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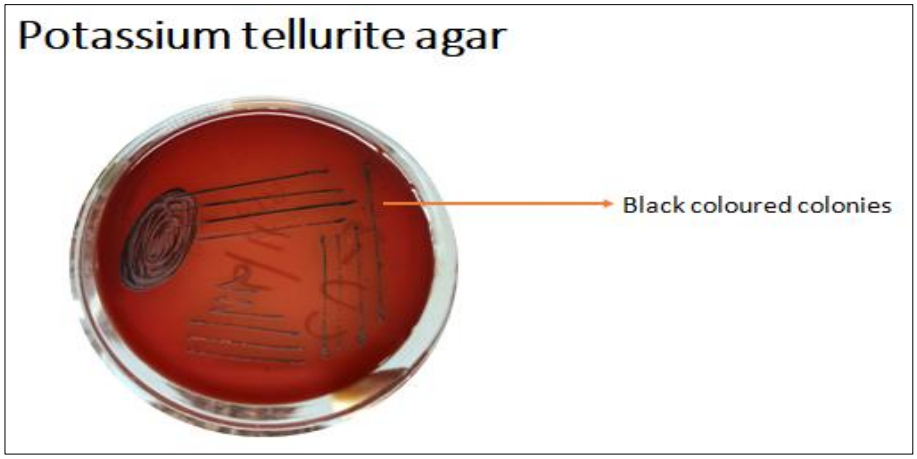

Fig 2: Growth on Potassium Tellurite Agar

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

Fig 3: Growth on Tinsdale agar

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

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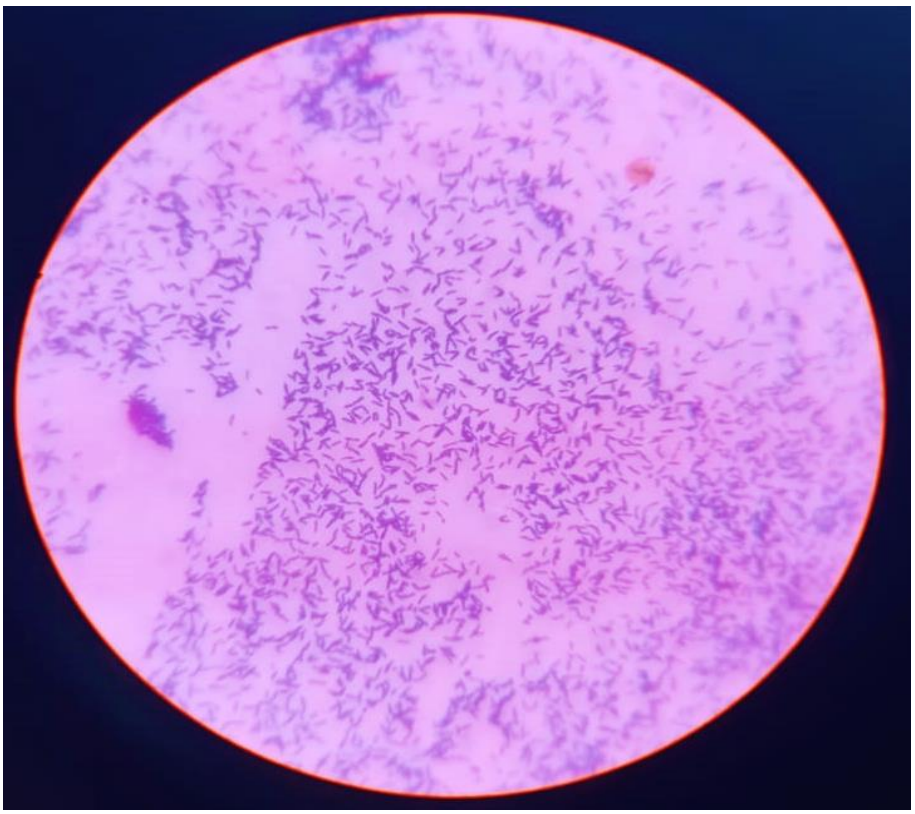

