Assessment of Lymphocytosis in Patients with Pertussis in the Amhara Regional State, Ethiopia

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Abstract

Background: Pertussis is a severe and prolonged coughing disease caused by *Bordetella pertussis*. In 2014, an estimated 24.1 million pertussis cases, resulting in 160,700 deaths were reported worldwide. The aim of this study was to assess lymphocytosis in patients with pertussis in the Amhara Regional State, Ethiopia.

Methods: An institution-based cross-sectional study was conducted among pertussis patients who met the clinical case definition for pertussis in the Amhara Regional State, Ethiopia. The study was conducted from July 2018 to February 2019 and nasopharyngeal swabs were collected from 321 participants, and samples were analyzed using real-time PCR. Blood specimens were collected from 321 study participant and total lymphocyte count was conducted using fully automated haematology analyzer.

Results: One hundred nine study participants were positive for *Bordetella* species. Lymphocytosis was found in 15/109 (13.8%) of the PCR confirmed pertussis patients. Of those 15 PCR confirmed participants, 53.3% had lymphocytosis grading 'high' (>51%) followed by 20% 'moderately high' (>70%) and 26.7% had 'very high' (>85%). Amongst participants with lymphocytosis and PCR confirmed pertussis, 11/15 (73.3%) were less than 1 year old.

Conclusions: This study revealed the importance of lymphocyte count in the diagnosis of pertussis. Thus, in combination with other laboratory tests, incorporation of lymphocyte count in pertussis suspected patients is recommended.

Background

Pertussis (whooping cough) is a severe and prolonged coughing disease caused by infection of the respiratory tract with *Bordetella* pertussis. *Bordetella pertussis*, the main causative agent of whooping cough, was first isolated by Jules Bordet and Octave Gengou in 1906 [1, 2]. In addition to *B. pertussis*; *B. parapertussis* and *B. holmesii* cause illness similar to whooping cough in humans. *Bordetella bronchiseptica* primarily an animal pathogen can occasionally cause respiratory illness in humans [2, 3].

Globally, there were an estimated 24.1 million pertussis cases and 160,700 deaths from pertussis in children younger than 5 years in 2014, with the African region contributing the largest proportions of 7.8 million (33%) cases and 92,500 (58%) deaths. Of the above, an estimated 5.1 million (21%) pertussis cases and 85,900 (53%) deaths were among infants younger than 1 year [4]. Even in countries where pertussis vaccination is widely available, there is still a significant burden of pertussis disease, particularly in adolescents and adults [3, 4].

Despite the availability of effective vaccines, resurgence of pertussis disease has been observed in several countries [5]. Several explanations have been suggested for this resurgence including vaccine type used (suboptimal vaccines), decreased vaccination coverage, improved laboratory diagnostic tools, increased awareness of the disease and genotype changes in the circulating *Bordetella* species

population [5, 6]. A further possible factor contributing to the resurgence of pertussis is the growth of a susceptible adult population, due to waning immunity, since the limited duration of protection induced by childhood immunization wanes as children reach adolescence [6].

Infection with *B. pertussis* in infants and young children is frequently characterized by a significant rise in the number of circulating lymphocytes (lymphocytosis) [7]. However, lymphocytosis is rarely seen in adolescents [8] and adults [9] with pertussis. It is caused by *B. pertussis*-induced blockage of lymphocytes re-entry into lymph nodes from the blood. The definition of leukocytosis (increased number of leukocytes) varied from 9400 to 13,500 cells/μl for infants, although the most common cut-off is 10, 000 cells/μl [7, 9].

Various diseases, such as leukemia, chemicals, drugs or other infections, can also cause leukocytosis [7, 10], but the more well-defined lymphocytosis is not typically observed in these cases as it is in pertussis. In infants suffering from pertussis, hyperleukocytosis (> 100,000/μl) can occur, and although this is a prognosticator of poor outcome [11], it is still unclear whether it is a contributor to fatality or just an associated marker of severe disease. Studies of pertussis in children show absolute lymphocytosis in > 50% of patients, and characteristic small, mature lymphocytes with hyperchromatic, cleaved nuclei may account for as much as 56% of total lymphocytes [12, 13].

