COMPARATIVE EVALUATION OF GENOTYPING AND CULTURE-BASED TECHNIQUES FOR FUNGAL KERATITIS DETECTION

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Abstract:
The study aims to compare the direct polymerase chain reaction with microbial culture for the detection and fungal pathogens in infectious keratitis. A total of 81 corneal ulcers were culture and analyzed prospectively. PCR was performed with all corneal scrapping with fungal and bacteria specific primers. PCR products were analysed and compared with the culture results using standard methods. Of the 81 samples, 80 were positive by PCR, 51 for fungi and 29 for bacteria. Out of 51 PCR positive samples, 22 samples were culture positive and 29 were culture negative. The majority of PCR genotyped samples matched the positive culture results. The positive detection rate of 80/81 (98.8%) with high suspicion of fungal keratitis and positive detection rate of direct PCR 50/51(98.0%) were observed. The sensitivities for the diagnosis of fungal keratitis with direct PCR and culture were 98.0% (50/51) and 43.1% (22/51) ($p< 0.001$) whereas the specificities were 100.0% (2/2) and 100.0% (1/1) respectively. The time required to complete the direct PCR was only 3 hours. The direct PCR assay is a rapid diagnostic technique with high sensitivity and specificity for infectious keratitis and it is expected to have impact on the diagnosis and treatment of infectious keratitis.

***Keywords*: Infectious, pathogens, detection, universal, strains**

1. Introduction

Infectious keratitis is a significant public health problem caused by bacteria, fungi, viruses and parasites [1]. World Health Organization estimated that in every year, about 1.5-2.0 million new cases of monocular blindness that occurred in developing countries are secondary to corneal ulceration and are common among outdoor workers and densely populated continents of Africa and Asia [2,3]. Rapid and aggressive treatment is needed to halt the progression and prevent loss of vision [4]. The principal causes of microbial keratitis are bacteria and fungi [5], more than 50% of infectious keratitis cases are fungal keratitis (FK), especially in developing countries [6]. Fungal keratitis develops rapidly, and easily induces corneal perforation which usually requires surgical intervention [6,7]. The predisposing factors for infectious keratitis varies with geographical location, corneal abrasion and ocular surface disorders such as dry eye, trichiasis are commoner in Iraq [8] while previous trauma to cornea and corneal opacity is prevalent in Nigeria [9]. Cornea scrapping which is the mainstay sample for diagnosis of infectious keratitis is obtained in small quantity from the patient eye and is usually insufficient for the conventional microbiological diagnosis hence; there is need for a fast and accurate diagnostic method of infectious keratitis. Definitive diagnosis of an infectious keratitis is generally confirmed by microbiological evaluation [10,11] which includes cultures, smear examination with microscope have limitations despite the suggestive appearance and course of corneal ulcer produced by certain pathogens.

Newer techniques like Analytical Profile Index, a commercially available phenotypic qualitative miniaturized systems API 20E (Bio Merieux, France) for Enterobacteriaceae and API Staph (Bio Merieux, France) have been used for biochemical identification of the bacterial isolates [12]. Polymerase chain reaction (PCR) is an advanced laboratory technique that involves enzymatic amplification of specific sequences of DNA [13]. The major advantage of PCR is its ability to detect DNA from microorganisms which cannot be cultured easily or require a longer time [14,15,16]. Hence, PCR can detect microbial DNA and is much more sensitive and specific than conventional methods [17,18,19].
In this study we evaluated the use of culture-based techniques for phenotypic characterization and PCR for genotyping fungal isolates associated with corneal keratitis.

2. Materials And Methods

Clinical samples totaling 81 were collected between July 2015 and July 2018 from patients attending the Ophthalmology

Department of the University of Ilorin Teaching Hospital (UITH), Sobi Specialist Hospital and Civil Service Clinic which serve as referral hospitals in Kwara State, North Central, Nigeria. Ethical permission for the study was obtained.

Eyes with clinically suspected viral and parasitic corneal ulcers were excluded. History of pain, photophobia, watering, and redness were taken as inclusion criteria. Duration of symptoms and history of predisposing factors like trauma, contact lens wear, dry eye, surgery, etc. were noted. Ocular examination included visual acuity (VA) of both eyes, and slit lamp examination of the cornea for size, site, and depth of the ulcer, presence or absence of perforation. Fluorescein staining of the corneal ulcer for epithelial defect and the presence or absence of hypopyon were determined. After taking informed consent and explaining the procedure to the patient, the corneal scrapings were collected by the Ophthalmologist from infected cornea using 23G sterile needle under Slit Lamp Biomicroscope after instillation of non-preservative topical anaesthesia into the infected eye. Immediately after collection, the samples were introduced into brain heart infusion broth (BHB), yeast extract broth (YEB), and Tris EDTA buffer which was stored at temperature of -80^{0}C. Also smears of the samples were made on the slide for Gram staining and the remaining sample was streaked directly on Blood agar, Chocolate and MacConkey agar.

