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Isolation and Molecular Characterization of *Klebsiella* And *Enterobacter* Species Recovered in Sunflower Seed Agar from Cases Resembling Respiratory Cryptococcosis

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Abstract: Respiratory cryptococcosis caused by Cryptococcus species can present with symptoms indistinguishable from bacterial or viral etiology. *Cryptococcus* species produce typical colonial features on Sunflower Seed Agar (SSA), which aids in rapid diagnoses of cryptococcosis. In studying respiratory cryptococcosis, we observed bacterial growths on SSA that resembled *Cryptococcus* species in colonial characteristics. This study aimed at identifying and characterizing those bacterial isolates for documentation. Sputum samples were collected from 201 patients with symptoms suggestive of respiratory cryptococcosis. The samples were inoculated onto SSA, incubated at 37^{o}C for two weeks. Suspected colonies were further evaluated. Of the samples, none yielded Cryptococcus species, although a total of twenty Cryptococcus-resembling bacterial colonies were encountered and isolated. Eight of the isolates could not amplify by PCR techniques. The other twelve were identified as follows: *Klebsiella pneumonia* (8 or 67%), *Klebsiella ozaneae* (3 or 25%), and *Enterobacter ludwigii* (1 or 8%). All isolates were susceptible to Ertapenem, Meropenem, and Fosfomycin but resistant to ampicillin. Results show that Klebsiella and Enterobacter pneumonia-like illnesses can be misidentified as cryptococcosis using SSA. Reliance on microscopic rather than macroscopic, colonial features on SSA will prevent misdiagnosis.
Keywords: *Klebsiella* sp; *Enterobacter* sp; sunflower seed agar.

INTRODUCTION

Respiratory infections are a significant cause of morbidity and mortality in different parts of the world[1]. They have posed a significant problem in our hospital (Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria). Respiratory infections can be as mild as the common cold, otitis media, and pharyngitis; it may be severe or sometimes invasive and fatal – as with pneumonia[2]. Pneumonia-like diseases usually present with cough, dyspnea, fevers, among other symptoms, and can be caused by bacteria, viruses, or fungi. *Cryptococcus species* are an important fungal cause of respiratory infection, and the infections they cause can present as pneumonia-like illnesses, indistinguishable from those of bacterial or viral etiology.

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Reports from Nigeria on cryptococcosis are sketchy and were mainly based on Cryptococcal antigenemia screening[3,4]; report on cultural isolation of Cryptococcus from throat samples is scanty[5]. Cultural isolation of Cryptococcal agents of pneumonia-like diseases has not been investigated in our region. This prompted us to investigate the prevalence and pattern of respiratory cryptococcal infection among patients in our hospital – using the Sunflower Seed Agar medium. Sunflower Seed Agar (SSA) is a selective and differential mycological medium designed for isolation of Cryptococcus species while suppressing the growth of bacteria[6]; it presumptively identifies Cryptococcus species as brownish, mucoid colonies.

In the course of our study on respiratory cryptococcal infection among patients presenting with suggestive symptoms in our hospital (Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria), we encountered bacterial growths on SSA medium that resembled *Cryptococcus species* in cultural characteristics; we could not find any similar report in the literature. Therefore, this study aimed to subject the bacterial isolates to thorough microbiological and molecular analyses for proper Identification and documentation.

MATERIALS AND METHOD

A total of 201 patients (95 HIV positive and 106 HIV negative) who visited Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nigeria, with symptoms suggestive of respiratory cryptococcosis, were recruited into the study between 2017 and 2018. Informed consent was sought, and questionnaires were administered. Ethical consent for the work was also sought, and approval was obtained from the NAUTH ethics committee. Sputum samples were collected from the patients by standard methods[7]. The samples were transported to Prof. F.E. Emele's research laboratory, Faculty of Medicine, NAU, Nnewi, with minimal delay. In the laboratory, the samples were inoculated onto Sunflower Seed Agar[8] and incubated aerobically at 37^{o}C for two weeks[6]. Colonies were evaluated for melanization on SSA and suspected colonies subjected to other preliminary analyses, including Gram staining, catalase, oxidase, citrate utilization, and other necessary bacteriological tests[9].