Many researches had indicated that pertussis toxin especially during paroxysmal stage is accompanied by lymphocytosis. The pertussis toxin modifies G proteins of lymphocytes via an Adenosine Diphosphate (ADP)-ribosylation activity. Thus, the G protein modification inhibits/impairs lymphocyte entry from blood into lymphoid tissues and responsible for lymphocytosis [7, 8]. However, the exact mechanisms by which this occurs are still unclear. Thus, marked lymphocytosis is partially helpful to guide the diagnosis of pertussis [7, 14, 15]. In Ethiopia, there are no reports which show the haematological changes in patients with pertussis. Therefore, the aim of this study was to assess lymphocytosis in patients with pertussis in the Amhara Regional State, Ethiopia.

Materials And Methods

Study Area and Design

The study was conducted in the Amhara Regional State, the second most populous region in Ethiopia. The region is divided into 10 Zones and 3 administrative cities and has a population of 21.5 million with 69 governmental hospitals [16]. University of Gondar comprehensive specialized hospital, Felege-Hiwot comprehensive specialized hospital in Bahirdar town, Masha hospital in Mekdela Woreda (South Wollo Zone) were selected based on convenience and laboratory infrastructure.

An institution-based cross-sectional study was conducted among pertussis patients who met the clinical case definition for pertussis using criteria established by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) [17] to assess lymphocytosis. The study was conducted from July 2018 to February 2019 on patients of all ages meeting the clinical case definition

for pertussis and who visited any of the hospitals were recruited. A total of 321 participants were enrolled in the study using convenient sampling method. Socio-demographic and clinical characteristics of each study subject were recorded using validated and structured questionnaires. Patients were clinically defined as pertussis cases when they had cough illness lasting ≥2 weeks, with at least one of the following signs or symptoms: paroxysms of coughing, inspiratory whoop, post-tussive vomiting without other apparent cause, apnea (with or without cyanosis) (for infants <1 year old only).

Blood and Nasopharyngeal Swab Collection, and Lymphocyte Count

Nasopharyngeal swabs were collected from all 321 patients using Dacron swabs (COPAN, Floq Technologies, Brescia, Italy). Samples were transported in a cold container with dry ice to the microbiology laboratory of University of Gondar hospital for analysis. Samples were stored at -20^{0}C until Deoxyribonucleic Acid (DNA) extraction was conducted. Three milliliters (3ml) of venous blood were collected from 321 study participant in to Ethylenediaminetetraacetic Acid (EDTA) tube. Total lymphocyte count was conducted using fully automated haematology analyzer (Sysmex KX-21, Japan) according to the manufacturers' manual.

Real-Time-Polymerase Chain Reaction (PCR) Assay Protocol

DNA extraction was performed at the University of Gondar Medical Microbiology laboratory. DNA was directly extracted from nasopharyngeal swabs using a commercial DNA extraction kit (QIAamp Minikits, Qiagen, Hilden, Germany) according to the manufacturer's instructions. All 321 extracted DNA samples were transported to Division of Medical Microbiology, University of Cape Town (UCT), South Africa for analysis. Detection of *Bordetella* species was performed using multiplex RT-PCR assays for the amplification of the insertion sequences (IS)*481* and *1002* modified from Roorda *et al* [18]. DNA was amplified with the CFX96® thermal RT-PCR System (Biorad). The PCR protocol used was as follows: at 50°C for 2 minutes, 95°C for 10 minutes; followed by 45 cycles of 95°C for 15 seconds, 60°C for 1 minute.

A positive result was defined as a cycle threshold (Ct) value below 38 cycles. A Ct value of >38 or no detection was considered negative. No-template controls were included in each PCR run as a negative control. A laboratory prepared and sequence confirmed control vector using known reference strains of *B. pertussis* and *B. parapertussis* was used as positive control. Furthermore, an internal amplification control using an internal fragment of the green fluorescent protein (GFP) was used to monitor possible reaction inhibition during PCR amplification [19].

