Candida spp Complications in Acute Myeloid Leukemia Patients in Erbil City

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ABSTRACT

This study has been carried out in Erbil City -Iraq, 30 patients were diagnosis by flow cytometry as AML which they were admitted to Nanakaly Hospital for Blood Disease and submitted to chemotherapy. Number of adults/children 22 (73.3%) / 8 (26.7%), female / male 13 (43.3%) / 17(56.7%). Only 20 (66.7%) patients were smoker. The highest number was subtyping M4 10 (33.3%) patients, followed by M1 7(23.3%), M3 6(20%), M2 were 3(10%) patients. M5 and M6 were 2 (6.7%). Ten candida spp isolates before induction phase chemotherapy show 9(90%) C.albicans, 1(10%) C. lusitaniae, and from 32 isolates after induction phase chemotherapy, 17(53.1%) C.albicans, 7(21.9%) C. lusitaniae, and 8(25%) C.famata. According to the site of infection, before starting chemotherapy 8(88.9%) C. albicans isolates were in the oral cavity and 1(11.1%) on the skin, while C. lusitaniae was 1 (100%) in oral cavity only. While after chemotherapy 12(70.6%) C.albicans in the oral cavity and 5(29.4%) on the skin, C. lusitaniae was 2 (28.6%) in the oral cavity and 5(71.4%) on the skin, while all C. famata was 8 (100%) on the skin.

The immunophenotype shows that the number and percentage of patients which the myeloid cells expressed cyMPO was 27(90) with mean percentage of positivity 59.38, while all patients expressed CD33 and CD13 on their blast cell 30(100) with a mean percentage of positivity 59.81 and 30.83 respectively.

Keywords: Blood cancer, flow cytometry, *candida spp*, chemotherapy.

تعقيدات فطر .Candida spp في مرضى سرطانِ الدم المايلودي الحاد في مدينة أربيل

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الملخص

أجريت هذه الدراسةِ في محافظةِ أربيل , العراق. تم تشخيص 30 مريض بواسطة جهاز flow cytometry مصاب بسرطانِ الدمِّ من نوع AML والذين أُدخلوا إلى مستشفى نانةكفلي لامراضِ الدمِّ وخضعوا للعلاج الكيمياوي. عدد المرضى بالغين/اطفال 22 (73.3 %) / 8 (26.7 %) ، عدد الأناث/ الذكورِ كان 13 (43.3 %) / 17 (56.7 %). فقط 20 (66.7 %) مرضى كانوامن المدخِّنينَ.ان العددَ الأعلى من المرضى كانت M 4(33.3%)10 مريض، تَليها 7M1(23.3)، 6 M3(20%) و M 2 كانت 3 (10 %). كُلّ من مرضى 5 M و 6 M كانت 2 (6.7 %). عشرة عزلات فطرَ Candida spp قَبْلَ العلاج الكيمياوي كانت 9 (90 %) عزلة C albicans،و(10%)1عزلة C. lusitaniae، ومِنْ 32 عزلة وبعد العلاج الكيمياوي 17 (53.1 %) عزلة كانت C. albicans، 7(21.9%) كانَ C. lusitaniae، و8 (25 %) كانَ C. famata. طبقاً لموقعِ العدوى، قبل بَدْءِ العلاج الكيمياوي 8 (88.9 %) C. albicans في التجويفِ الفمي و1 (11.1 %) على الجلدِ، بينما C. lusitaniae 1 (100 %) في التجويفِ الفمي فقط. بعد العلاج الكيمياوي 12 (70.6 % C. albicans في التجويفِ الفمي و5 (29.4 %) على الجلدِ، C. lusitaniae كانَ 2 (28.6 %) في التجويفِ الفمي و5 (71.4 %) على الجلدِ، بينما كُلّ C. famata 8 (100 %) على الجلدِ.

بينت التمطيط المناعي immunophenotype بأنَّ العددَ والنسبة المئويةَ مِنْ المرضى الذي اظهرت خلايا myeloid cyMPO كَانتْ 27 (90 %) وكانت معدل النسبة المئويةِ للإيجابيةِ 59.38، بينما اظهر CD33و 13 CDعلى خليةِ السرطانية لجميع المرضى 30 (100 %) وكانت معدل النسبة المئويةِ للإيجابيةِ 59.81 و30.83 على التوالي

الكلمات الدالة: سرطان الدم ،فلوسايتوميتر ،.candida spp, العلاج الكيميائي .