Molecular Identification:

DNA Extraction

The DNA of 24 hrs. The culture of each isolate was extracted using *Quick*-DNA™ Fungal/Bacterial Miniprep Kit, following the manufacturer's protocol.

Oligonucleotide primers and PCR

The 16S rRNA genes were amplified (by PCR technique) from the genomic DNA, using the following primer pair: 16S-F: GTGCCAGCAGCCGCGCTAA; 16S-R: AGACCCGGGAACGTATTCAC.

The PCR involved the following cocktail mix and condition: 10× PCR buffer (1.0 µL), 25mM $MgCl_2$ (1.0 µL), 5pMol forward primer (0.5 µL), 5pMol reverse primer (0.5 µL), DMSO (1.0 µL), 2.5Mm dNTPs (0.8 µL) Taq DNA polymerase 5u/ul (0.1 µL), 10ng/µl DNA (2.0 µL), Nuclease free water (3.1 µL), making a total of 10µL. Amplifications were carried out in a thermocycler under the following thermal profile: 95°C for 5 min, 35 cycles consisting of 94ºC for 30sec (denaturation), 56ºC for 30sec (annealing), 72ºC for 45sec, and final elongation of the amplified DNA at 72°C for 5 min. The holding temperature was 10˚C. After amplifying the 16SrRNA gene, the PCR amplicons were resolved on 1.5%

agarose gel electrophoresis. Sequencing was done using BigDye® Terminator v3.1 Cycle Sequencing Kit following the manufacturer's instruction.

Data Analysis

Bio- Edit software and MEGA 6 were used for all the analyses. First, to edit and clean up the sequences, sequences were opened with the Bio-edit software, and with the help of the chromatograph, the most likely nucleotide sequence for each isolate was established. These were all compared with already established 16SrRNA gene sequences on the database by using the Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database, and the most likely organism was established. The edited sequences of the bacteria isolate (together with that of the different strains already established in the NCBI) were clustered using Bio-edit, after which multiple sequence alignment was performed. The multiply aligned sequences were also imported to MEGA 6 for phylogenetic analysis. A dendrogram was constructed using the maximum likelihood tree with 1000 bootstrap replicates based on Single Nucleotide Polymorphisms (SNPs), insertions/deletions (INDELS), and length diversity in the 16S rDNA regions.

Antimicrobial susceptibility testing

The antimicrobial drug susceptibility test on the isolates was performed by agar diffusion method, using commercial antibiotic paper discs (Oxoid), following Clinical and Laboratory Standards Institute guideline and interpretative criteria[10]. The antimicrobial drugs used, and their disc contents, were as follows: Ceftazidime (30 µg), Cefuroxime (30 µg), Cefepime (30 µg), Ceftriaxone (30 µg), Gentamicin (30 µg), Ertapenem (10 µg), Meropenem (10 µg), Fosfomycin (50 µg), Ampicillin (10 µg), Amoxicillin-Clavulanic acid (30 µg), and Levofloxacin (5 µg).

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS), version 20. A Chi-square test was used to compare information on the patients age groups, gender, and HIV status. Statistical significance was set at P-values less than 0.05 ($P<0.05$).

RESULTS AND DISCUSSION

Of the 201 samples, none yielded Cryptococcus species, but twenty (10%) samples yielded mucoid, brown-appearing, bacterial colonies that showed Gram-negative reaction. According to the age, gender, and HIV status of the patients from whom isolates were recovered, the distribution of the isolates is as shown in Table 1.