Data Analysis

Questionnaires were checked for completeness and data was entered to Epi-info version-7 and then exported to SPSS version-20 for statistical analysis. Results were presented using percent, mean, median and frequencies.

Ethical issue

Ethical approval was obtained from the University of Gondar institutional review board (IRB) (Ref. No. O/V/P/RCS/05/376/2017). Each hospital, laboratory and the out-patient department were communicated through written letter obtained from the University of Gondar IRB. Each study subject was informed about the objectives of the study that would contribute towards the understanding of lymphocyte count for the diagnosis of pertussis. The study subjects were informed that it is their right not to participate or withdraw from the study at any time during the study. Each study subject was requested to give written informed consent. An assent form by parents or legal guardians was completed for anyone less than 18 years. Specimens were collected and disposed of following standard operating procedures. For each clinically suspected case, the responsible clinician of the patient was informed, and treatment was initiated as per national guidelines. Data taken from each study subject was numerically coded, and results obtained were used only for study purposes and kept confidential.

Results

Socio-demographic Characteristics of the Study Participants

Of the total 321 study participants, 162 (50.5%) were male. The age of the study participants ranged from 1 month to 72 years with median age of 7 years. Seventy seven (24%) of the study participants were 6-9 years old, 21.1% were less than 1 year old and 21.2% were greater than 14 years old. Of the study participants, 58.6% were from urban settings while the remaining were rural dwellers. Only 8.1% of the study subjects were literate (grade 9+), followed by 34% 1-8 school grades and 57.9% illiterate. Two hundred sixty-four (82.2%) of the participants had lymphocyte count within the reference range (15% to 50%). Before determination of *Bordetella* species using PCR, lyphocytosis was observed in 57/321 (17.7%) of the study participants. Of those participants, 11.8% had lymphocytosis grading ‘high’ (>51%) followed by 3.4% ‘moderately high’ (>70%) and 2.5% had ‘very high’ (>85%). Data on the family size of the study participants showed that 42.4% of them had six and more family members, and 44.2% of participants were farmers by occupation (Table 1).

Lymphocytosis in Patient with Pertussis

The PCR results, using RT-PCR assay, showed a total of 109/321 (34%) participants positive for *Bordetella* species. Of these, 56/109 (17.4%) were positive for *B. pertussis*, 52/109 (16.2%) were indeterminate *B. pertussis* and 1/109 (0.3%) were *B. parapertussis*. Lymphocytosis (>51%) was found in 15/109 (13.8%) of the PCR confirmed pertussis patients. Of those 15 PCR confirmed participants, 53.3% had lymphocytosis grading ‘high’ (>51%) followed by 20% ‘moderately high’ (>70%) and 26.7% had ‘very high’ (>85%). Amongst participants with lymphocytosis and PCR confirmed pertussis, 11/15 (73.3%) were less than 1 year old (Table 2).

Lymphocytosisand Clinical Symptoms

In this study, 10/22 (45.5%) of the participants with apnea had lymphocytosis, followed by cyanosis 1/5 (20%) and paroxysms of cough 15/108 (13.9%). On the other hand, 12/88 (13.6%) of those who reported

post-tussive vomiting and 4/79 (5.1%) of those who reported inspiratory whoop had lymphocytosis. In our study, lymphocytosis was not detected in patients with syncope (Table 3).

Discussion

The significant and dramatic rise in the number of circulating lymphocytes (lymphocytosis) in infants suffering from pertussis has been recognized for over a century [7]. Although pertussis is a disease that afflicts people of all ages, it can be particularly severe in young infants, and these are the individuals in whom lymphocytosis is most pronounced. Very high levels of lymphocytosis are associated with poor outcome in infants hospitalized with pertussis [7, 11]. This is the study in the Amhara Regional State, Ethiopia, aimed to assess lymphocytosis in patients with pertussis. In this study, lymphocytosis was detected in 15/109 (13.8%) PCR confirmed pertussis patients. Our finding was similar with studies conducted in Canada [23], Argentina [24], and New Zealand [25].

