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Detection of Candidemia in a Sample of Iraqi Neonates Admitted to the Neonates Intensive Care Unit (NICU) by Molecular Methods.

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Abstract

Candidemia is a leading cause of sickness and mortality in neonatal care. Although current diagnostic methods are beneficial, a better knowledge of molecular pathways is necessary for enhancing detection. This study used molecular techniques to assess the incidence of candidemia in infants being treated in Iraqi NICUs. Using a cross-sectional experimental design, blood samples from newborns exposed to different risk factors were analyzed. The use of polymerase chain reaction (PCR) that targeted the ITS1 and ITS2 regions facilitated the identification of fungi. Several Cladosporium species, such as Cladosporium macrocarpum, Cladosporium allicinum, Cladosporium limoniforme, Cladosporioides, and Cladosporium tenuissimum, were found, which was unexpected. A phylogenetic study indicated the widespread distribution of these strains throughout Asia and North America. Cladosporium's unexpected appearance necessitates a broadening of infection control measures and diagnostic perspectives in healthcare facilities. The findings of this research stress the need for constant vigilance and an all-encompassing approach to infection and diagnosis management in NICUs.

Keywords: Neonatal Candidemia, Cladosporium, ITS1/ITS2 regions, Molecular diagnostics, NICU and Phylogenetic analysis.

Introduction:

Candidaemia, a bloodstream infection caused by a type of Candida, is a serious global health concern, especially for infants in Neonatal Intensive Care Units (NICUs) due to their immature immune systems and prolonged hospital stays (1). Late-onset sepsis is associated with increased morbidity, mortality, and the likelihood of neurodevelopmental impairments in survivors, and recent investigations reveal that Candida species are a substantial contributor (2, 3). Newborn candidemia is still a major issue, especially in Iraq, despite advances in medical treatment. Environmental factors and healthcare delivery delays due to ongoing conflicts

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exacerbate the situation. Most current studies only look at metropolitan and tertiary hospitals; therefore, there is a dearth of comprehensive information, especially in different healthcare settings (4). The diagnostic problem only adds to the complexity of the situation; conventional blood cultures, although necessary, are slow and may miss early infections in neonates due to their tiny blood volumes (5). This motivates the present work by highlighting the critical need for better diagnostic methods and a better understanding of the neonatal candidemia situation in Iraq.

The Materials and Methods

A Sample Collection

Between December 2022 and April 2023, 100 blood samples of newborns admitted to the neonatal intensive care units of Children's Welfare Teaching Hospital and Baghdad Teaching Hospital were collected (67 male and 33 female). Low birth weight, preterm, use of invasive procedures, delay in enteral feeding of more than three days, risks associated with antibiotics, hospitalization lasting more than seven days, and thrombocytopenia were required as inclusion criteria for neonates with candidemia. The study excluded neonates with no risk factors or who had been treated with antifungal drugs. This study employed a combination of data from infant medical records and a custom-designed questionnaire to draw its conclusions. The left-over blood (0.5 ml) was placed in EDTA tubes and frozen for later molecular analysis.

Molecular techniques for identifying Candida species:

Blood samples' genomic DNA was extracted using Geneaid's gSYNCTM DNA extraction kit according to the manufacturer's instructions. Conventional polymerase chain reaction (PCR) was performed to identify Candida spp. using a primer set (forward: TCCGTAGGTGAACCTGCGG, reverse: GCTGCGTTCTTCATCGATGC) designed based on the nucleotide sequence of the ITS1 and ITS2 ribosomal DNA sections of fungi. The MyGenie 96/384 Gradient Thermal Block (Bioneer, Korea) was utilized to perform PCR reactions using the AccuPower® PCR PreMix kit.

Analysis and Sequencing of Nucleic Acids

In accordance with their methods, Macrogen Inc. (Geumchen, Seoul, South Korea) sequenced PCR amplicons in both forward and reverse. To confirm that annotations and variations were not the results of PCR or sequencing artifacts, further analysis was performed on clear chromatographs acquired from Applied Biosystems (ABI). Using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA), sequencing data were edited, aligned, and analyzed. SnapGene Viewer 4.0.4 was used to annotate the observed nucleic acid changes.

Deposition of Sequences and Phylogenetic Analysis

Following the instructions given by the portal Benson et al., (2016) (6), sequences were uploaded to the NCBI Bankit portal to receive distinct GenBank accession numbers for the sequences under investigation. Based on the neighbor-joining procedure outlined by Alwan et al., (2023) (7), a phylogenetic tree was created. Using the NCBI-BLASTn service, variations were compared to homologous reference sequences Zhang et al., (2000) (8). Using the iTOL package, a conventional circular phylogenetic tree was created and annotated as a cladogram tree Letunic and Bork, (2019) (9). To determine the precise identification of the fungal samples under investigation, reference sequences for several fungal taxa were employed.

Results

Traditional Polymerase Chain Reactions and DNA Extraction

Out of the 100 samples examined, PCR analysis found 20 positive cases (Figure 1).

Sequencing Outcomes

To identify various species, the ribosomal sequence differences in a total of 20 samples were examined. Through NCBI BLASTn analysis, the sequencing reactions verified the PCR amplicons' identities.

The full sequence similarity between samples S1, S5, S6, and S14 and Cladosporium macrocarpum (GenBank acc. MH8631325.1) was observed (Figure 2a).

• Cladosporium allicinum (GenBank accession number MT57347.1) and Sample S2 had a full sequence similarity (Figure 2b).