Fig 4: Gram positive bacilli on Gram stain

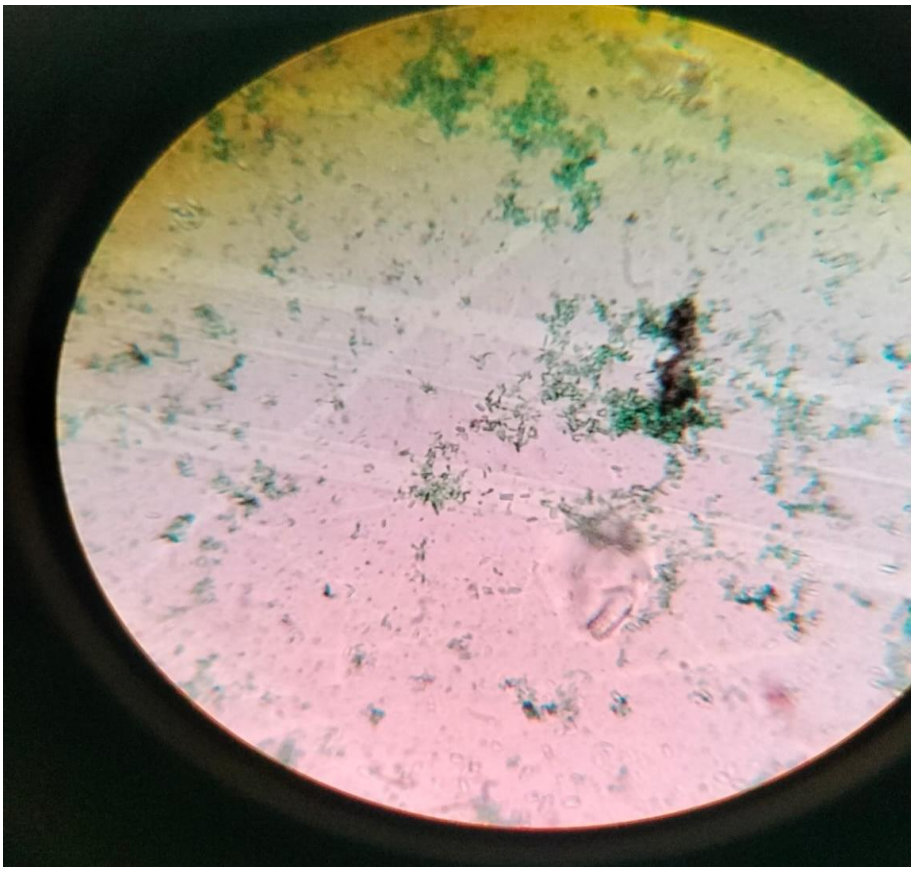

Fig 5: Green coloured bacilli on Albert stain

Albert staining was done for confirmation which showed greencoloured bacilli with metachromatic granules at the end. Further biochemical testing was done with Hiss Serum sugars - Glucose, Maltose, Sucrose, Ribose, Christensens Urease agar, Nitrate Reduction Test. Antibiotic susceptibility testing was carried out for selecting appropriate antibiotic.

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The following antibiotics were tested – Penicillin, Erythromycin, Azithromycin, Ceftriaxone, Cefotaxim, Rifampin. The other swab was used for molecular testing for detection of tox gene and rpoB gene for Corynebacterium diphtheriae. Extracted DNA from isolate can be used as the template for detecting diphtheria toxin (tox) gene using oligonucleotide primers.