Leukocytosis in pertussis patients has been described since the late 1800s, when leukocytosis to be present in the large majority of pertussis cases examined and considered it to be of diagnostic value [7, 10, 11]. Leukocytosis was more pronounced at younger ages and at early stages of the disease. In addition, lymphocytosis is also frequently present and characterizes pertussis but not other diseases that could be confused for whooping cough [20]. However, there was considerable debate about the time of appearance of leukocytosis and lymphocytosis relative to disease symptoms, the specific cell types showing increased numbers and percentages and the diagnostic value of these blood cell counts [21]. The absence of leukocytosis early during cough disease should not discount pertussis as the diagnosis in children with pertussis before peak leukocytosis (up to 65, 000/μl) during the paroxysmal phase. Lymphocytosis was highest at the paroxysmal stage of disease, and that the highest levels of leukocytosis were observed in children with fatal complications [7, 22]. Similar pattern of lymphocytosis was detected in pertussis patients in the present study.

In our study, amongst patients with lymphocytosis and PCR confirmed pertussis, 11/15 (73.3%) were participants with less than 1 year old. Our finding was in line with other studies [22–26]. Furthermore, in this study, 45.5% of the participants with apnea, 20% with cyanosis, 13.9% with paroxysms of cough, 13.6% with post-tussive vomiting and 5.1% who reported inspiratory whoop had lymphocytosis. Similar to other studies, the correlation of these clinical symptoms with lymphocytosis in our study could be responsible for disease severity in infants less than one year old.

Many studies have firmly established that pertussis toxin is the leukocytosis promoting factor. Pertussis toxin (PT) acts on the circulating cells rather than the tissues to induce lymphocytosis. Several studies found that an important aspect of PT induction of leukocytosis is its inhibition of lymphocyte extravasation. The lymph node homing marker L-selectin (CD62L) on lymphocytes mediates their initial attachment and rolling on lymph node high endothelial venules, and upregulation of leukocyte function antigen (LFA-1) (CD11a/CD18) on lymphocytes mediates arrest for subsequent extravasation [7, 14, 15]. Furthermore, pertussis toxin inhibits Gi protein-linked signaling necessary for the sticking of lymphocytes

prior to extravasation at high endothelial venules in lymph nodes [7]. Phenotypic analysis of leukocytes from infants with pertussis revealed a dramatic reduction in L-selectin expression on all leukocyte subsets, possibly contributing to the lack of extravasation of these cells [15]. However, the specific mechanisms by which pertussis toxin induces leukocytosis are still not completely elucidated [7, 22].

Lymphocytosis is strong indicator of pertussis infection, however, the presence of lymphocytosis was not very specific to pertussis and that approximately 50% of children with suspected pertussis and raised lymphocyte counts will not have pertussis [27]. Contrary to the above study, our study showed only 13.8% PCR confirmed pertussis patients had lymphocytosis. Furthermore, raised lymphocyte is not always a specific predictive of death among pertussis infants [23–26]. However, lymphocytosis above 70,400 was particularly predictive of death, especially if combined with low birth weight [28].

Limitations

This study had certain limitations. Firstly, this study did not correlate lymphocytosis with death. Secondly, the sample size was relatively small. Thirdly, hospital and sample selection using the convenience sampling method and the limited variables included in the study might limit generalizability of our findings.

Conclusions

This study revealed the importance of lymphocyte count in the diagnosis of pertussis. Lymphocytosis is strongly associated with pertussis infection, particularly in infants, although the timing of measurement versus onset of infection and disease can influence the diagnostic value of this finding. Thus, in combination with other laboratory tests, lymphocyte count in pertussis suspected patients is recommended.