1. Introduction

Blood cancer or leukemia is a type of blood or bone marrow (BM) cancer characterized by an abnormal increase of cells called "blasts" [1]. All hematopoietic neoplasms can be classified to acute and chronic leukemia. Acute leukemia which is characterized by a rapid and uncontrolled proliferation of hematopoietic precursor cells, with a loss of maturation and differentiation was classified to acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) [2][3]. AML is a hematological malignancy resulting from the proliferation of a clone of myeloid stem cells that show defective or absent maturation. The French-American-British (FAB) classification system divided AML into 8 subtypes(M0- M7) based on the type of cell from which leukemia developed and the degree of its maturity [4][5]. The AML occurs at all ages, but it is predominantly a disease of adults. The incidence rises progressively with age, reaching 12.6 per 100,000 in adult ≥65 years. Approximately 85% of acute leukemia in adults are AML, compared to 15% of acute leukemia in children [6].

In AML the blast cells often differ from more mature cells by expressing markers of immaturity and lacking antigens expressed by more mature cells. Immunophenotype by flow cytometry is most useful in identifying myeloid lineage and distinguishing between AML and ALL. Myeloid lineage–associated markers include CD13, CD15, and CD33.CD7 is often found on AMLs (M0 and M1) [7].

Infectious complications Infections are an important cause of morbidity and mortality in leukemia patients, which are at high risk of infection, likely related to the intensity of their induction phase chemotherapy resulting in repeated episodes of prolonged and profound

neutropenia. Infections also prolong hospitalization, compromise chemotherapy delivery, affect the quality of life, and increase health care utilization. Furthermore, protracted empiric and therapeutic use of broad-spectrum antibiotics may contribute to the evolution of resistant microbiologic flora (Lange *et al.*, 2004). Fungal infections with *Candida albicans C. tropicalis C. krusei C. glabrata* are a threat to patients who have prolonged neutropenia [8].

Systemic fungal infections including those by *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients. Nosocomial candidemia occurs predominately in patients who have hematological malignancies and/or have undergone stem cell transplantation and is associated with a high mortality rate [9]. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns. Patients with AML developed cervical lymphadenitis and chronic disseminated infection due *to C. albicans* [10]. *C.albicans* appendicitis was reported in a neutropenic patient after induction chemotherapy [11]. *Candida lusitaniae* was first identified as a human pathogen in 1979. There has been a marked increase in the number of recognized cases of candidemia due to this organism in the last two decades, bone marrow transplantation and high dose cytoreductive chemotherapy have both been identified as risk factors for infections with this organism [12]. *Candida Famata* is a hemiascomycetous yeast commonly found in natural substrates and in cheese [13]. It has been described in human infections [14][15]. *C. famata* has been reported in the patient undergoing chemotherapy for Hodgkin's disease and caused bloodstream infections [16][17].

2. Material and Methods

A- Patients

This study included 30 newly diagnosed blood cancer patients type AML before treatment and after the first induction phase chemotherapy, they admitted to the" Nanacally Hospital for Blood Disease" in Erbil province A questionnaire form was provided to each patient which included age, sex, family degree, education level, smoking, residence (geographical area).

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B- Sample collection

1-Bone marrow aspiration

Two ml bone marrow samples (BM) were collected aseptically in collaboration with senior hematopathologists at Nanakaly Hospital. (BM) from patients with newly diagnosed blood cancer were studied with flow cytometry FC500 with a battery of monoclonal antibodies. For the FC evaluation, the BM was diluted with phosphate buffered saline (PBS) to maintain cells *in vitro* for brief periods. The immunophenotyping staining procedures are:

A-Surface membrane staining of the blast cells

1- The combination of fluorochrome-conjugated antibodies for each panel designed for an FC study was added to 12×75 mm plastic test tubes supplied by Beckman Coulter.

2- Then 100 μL of single-cell BM suspension was pipetted into the tubes and incubated at room temperature (18-25°C) for 15-20 minutes in the dark.

3- Two ml VersaLysisR lysing solution was added to each tube to destroy RBCs in the sample. The samples were gently vortexed and incubated for 10 minutes in the dark at room temperature.

4- The mixture was then centrifuged for 5 minutes at 150 rpm and the supernatant discarded.