• According to Figure 2c, samples S3, S8, and S17 have a lot of sequence similarities with Cladosporium limoniforme (GenBank accession MF473139.1).

• According to Figure 2d, samples S4, S7, S9, S11, S13, S15, S16, S18, S19, and S20 have a lot in common genetically with Cladosporium cladosporioides (GenBank accession ON712429.1).

• According to Figure 2e, samples S10 and S12 have a lot of sequence similarities with Cladosporium tenuissimum (GenBank acc. OQ165279.1).

After positioning the ribosomal amplicons' sequences within the genomic sequences of *Cladosporium macrocarpum, Cladosporium allicinum, Cladosporium limoniforme, Cladosporium cladosporioides,* and *Cladosporium tenuissimum*. The details of its sequences were highlighted (Table 1).

The alignment results of the investigated ribosomal samples revealed the presence of variable numbers of nucleic acid variants in some of the analyzed samples in comparison with the most similar referring reference nucleic acid sequences (Fig. 3a-e)

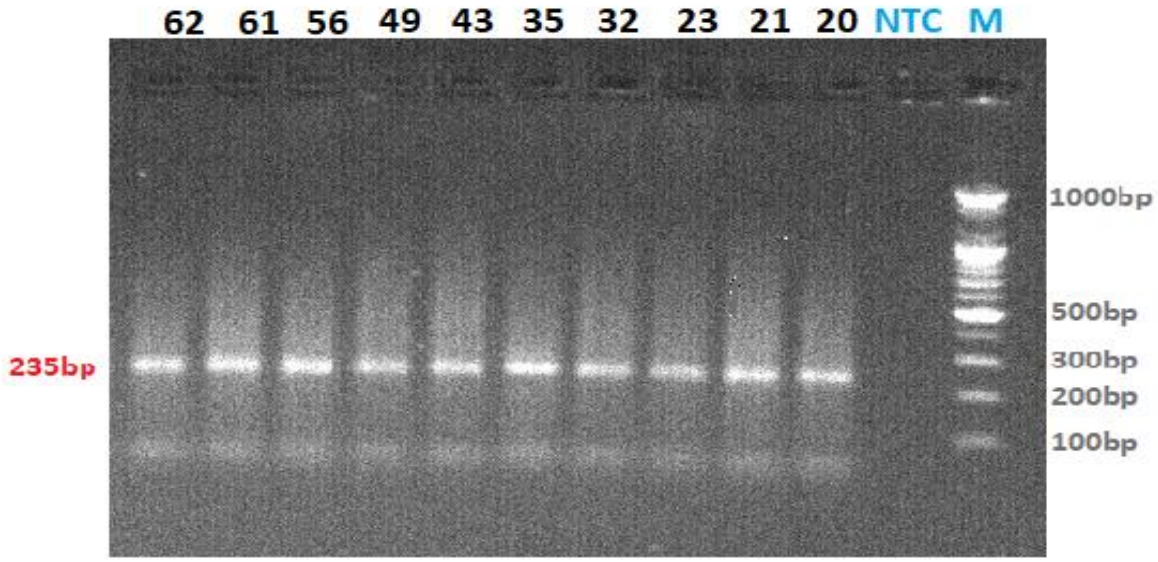


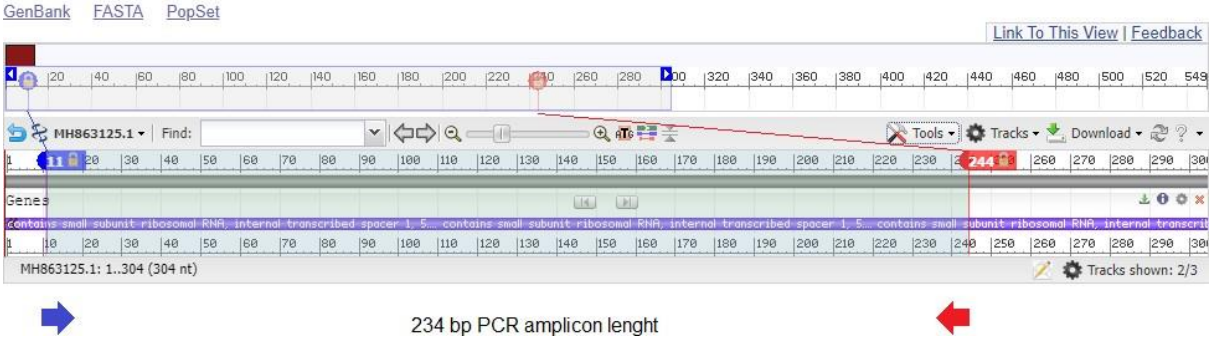
Figure 1: Results of traditional PCR on gel electrophoresis (1.5% agarose, 100v/mAmp, 1 hour) indicating optimum annealing temperature. Bands at 235 bp, M: 100 bp ladder marker, NTC: non-template control.