List Of Abbreviations

ADP: Adenosine Diphosphate; CD: Cluster of differentiation; CDC: Centers for Disease Control and Prevention; Ct: Cycle Threshold; DNA: Deoxyribonucleic Acid; EDTA: Ethylenediamine tetraacetic Acid; GFP: Green Fluorescent Protein; IRB: Institutional Review Board; IS: Insertion Sequences; LFA: Leukocyte Function Antigen; PCR: Polymerase Chain Reaction; PT: Pertussis Toxin; UCT: University of Cape Town; WHO: World Health Organization

Declarations

Ethics approval and consent to participate

This study was ethically approved by the University of Gondar institutional review board (Ref. No. O/V/P/RCS/05/376/2017). Permission was sought from each hospital. Each study subject was requested to give written informed consent. An assent form by parents or legal guardians was completed

for anyone less than 18 years. Patients data were kept in full confidentiality and was not be disclosed to an unauthorized person.

Consent for publication

Not applicable.

Availability of data and material

The original data for this study is available from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ST* conceptualized and designed the study, carried out data collection and PCR assay. ST*, BT, BG and FM analyzed and interpreted the data, drafted the manuscript and critically reviewed the manuscript. All the authors' read and approved the manuscript.

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Tables

Table 1
Socio-demographic characteristics of study participants, Amhara regional state, Ethiopia, 2019.

Characteristics	Category	Frequency	%
Gender	Male	162	50.5
	Female	159	49.5
Age group	≤ 1 year	71	22.1
	2–5 years	61	19.0
	6–9 years	77	24.0
	10–13 years	44	13.7
	≥ 14 years	68	21.2
Residence	Rural	133	41.4
	Urban	188	58.6
Educational status of participants	Illiterate	186	57.9
	Grade 1–8	109	34.0
	9+	26	8.1
Religion	Orthodox	242	75.4
	Muslim	68	21.2
	Protestant	11	3.4
Family size	≤ 4 people	88	27.4
	5 people	97	30.2
	≥ 6 people	136	42.4
Family occupation	Governmental	75	23.4
	Non-governmental	19	5.9
	Merchant	85	26.5
	Farmer	142	44.2
Lymphocytosis	Present	57	17.7
	Absent	264	82.3
Lymphocytosis grading	In ref. range (15%-50%)	264	82.3
	High (> 51%)	38	11.8
	Moderately high (> 70%)	11	3.4

	Very high (> 85%)	8	2.5

Table 2
Distribution of lymphocytosis in patient with pertussis, Amhara regional state, Ethiopia, 2019.

Variable	Category	Frequency	%
PCR confirmed pertussis	Yes	109	34.0
	No	212	66.0
Lymphocytosis in PCR confirmed pertussis patients	Yes	15	13.8
Lymphocytosis grading among PCR confirmed pertussis patients	No	94	86.2
	High (> 51%)	8	53.3
Age group	Moderately high (> 70%)	3	20.0
	Very high (> 85%)	4	26.7
	≤ 1 year	11	73.3
	2–5 years	0	0.0
	6–9 years	3	20.0
	10–13 years	1	6.7
	≥ 14 years	0	0.0

Table 3
Correlation of lymphocytosis with clinical symptoms in PCR confirmed pertussis patients in the Amhara regional state, Ethiopia, 2019.

Variable	Category	Lymphocytosis	Total (%)
		Present (%) Absent (%)	
Lymphocytosis	Yes	15 (13.8) 94 (86.2)	109 (34.0)
Paroxysms of cough	No	42 (19.8) 170 (80.2)	212 (66.0)
	Yes	15 (13.9) 93 (86.1)	108 (99.1)
	No	0 (0.0) 1 (100.0)	1 (0.9)
Post-tussive vomiting	Yes	12 (13.6) 76 (86.4)	88 (80.7)
Apnea	No	3 (14.3) 18 (85.7)	21 (19.3)
Cyanosis	Yes	10 (45.5) 12 (54.5)	22 (20.2)
Syncope	No	5 (5.7) 82 (94.3)	87 (79.8)
Inspiratory whoop	Yes	1 (20.0) 4 (80.0)	5 (4.6)
	No	14 (13.5) 90 (86.5)	104 (95.4)
	Yes	0 (0.0) 2 (100.0)	2 (1.8)
	No	15 (14.0) 92 (86.0)	107 (98.2)
	Yes	4 (5.1) 75 (94.9)	79 (72.5)
	No	11 (36.7) 19 (63.3)	30 (27.5)