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Sanger Sequencing Implementation in Clinically Ill Patients for Bacterial and Fungal Pathogens Identification

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Abstract

The diagnosis of bacterial or fungal infections requires the identification of the pathogen etiology in the shortest time possible. Although some biomarkers are used as indicators of bacterial infections, their specificity and sensitivity are highly variable, and there is no direct relationship between the level increase of these biomarkers for mycosis. It is common to obtain negative microbiological cultures in patients infected by non-culturable, intracellular bacteria or mycosis, even though there is a high clinical suspicion of infection. This study identifies the pathogen present in critically infected patients through 16S and 18S/eEF1 genes detection by polymerase chain reaction (PCR) coupled with Sanger sequencing. Thirty clinical samples were evaluated by PCR, of which 40% were positive for fungi, 23.33% for bacteria, 26.7% for fungi and bacteria, and 10% for no pathogen. The PCRs outcomes period for bacteria or fungi was one day compared to seven and up to 14 days (on average) of microbiological culture for bacteria and fungi. Then, we assessed the relationship with the most used biomarkers (procalcitonin, C-reactive protein, globular sedimentation velocity, and the neutrophil-lymphocyte index). This combination of molecular techniques has been shown as helpful in identifying intracellular bacteria and fungi that are difficult to culture by conventional methods. Screening with genomic markers 16S and 18S/eEF1 by PCR allowed us to optimize the time to obtain the result of the infection caused by bacteria or fungi. Also, identifying the specific etiological microorganism by Sanger sequencing was very helpful in avoiding the progression of the disease and setting targeted treatment with better clinical outcomes.

Keywords

Pathogen, Identification, Bacteria, Fungi, PCR, Sanger, Sequencing

1. Introduction

Sepsis is a life-threatening condition in which organ dysfunction is caused by a dysregulated body response to infection [1]. When this condition worsens, it leads to septic shock, characterized by severe circulatory dysfunction and metabolic abnormalities, which are enough to improve complications and may be deathful. In Mexico, mortality associated with septic shock is related to 60%, and the worldwide mean is approximately 37% [2]. Early diagnosis and etiological microorganism identification for sepsis are essential to reduce patients' morbidity and mortality rates dramatically. However, in sepsis, usually, we make late diagnoses due to signs and symptoms being non-specific and not always related to the infectious microorganism. Albeit biomarkers such as procalcitonin (PCT), erythrocyte sedimentation rate (ESR), and C-reactive protein (Reactive-CRP) are habitually used as parameters to determine the range and severity of infectious disease, besides severity scales such as SOFA, among other [1].

Fast, efficient, and accurate identification of etiological microorganisms is a fundamental task in clinical microbiology. It provides valuable and helpful information on etiologies for infections to choose the appropriate antibiotic treatment. Furthermore, phenotypic methods are based on obtaining microbiological cultures, which depend on growth characteristics and the microorganisms' biochemical profile [3]. Therefore, identifying slow-growing bacteria requires considerable time for complete identification, which may take as long as seven or more days. In septic patients, acute and chronic infections by fungus are not usually considered to be primary etiological microorganisms; according to IDSA Surviving Sepsis 2021 guidelines [1], fungal agents represent up to 17% of microorganisms that may cause sepsis. Nonetheless, risk factors for fungemia may be non-specific, very diverse, and therefore exclude patients potentially infected by fungi [4]. It is worth noting that there are different tools for detecting, differentiating, and identifying fungal organisms. Each technique presents difficulties; the diagnosis of mycoses has been based on direct microscopic examination of clinical specimens, histopathology, and microbiological culture. The diagnostic performance of microscopic examination and microbiological culture is highly dependent on the quality of the samples and the experience of the microbiologist. Also, these methods have previously shown less sensitivity in detecting fungi than molecular methods [5]. Despite the low sensitivity and highly dependent on the microbiologists' expertise, conventional methods are relatively inexpensive for each microbiological identification. Nonetheless, we must consider whether a patient needs more than one microbial identification and the number of patients in a healthcare facility requiring microbiological identification per

day with the attendant costs increase. These techniques allow the identification of cultivable bacteria and continually fail in cases of rare, intracellular, nonculturable microorganisms or microorganisms with ambiguous taxonomic profiles [6].

The molecular techniques offer an alternative for fast, efficient, and accurate microorganisms' identification, which are microbiological culture-independent. They also provide competitive costs for their scalability and the recent decades' high demand for these techniques. As part of molecular techniques, polymerase chain reaction (PCR) allowed fast, efficient, and accurate detection of bacteria and fungi [7]. Detection of the 16S gene (16S rDNA gene) has been widely used to identify bacteria through PCR and coupled with Sanger sequencing or next-generation sequencing (NGS). 16S gene sequencing is beneficial in identifying unusual bacteria, which are difficult to culture by conventional microbiological methods, hence providing identification at the genus level in over 90% and identification of these organisms to the species level among 65% - 83% [7]. Molecular identification of fungi is carried out by several genomic markers, including 18S (18S rDNA gene), 28S D1/D2 (variable domains D1/D2 of the 28S rDNA gene), ITS (internal transcribed regions [ITS1-5.8 S-ITS2]), eEF1 (eukaryotic translation elongation factor alpha subunit), RPB1 and RPB2 (RNA polymerase subunit I and II), CHS (chitin synthase), and Chi18-5 (chitinase 18 - 5) [8] [9]. Currently, no genomic marker option has been found to discriminate taxa at the species level for fungi. Therefore, for more specific identification, several genomic markers are used simultaneously to achieve a combination of segments conserved between species. In addition to the PCR amplification of these genomics, Sanger sequencing is already recommended [10]. Sanger sequencing is a valuable tool in the clinical setting due to its low cost and fast turnaround time relative. These features make it possible to have a faster identification of a pathogen and contribute substantially to the diagnosis [11] [12].

Determining the etiologic microorganism in the shortest outcomes possible is very useful for patients with high-risk factors for bacterial and fungal infections. In this study, the population assessed from Baja California Sur, Mexico, has environmental characteristics, such as heat, humidity, dust, and air transport of contaminated particles of the semi-arid region, that lead to the development of bacteria and especially fungi infections. In this study, we proposed to use the molecular PCR technique for the genomic markers 16S and 18S/eEF1 coupled with Sanger sequencing to identify the infective microorganisms.

2. Materials and Methods

Study design and clinical samples

A prospective, cross-sectional, analytical and observational relational study was carried out. Patients diagnosed with infectious processes with pathologies of infectious and inflammatory types hospitalized at the Benemérito General Hospital with Specialties Juan María de Salvatierra (La Paz, BCS, Mexico) were eva-

luated. Thirty patients diagnosed with an infection on admission were included in the study, from whom a clinical sample was taken from the primary site of infection (Figure 1).