5- The cell pellet was washed by re-adding 2 ml of PBS to each tube and centrifuged at 150 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended with 0.5 ml of PBS to each tube. The sample will be ready for FC study within 2 hours.

b- Intracytoplasmic and nuclear staining procedures

1- Hundred μl of reagent 1IntraPrepR (fixative reagent) was added to a 50μl BM sample to fix the cells. The specimen was then incubated for 15 minutes in the dark.

2- Four ml PBS solution was added and centrifuged for 5 minutes at 300 rpm, and then the supernatant was discarded.

3- Hundred μl of reagent 2 (IntraPrepR permeabilization solution) was added to the separated cell pellet and incubated for 5 minutes in the dark. This procedure creates holes in the cytoplasmic and nuclear membrane that allowed monoclonal antibodies (MoAbs) to enter the internal structures of the cell.

4- The fluorescent-conjugated monoclonal antibodies for cytoplasmic and nuclear antigen detection were added and allowed to incubate for 15-20 minutes in the dark. In this step, antibodies diffuse into the interior of the cell.

5- Four ml PBS solution was added for washing. After 5 minutes centrifugation at 300 rpm, the cell pellet was suspended in 0.5 to 1 ml PBS. The sample was ready for FC study. After FC was completed, the cases were given a blast lineage assignment as myeloid or lymphoid classification based upon antigen expression.

2- Skin and abscess swabs

The swab tip was moistened with normal saline and was rotated in the center of the skin or abscess for 30 seconds. Then the swab was placed in the appropriate container and cultured on Sabuorate agar media.

3- Oral cavity swabs

The cotton tip of the swab was typically rubbed on the inside of the oral cavity for several seconds then placed into a sterile container to cultured on Sabuorate agar media.

Skin or abscess swabs and oral cavity swabs were streaked on Sabouraud agar plates gently. plates were incubated at 25°C for 5-7days and identified by VITEK 2 directly in which a sterile swab on applicator stick was used to transfer a sufficient number of pure culture and to suspend the microorganism in 3 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic polystyrene test tube. The turbidity was measured using a turbidity meter (Denis Chek$_{TM)}$. Suspension turbidities used for card

inoculation (McFarland Turbidity Range) for fungi were 1.80 - 2.20. For Card Sealing and Incubation, the inoculated cards were passed by a mechanism, which cut off the transfer tube and seals the card prior to loading into the carousel incubator. Identification levels of unknown biopattern were compared to the database of reactions for each taxon, and a numerical probability calculation was performed. Various qualitative levels of identification were assigned based on the numerical probability calculation. The different levels were Excellence 96-99%, Very good 93 -95%.

3. Results and Discussion

Table1 shows the distribution of patients according to some risk factors. Most of the AML patients were adults 22 (73.3) and children were 8 (26.7). Another study on the prevalence of acute leukemia among a group of Iraqi patients with acute leukemia registered and treated in Oncology Department attached to the Al-Yarmuk Teaching Hospital and Child Central Hospital in Baghdad were showed that the incidence with AML in adults was 27.5% (Mohammad *et al.*, 2009). The result shows that the number of female to male patients was 13 (43.3)/17(56.7). In another study included 115 Iraqi patients with AML, there were 63 males and 52 females with a median age of 35 years [18]. Prevalence and comparison of male and female patients with different types of leukemia during 2001-2010 in the multiethnic population of North Karnataka, there were 122,000 males 78,000 female in AML [19]. [20] found 8/22 females to male in AML patients. [21] found 14 females and 25 males AML patients. Most of the patients were without family relationship 15 (50%). Most of AML patients10 (33.3) were of a bachelor level. 20 (66.7) of AML patients were a cigarette smoker. This result agrees with [22] who found that among the 161 males and 119 females with AML, 101 (36.1%) had never smoked and 179 (63.9%) were ever smokers.

Table 1 showed the distribution of patients according to some risk factors.