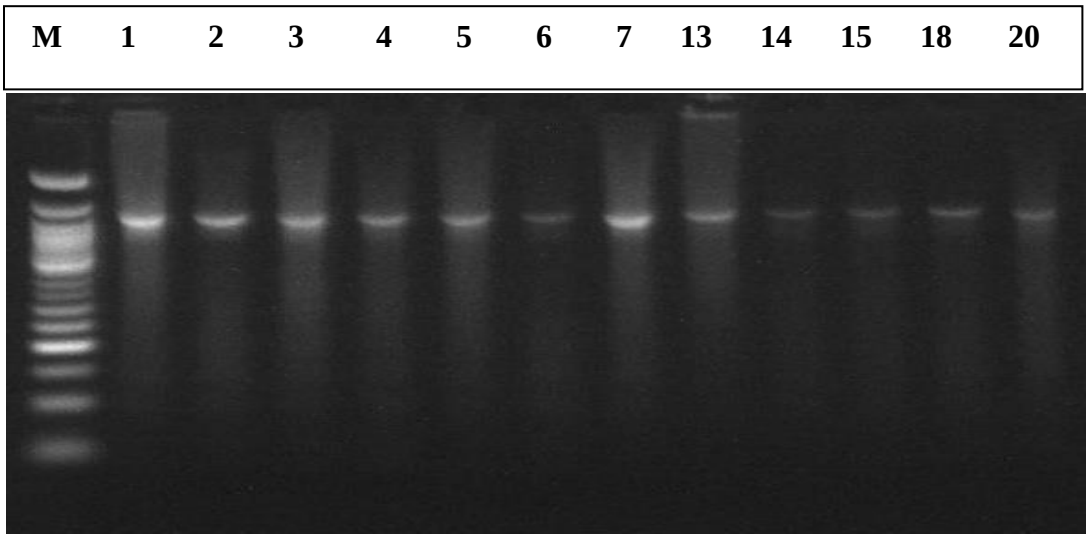
Identity of isolates, as revealed by BLAST analysis of the 16S rDNA gene homology, is shown in Table 2: Isolates no. 2 and 20 showed maximum identities of 99% to *Klebsiella pneumoniae* strain ATCC 13883 (NR 114506.1), 5 and 14, 99% to *Klebsiella pneumoniae subspecies ozaenae* ATCC 11296 (NR 119276.1); 3, 13, and 18 showed the maximum identity of 99% to *Klebsiella pneumoniae* DMS 30104 (NR 11768.1), 7 and 15 exhibited sequence homology level of 98% with, *Klebsiella pneumoniae* DMS 30104 (NR 11768.1) and 1 with *Enterobacter ludwigii* strain EN-119 (NR 042349.1). Isolate 4 and 6 showed the maximum identity of 97% and 93% to *Klebsiella pneumoniae subspecies ozaenae* ATCC 11296 (NR119276.1) and *Klebsiella pneumoniae* DSM 30104 (NR 114715.1), respectively.

Table 1. Frequency of Isolation of Bacteria According to Age, Gender, and HIV Status of Individuals with Respiratory Symptoms

Age Range (Years)	Number Sampled	No. (%) of Isolates	X^2	P-value
17-26	25	4 (16.0)		
27-36	40	4 (10.0)		
37-46	62	5 (8.1)		
47-56	39	4 (10.3)		
			2.072	0.913
57-66	18	1 (5.6)		
67-76	15	2 (13.3)		
77-86	2	0(0.0)		
Gender				
Male	90	11 (12.2)		
Female	111	9 (8.1)	0.939	0.333
HIV Status				
Positive	96	9 (9.4)		
Negative	105	11 (10.5)	0.068	0.794

*Made from Chi-Square Test on SPSS version 20

The frequency of isolation in HIV negative was slightly higher (11 or 10.5%) than in HIV positive patients (9 or 9.4%). Figure 1 shows agarose gel electrophoresis of the DNA fragments amplified from genomic DNA.