Biological samples were taken using a sterile technique. Peripheral blood samples were taken before asepsis with 5% povidone-iodine, performing 3-stage asepsis with the placement of a sterile tourniquet; venous puncture was served with a sterile needle, and the sample was placed in a sterile tube with EDTA. Bronchial aspirate samples were collected using a Müller trap with a closed circuit without previous aspiration in 12 h. Cerebrospinal fluid samples were taken through lumbar puncture after asepsis and antisepsis with 5% iodopovine three times, infiltration with lidocaine was performed, and root space was removed and punctured to obtain cerebrospinal fluid. A blood sample was taken from all patients at admission to perform a blood count, liver function test, blood chemistry, acute phase reactants (ESR, CRP), and NLR and PCT measurement. In addition, the systemic inflammatory response and the response that occurs with the initial treatment were assessed based on the concomitant pathology.

The same day the samples were taken, they were processed to obtain total DNA in the Biotechnologika A2 facilities.

Extraction of DNA

Four hundred µL of whole blood, cerebrospinal fluid, bronchial aspirate, and ascites samples were taken and mixed with 400 µl of DNA/RNA Shield™ 2× (Zymo Research, Irvine, CA, USA). From tissue samples, we took 25 mg to biopsies. Then, we followed the manufacturer's instructions for the DNA Quick Miniprep kit (Zymo Research, Irvine, CA, USA) for DNA extraction and purification. The quality and concentration of the DNA were determined with the Qubit dsDNA BR assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

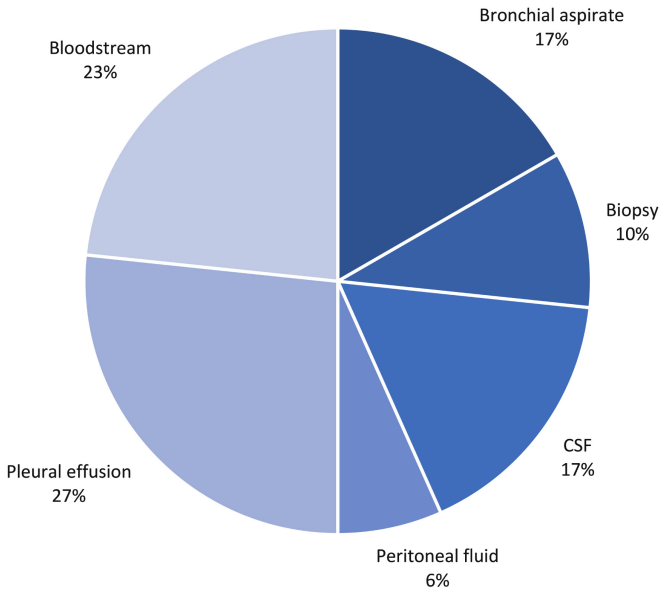


Figure 1. Clinical samples. Types of the clinical samples collected and analyzed in the study ($n = 30$).

Detection of bacteria and fungi by semi-quantitative PCR and Sanger sequencing

For the detection of bacteria and fungi in clinical samples, specific oligonucleotides were used for the 16S rDNA genes (V3 - V4 region) (forward: CCGTCAATTCCTTTGAGTT reverse: CAGCAGCCGCGCTAATAC), eEF1 (forward: GAYTTCATCAAGAACATGA reverse: GACGTTGAADCCRACRTTG) and 18S rDNA (forward: GATCACACCGCCCGTC reverse: TGATCCTTCTGCAGGTTCA). For the detection of these genes, 1 µl of DNA and 2 µl of primers (10 pmol) were added to 47 µl of GoTaq® Green Master Mix (M712) PCR reaction mix (Promega, Madison, WI, USA). Amplification was performed with an initial denaturation for 5 min at 95˚C, followed by 25 cycles of 5 s, 95˚C; 15 s, 60˚C, and 15 s, 72˚C. The expected PCR products (16S, 400 bp; 18S, 150 bp, and eEF1, 600 bp) were visualized by 2% agarose gel electrophoresis. The DNA of the agarose gel bands was purified using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA, USA).

The amplicons were sequenced using the Big Dye-terminator technique sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) on the 3500 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions at the Sequencing Facilities of the Instituto de Biotechnología from the National Autonomous University of Mexico (UNAM).

The electropherograms were visualized, and the qualities were assessed based on the regularity of the separation between bases, the height of the peaks and the presence of secondary peaks with the Geneious Prime v2019.2.3 bioinformatics package (https://www.geneious.com/). For the quality analyses, we took the quality of each nucleotide (quality score or Phred value) into account. The sequences obtained were analyzed using the GeneBank nt/nr database. (http://www.ncbi.nlm.nih.gov/) as a reference. The highest identity, sequence coverage, and expectation value were selected as parameters for identifying genus or species.

Microbiological cultures

Microbiological cultures were performed in the clinical and microbiological facilities of the Benemérito Hospital General de Especialidades "Juan María de Salvatierra" (BHGEJMS). Samples were grown on agar plates (blood, chocolate, and Macconkey agar) and BHI broth. The plates were incubated at 37˚C for 48 h. Growth was visually evaluated at 24 h, and if there was no growth, the BHI broth was subcultured for 5 days. For fungi, culture was performed in a Sabouraud medium, with thermal incubation at 37˚C for seven days with subsequent microscopic identification with lactophenol blue and 10% potassium hydroxide.

Statistical analysis

The characteristics of the clinical samples were reported as mean or frequencies and percentages. The difference corresponding to days of hospital stay, the differences between positivity between microbiological cultures and Sanger sequencing, and the efficiency of results between Sanger sequencing and use of

culture media were statistically analyzed using the chi-squared (X^2) test. Statistical differences between acute phase reactants between groups of patients with the identified pathogen of bacteria, fungi, and bacteria/fungi and the differences between quality (Q) values of sequence reads for 16S, 18S, and genomic markers eEF1 were analyzed with the Kruskal-Wallis test.

3. Results

Demographic data, the association of groups, and comorbidities

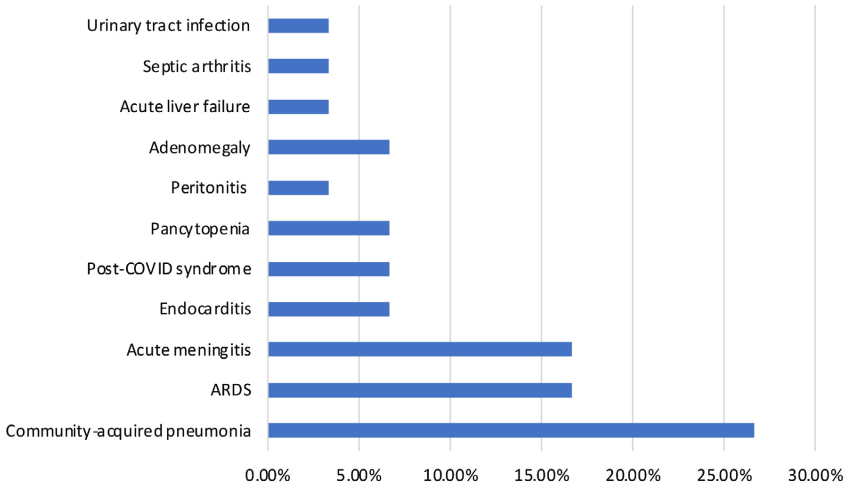
The study cohort consisted of 30 patients, of whom 63.3% were male, and 36.7% were female (Table 1). The mean age was 43.67 years (SD ± 13.57 years);

Table 1. Characteristics of the study population.