Risk factors	AML	
	N	%
Child(≤ 18 years)	8	26.7
Adult(>18 years)	22	73.3
Male	17	56.7
Female	13	43.3
Family relationship		
First degree	7	23.3
Second degree	6	20.0
Third degree	2	6.7
None	15	50.0
Education level		
Primary	2	6.7
Secondary	6	20
Diploma	5	16.7
Bachelor	10	33.3
None	7	23.3
Smoking		
Non smoker	10	33.3
Smoker	20	66.7
Total	30	100%

Exposure to ionizing radiation such as the atomic bombings of Hiroshima and Nagasaki had an increased rate of AML [23]. Alkylating agents and inhibitors of DNA topoisomerase II enzyme, increase the risk of leukemia both in children and adults. The disease occurs mostly as AML, in some cases also as ALL [24][25]. The chemical classes most commonly associated with childhood leukemia are hydrocarbons and pesticides. The most widely recognized hydrocarbon is benzene which has a strong relationship with leukemia [26]. On the other hand, [27] suggested a link between pesticides exposure and childhood leukemia. Chronic exposure to certain chemicals such as benzene, embalming fluids, ethylene oxides, and herbicides also appears to be at increased risk [28]. Workers exposed to benzene were found to have increased levels of cytogenetic abnormalities associated with AML [29]. Smoking has been increased risk of developing AML

especially in those persons aged 60–75 [30]. Smoking increases the risk of AML blood cancer and causes around 6% of all leukemia cases in the United Kingdom [31].

Table2 describes the immunophenotype or the CD marker of AML leukemia. The number and percentage of patients which the myeloid cells expressed cyMPO 27(90) with mean percentage of positivity 59.38,while all patients expressed CD33 and CD13 on their blast cell 30(100) with mean percentage of positivity 59.81 and 30.83 respectively, while CD 117,CD15, CD34, CD64, CD36 and CD14 positivity were 21(70), 13(43.33), 8(26.67), 10(33.33), 11(36.67) and 11(36.67) respectively, with mean percentage of positivity 48.33, 24.66, 45.11, 56.22, 43.14 and 23. Fig.1 shows the passivity of AML case to some CD markers by flow cytometry.

Table 2 Immunophenotype and CD marker of AML leukemia.

CD marker of AML Leukemia	N(%) of positive case	Mean of positivity	Rang of positivity	conjugated fluorochromes
cyMPO	27(90)	59.38	24-92	FITC
CD 33	30(100)	59.81	21-94	FITC
CD 13	30(100)	30.83	20-45	PC5
CD 117	21(70)	48.33	20-70	PE
CD 15	13(43.33)	24.66	20-30	PC5
CD 34	8(26.67)	45.11	20- 69	ECD
CD 64	10(33.33)	56.22	25-90	PE
CD 36	11(36.67)	43.14	20-88	FITC
CD14	11(36.67)	23	21-25	ECD

Acute Myeloid Leukemia without maturation (M1) subtype usually presents with blasts expressing cyMPO and one or more of myeloid-associated antigens such as CD13, CD33, and CD117. CD34 is positive in approximately 70% of cases. There is generally no expression of markers associated with granulocytic maturation such as CD15 and CD65 or monocytic markers such as CD14 and CD64. In AML with maturation (M2) the blast cells usually express one or more of the myeloid-associated antigens (CD13, CD33, and CD15). There is often an expression of CD34 and/or CD117, which may be present only in a fraction of blasts. Monocytic markers such as CD14 and CD64 are usually absent. While in acute promyelocytic leukemia (M3) the FC immunophenotyping shows a lack of expression of CD34, with positive CD33, cytoplasmic MPO, and variable expression of CD13. CD15 is typically absent [32].Acute Myelomonocytic Leukemia

