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Molecular detection of *hupB* gene of *Mycobacterium tuberculosis* in young patients with acute lymphoblastic leukemia (ALL) on Chemotherapy

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Abstract: Cancer chemotherapy can lead to the mycobacterial infections. Tuberculosis has been reported a serious complication in leukemia patients who undergo chemotherapy. The study was focused to find mutations in *hupB* gene of *M. tuberculosis* in 50 acute lymphoblastic leukemia (ALL) patients through semi multi complex PCR. A column based DNA isolation method was adopted for DNA isolation. The gene for histone-like protein (*hupB* [*Rv2986c*]) of *M tuberculosis* was amplified to detect two closely related mycobacterial species. Primers M and S (histone like protein *HupB*) were utilized to generate amplicons of 318 bp and 291 bp for *M. tuberculosis* and *M. bovis,* respectively. Out of fifty ALL patients, 21 (42%) were females and 29 (58%) were males. The prevalence of ALL was found higher in males as compared to females. The prevalence of ALL was higher in patients of age group 5-10 years. The results of the amplification showed that, the 318 bp fragment specific for *M. tuberculosis* was observed in seven samples (14%), while 291 bp fragment specific for *M. bovis* was not observed in any sample. Children with ALL were found at higher risk for tuberculosis. A risk evaluation of tuberculosis infection must be conducted before managing chemotherapy.

Keywords: Acute Lymphoblastic leukemia (ALL), *Mycobacterium tuberculosis*, *hupB* gene, chemotherapy, semi multi complex PCR.

INTRODUCTION

Cancer chemotherapy had long been considered as a cause of mycobacterial infections (Lancioni *et al.,* 2009). Tuberculosis (TB) is a life threatening bacterial disease caused by *Mycobacterium tuberculosis* and *Mycobacterium bovis,* which can also lead to the secondary infection (Park, 2009). The tuberculosis in acute lymphoblastic leukemia (ALL) have not been clearly understood, due to the complex pharmacological interactions of chemotherapy drugs with the drugs such as Amikacin and Quinolones (Mishra *et al.,* 2005). In 2012, about 8.6 million people suffered from this disease and among them, 1.3 million people died (Wang *et al.,* 2018). The incidence of leukemia is increasing around the world and being spreading rapidly due to certain environmental and genetic factors (Hughes, 1990). Tuberculosis can also prevail in different cancers, including blood cancer patients (Anazi, 2007). The incidence of Tuberculosis in patients with hematological malignancies is found between 2.1-2.6% (Iuldasheva *et al.,* 2002). Tuberculosis are serious and life threatening complication as infection in patients with hematological malignancies (Anazi, 2007). Fungal and bacterial infections are considered the main cause of morbidity and mortality in patients of ALL undergoing chemotherapy (Mishra, 2005). In different malignancies of lung, head/neck and in Hodgkins and non-Hodgkins lymphomas, the elevated risk of tuberculosis has been reported (Lancioni *et al.,* 2009). The overuse of the anti-neoplastic drugs to treat ALL is linked with an increased prevalence of *M. tuberculosis* (Misonou *et al*., 1987). This study was aimed to evaluate the prevalence of tuberculosis in young acute lymphoblastic leukemia (ALL) patients. A semi multi complex PCR was used to detect the *hupB* gene of *Mycobacterium tuberculosis* in ALL patients. Histone-like proteins (Hlps) are small and fundamental bacterial proteins involved in regulating DNA architecture and transactions (Gupta *et al.,* 2014). The absence of mtp40 and mpb70 genes was also found in certain strains of *M. tuberculosis* and *M. bovis* (Jabbar *et al.,* 2015). Previously, 185bp fragment specific to *M. tuberculosis* and 500bp fragment specific to *M. bovis* have been identified via. normal PCR amplification techniques (Jabbar *et al.,* 2015). Leukemia is a cancer caused by abnormal white blood cells. White blood cells are the part of the immune system, which play a key role in body defense mechanism. Cancer weakens the immune system as it extends into the bone marrow that generates the red and white blood cells. Cancer treatments such as chemotherapy, radiotherapy, surgery and bone marrow & stem cell transplants can weaken immunity by causing a

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drop in the number of WBC and hence, the infection risk can be increased in cancer patients (Gea-Banacloche and Segal, 2011). Chen *et al.,* (2011) reported that patients with acute myeloid leukemia (AML) suffered from higher rate of tuberculosis from the spread of *Mycobacterium tuberculosis* (Mishra *et al.*, 2005).