	Number (n)	Percentage (%)
Gender		
Masculine	19	63.3
Feminine	11	36.7
Age (years)		
20 - 30	8	26.7
31 - 40	5	16.7
41 - 50	6	20.0
51 - 60	7	23.3
61 - 70	4	13.3
Days of hospital stay		
0 - 10	8	26.6
11 - 20	10	33.3
21 - 30	9	30
31 - 40	1	3.3
41 - 50	1	3.3
>50	1	3.3
Comorbidities		
Arterial hypertension	9	30
Diabetes	4	13.3
HIV virus infection	3	10
Post-transplant	3	10
Obesity	2	6.6
Chronic kidney disease	2	6.6
Hyperthyroidism	1	3.33
Pulmonary tuberculosis	1	3.33
Congenital heart disease	1	3.33
Hepatitis C virus infection	1	3.3
None	4	13.3

by age ranges, the majority percentages of 26.7% and 23.3% corresponded to 20 - 30 years and 51 - 60 years, respectively (Table 1). The average hospital stay was 14.7 days (SD ± 10.2 days), with 11 to 20 days of more extended hospital stay (Table 1). Although no statistical differences were determined between the days of hospital stay between patients who had a bacterial infection and fungal infection ($p = 0.06$), it is noteworthy that more patients with fungal infection recorded a hospital stay greater than seven days recorded.

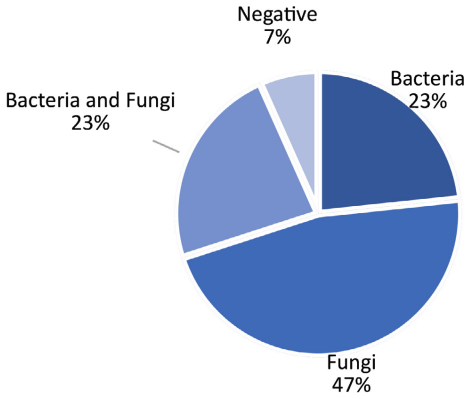
The 30 clinical samples were distributed as follows: bronchial aspirate (17%), biopsy (10%), CSF (17%), peritoneal fluid (6%), pleural fluid (27%), and peripheral blood (23%). (Figure 1). Of the total samples, 46.6% were positive for fungi, 23.3% for bacteria, 23.3% for bacteria and fungi, and 6.7% for no pathogen (Figure 2(c)). In addition, comorbidities present in patients in this study cohort were recorded. The most prevalent comorbidity was arterial hypertension



(a)

	Sanger Sequencing	Culture
Positive	27	18
Negative	3	12

(b)



(c)

Figure 2. Pathogens identified in clinical samples. (a) Diagnosis of the 30 patients, (b) Testing results for Sanger sequencing and culture assays; (c) Classification of identified pathogen in clinical samples by PCR and Sanger sequencing. UTI = Urinary tract infection, ARDS = Acute respiratory distress syndrome.

in 30%, followed by diabetes mellitus in 13.3%, and the rest of the comorbidities were presented in a range of 10% to 3.33% (Table 1). Only 13.3% of the patients studied did not present any comorbidity at the study time. We recorded all patients' clinical pictures corresponding to infection in the study cohort were recorded. ARDS diagnosis corresponds to 15.63%, meningitis 9.38%, and coagulopathy 6.25% (Figure 2(a)). The remaining diagnoses presented a prevalence of less than 5% (Figure 2(a)). The study's overall mortality was 36.6% associated with the severity of the clinical symptoms and their presentation, as determined by the SOFA scale (Sepsis-related Organ Failure Assessment) (Table 2).

Clinical presentation

Within the spectrum of clinical presentations described in this study, pneumonia was the most prevalent (Table 2 and Figure 2(a)), which was associated with a higher quantity of infections caused by agents of fungal nature. The infections in that study cohort showed a quite diverse clinical presentation and, in most cases, were directly related to the agent isolated and identified by Sanger sequencing (Table 2). The assessment of the genomic markers (16S, 18S/eEF1) and the clinical picture was very useful because, in most cases, it presented a non-specific picture of meningoencephalitis that, according to the analysis of cerebrospinal fluid, the orientation into mycotic agents would not have taken into account. The diagnosis would not have been timely with the progression of the disease to severe or even fatal sequelae. In several of the patients in this study, fungal infections were not considered in the first instance. The fungi tended not to be regarded as the main microorganism in severe infections. However, due to comorbidities and public hospital records, a high frequency of systemic mycosis has been found.

PCR for 16S, 18S, eEF1, and Sanger sequencing

To identify the presence of bacteria or fungi in the 30 clinical samples of this study cohort, semi-quantitative PCR was performed for the genomic markers 16S and 18S/eEF1, respectively. The amplification products of these genes (16S, 18S, and eEF1) were subjected to the Sanger sequencing technique. The resulting electropherograms of each of the sequences obtained were evaluated according to the criteria of the regularity of base separation, distribution of peak heights and appearance of secondary peaks, and the quality of the sequences (Figure 3(a)). The quality (Q) values distribution is shown for each position for all reads sequenced for each genomic marker (Figures 3(b)-(d)). The sequences generated presented an average quality value (Q) of the sequence per base for the genes of 36.39 (SD ± 17.72) (Figure 3). We found no statistical differences in the quality of the reads between the assessed genes (Figure 3(e)). The sequences were analyzed using the "bacteria" or "fungus" filter with the BLAST algorithm (http://www.ncbi.nlm.nih.gov/). The criteria of coverage cut-off > 90%, expectation value (E-value) < 0.001, and an identity percentage > 85% were used. All the sequences from the 30 clinical samples were within the established cut-off parameters (Table 3).

Table 2. Description of the cases of critically ill patients (with immunosuppressive states and suspicion of infection), and the result of pathogen identification (by classical culture, PCR for the 16S, 18S/eEF1 genes, and subsequent Sanger sequencing).