A)

Cladosporium macrocarpum culture CBS:121623 strain CBS 121623 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MH863125.1

GenBank FASTA PopSet



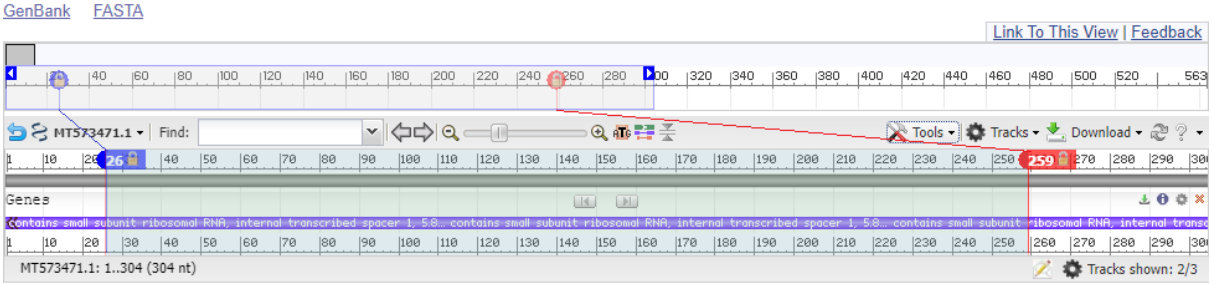
234 bp PCR amplicon lenght

B)

Cladosporium allicinum isolate 9 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: MT573471.1

GenBank FASTA



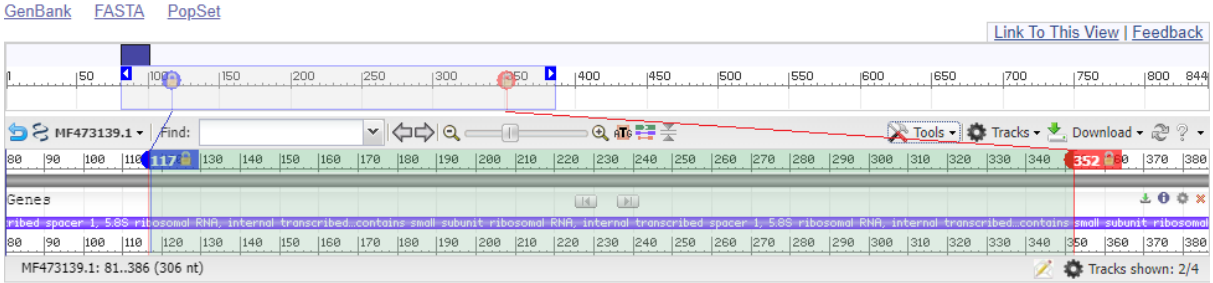
234 bp PCR amplicon lenght

C)

Cladosporium limoniforme culture personal:Jos:Houbraken:DTO:305-G4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA...

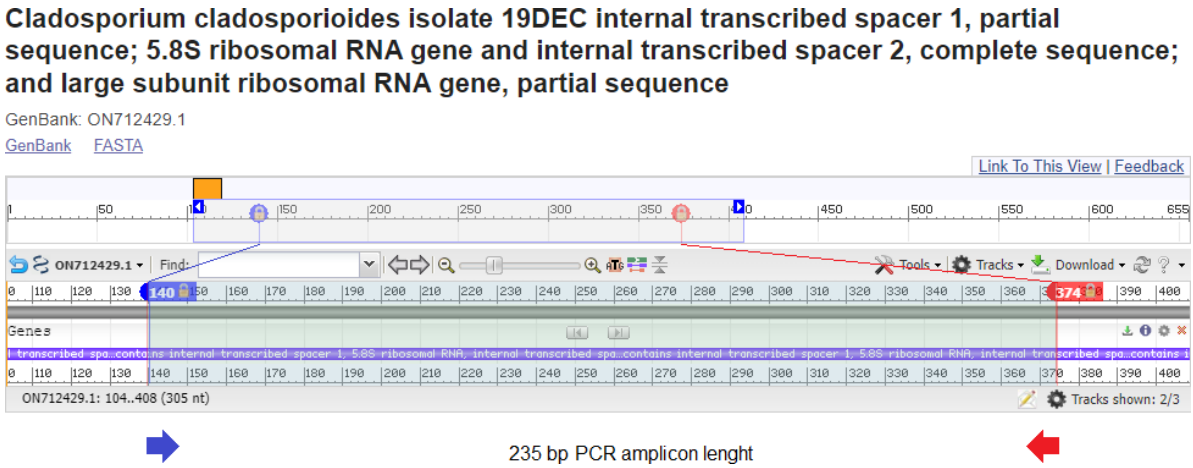
GenBank: MF473139.1

GenBank FASTA PopSet



236 bp PCR amplicon lenght

D)



E)