Lane M: Size marker (850bp Molecular DNA Ladder)
Lane 1: *Enterobacter ludwigii* isolate
Lanes 2-7, 13-15, 18, 20: *Klebsiella pneumoniae* isolates

Figure 1. Agarose Gel Electrophoresis of DNA Fragments Amplified from Genomic DNA with 16S rDNA Primer

Table 2. 16S rDNA-Based Identification of the Isolates

ab No.	Genus/Strain	BLAST E-value	% ID	Gen Bank Accession No.
1	*Enterobacter ludwigii* strain EN-119	0.0	98	NR 042349.1
2	*Klebsiella pneumoniae* strain ATCC 13883	0.0	99	NR 114506.1
3	*Klebsiella pneumoniae* strain DMS 30104	0.0	99	NR 036794.1
4	*Klebsiella pneumoniae subsp. ozaenae* ATCC 11296	0.0	97	NR 119276.1
5	*Klebsiella pneumoniae subsp ozaenae* ATCC 11296	0.0	99	NR 119276.1
6	*Klebsiella pneumoniae strain* DSM 30104	0.0	93	NR 114715.1
7	*Klebsiella pneumoniae* strain DSM 30104	0.0	98	NR 117686.1
13	*Klebsiella pneumoniae* DSM 30104	0.0	99	NR 117686.1
14	*Klebsiella pneumoniae subsp ozaenae* ATCC 11296	0.0	99	NR 119276.1
15	*Klebsiella pneumoniae* strain DSM 30104	0.0	98	NR 117686.1
18	*Klesiella pneumoniae* strain DSM 30104	0.0	99	NR 117686.1
20	*Klesiella pneumoniae* strain ATCC 13883	0.0	99	NR 114506.1

All isolates, except one, clustered with *Klebsiella pneumonia* (Fig 2). Also, the NCBI blast results indicated substantial similarity with different strains of *Klebsiella pneumonia*; most isolates did not cluster closely to already established *Klebsiella pneumonia* species. Overall, eleven (92%) of the twelve identified isolates were *Klebsiella species*, while the twelfth was *Enterobacter ludwigii.* Of the *Klebsiella* species, 8 (72.7%) were *K. pneumoniae*, while 3 (27.7%) were *K. ozaenae*.

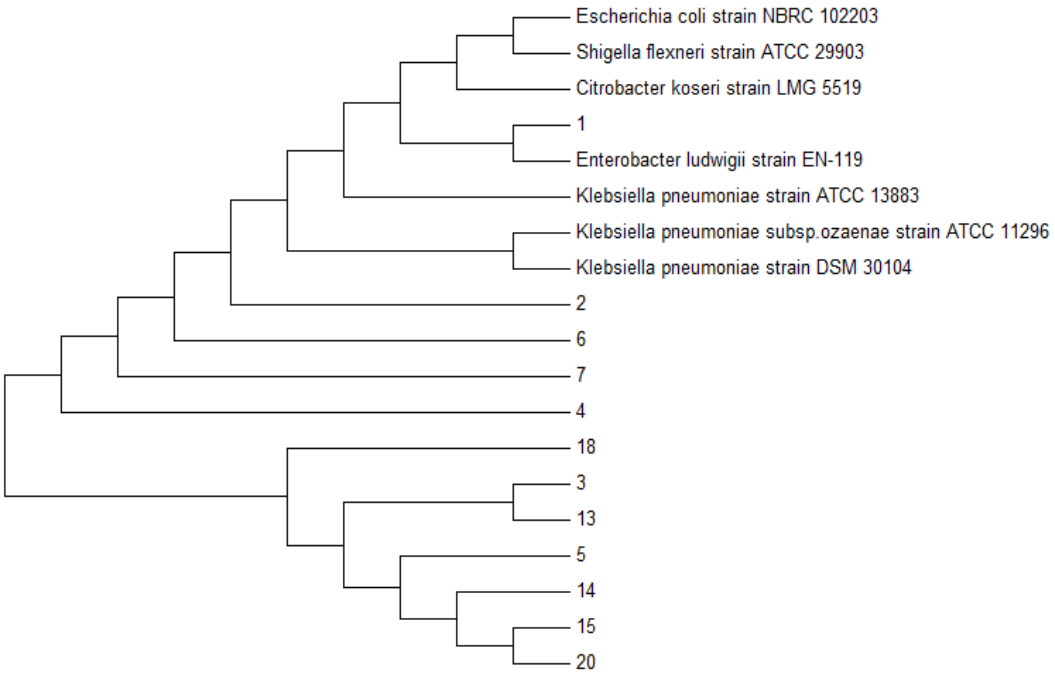


Figure 2: Phylogenetic Tree of Respiratory Tract Bacterial Isolates in Nnewi, Nigeria