No.	Clinical sample	Medical diagnosis	Gene 16s	Gene 18s	*Microbiological culture*	*Sanger sequencing*	Clinical manifestations	SOFA
1	Bloodstream	Endocarditis	+	-	*Staphylococcus aureus*	*Staphylococcus aureus*	Fever, acute heart failure, dyspnea.	10
2	Bloodstream	Acute liver failure	+	-	*Staphylococcus aureus*	*Staphylococcus aureus*	Abdominal pain, fever, septic shock, jaundice, transamineemia, coagulopathy.	9
3	CSF	Acute meningitis	+	-	*Staphylococcus aureus*	*Staphylococcus aureus*	Headache, fever, seizures.	5
4	CSF	Acute meningitis	+	-	*Negative*	*Pseudomonas putida*	Headache, oral intolerance, fever, impaired state of consciousness.	3
5	Peritoneal fluid	Peritonitis	+	-	*Negative*	*Salmonella enterica*	Septic shock, abdominal pain, diarrhea, fever.	9
6	Bronchial aspirate	Community-acquired pneumonia	+	-	*Candida spp.*	*Candida krusei*	Septic shock, pleural effusion, fever, ARDS.	8
7	CSF	Acute meningitis	+	-	*Cryptococcus spp.*	*Pseudomonas putida*	Headache, oral intolerance, fever, and impaired state of consciousness.	4
8	Pleural effusion	Community-acquired pneumonia	-	+	*Mixed microbiota*	*Aspergillus fumigatus*	Septic shock, fever, pleural effusion, and ARDS.	1
9	Bronchial aspirate	Community-acquired pneumonia	-	+	*Negative*	*Candida krusei*	Pleural effusion, cough, dyspnea, and fever.	0
10	Pleural effusion	Post COVID syndrome	-	+	*E. Coli BLEE*	*Aspergillus niger*	Hemoptysis, fever, dyspnea, and cough.	0
11	Pleural effusion	Post COVID syndrome	-	+	*Negative*	*Cryptococcus neoformans*	Pleural effusion, fever, septic shock, and dyspnea.	0
12	Pleural effusion	Community-acquired pneumonia	-	+	*Pseudomonas aeruginosa*	*Rhizopus oryzae*	Septic shock, pleural effusion, dyspnea, and fever.	8
13	CSF	Acute meningitis	-	+	*Negative*	*Aspergillus flavus*	Ataxia, headache.	5

Continued

14	Pleural effusion	Community-acquired pneumonia	-	+	*Acinetobacter spp./SARM*	*Candida krusei*	Septic shock, pleural effusion, fever, ARDS.	9
15	Bloodstream	Pancytopenia	-	+	*Negative*	*Candida krusei*	Septic shock, fever, pancytopenia, coagulopathy.	3
16	Pleural effusion	Community-acquired pneumonia	-	+	*Streptococcus pneumoniae*	*Candida krusei*	Pleural effusion, cough, dyspnea, fever.	3
17	Pleural effusion	Community-acquired pneumonia	-	+	*Negative*	*Cryptococcus neoformans*	Pleural effusion, cough, fever, dyspnea.	0
18	CSF	Acute meningitis	-	+	*Negative*	*Aspergillus flavus*	Headache, oral intolerance, and impaired state of consciousness.	6
19	Bronchial aspirate	ARDS	-	+	*Negative*	*Aspergillus flavus*	Septic shock, pleural effusion, dyspnea.	4
20	Bronchial aspirate	ARDS	-	+	*Negative*	*Leptotrichia shahii*, *Lichteimia spp.*	Pleural effusion, fever, septic shock, and dyspnea.	3
21	Pleural effusion	Community-acquired pneumonia	+	+	*Rhyzosporum*	*Aspergillus flavus*, *Klebsiella pneumoniae*	Septic shock, cough, pleural effusion, fever	4
22	Biopsy	Adenomegaly	+	+	*Negative*	*Talaromyces pinophilus*, *Klebsiella pneumoniae*	Adenomegaly, dysphagia, intermittent fever.	0
23	Bloodstream	Septic arthritis	+	+	*Staphylococcus aureus*	*Staphylococcus aureus*, *Aspergillus flavus*	Septic shock, coagulopathy, fever, seizures, phlogosis.	13
24	Bloodstream	Urinary tract infection	+	+	*Candida glabrata*	*Staphylococcus aureus*, *Aspergillus flavus*	Fever, dysuria, pyuria.	7
25	Biopsy	Adenomegaly	+	+	*Mixed microbiota*	*Phyllobacterium myrsinacearum*, *uncultured fungus sp. 5OPS3-3AITS1*	Adenomegaly, fever and dysphagia.	0
26	Bloodstream	Endocarditis	+	+	*Staphylococcus aureus*	*Staphylococcus aureus*, *Aspergillus flavus*	Fever, acute heart failure, dyspnea.	5

Continued

27	Bronchial aspirate	ARDS	+	+	*Candida spp.*	*Delftia sp., Aspergillus flavus*	Septic shock, dyspnea, ARDS.	8
28	Bronchial aspirate	ARDS	-	-	*Candida spp.*	*Negative*	Septic shock, dyspnea, pleural effusion, fever.	2
29	Bloodstream	Pancytopenia	-	-	*Shigella spp.*	*Negative*	Abdominal pain, fever, septic shock, jaundice, transamineemia, coagulopathy.	9
30	Bronchial aspirate	ARDS	-	-	*Negative*	*Negative*	Septic shock, pleural effusion, dyspnea, cough, fever	0

CSF: Cerebrospinal Fluid, ARDS: Acute Respiratory Distress syndrome, SOFA: Sepsis related Organ Failure Assessment.

Table 3. Quality score parameters for identified sequences in clinical samples.

		E value	Query cover	% identity	Specie	Specie	E value	Query cover	% identity
Bacterium and fungi	17	0.00E+00	96%	99.26%	*Staphylococcus aureus*	*Aspergillus flavus*	5.00E−61	94%	99.24%
	18	0.00E+00	98%	98.92%	*Staphylococcus aureus*	*Aspergillus flavus*	4.00E−62	96%	99.25%
	19	6.00E−179	52%	99.15%	*Phyllobacterium myrsinacearum*	*Uncultured fungus sp. 5OPS3-3AITS1*	0.00E+00	79%	98.48%
	20	0.00E+00	99%	98.92%	*Staphylococcus aureus*	*Aspergillus flavus*	6.00E−47	97%	93.28%
	28	4.00E−140	98%	88.04%	*Delftia sp.*	*Aspergillus flavus*	6.00E−22	10%	79.17%
	29	4.00E−61	97%	69.13%	*Leptotrichia shahii*	*Lichteimia spp.*	4.00E−71	98%	68.17%
	5	7.00E−65	93%	99.29%	*Klebsiella pneumoniae*	*Talaromyces pinophilus*	1.00E−02	1%	96.97%
Bacterium	6	2.00E−170	94%	97.45%	*Klebsiella pneumoniae*				
	4	1.00E+128	93%	89.60%	*Pseudomonas putida*				
	23	4.00E−109	93%	85.28%	*Pseudomonas putida*				
	16	3.00E−160	86%	97.87%	*Salmonella enterica*				
	1	0.00E+00	99%	96.43%	*Staphylococcus aureus*				
	2	3.00E−22	37%	77.51%	*Staphylococcus aureus*				
	3	0.00E+00	85%	90.64%	*Staphylococcus aureus*				
Fungi	7	2.00E−08	39%	87.27%	*Aspergillus fumigatus*				
	9	2.00E−03	4%	80.65%	*Aspergillus niger*				
	12	4.00E−62	93%	99.28%	*Aspergillus flavus*				