MATERIALS AND METHODS

Sample Collection

With informed consents, the blood samples (n=50) from clinically confirmed acute lymphoblastic leukemia (ALL) patients were obtained from University of Health Sciences (UHS), Lahore, in collaboration with Children Hospital, Lahore, for the tuberculosis screening. The blood samples were obtained in EDTA containing tubes. The blood samples were collected after taking the history of patients that were diagnosed as acute lymphoblastic leukemia (ALL). The patients were undergoing a chemotherapy. Stored the sample at 4^{o}C in fridge for subsequent molecular analysis.

Genomic DNA Isolation

To diagnose the type of Mycobacterium involved in tuberculosis, the genomic DNA was isolated from column based method from the blood samples of 50 ALL patients. table 1 shows the detail on the used reagents. TIANamp genomic DNA kit is a column based DNA isolation kit. This kit was basically based on a silica membrane technology and a special type of buffer for the isolation of genomic DNA.

Differentiation of M. Tuberculosis and M. Bovis

The gene for histone-like protein (*hupB* [*Rv2986c*]) of *M. tuberculosis* was amplified to differentiate two closely related Mycobacterial species; *M. tuberculosis* and *M. bovis* by a method described by Prabhakar *et al*., (2004). The *M. tuberculosis* and *M. bovis* were detected by the PCR based test. Primers M of nucleotide length 20 (5'GCAGCCAAG AAGGTAGCGAA 3') and S of nucleotide length 24 (5'GTATCCGTGTGTCT TGACCTATTTG 3') were used to generate amplicons of sizes 318 bp and 291 bp for *M. tuberculosis* and *M. bovis* respectively (Prabhakar *et al*., 2004). The primers are histone like protein (*Hup B*) gene specific of *Mycobacterium tuberculosis* and *Mycobacterium bovis.* The optimized annealing temperature for primers set M and S was 59^{o}C.

Polymerase Chain Reaction (PCR)

A semi multi complex PCR was used to detect the *hupB* gene of *M. tuberculosis* in ALL patients. The master mixture was prepared by mixing the following reagents: PCR buffer, $MgCl_2$, dNTPs and dH_2O. After the preparation of the master mixture, 46µl of Master Mixture was pipetted into the PCR reaction tube. A 2.5µl of DNA extract, Taq DNA polymerase 0.5 (µl), forward M and reverse primers S (0.5µl each) were added into the PCR tube and mixed with 46µl Master Mixture. All reaction tubes were then transferred into the PCR system to perform PCR amplification with preset PCR conditions and run the PCR. After completion of 40X cycles, the PCR system automatically stopped. After PCR amplification, the products of the *hupB* gene were obtained by using both forward and reverse primers. Fragments of 291bp and 318bp were amplified by these primers. The different PCR cycles were checked with different melting temperatures (50°-59°C) for one minute, and 59°C was found as an optimum temperature (Prabhakar *et al*., 2004).

Ethical approval

The study was conformed to the Institutional Ethical Standards.

STATISTICAL ANALYSIS

The data was analyzed for the descriptive parameters sand prevalence statistics for number of positive/negative samples in *hupB* gene with respect to amplified gene products in IBM SPSS version 25.

RESULTS

Age and Gender Distribution Female in ALL Patients

Out of fifty (n=50) ALL patients, 21 (42%) were females and 29 (58%) were males. The prevalence of ALL was higher in males as compared to females. There were 46% patients in the age group 1 (5-10 years), 32% patients in the age group 2 (between 10-15 years) and 22% patients in the age group 3 (15-20 years). The prevalence of acute lymphoblastic leukemia was higher in patients of age group 1 as compared to other age groups.

Detection of Mycobacteria Species in Blood Samples through PCR

Table 2 shows the quantification of samples by UV Spectrometer. The results of the amplifications showed that, the 318 bp fragment specific for *M. tuberculosis* was observed in seven samples (14%) out of total fifty samples, while 291 bp fragment specific for *M. bovis* was not observed in any sample (table 3). These results were checked on 1 % agarose gel indicating that these samples were positive for *M. tuberculosis*. However, *M. bovis* was not present in ALL patient samples by using M and S primers.