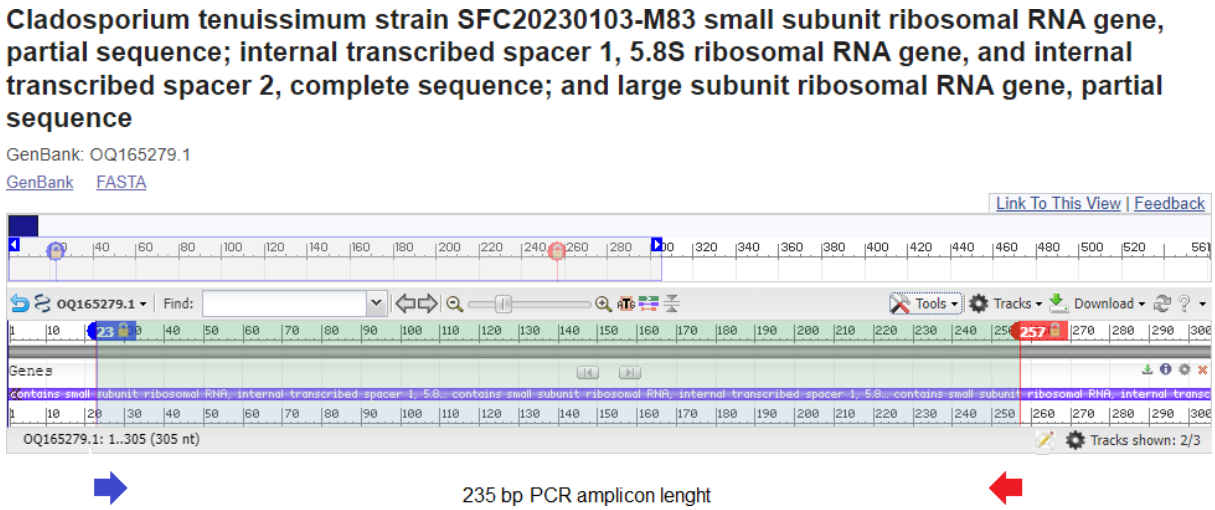


FIGURE (2) Positions of the recovered amplicons of the IST1-5.8S-IT2 ribosomal sequences inside the Cladosporium genomic sequences are shown in Figure 2; (a) to (e) indicate several species identifications.

Table 1A – G. The position and length of the ribosomal PCR amplicons that used to amplify ITS1-5.8S-ITS2 rRNA sequences within the genomic sequences of *Cladosporium macrocarpum*, *Cladosporium allicinum*, *Cladosporium limoniforme*, *Cladosporium cladosporioides*, and *Cladosporium tenuissimum* in branches a, b, c, d, and e, respectively. The green color refers to the forward primer that is placed in the forward direction. The red color refers to the reverse primer that is placed in the reverse complement direction.

Amplicon	Reference locus sequences (5′ - 3′)	length
A) Cladosporium macrocarpum	TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGAACGCCCGGGCTTCGGCCTG GTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCT TCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTG AGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGC ATCGATGAAGAACGCAGC	234 bp
B) Cladosporium allicinum	TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGAACGCCCGGGCTTCGGCCTG GTTATTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCT TCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTG AGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGC ATCGATGAAGAACGCAGC	234 bp
C) Cladosporium limoniforme	TCCGTAGGTGAACCTGCGGAGGGATCATTATAAGTTCACCCAGGCTTGTACAGC TGGGGACTGACAACCCTTTGATTTCCGACTCTGTTGCCTCCGGGGCGACCCTGC CTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTC TGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTG GCATCGATGAAGAACGCAGC	236 bp
D) Cladosporium cladosporioi des	TCCGTAAGGGTGACCTGCGGAGGGATCATTACAAGTGACGCGGTCTAACCACCG GGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGCGGCGACCCTGCC TTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCT GAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGG CATCGATGAAGAACGCAGC	235 bp
E) Cladosporium tenuissimum	TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCG GGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCC TTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCT GAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGG CATCGATGAAGAACGCAGC	235 bp

A)

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                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....| ..........................................................|
ref.
TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGAACGCCCGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTG
CCTCCGGGGCGA S1          ..........................................................
S5      ..........................................................
S6      ..........................................................
S14     ..........................................................

               110       120       130       140       150       160       170       180       190       200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....| ..........................................................|
ref.
CCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACT
TTTAACAACGG S1 ..........................................................
S5      ..........................................................
S6      ..........................................................
S14     ..........................................................

                210       220       230
        ....|....|....|....|....|....                ref.
ATCTCTTGGTTCTGGCATCGATGAAGAACGCAG
C S1    ..............................
S5      ..............................
S6      ..............................
S14     ..............................
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B)

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                10        20        30        40        50        60        70        80        90       100

        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....| ..........................................................|
ref.
TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGAACGCCCGGGCTTCGGCCTGGTTATTCATAACCCTTTGTTGTCCGACTCTGTTG
CCTCCGGGGCGA S2          ..........................................................

               110       120       130       140       150       160       170       180       190       200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....| ..........................................................|
ref.
CCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACT
TTTAACAACGG S2 ..........................................................

                210       220       230
                  ....|....|....|....|....|.... ref. ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC S2     ..............................
```

C)

```
                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....| ..........................................................|
ref.
TCCGTAGGTGAACCTGCGGAGGGATCATTATAAGTTCACCCAGGCTTGTACAGCTGGGGACTGACAACCCTTTGATTTCCGACTCTGTTG
CCTCCGGGGC S3  ..........................................................
S8      ..........................................................
S17     ..........................................................

               110       120       130       140       150       160       170       180       190       200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....| ..........................................................|
ref.
GACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACT
TTTAACAAC S3      ..........................................................
S8      ..........................................T..............
S17     ..........................................................

                210       220       230
        ....|....|....|....|....|.....................................................|.
ref.
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
S3      ..................................
S8      ..................................
S17     ..................................
```

D)

```
                10        20        30        40        50        60        70        80        90        100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
TCCGTAAGGGTGACCTGCGGAGGGATCATTACAAGTGACGCGGTCTAACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGC
CTCCGCGGCG S4  ..................................................................................
S7            ..................................................................................
S9            ..................................................................................
S11           ..................................................................................
S13           ..................................................................................
S15           ..................................................................................
S16           ..................................................................................
S18           ..................................................................................
S19           ..................................................................................
S20           ..................................................................................