Antimicrobial susceptibility test results showed that all isolates were susceptible to Ertapenem, Meropenem, and Fosfomycin but resistant to Ampicillin (Table 3). Eight isolates had their DNA extracted but failed to amplify, even with repeated trials; these could not be sequenced.

Table 3: Antimicrobial Susceptibility Pattern of Bacterial Isolates Recovered on Birdseed Agar

	No. (%) of isolates susceptible or resistant.								
Drug	***K. pneumonia* (n = 8)**			***K. ozaenae* (n = 3)**			***E. ludwigii* (n = 1)**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Cft	3 (37.5)	0 (0)	5 (62.5)	1 (33.3)	0 (0)	2 (66.7)	1 (100)	0 (0)	0 (0)
Cfx	1 (12.5)	1 (12.5)	6 (75)	1 (33.3)	0 (0)	2 (66.7)	0 (0)	0 (0)	1 (100)
Cfp	2 (25)	0 (0)	6 (75)	1 (33.3)	0 (0)	2 (66.7)	1 (100)	0 (0)	0 (0)
Ctx	2 (25)	0 (0)	6 (75)	1 (33.3)	0 (0)	2 (66.7)	1 (100)	0 (0)	0 (0)
Gent	2 (25)	0 (0)	6 (75)	1 (33.3)	0 (0)	2 (66.7)	0 (0)	0 (0)	1 (100)
Ertp	8 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Mrpn	8 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Fos	8 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Amp	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	1 (100)
Am/Clav	0 (0)	4 (50)	4 (50)	2 (66.7)	0 (0)	1 (33.3)	0 (0)	0 (0)	1 (100)
Levo	7 (87.5)	0 (0)	1 (12.5)	2 (66.7)	0 (0)	1 (33.3)	1 (100)	0 (0)	0 (0)

Key: S = Susceptible; I = Intermediate; R = Resistant

a. Cft = Ceftazidime (30 µg); Cfx = Cefuroxime (30 µg); Cfp = Cefepime (30 µg); Ctx = Ceftriaxone (30 µg); Gent = Gentamicin (30 µg); Ertp = Ertapenem (10 µg); Mrpn = Meropenem (10 µg); Fos = Fosfomycin (50 µg); Ampicillin (10 µg); Am/Clav = Amoxicillin-Clavulanic acid (30 µg); Levo = Levofloxacin (5 µg)

Sunflower Seed Agar (SSA) is a selective medium that is designed to enhance melanin production by *Cryptococcus* species[8]. In this study, twenty bacterial isolates (belonging to *Klebsiella* and *Enterobacter species*) were recovered in the medium. No C*ryptococcus* species was isolated, although the patients manifested suggestive symptoms, and that 48% of the patients had immunocompromising medical conditions, such as HIV infection. It should be pointed out that many in the past have sought

Cryptococcus from the throat of patients with suggestive symptoms in this locality and found none, as was also the case in this study. It would appear that cryptococcosis is not common in this region.

There was no statistically significant relationship between the frequency of isolation of bacterial isolates and any of the following factors- age, gender, and HIV status ($X^2 = 2.072$; $P > 0.05$). Of the 20 isolates, only 12 were sequenced. The remaining eight had their DNA extracted, but did not amplify, even with repeated trials. As a result, the eight isolates could not be sequenced, although they were conventionally identified as *Klebsiella spp*. The reason for this failure was not investigated; however, some factors have been previously reported to influence amplification efficiency – factors such as target sequence length, sequence base composition, primer sequences, and specificity, buffer compositions, presence of PCR inhibitors in the template DNA solution, cycling conditions, and thermostable DNA polymerase[11,12].