Table 1: Reagents

Non-column based method	
Name	Concentration (µl)
RL buffer	300µl
AL buffer	300µl
PP buffer	100µl
Proteinase K	3-4µl

Column based method	
Name	Concentration (µl)
Buffer GA	300µl
Buffer GB	300µl
Buffer PW	600µl
Buffer TE	150µl
Buffer GD	500µl
Proteinase K	3-4µl

Table 2: Quantification by UV spectrometer

S. No.	Sample No.	DNA Quantity (ng/µL)	Total volume (ng/100)
1	ALL- 1	30	3000
2	ALL- 2	33	3300
3	ALL- 3	55	5500
4	ALL- 4	46	4600
5	ALL- 5	47	4700
6	ALL- 6	49	4900
7	ALL- 7	55	5500
8	ALL- 8	23	2300
9	ALL- 9	20	2000
10	ALL- 10	39	3900
11	ALL- 11	30	3000
12	ALL- 12	52	5200
13	ALL- 13	65	6500
14	ALL- 14	56	5600
15	ALL- 15	49	4900
16	ALL- 16	37	3700
17	ALL- 17	44	4400
18	ALL- 18	37	3700
19	ALL- 19	25	2500
20	ALL- 20	38	3800
21	ALL- 21	22	2200
22	ALL- 22	46	4600
23	ALL- 23	33	3300
24	ALL- 24	55	5500
25	ALL- 25	51	5100
26	ALL- 26	49	4900
27	ALL- 27	22	2200
28	ALL- 28	50	5000
29	ALL- 29	21	2100
30	ALL- 30	47	4700
31	ALL- 31	52	5200
32	ALL- 32	43	4300
33	ALL- 33	67	6700
34	ALL- 34	35	3500
35	ALL- 35	56	5600
36	ALL- 36	47	4700
37	ALL- 37	38	3800
38	ALL- 38	24	2400
39	ALL- 39	35	3500
40	ALL- 40	36	3600
41	ALL- 41	46	4600
42	ALL- 42	41	4100
43	ALL- 43	45	4500
44	ALL- 44	34	3400
45	ALL- 45	37	3700
46	ALL- 46	40	4000
47	ALL- 47	42	4200
48	ALL- 48	35	3500
49	ALL- 49	48	4800
50	ALL-50	56	5600

Table 3: Molecular differentiation of *M. tuberculosis* in ALL patients (n= 50)

Mycobacterium Type	No. of Positive Samples	No. of Negative Samples	Pre-valence (%)	Amplified product size (bp)
M. tuberculosis	7	43	14	318
M. bovis	0	50	0	291

DISCUSSION

Identification of the *M. tuberculosis* complex organisms to the species level is necessary for diagnostic purposes and to identify the proper cancer treatments (Kahla *et al.,* 2011). The prevalence of tuberculosis in patients with hematologic malignancies is observed higher, which is mainly due to the intake of steroids or chemotherapeutical drugs (Lancioni *et al.,* 2009). In our study, there were more male, child ALL patients in age 5-10 years. *M. tuberculosis* specific PCR described that only 318 bp fragment was amplified in 14% ALL samples.

An occurrence of tuberculosis has been reported during chemotherapy treatments for leukemia (Chen *et al*., 2011; Fusegawa *et al.,* 2006). There is a challenge in the treatment of those cancer patients, who are diagnosed with tuberculosis as a secondary infection (David, 2011). Although, the major cause of TB in humans is *M. tuberculosis*, but there some cases are reported from *M. bovis* (Cosivi *et al*., 1998). Children are at more risk of ALL than adults, because their immune system used to be the developing stage (Hughes, 1990). There is a paucity of data related to the incidence and clinical presentation of TB in pediatric ALL patients (Stefan *et al.,* 2013). Child patients who receive cancer treatments are more susceptible to severe infections of tuberculosis (Lancioni *et al.,* 2009). In a case report, the pediatric primary pulmonary tuberculosis with precursor B-cell ALL was discussed. The immunosuppressed patient was on chemotherapy and he diagnosed with TB. It was emphasized that tuberculosis risk assessment, including TST (Tuberculosis skin test) must be done before any initiation of any immune therapy (Lancioni *et al.,* 2009). The identification of *M. tuberculosis* genes, is an important step in understanding the molecular mechanism of mycobacterial pathogenesis. Al-Anazi *et al.,* (2007) reported that tuberculosis was a significant cause of morbidity and mortality in different benign and malignant hematological disorders and in those patients who underwent a hematopoietic stem cell transplant. They reported a suppressed immune response from primary hematological disorder in male patients who were treated with radiotherapy, cytotoxic chemotherapy or steroids. An early diagnosis of tuberculosis was found in patients who were treated with intravenous cytotoxic chemotherapy as compared to patients who were on oral chemotherapy. A proper TB therapy management for successful outcomes is required to avoid multidrug resistance and military