Target Primer / Probe	Primer/ Probe	Sequences 5'-3'	Amplicon length (bp)
C. diphtheriae rpoB	dip _rpob_F dip_rpob_R dip_probe	CGTTCGCAAAGATTACGGAACCA CACTCAGGCGTACCAATCAAC HEX_AGGTTCCGGGGCTTCTCGATATTCA-BHQ1	97
C.ulcerans rpo B	ulc_rpob_F ulc_rpob_R ulc_probe	TTCGCATGGCTCATTGGCAC TCCAGGATGTCTTCCAGTCC FAM_CCAGCAGGAGGAGCTGGGTGAA_BHQ1	98
Toxin A	tox A_F tox A_R toxA_probe	CTTTTCTTCGTACCACGGGACTAA CTATAAAACCCTTTCCAATCATCGTCCY5- AAGGTATACAAAAGCCAAAATCTGGTACAC- BHQ2	117

PCR Master Mix

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

Reaction Mixture (1rxn)

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

Total 20 µl

PCR Program AB7500:

Thermal Cycle Conditions;

Initial Activation: 95 °C for 10 mins

Combined 45 cycles of:

Denaturation 95 °C for 15 secs

Annealing and Extension 60 °C for 30 secs.

Data Interpretation for real time PCR based on Ct values

Ct cut off for positivity	*C. diphtheriae*	*C. ulcerans*	*ToxA*
Minimum	<31.24	< 28.96	< 31.05
Maximum	34.06	31.12	35.03

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

Testing for *B. Pertussis*

Two nasopharyngeal swabs were collected from patients with clinical suspicion of Pertussis and they were transported in Amies transport medium with Charcoal to Guntur medical college, Guntur where they were processed on Blood agar, Bordet Gengou medium, Charcoal Agar with cephalexin and Macconkey agar. Gram stain was also done. Bio chemicals were set up and Antibiotic susceptibility testing was carried out.

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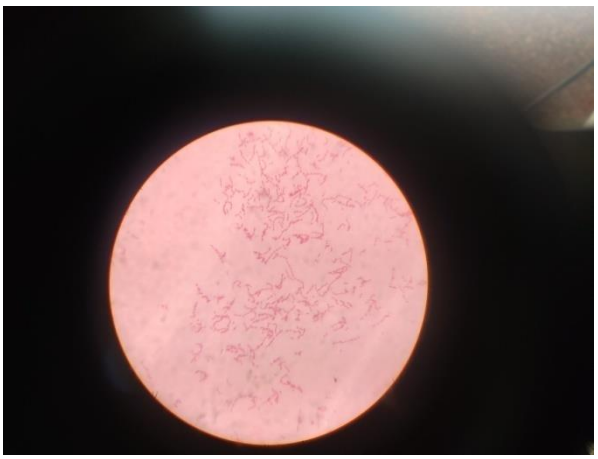

Molecular testing for *B. Pertussis*

Nucleic acid extraction

The Nasopharyngeal swab is resuspended in at least 400 µl of 0.85% saline for optimal yields. DNA is extracted using QIASYMPHONY, automated purification system.

Real-Time PCR

Extracted DNA is used as the template for qualitative detection of IS481, pIS1001, hIS1001 and *ptxS1* gene by multiplex and uniplex PCR using published oligonucleotide primers to detect *B. Pertussis, B. parapertussis, B. holmesii* and its toxin from the suspected specimen

Preparation of Primers and Probes

Primers and probes (Table 3) are re-suspended in 10mM Tris pH 7.5 or nuclease free water. Stock (10X) should be stored at -20 to -70 °C. The working primers and probes (1X) can be maintained at 4 °C, if used frequently.