                110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
ACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTT
TTAACAACG S4   ..................A.............A.....................................................
S7            ..................A.....................................................................
S9            ..................A.............A.....................................................
S11           ..................A.....................................................................
S13           ..................A.............A.....................................................
S15           ..................A.............A.....................................................
S16           ..................A.............A.....................................................
S18           ..................A.............A.....................................................
S19           ..................A.............A.....................................................
S20           ..................................................................................

                210       220       230
          ....|....|....|....|....|....|.....................................|
ref.
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC S4
              ..............................
S7            ..............................
S9            ..............................
S11           ..............................

S13           ..............................
S15           ..............................
S16           ..............................
S18           ..............................
S19           ..............................
S20           ..............................
```

E)

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                10        20        30        40        50        60        70        80        90        100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
TCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCT
CCGGGGCG S10  ..................................................................................
S12           ..................................................................................

                110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
ACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTT
TAACAACG S10   ..................A.................................................................
S12           ..................................................................................

                210       220       230
          ....|....|....|....|....|....|.....................................|
ref.  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
S10           ..............................
S12           ..............................
```

Fig. 3. Nucleic acid sequences alignment of twenty samples with their corresponding reference sequences of the ribosomal sequences within *Cladosporium macrocarpum*, *Cladosporium allicinum*, *Cladosporium limoniforme*, *Cladosporium cladosporioides*, and *Cladosporium tenuissimum* in branches a, b, c, d, and e, respectively. The symbol "ref" refers to the NCBI referring sequence, letter "S", followed by a number refers to the sample number.

Fungal Infection Among Neonates Admitted to NICU in Relation with Demographic Information:

Sex, Weight at Birth, Gestational Age, and Mode of Delivery:

Regarding the relation of fungal infection among neonates admitted to NICU with sex, weight at birth, gestational age, and mode of delivery, it was found that sex and mode of delivery have no role with molecular method (p=0.457 and 0.263, respectively),

It was also found each weight at birth and gestational age is highly statistically significant with fungal infection (p=0.001 and 0.000, respectively), table (2).

Relation of Fungal Infection and Antibiotic Consumption as Risk Factors Among Neonates Admitted to Neonates Intensive Care Unit:

Regarding the relation of fungal infection among neonates admitted to NICU and antibiotic consumption as a risk factor, it was found that fungal infection detected was not significantly associated with the consumption of the broad-spectrum antibiotic and those not responding to antibiotics (p=0.091 and 0.011, respectively), table (3).

Relation of Fungal Infection and Invasive Procedures as a Risk Factor Among Neonates Admitted to Neonates Intensive Care Unit:

Regarding the relation of fungal infection among neonates admitted to NICU and the invasive procedures, it was found that fungal infection was not significantly associated with the use of central venous line and surgery (p=0.117 and 0.644, respectively), while significantly associated with the use of an endotracheal tube (p=0.000) Table (4).

Relation of Fungal Infection and Hospital Care, Management as a Risk Factor Among Neonates Admitted to Neonates Intensive Care Unit:

Regarding the relationship between fungal infection among neonates admitted to NICU and hospital Care, management, it was found that fungal infection was not significantly associated with prolonged hospitalization (p=0.161), while significantly associated with delay in enteral feeding (p=0.002) Table (5).

Relation of Fungal Infection and Hematological Abnormalities as a Risk Factor among Neonates Admitted to Neonates Intensive Care Unit:

Regarding the relationship of fungal infection among neonates admitted to NICU and the hematological abnormalities, it was found that fungal infection was not significantly associated with the use of thrombocytopenia (p=0.078) Table (6).

Table (2): Relation between fungal infections among neonates admitted to NICU concerning gestational age, sex, weight at birth, and mode of delivery:

		Fungal Infection			
		Negative (NO.)	%	**Positive (NO.)**	%
Gestational age	**Extremely preterm**	1	33.3%	2	66.7%
	Early preterm	6	66.7%	3	33.3%
	Very preterm	6	50.0%	6	50.0%
	Late preterm	24	72.7%	9	27.3%
	Term	43	100.0%	0	0.0%
P value		0.000			
Sex	**Female**	25	75.8%	8	24,2%
	Male	55	82.1%	12	17.9%
P value		0.457			
Weight at birth	**ELBW**	1	25.0%	3	75.0%
	VLBW	6	66.7%	3	33.3%
	LBW	19	67.9%	9	32.1%
	NBW	54	91.5%	5	8.5%
P value		0.001			
Mode of delivery	**NVD**	35	85.4%	6	14.6%
	C/S	45	76.3%	14	23.7%
P value		0.263			

ELBW: extremely low birth weight; VLBW: very low birth weight; LBW: low birth weight; NBW: normal birth weight; NVD: normal vaginal delivery; C/S: caesarian section.