Continued

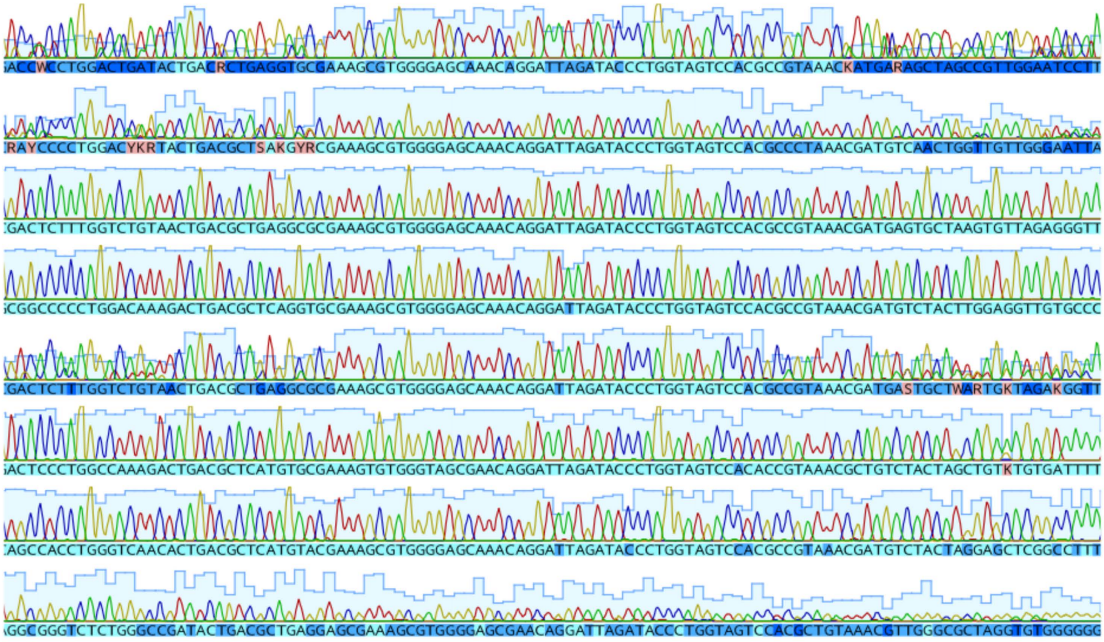
	26	4.00E−62	97%	99.25%	*Aspergillus flavus*
	30	6.00E−60	97%	98.51%	*Aspergillus flavus*
	8	5.00E−26	81%	74.37%	*Candida krusei*
	13	1.00E−62	92%	98.57%	*Candida krusei*
	15	1.00E−104	96%	82.73%	*Candida krusei*
	22	3.00E−24	45%	74.11%	*Candida krusei*
	25	1.00E−26	65%	74.62%	*Candida krusei*
	10	3.00E−08	3%	90.38%	*Cryptococcus neoformans*
	24	3.00E−08	3%	90.38%	*Cryptococcus neoformans*
	11	1.00E−53	84%	95.15%	*Rhizopus oryzae*
	27				*Negative*
Negative	14				*Negative*
	21				*Negative*

Association of acute phase reactants with Sanger sequencing

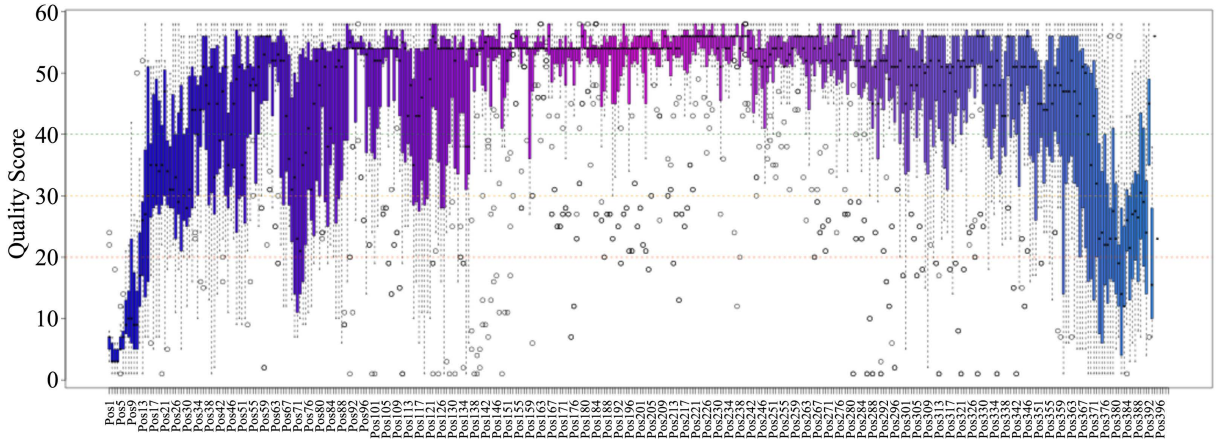
For the inclusion of patients in the protocol, acute-phase reactants were measured. These parameters are usually taken into account to assess the presence of infectious agents, severity, and evolution of infectious diseases; however, sometimes, the clinical association is not related directly to the causal agent, as is the case of encapsulated, intracellular bacteria, and fungi. At hospital admission, acute phase reactants and NLR were immediately measured. We grouped these values according to the result of the pathogen identified by semi-quantitative PCR and Sanger sequencing into three groups: positive for the 16S gene or bacterial infection, positive for the 18S gene/eEF1, or fungal infection, and positive for the 16S/18S/eEF1 genes or co-infection (**Figure 4**). Serum markers of infection (CRP, ESR, NLR, and PCT) commonly used in critically ill patients were analyzed, and we found no statistically significant differences were found between the three groups (**Figure 4**).