Primers and Probes used in real time PCR assay

Target	Primer/Probe	Sequence 5'- 3'	Amplicon length (bp)
IS 481	825U18 894L24 871U22P	CAAGGCCGAACGCTTCAT GAGTTCTGGTAGGTGTGAGCGTAA CAGTCGGCCTTGCGTGAGTGGG	66bp
pIS1001	135U17 199L20 157U21P	TCGAACGCGTGGAATGG GGCCGTTGGCTTCAAATAGA AGACCCAGGGCGCACGCTGTC	65bp
hIS1001	BHIS41U20 BHIS91L17 BHIS62U28P	GGCGACAGCGAGACAGAATC GCCGCCTTGGCTCACTT CGTGCAGATAGGCTTTTAGCTTGAGCGC	67bp
ptx S1	402U16 422L15 410U22P	CGCCAGCTCGTACTTC GATACGGCCGGCATT AATACGTCGACACTTATGGCGA	55bp
Rnase P	RnaseP F RnaseP R RnaseP P	AGATTTGGACCTGCGAGCG GAGCGGCTGTCTCCACAAGT TTCTGACCTGAAGGCTCTGCGCG	

Controls

Positive control

Genes used as a positive control

Target	DNA	Name designated
PtxS1	A639	A639
IS481 hIS1001 pIS1001	Mixture of A639, C690, F585	ACF
rnaseP	Human	hDNA

Negative control

DNA of a known negative specimen is included in the PCR setup to determine whether there is non-specific amplification or contamination.

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Water control: A control of nuclease free water is included during the PCR setup to rule out contamination while performing PCR.

PCR Master Mix

PCR master mix setup for IS481/pIS1001/hIS1001

	Number of Reactions		
Reagent (µl)	**1**	**6**	**10**
2X Gene expression Mix	12.5	75	125
Water	1.0	6	10
3 µMIS481 Forward Primer	0.84	5.0	8.4
3 µMIS481 Reverse Primer	0.84	5.0	8.4
9 µMIS481 Probe	0.84	5.0	8.4
9 µMpIS1001 Forward Primer	0.84	5.0	8.4
9 µMpIS1001 Reverse Primer	0.84	5.0	8.4
3 µMpIS1001 Probe	0.84	5.0	8.4
3 µMhIS1001 Forward Primer	0.84	5.0	8.4
3 µMhIS1001 Reverse primer	0.84	5.0	8.4
3 µMhIS1001 Probe	0.84	5.0	8.4
Total Volume	21	126	210
Use 4 µl of DNA per reaction			

PCR master mix setup for *ptxS*1

	Number of Reactions		
Reagent (µl)	**1**	**6**	**10**
2X Gene expression Mix	12.5	75	125
Water	1.0	6	10
7 µM Forward Primer	2.5	15	25
7 µM Reverse Primer	2.5	15	25
3 µM Probe	2.5	15	25
Total Volume	21	126	210
Use 4 µl of DNA per reaction			

PCR Sample Template for the different reactions

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A	Specimen 1		Specimen 3		Specimen 5		Specimen 7		Specimen 9	
B	Specimen 1		Specimen 3		Specimen 5		Specimen 7		Specimen 9	
C	Specimen 1 dil 1:5		Specimen 3 dil 1:5		Specimen 5 dil 1:5		Specimen 7 dil 1:5		Specimen 9 dil 1:5	
D	NTC		NTC		NTC		NTC		NTC	
E	Specimen 2		Specimen 4		Specimen 6		Specimen 8		Specimen 10	
F	Specimen 2		Specimen 4		Specimen 6		Specimen 8		Specimen 10	
G	Specimen 2 dil 1:5		Specimen 4 dil 1:5		Specimen 6 dil 1:5		Specimen 8 dil 1:5		Specimen 10 dil 1:5	
H	NTC		NTC		NTC		NTC		NTC	

NTC – No template control

PCR Program AB7500
IS481/pIS1001/hIS1001 and RnaseP
50 °C 2 min

Denature	95 °C 10 min	1 cycle
Amplification	95 °C 15 sec	45 cycle
	60 °C 60 sec	
ptxS1	50 °C 2 min	
Denature	95 °C 10 min	1 cycle
Amplification	95 °C 15 sec	45 cycle
	57 °C 60 sec	

Data Interpretation
Cut-off values Ct>40 is negative for all targets. Cut-off values are used for IS481 only.
Positive = Ct < 35
Negative = Ct ≥40
Indeterminate = 35≤ Ct<40

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Note: Specimens with indeterminate results should be re-tested by using a different DNA extract.