Table (3): Relation between fungal infection and antibiotic consumption as a risk factor:

		Fungal Infection			
		Negative	%	**Positive**	%
Consumption of the broad-spectrum antibiotic	**YES**	63	76,8%	19	23.2%
	NO	17	94.4%	1	5.6%
P value		0.091			
Not responding to antibiotics	**YES**	27	67.5%	13	32.5%
	NO	53	88,3%	7	11.7%
P value		0.011			

Table (4): Relation between fungal infection and invasive procedures as risk factor:

		Fungal Infection			
		Negative	%	**Positive**	%
Use of central venous line	**YES**	4	57.1%	3	42.9%
	NO	76	81.7%	17	18.3%
P value		0.117			
Surgery	**YES**	9	75.0%	3	25.0%
	NO	71	80.7%	17	19.3%
P value		0.644			
Use of endotreacheal tube	**YES**	27	61.4%	17	38.6%
	NO	53	94.6%	3	5.4%
P value		0.000			

Table (5): Relation between fungal infection and the hospital Care, management as risk factor:

		Fungal Infection			
		Negative	%	**Positive**	%
Prolonged Hospitalization	**YES**	38	74.5%	13	25.5%
	NO	42	85.7%	7	14.3%
P value		0.161			
Delay in Enteral Feeding	**YES**	11	55.0%	9	45.0%
	NO	69	86.3%	11	13.8%
P value		0.002			

Table (6): Relation between fungal infection and hematological abnormalities as risk factor:

		Fungal Infection			
		Negative	%	**Positive**	%
Thrombocytopenia	**YES**	20	69.0%	9	31.0%
	NO	60	84.5%	11	15.5%
P value		0.078			

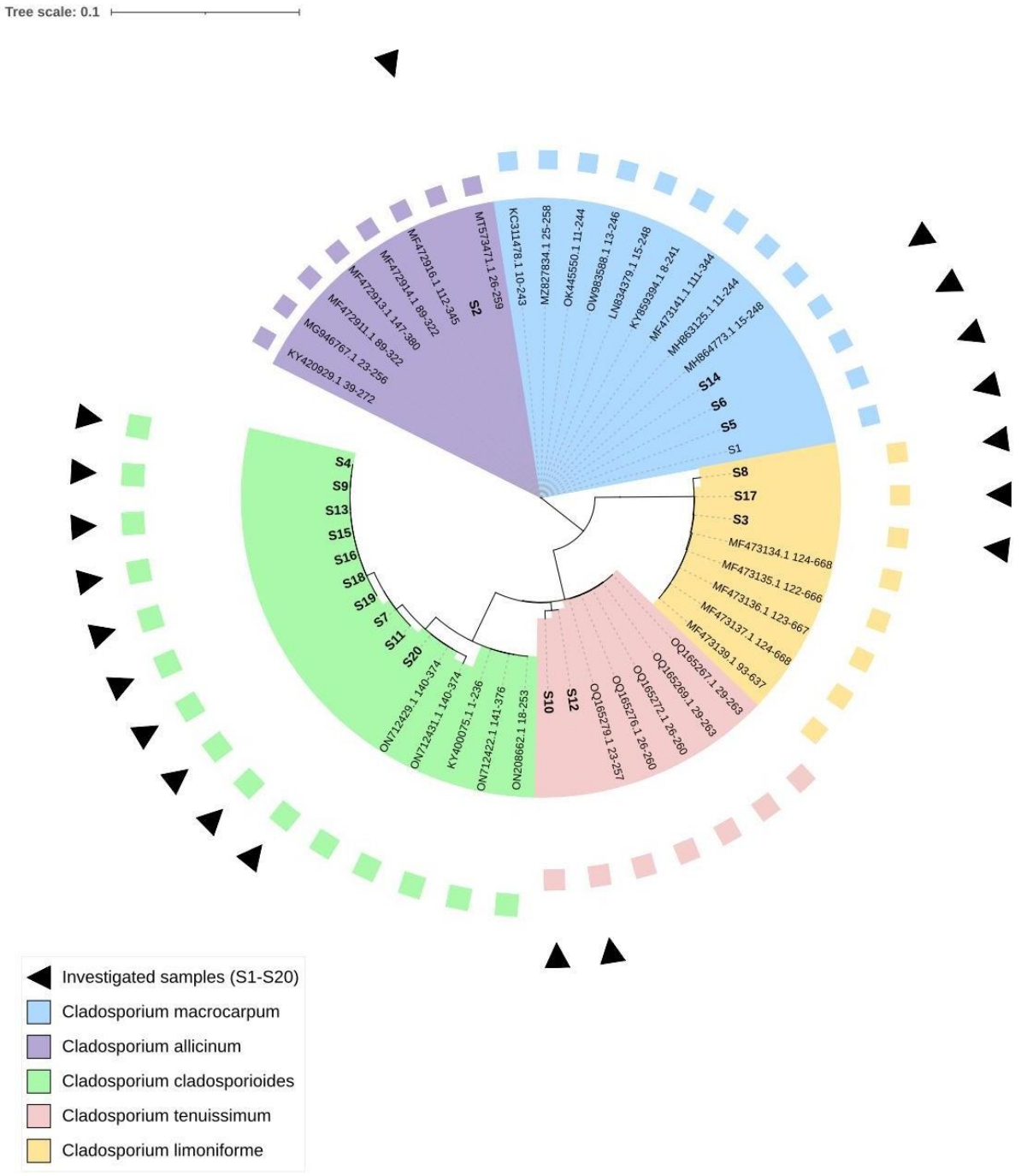

Fig. 4. A comprehensive circular cladogram phylogenetic tree of genetic variants of the ITS1-ITS2 sequences of five different species of *Cladosporium*. The black-colored triangle refers to the analyzed sample. All the mentioned numbers referred to the GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letters "S#" refer to the code of the investigated samples.

Discussion

Candidemia occurring in neonatal intensive care units (NICUs) pertains to bloodstream infections caused by Candida species, presenting a noteworthy health issue characterized by elevated morbidity and fatality rates. Frequently involved species are *Candida parapsilosis* and *Candida albicans*. The occurrence of candidemia exhibits variability, as seen by a documented rate of 3.0 cases per 100 admissions in a specific research investigation. The presence of neonatal candidiasis has been linked to adverse consequences, such as significant cognitive deficits and harm to vital organs. There is observed regional heterogeneity in the distribution of pathogenic Candida species, whereby some places demonstrate a higher prevalence of non-albicans species (10, 11, 12, and 13).