infections leading high mortalities (Al Anazi *et al.,* 2007). The prevalence and mortality of cancer patients from tuberculosis is higher. Altered humoral and cellular immunity are often reported from a chemotherapy treatment. The hematopoietic stem cell patients, who treat with an intense immunosuppressant regimen; are considered at higher risk of having infections (Chen *et al.,* 2011). Jabbar *et al.,* (2015) reported that 98% isolates were found *M. tuberculosis* through mPCR. They found that *M. bovis* accounts for 2% cases of pulmonary tuberculosis. El-Sabban *et al*., (1992) reported that *M. bovis* was detected in up to 5.4% isolates, Idigbe *et al*., (1986) reported 4% detection of *M. bovis* and Nawaz *et al.,* (2012) reported a 5% *M. bovis* detection. Jabbar *et al.,* (2015) and Shah *et al.,* (2004) have used mPCR assays based on the amplification for the identification of a 500bp *M. bovis* specific product and 185bp *M. tuberculosis* specific product. The mPCR based amplifications have a potential role to differentiate *M. bovis* from *M. tuberculosis,* with one primer set targeted *hupB* gene, whereas, another primer set targeted to *pncA* gene (Kidane *et al*., 2002; Jabbar *et al.* (2015). The assays prepared by Kahla *et al.* (2011) revealed a high prevalence of *M. tuberculosis.* They identified 11 putative *in-vivo* induced genes encoding for immunogenic proteins of diverse functions; these included transcriptional regulators, biosynthesis and macromolecule metabolism, polyketide synthases, cell processes etc. The increased risk of tuberculosis has frequently been observed in patients with hematological malignancies due to the immunodeficiency of T-cells from either the disease itself or from the intake of Fludarabine or Corticosteroids etc. (Silva *et al.,* 2005). In ALL cases, host defense, including cell-mediated immunity used to be depressed from either disease or from therapies. A study was conducted by Mishra *et al.,* (2005) to evaluate consecutive cases of ALL who were receiving conventional chemotherapy, they identified around 7% prevalence of active TB. The successful course of treatment was followed in ALL-TB patients by considering careful clinical management. Moreover, the treatment of immunosuppressive therapies with steroids can lead to cell mediated immunity. Patients on stem cell transplant were also found on two fold risk of developing TB (Chimara *et al.,* 2005). Prabhakar *et al.,* (2004) discovered a 27bp difference in the C-terminal parts of the *hupB* genes of *M. tuberculosis* and *M. bovis*. Prasad *et al.,* (2005) reported a pre-dominance of *M. tuberculosis* in humans. Stefan *et al.,* (2013) mentioned active infections (47%) in the form of latent tuberculosis were found in those child patients who were on the initial stages of chemotherapy treatment. Verma *et al.* (2010) performed a PCR assay for *hupB* gene and found that, 82% patients were positive for *M. tuberculosis*. 1655 genes have significantly differentially been expressed during active tuberculosis infection (Wang *et al.,* 2018). Wang *et al.,* (2018) proposed new targets to re-design drugs by including following intravenous immunoglobulin, ion-channel blockers and immune-drugs as adjuvant to different other therapeutics along with anti-mycobacterial agents.

CONCLUSION

Tuberculosis is a serious threat as infection in ALL young patients with defective immune systems due to chemotherapies. Diagnosis of tuberculosis infections must be considered while recommending chemotherapy. Further evaluations are required to optimize the screen and management of TB in ALL pediatric patients prior to start of any cancer therapy.

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