Comparison with culture results

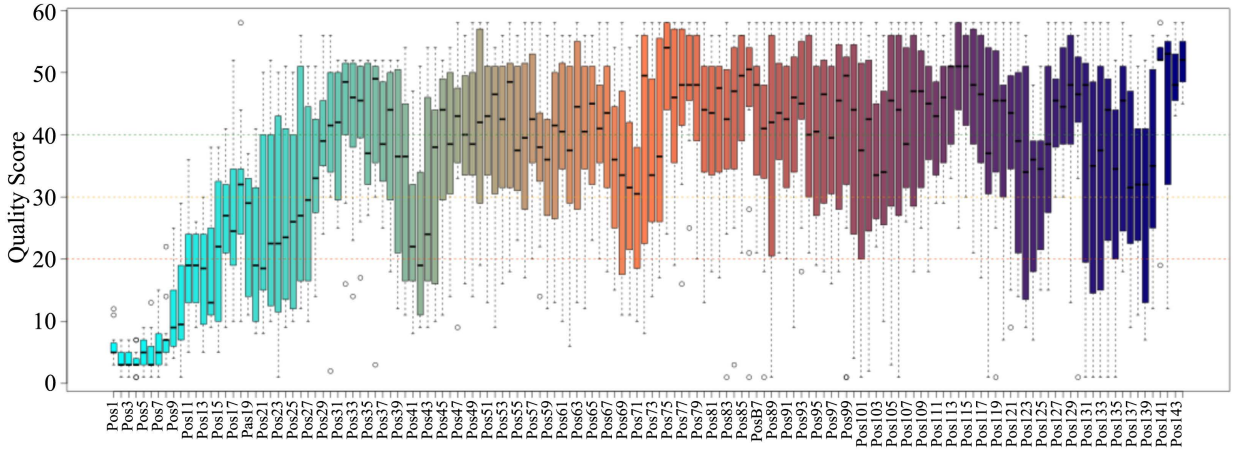
The relationship between the microbiological cultures and the Sanger sequencing was low; we found no growth in most of the cultures. It did not agree with the sequencing results nor with the clinic of the studied patient. Unlike the result reported by cultures, the results of pathogens identified by Sanger sequencing showed direct agreement with the patient's clinical symptoms. Regarding the efficiency of results between Sanger sequencing and cultures, a statistical difference was found ($p = 9.47 \times 10^{-8}$). By Sanger sequencing, in 28 of 30 (93.33%), the presence of the causal agent of the infection could be determined, while in the cultures, it was determined in 18 of 30 (60%) (**Figure 2(b)**). Only was a coincidence of identification results of the causal infection agent by Sanger



(a)


Quality Score
60
50
40
30
20
10
0

(b)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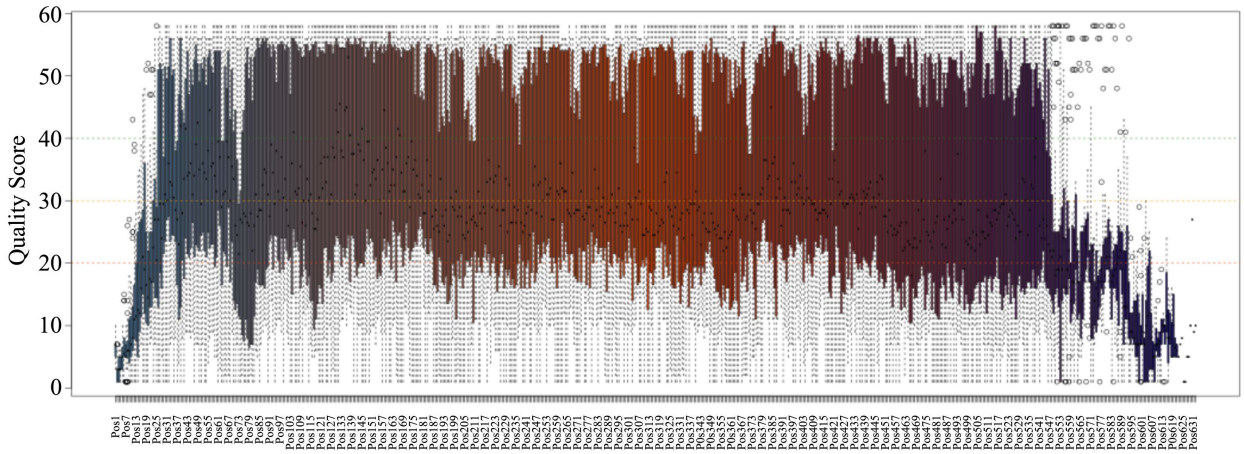

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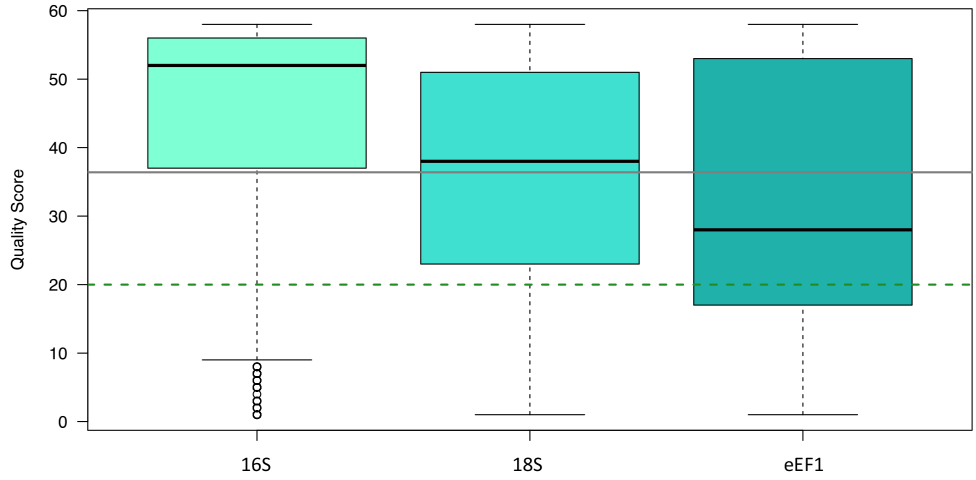

(e)

Figure 3. Quality analysis of Sanger sequencing reads for all genomic marker employed in this study. (a) Representative electropherograms; analysis of the quality (quality score) by nucleotide for the sequences of (b) 16S, (c) 18S and (d) eEF1; and (e) quality score distribution for all nucleotide position of 16S, 18S, and eEF1 sequences.

sequencing with the culture in 5 samples (16.66%); with both techniques, *Staphylococcus aureus* was identified in the clinical samples analyzed. Interestingly, *Aspergillus spp.* was also identified in these samples, suggesting a co-infection.

4. Discussion

In this work, we assessed the application of the infectious pathogen molecular identification through PCR coupled with Sanger sequencing of the 16S and 18S/eEF1 genomic markers for bacteria and fungi, respectively, from hospitalized patients with suspicious acute infections to obtain a fast, efficient, and accurate diagnostic against traditional microbiological cultures. Of the assessed patients, 63% were males, 37% were females, and the most frequent age range was from 20 to 30 years old, and the main comorbidities were high blood pressure and diabetes, which are often associated with acute infections.