Unfortunately, in the current study and from the whole results of infection, no Candida isolates were obtained, but a common agent seen among infected neonates was *Cladosporium* spp., there discussion included this agent instead of Candida fungus.

Fungal Infection Among Neonates Admitted to NICU in Relation with Demographic Information:

The results in Table 2 show that there was a strong and stable significant link between the rate of *Cladosporium* infection in newborns admitted to the NICU and their gestational age and weight at birth. However, there was no significant link between infection rates and sex or mode of delivery.

These results agree with the idea that there is a strong link between having a low-birth-weight baby, being born early, and getting fungus illnesses. First, a study by Manzoni *et al*. (2006) found that babies born before their due dates were more likely to get an invasive fungus illness if they were small at birth or had a short gestational age (14). Another review by Weimer *et al. (2022)* agrees with this opinion; they found that babies with very low birth weight (VLBW) or extremely low birth weight (ELBW) are more likely to get invasive fungal infections because their immune systems aren't fully developed yet. There's also a negative relationship between birth weight and the incidence of invasive candidiasis (15). In the same way, Luparia *et al*. (2019) found that babies who were born very early and had a very low birth weight were more likely to get fungus infections (16). Also, David A. Kaufman *et al*. (2020) said that babies born at the earliest gestational ages who live have the biggest chance of invasive fungus infections (17). Due to their immature immune systems, preterm neonates show reduced innate and adaptive immunity. They also have a smaller pool of monocytes and neutrophils, which are crucial for fighting infections but have impaired pathogen-neutralizing potential (18). Additionally, as the skin is the body's first line of defense against infections, fungal pathogens are more likely to penetrate it when the skin barrier is underdeveloped in extremely immature infants (19). Therefore, a compromised skin barrier and an immature immune system make premature infants more susceptible to invasive fungal infections, especially those who are extremely preterm. This risk is increased in hospital settings due to the potential for increased exposure to fungal pathogens (20).

Relationship of Fungal Infection and Antibiotic Consumption as Risk Factors Among Neonates Admitted to the Neonates Intensive Care Unit:

The data in Table 3 shows that there is no significant link between using broad-spectrum antibiotics and not responding to antibiotics. This result is interesting because it disagrees with some general trends that have been seen in research about fungus diseases in newborns, especially in the NICU.

A study done by Hsieh, E. *et al. (*2012) published in Nature says that being born before their due date and having used drugs in the past can make them more likely to get Candida infections, which are a major cause of illness and death in the NICU (21). Another study prepared by Benjamin Jr. *et al.* (2010) backs this up even more by saying that newborns who have been exposed to third-generation cephalosporins or carbapenems should be given antifungal treatment on the spot (22). This disagreement may be caused by the fact that the number of samples included in this study

is not enough or because the fungal infection here is not Candida. It is known that patients who have undergone a long course of antibiotics predispose to fungal infection, particularly Candiasis. This fungus is a normal flora, while the fungal infection in the current study is a saprophytic agent rather than an opportunistic one.

Relation of Fungal Infection and Invasive Procedures as a Risk Factor Among Neonates Admitted to Neonates Intensive Care Unit:

The results in Table (4) show that using central venous lines or having surgery did not have a strong link to fungal infections. However, using intubation tubes did have a strong link. Regarding central venous line and surgery, the results show that there isn't a strong link between having a fungal infection and having surgery or a central venous line. The research done by De Rose *et al.* (2021) agrees with the current results that there isn't a clear picture of how common fungus growth and invasive illnesses are in newborns in NICUs who need major surgery (23). The study done by Auriti, C *et al.* (2022) finds a strong link between neonatal fungus infections and surgery. In particular, invasive fungal infections (IFIs) are more likely to occur in neonates following major surgery, especially abdominal surgery, especially if there has been fungal colonization previously (24). Regarding endotracheal tubes, the results show a strong link between the use of this appliance and fungus infections. This agrees with other results obtained by Adair, C. G., *et al.* (1999), who discussed the fact that biofilm formation on endotracheal tubes can serve as a reservoir for pathogens including fungi, which may lead to ventilator-associated pneumonia (25). Their use can predispose patients to infections due to multiple factors including the breach of natural barriers, biofilm formation on the surface of the tubes, and the potential for microaspiration of contaminated secretions.

Relation of Fungal Infection and Hospital Care, Management as a Risk Factor Among Neonates Admitted to Neonates Intensive Care Unit:

According to the results in Table (5), fungal infection is significantly linked to the delay in enteral nutrition of newborns in the NICU (p=0.002) but not to the longer stay in the hospital (p=0.161). This reveals that different parts of hospital care and management may have various effects on the chance of getting a fungal infection.

Regarding long stays in the hospital, the current results agree with those obtained by Johnson. *et al.* (2022) proved that infection prevention and control (IPC) practices in NICUs are very important for lowering the risk of illnesses, and these practices often go hand-in-hand with efforts by hospitals to shorten hospital stays (26). Prolonged hospital stay is one of the predisposing factors of nosocomial infection represented by exposure to contaminants tools, appliances, and air contamination particularly in the case of Cladosporium infection which is considered to be one of the indoor contaminants.