(M4) generally show several populations of blasts variably an expressing myeloid antigens CD13, CD33, and CD15; and others expressing markers characteristic of monocytic differentiation such as CD14and CD36, CD64[33].
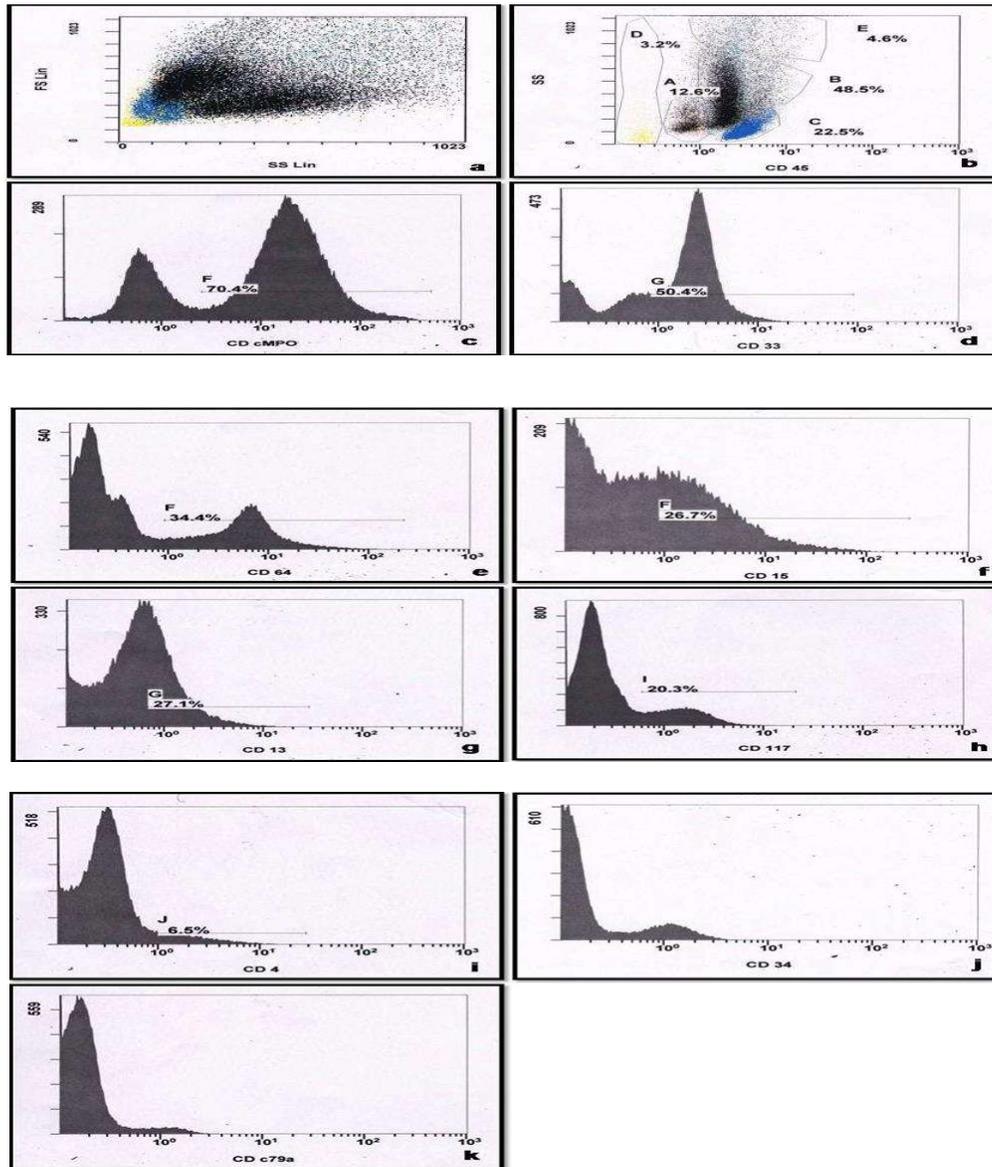

Fig. 1 Immunophenotype and CD marker of AML patients .(a)FS.Forward Scatter ,SS. Side Scatter distribution of the blast cells.(b) is CD45 and SSC,(c) is CD cMPO ,(d) is CD13,(e) is CD64,(f) is CD15, (g) is CD13 ,(h) is CD117 were positive .(i),(j) and (k) for CD4,CD34 and CD79 were negative.

Fig. 2 showed that the highest number was M4 10 (33.3%) patients, followed by M1 7(23.3%), M3 6(20%), M2 was accounted for 3(10%) of all AML patients. M5 and M6 were 2 (6.7%) patients which are the lowest number of AML subtypes. While there was no case of M0 and M7 in this study.
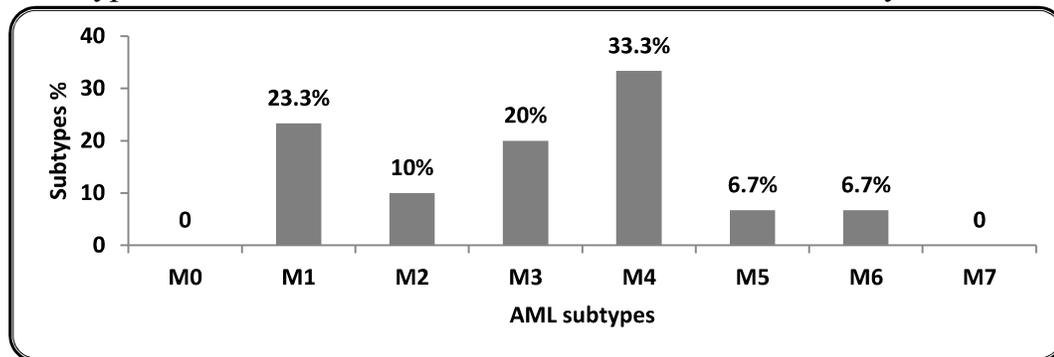

Fig.2 The percentages of AML subtype according to FAB classification.