Interpretation criteria for PCR results using multiple targets

Species	ptxS1	Multiplex		
		IS481	hIS1001	pIS1001
B. Pertussis	+	+	-	-
B. parapertussis	+	-	-	+
B. holmesii	-	+	+	-
B. Pertussis & *B. Parapertussis*	+	+	-	+
B. Pertussis & *B. holmesii*	+	+	+	-

Elisa was also performed with serum sample collected from the clinically suspicious case of Pertussis with EUROIMMUN Anti Bordetella Elisa kit to detect IgG antibodies. Interpretation is as follows. <40 IU/ml is negative 40-100 IU/ml is indeterminate and >100IU/ml is positive indicating recent infection or vaccination.

Results

A total of 53patients with clinical suspicion of Diphtheria were involved in the study from February 2022 to August 2024. 35 were males whereas 18 were females. Male to female ratio is 1.9:1. Patients presented with varied symptoms - fever, sore throat, membrane over tonsil, Bull neck, difficulty in breathing. The patients included in the study ranged from 1 month old baby to 15 years old child. Most of the positives were in the age group of 1-5 years. Out of 53 samples processed, 6 were positive for Corynebacterium diphtheriae. Molecular testing was also done for testing of rpo B gene and Tox A gene. 6 samples came to be positive for C. diphtheria rpo B gene where as one was positive for tox gene production.

A total of 20 samples with strong suspicion of Pertussis were received from February 2022 to August 2024. Out of these, 12 were female children and 8 were male children. Age criteria ranges from 1 month baby to 12 years child. The maximum cases were in the age group of 0-6 months followed by 2-5 years. Maximum cases presented with Cough with Paroxysms and Apnoea. Out of 20 cases, we have cultured Bordetella pertussis in 2 samples. Molecular testing came out to be positive for one sample. Testing for the antibodies by ELISA two patients were serologically positive for Pertussis. Both Culture and molecular testing was positive in one sample. Both culture and serology was positive in one sample. Only serology was positive in one sample.

Discussion

Infection with vaccine-preventable diseases (VPD) can cause life-threatening episodes, including deaths. Diphtheria being a Vaccine Preventable Disease, is caused by the toxin-producing gram-positive bacterium, *Corynebacterium diphtheria*. It mainly affects the respiratory mucosa and skin, causing respiratory diphtheria and cutaneous diphtheria. Transmission is from person to person by respiratory droplets or contacting respiratory secretions, discharges from a skin lesion, or rarely fomites.

With the introduction of vaccines and adoption of the diphtheria vaccine in universal immunization programs by various national governments, morbidity and mortality of diphtheria have significantly reduced. Diphtheria, pertussis, and tetanus (DPT) surveillance is part of a Universal Immunization Program (UIP) as per the DPT surveillance guidelines issued by the Ministry of Health and Family Welfare (MoHFW), New Delhi, Government of India. Under the UIP national program, the DPT vaccine has been replaced with pentavalent (DPwT-HBV-Hib) for infants with schedules of 6 weeks (first dose), ten weeks (second dose), and 14 weeks (third dose) after birth since 2012. The diphtheria vaccine is given at 6, 10, and 14 weeks of age; the diphtheria booster dose is between 16-24 months (first booster) and 5-6 years (second booster), and the Td is given at the age of 10 and 16 years of age and to the pregnant women [6].

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

A paroxysmal cough and whooping were clinical symptoms of pertussis. Pediatric patients tend to develop into more severe cases and present with poor prognosis. In the present study there is no major resistance among the isolates. Given the fragility of *B. Pertussis*, the sensitivity of culture depends on the quality of the specimen, the vaccination status, the duration of the disease and the previous antibiotics therapy. And the culture of *B. Pertussis* takes longer time and needs more experience than the polymerase chain reaction (PCR) [11].

Conflicts of Interest: Nil.

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