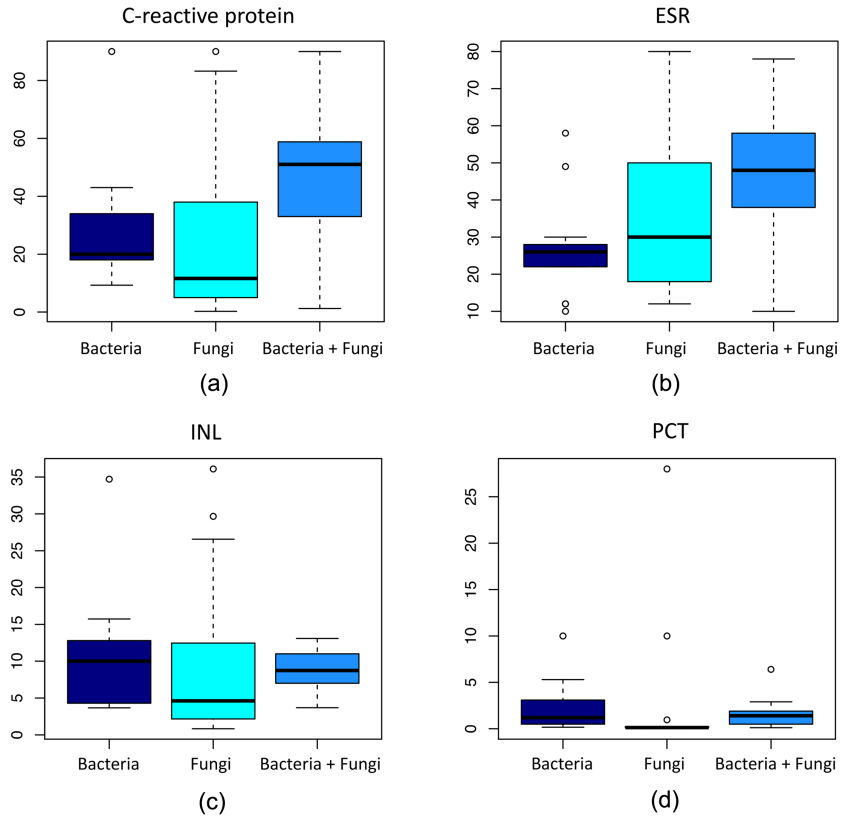

Figure 4. Levels of acute phase reactants. Distribution level analyses of the four biomarkers more widely used for infection diagnosis: (a) C-reactive protein (CRP), (b) Erythrocyte sedimentation rate (ESR), (c) neutrophil-lymphocyte ratio (NLI) and (d) Procalcitonin (PCT).

The required time from the sample collection to the result delivery for bacterial or fungal detection through PCR for all patients from this study cohort was one day against the seven days required for traditional microbiological cultures for bacteria identification and 14 days for fungal identification. From the PCR positive samples for 16S and 18S/eEF1, we proceeded with Sanger sequencing as part of the pathogen identification workflow (**Figure 5**). After sequencing, we were able to identify the microbial pathogen in 93.3% of the samples analyzed, which in turn did not show statistical differences in the sequence-associated Phred values (Q) among the sequences of 16S and 18S/eEF1 ($p = 0.648$). We established and proposed this fast, efficient, and accurate PCR and Sanger sequencing-based pathogen identification workflow showing a lesser required time (five days) than conventional microbiological cultures (seven days for bacterial identification and 14 days for fungal identification); with a total cost of USD $150.00, that in turn allow reaching an accurate diagnosis to start with a directed treatment with beneficial therapeutic and clinical outcomes for the patient. Otherwise, the conventional microbiological cultures showed pathogen identification for 18 of the 30 samples (60%) analyzed, and only six samples (20%) were concordant with Sanger sequencing molecular approach. The patient's clinical