In the case of delay in enteral Feeding, it is interesting that there is a strong link between delay in enteral feeding and fungal illness. The study done by Morgan, *et al.* (2011) agrees with the current ones that delayed enteral feeding may make it harder to build good gut bacteria, which could make people more likely to get fungal illnesses. This is very important in NICUs, where babies are often born early and have immune systems that aren't fully developed yet, making them more likely to get infections while going through invasive procedures (27).

Relation of Fungal Infection and Hematological Abnormalities (thrombocytopenia) as a Risk Factor among Neonates Admitted to Neonates Intensive Care Unit:

Looking at the results in Table (6), show that there is no significant link between fungal infection and the development of thrombocytopenia (p=0.078) in newborns in the NICU.

A study done by (Hsieh, E. *et al.* 2012) shows that neonates with unclear low platelets might be good

candidates for antifungal medicine in the nursery revealing an arrangement between this result and the current one (28), but the latter disagrees with another study done by Guida, et al. (2003) expected that sever thrombocytopenia could be seen as a sign of fungal diseases in some cases (29).

Phylogenetic Tree:

Within this tree, five clades of incorporated sequences within the cladogram were constituted by the incorporation of the samples along with the other closely related sequences. The presence of variable phylogenetic positions among the incorporated clades within the generated tree was shown by the data for this tree. The ability of ITS1-ITS2 sequences-based amplicons to differentiate among the currently investigated Cladosporium species within the same tree was indicated by these data. Fifty was the total number of aligned nucleic acid sequences in this comprehensive tree.

Representations of the incorporated fungal sequences were explained by a cladogram that was generated, a circular cladogram (Fig. 4). Five main clades to represent Cladosporium were formed by the clustering of the investigated samples. The most interesting fact observed in our investigated isolates is correlated with the ability of the utilized ITS1-ITS2-based amplicons to categorize the investigated sequences into diverse phylogenetic distributions of numerous phylogenetic positions. Five major clades were comprised in the currently constructed tree. The ability to infer ancestral characteristics and evolutionary relationships among them becomes possible by comparing the incorporated taxa of Cladosporium.

Eight sequences of Cladosporium allicinum were comprised in one of the major clades of this tree. The investigated sample of S2 was incorporated within this clade. The positioning of this sample in the vicinity of variable strains of Cladosporium allicinum that were deposited from Poland (GenBank MT573471.1), China (GenBank MF472916.1 and KY420929.1), Hungary (GenBank MF472914.1), Georgia (GenBank MF472913.1), Denmark (GenBank MF472911.1), and Italy (MG946767.1) resulted in the determination of the Asian – European sources of the S2 sample.

Thirteen sequences of Cladosporium macrocarpum were comprised in the other major clades of this tree. Our investigated samples of S1, S5, S6, and S14 were incorporated within this clade. The positioning of these samples in the vicinity of variable strains of Cladosporium macrocarpum that were deposited from the USA (GenBank MH864773.1, MH863125.1, and LN834379.1), Denmark (GenBank MF473141.1), Turkey (GenBank KY859394.1), Belgium (GenBank OW983588.1), Hungary (GenBank MF472914.1), Spain (GenBank MZ827834.1), and China (OK445550.1) resulted in the determination of the Asian, European, and American sources of the S1, S5, S6, and S14 samples.

Eight sequences of Cladosporium limoniforme were comprised in the other major clades of this tree. Our investigated samples of S3, S8, and S17 were incorporated within this clade. The positioning of these samples in the vicinity of variable strains of Cladosporium limoniforme that were deposited from the USA (GenBank MF473134.1, MF473135.1, MF473136.1, MF473137.1), and Australia (MF473139.1) resulted in the determination of the American - Australian sources of the S3, S8, and S17 samples.

Seven sequences of Cladosporium tenuissimum were comprised in the other major clades of this tree. Both of our investigated samples of S10 and S12 were incorporated within this clade. The positioning of these samples in the vicinity of variable strains of Cladosporium tenuissimum that were deposited from South Korea (GenBank OQ165279.1, OQ165276.1, OQ165272.1, OQ165269.1, and OQ165267.1) indicated the Asian sources of the investigated S10 and S12 samples. Fourteen

sequences of Cladosporium cladosporioides were comprised in the last major clades of this tree. Our investigated sample of S4, S7, S9, S11, S13, S15, S16, S18, S19, and S20 were incorporated within this clade. The positioning of these samples in the vicinity of two strains of Cladosporium cladosporioides that were deposited from China (GenBank ON712429.1 and ON712431.1) also indicated the Asian sources of the investigated S4, S7, S9, S11, S13, S15, S16, S18, S19, and S20 samples.

No phylogenetic positioning between Cladosporium allicinum and Cladosporium macrocarpum was identified based on the investigated ribosomal sequences. Variable levels of similarities with the other three clades of Cladosporium limoniforme, Cladosporium tenuissimum, and Cladosporium cladosporioides were identified. Due to their positioning toward the roots of the tree, it was inferred from this tree that two similar ancestral positions were occupied by the sequences of Cladosporium allicinum and Cladosporium macrocarpum compared with the sequences of the other clades of Cladosporium tenuissimum, Cladosporium limoniforme, and Cladosporium cladosporioides, respectively.

Conclusions

The study found that no Candida spp. was found in NICU-admitted neonates with fungemia. Cladosporium spp. was found to be a common fungal infection, likely due to airborne contamination of appliances and endotracheal tubes.

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