A study done by [34] supports the results of our study. They found that the percentage of patients with M4 was the most frequent subtype 40%. Of the overall 1686 evaluable cases, 400 (23.7%) were diagnosed AML-M4 [35]. Numeric differences between the AML subtypes showed that M4 number was 68/300 which was the second number after M2 (73), while M1 49, M3 22, M0 18, M5 12, M6 7 and M7 6, although 45 cases were unclassified [36]. Other researchers demonstrated that the most common subtype was M1 (35%) followed by M3, M4, M2, M6 31%, 15%, 15%, and 4%, respectively. None of the patients were diagnosed as M5, M7 or M0 subtype [37].

Identification of several cytogenetic subgroups of AML, characterized by more or less distinct and specific clinical and morphologic features, has proved its great importance in the classification, understanding, and clinical management of this complex and heterogeneous disorder [38]. According to FAB classification patient with inversion (16) and translocation (16;16), translocations of chromosome 16 have been identified in AML subtype M4, and translocation (8;21) occurs most commonly in subtypes M1 and M3, and M4 has a high correlation with t(15;17) while M2 has a correlation with t(8;21). High frequency of M4, M1 and M3 subtype in our study may be related to environmental predisposing factors such as carcinogen in weather, diet, soil and industrial products as a

second event for the subsequent development of these subtypes, in addition to the genetic alteration to the occurrence of these subtypes in our community [39].

Table 3 shows distribution of total 10 *candida spp* isolates before induction phase chemotherapy shows that 9(90%)isolates were C.albicans,1(10%) was *C. lusitaniae*, and from 32 isolates after induction phase chemotherapy,17(53.1%) isolates were C.albicans, 7(21.9%) was *C. lusitaniae*,and 8(25%) was *C.famata*. According to the site of infection, before starting chemotherapy 8(88.9%) *C. albicans* isolates were in the oral cavity and 1(11.1%) on the skin, while *C. lusitaniae* was 1 (100%) in oral cavity only. While after induction phase chemotherapy 12(70.6%) *C.albicans* in the oral cavity and 5(29.4%) on the skin, *C. lusitaniae* was 2 (28.6%) in the oral cavity and 5(71.4%) on the skin, while all *C. famata* was 8 (100%) on the skin.

Table 3 Distribution of *Candida Spp.* isolates before and after chemotherapy according to the site of infection.

Blood cancer type	Candida albicans				Candida lusitaniae				Candida famata				Total			
	Before															
	Oral cavity		Skin		Oral cavity		Skin		Oral cavity		Skin		Oral cavity		Skin	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
AML	8	88.9	1	100	1	0	0	0	0	0	0	0	9	90	1	10
Total	8		1		1		0		0		0		10(100)			
	After															
Blood cancer type	Oral cavity		Skin		Oral cavity		Skin		Oral cavity		Skin		Oral cavity		Skin	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
AML	12	70.6	5	29.4	2	28.6	5	71.4	0	0	8	100	14	43.7	18	56.3
Total	17(53.1)				7(21.9)				8(25)				32(100)			

Candida spp. is the most common cause of invasive fungal infection in humans. *Candida* infection in immunocompromised hosts was frequently life-threatening [40]. Fungal infections were also observed in AML patients by [41]. *C. albicans,* other *Candida spp. Aspergillus, Fusarium,* and Mucor species were reported in the skin of Children with acute leukemia by [42]. There were 16 isolates of *Candida, C. albicans* 13 and other *Candida spp.* was 3. Nineteen of 22 fungal isolates were detected in the induction phase of chemotherapy as compared to 3 in the intensification/consolidation phase versus none in the maintenance chemotherapy [43]. The epidemiology of fungal infections in patients with hematologic malignancies was described by [44] who found candidemia in patients with hematologic malignancies. [45] represent to appendicitis in a neutropenic patient by *C. albicans* after induction chemotherapy.

Conclusions

Most of patients AML were adults in our community and M4 subtype was more prevalent when compared to another subtype. The number of Candida spp isolates increased after induction phase chemotherapy. The immunophenotype shows that most patients' myeloid cells expressed cyMPO.

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