The most frequently isolated organism was *K. pneumonia,* with a 67% prevalence. This agrees with reports of previous authors that *Klebsiella pneumoniae* is one of the predominant pathogens isolated from patients with Respiratory Tract Infection[13,14]. *Klebsiella pneumoniae* is known to be the most common cause of nosocomial and community-acquired infections[15]. The fact that these organisms (which are recognized respiratory pathogens) were recovered in pure culture from those throat samples (in the presence of respiratory symptoms) tended to suggest an etiologic role for the organism in those patients. However, we could not confirm the etiologic role in those patients – we probably needed haematologic, immunologic, and other correlates of infection to establish an etiologic role for the isolates.

Similarly, *Enterobacter ludwigii* is a recognized potential pathogen; *E. ludwigii,* with five other species of *Enterobacter* (*Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, and *Enterobacter nimipressuralis*), make up the *Enterobacter cloacae* complex (ECC)[16]. Members of ECC are increasingly isolated as hospital-acquired pathogens[17]. *Enterobacter ludwigii* and other members of the ECC complex are also known to cause a range of life-threatening infections, including catheter-associated urinary tract infections, bacteremia, and surgical site infections, and have the capacity for acquiring resistance genes, as previously reported by other authors[18,19,20]. This makes the isolation of *E*. *ludwigii* (in this study) significant, as it constitutes a potential threat, mainly because of its drug resistance pattern.

The result of the susceptibility assessment indicates that *Klebsiella pneumoniae* isolates were most susceptible to Ertapenem, Meropenem, Fosfomycin, and Levofloxacin, while less susceptible or resistant to antibiotics such as ampicillin, gentamycin, Ceftazidime, Cefuroxime, Cefepime, Ceftriaxone, and Amoxicillin-Clavulanic acid; the later set of drugs are among commonly available and readily abused drugs in Nigeria; this abuse could have resulted in antimicrobial drug selective pressure, favoring resistant mutants of these organisms. The molecular basis for this high level of resistance to cephalosporins was not determined. However, the increasing prevalence of β-*lactamases* (specifically cephalosporinases) among resistant strains of *Klebsiella pneumoniae* has been reported by other authors[21]. It may lead to poor clinical outcomes in using these drugs in the management of respiratory infections in this locality. The high rate of resistance of the isolates to penicillin antibiotics could be due to penicillinase

production, known to occur among these organisms encountered commonly[21,22]. However, penicillinase activity was not tested for by us.

The drug resistance pattern of *E*. *ludwigii* isolate seems to agree with the view of Mezzatesta et al.[16] that most isolates of the *E. cloacae* complex are intrinsically resistant to ampicillin amoxicillin-clavulanate, cephalosporins, and cefoxitin, owing to the production of constitutive AmpC β-lactamase. However, we could not carry out a β-lactamase test on the isolates because it was outside the scope of the work. The result also suggests that Carbapenems are the practical choice of drugs in empirical therapy of respiratory tract infections in this locality.

We found out that the “browning effect” on the bacterial colonies in SSA was not due to melanin production but possibly due to reflection of the medium on the colonies; this effect could lead to misdiagnosis. This underscores the need for reliance on microscopic (rather than macroscopic) colonial features in the use of SSA for presumptive Identification of *Cryptococcus* species. Limitations of the work we could not evaluate the organisms for resistance markers because that was outside the scope of this study.

CONCLUSION

Based on the results, it could be seen that *Klebsiella* and *Enterobacter* species can produce colonies on SSA that can be mistaken for *Cryptococcus* species, especially with a less experienced scientist; this can lead to misdiagnosis of bacterial pneumonia-like illnesses as cryptococcosis. In a medical emergency (when empirical treatment is needed), this could be of dire therapeutic consequences. In the light of the increasing level of resistance being displayed by bacterial species that are most likely to contaminate cultures of throat specimens in SSA, it is pertinent to suggest that the antibacterial content of the medium be reviewed.

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CONFLICT OF INTEREST

The author report no conflicts of interest and no funding resources in this study.

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