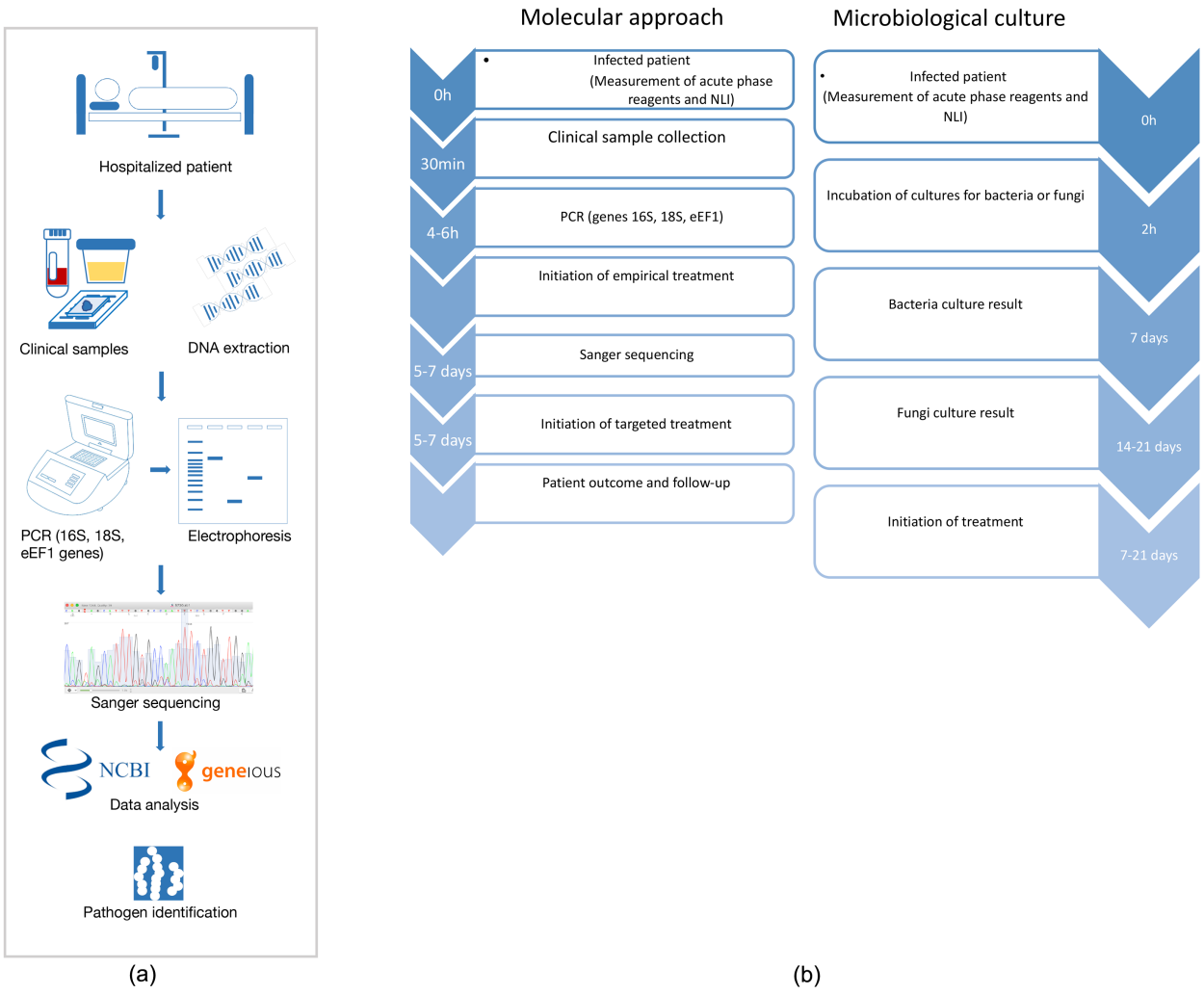

Figure 5. Study workflow. (a) Clinical samples were collected from hospitalized patients. After DNA extraction and PCR for 16S or 18S/eEF1 genes fon bacteria or fungi, respectively, Sanger sequencing was carried out. The analysis of data was performed in Geneious Prime v2019.2.3 (https://www.geneious.com/) using the bacterial and fungal available data bases in the GenBank (https://www.ncbi.nlm.nih.gov/), (b) Time-line comparison between the molecular and microbiological culture workflows for the management from of infected patients samples in to patients treatment.

behavior of the 28 identified pathogens through Sanger sequencing showed a direct relatedness. Those patients were addressed with immediate treatments with positive clinical outcomes showing a dramatic decrease in their symptomatology. On the other hand, due to the high severity of infections for some patients and their comorbidities, the global mortality at 30 hospitalization days was three patients (10%).

From the identified pathogens through Sanger sequencing in this work, the most abundant fungal pathogens belong to the *Aspergillus* genus, followed by the species *Candida krusei* and *Cryptococcus neoformans*; and the most abundant bacterial pathogens were *Staphylococcus aureus* and *Pseudomonas putida*. It is worth noting that it was possible to determine the bacterial and fungal co-infection in four patients; those co-infections were among *Staphylococcus*

aureus and *Aspergillus spp.* with a direct relatedness to the patients' clinical frame. This finding is in accordance with the rise of co-infection case reports in several unrelated illnesses patients since the next-generation sequencing (NGS) approach implementation has allowed the accurate bacterial and fungal co-infection characterization, such as immunocompromised patients and COVID-19 patients [13] [14] [15].

It is to highlight that most of the patients in this work showed the positive fungal pathogen 18S/eEF1 genomic marker identification; these results might directly relate to these infections' development due to immunosuppression triggered by the previous condition with HIV. Moreover, novel risk factors have been reported associated with positive mycosis development in patients exposed to fungal-rich environments, working conditions, environmental perturbations of plant and soil materials, and exposed to fecal material of wild mammals [16]. It is worth noting that those risk factors have also been identified in our study location, Baja California Sur, including patients with working conditions in caves or close contact with wild birds or poultry.

Among the most prevalent clinical illnesses found in this group of patients in this study, pneumonia-related infections were the most prevalent, which can develop high mortality levels without an early and reliable diagnosis. The neuro infection-related illnesses were the second most prevalent clinical frames, in which first symptomatology was primarily associated with headaches, without fever or any sign of meningism. For those patients, systemic mycosis in the central nervous system was identified thanks to the fungal genomic markers detection through the molecular workflow leading to the treatment application for these patients. Also, the patients' clinical frames with bacterial infections showed the fever- and bacteremia-associated classical inflammatory response most probably due to the bacterial-induced immunological response. On the other hand, some patients did not show either inflammatory or immunological responses, and then their diagnosis is often misleading with other causal agents [17]. According to surviving sepsis 2021 guidelines, mycotic infections are directly related to diabetes, immunosuppression, neutropenia, and wide-spectrum antibiotic therapy [1]. The diagnosis of mycotic infections becomes difficult since there are no accurate and specific clinical biomarkers. The β-glucan detection and fungal antibodies for systemic mycosis have shown a low diagnosis performance against molecular detection [18].

The conventional and regular procedure for patients' infection diagnosis is to assess the acute phase reactants and infection-related biomarkers that may lead us to determine the causal agent showing inflammatory response. However, today there is not any high-performance biomarker that could help us specifically differentiate between fungal or bacterial infections. In this work, we assessed the acute phase infection conventional biomarkers (C-reactive protein, VSG, INL, and PCT), which exhibited high variability and without statistical differences among those and between the infections' causal agents. These biomarkers have

shown low sensitivity and specificity and even yield false-positive identifications, notably contrasting with molecular identifications' high sensitivity and specificity through PCR and Sanger sequencing.

Moreover, the sepsis early symptoms such as fever, tachycardia, and leukocytosis are relatively unspecified and are very frequent, and these overlap with the systemic inflammation symptoms. Other symptoms such as high blood pressure, thrombocytopenia, and lactate concentrations increase altogether suggest an acute phase infection. However, these increased levels of biomarkers are often detected too late for a treatment application that might save the patients' lives or stop the organic dysfunction progression during microbial infection or sepsis. Thereby, diagnosis and treatment delay increases the sepsis mortality risk and extends the clinical stance, increasing costs. These facts highlight the need to apply early, efficient, fast, and accurate diagnosis biomarkers to decrease all patients' risk factors and use a directed treatment against the infection causal agent for a positive outcome for the patient. The pathogen identification through PCR coupled with Sanger sequencing can yield a fast, efficient, and accurate result for non-culturable and/or obligate intracellular bacteria. Also, this molecular workflow overcomes the intrinsic difficulties associated with traditional microbiological cultures and their morphological characterization for fungal identification. Altogether, the application of this molecular workflow offers a suitable diagnostic approach for Mexican healthcare facilities.

5. Conclusion

The pathogen identification of the infectious causal agent is essential to establish the patient's proper diagnostics, treatment, and a positive outcome. The application of molecular techniques, such as PCR and Sanger sequencing in this study has significantly decreased the time for pathogen identification from patients' samples, achieving faster and more accurate diagnostics reflected in better patient clinical outcomes. We propose the application of molecular techniques to improve the hospitals and health care facilities' diagnostics capabilities in Mexico and extend it to become a regular procedure in public health management.

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Ethical Statement

The protocol was approved by the Clinical Research Ethics Committee Board of the Benemérito Hospital General de Especialidades "Juan María de Salvatierra" (BHGEJMS), reference number 083-083-2021.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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