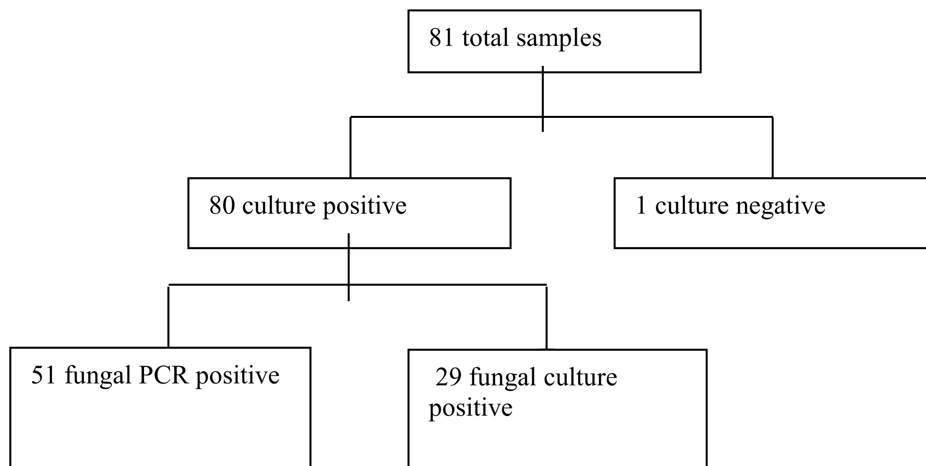

Fig. 1. PCR and Culture results of samples collected from infectious keratitis patients

Gram stained for cellular morphology, biochemical test carried out according to Sagar Aryal [20].

The yeast extract broths (YEB) were subcultured onto Sabouraud dextrose agar (SDA) and were incubated at 28 degree Celsius for five days. Lactophenol cotton blue wet mount preparation was carried out on the growth from Sabouraud dextrose agar (SDA) plates while Mycology Atlas and biochemical tests were used for the identification of the fungi. Suspected yeast isolates from Sabouraud dextrose agar (oxoid, UK) were Gram's stained and confirmed yeast isolates were further characterized as reported by Matare [21].

Phenotypical bacteria and fungi isolates

Bacterial DNA was isolated by boiling method according to the steps previously described by Grupta [18]. Isolates that were positive to phenotypic confirmatory test were grown on nutrient agar for 24 hours.

A single colony growth was picked, transferred to 0.1 mls of sterile water, boiled for 10 minutes to lyse the cells. The lysate was centrifuge and 3 ml of the supernatant was used as the DNA sample for the PCR. All primers were prepared by Iqaba Biotechnology, West African Limited. They were used in 4 sets of PCR as follows:

Extracted DNA was genotyped for bacterial and fungal isolates using the universal bacterial primer (27f, 1525r) (5'-AGAGTTTGATCCTGGCTCAG-3, 5'-AAGGAGGTGATCCARCC-3); Universal fungi (F, R) (GTG AAA TTG TTG AAA GGGAA, GAC TCC TTG GTC CGT GTT); Species Specific primer (Pseudomonas aeruginosa (PA-SS-F, PA-SS-R), Staphylococcus aureus (S4F, S4R) and Staphylococcus epidermidis (91E-F, 3B-R); Primers specific

for Candida albicans (CABF59F, CADBR125R) to identify samples that gave positive results in the second set of PCR. For all PCR protocols, a reaction mixture without sample DNA was used as a negative control, in addition to using DNA from the most common cause of microbial keratitis as a positive control. Amplification was performed in a thermal cycler for a total of 25 cycles (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA). After an initial denaturation at 95 degree Celsius for 5 minutes, each cycle consisted of denaturation at 94 degree Celsius for 1 minute, annealing at 50 degree. Amplification was performed in a thermal cycler for a total of 25 cycles (GeneAmp PCR System 9700; Applied

Biosystems, Foster City, CA, USA). After an initial denaturation at 95 degree Celsius for 1 minute, and extension at 72 degree Celsius for 5 minutes. The amplified product was then electrophorised in 1% of agarose gel then stained with E2-vision blue light DNA dye and visualized in an ultraviolet transilluminator [15].

3. RESULTS

The age group ranged from 10-65 years with a male to female ratio 3:1 as shown in Table 1 below.

Table 1: Age distribution for Infectious Cornea Keratitis among the patients

Age Group (Year)	Number of patients (n)	Overall percentage (%)	Male (n)	Percentage (%)	Female (n)	Percentage (%)	χ^2	P-value
10- 20	9	11.1	5	6.2	4	4.9		
21-49	48	59.3	38	46.9	10	12.4	2.793	0.259
≥ 50	24	29.6	16	19.7	8	9.9		
Total	81	100.0	59	72.8	22	27.2		

The majority of patients were outdoor workers more importantly the artisans, farmers and traders which accounted for 79.1% (Table 2). The single largest category among outdoor workers was farmers, constituting 38.3% of total patients.

Table 2: Occupation as risk factor for Infectious Cornea Keratitis among the patients

Occupation	Overall number	Percentage	Male		Female		χ^2	P value
	(n)	(%)	(n)	(%)	(n)	(%)		
Artisan	19	23.5	16	19.7	3	3.7		
Farmer	31	38.3	26	32.2	5	6.2	22.035	0.001
Civil servant	7	8.6	7	8.6	0	0.0		
Trader	14	17.3	6	7.4	8	9.9		
Student	7	8.6	4	4.9	3	3.7		
Others	3	3.7	0	0.0	3	3.7		
Total	81	100.0	59	72.8	22	27.2		

P <0.001 is significant

The mean duration of patients presenting to hospital was 7 days. Most patients had symptoms for at least 7-10 days. Centrally located corneal ulcers were evident in many patients (75.0%). Majority of the ulcers (87.3%) measured 1-3mm in size while 12.7% of patients had ulcer size > 4mm.

Table 3: PCR detection results for suspected infectious keratitis in the clinic

Clinical diagnosis	No of samples examined	Positive samples		Positive detection rate %	Total detection rate %
		Fungi	Bacteria		
Fungal keratitis	52	51	0	62.9%	98.8%
Bacterial keratitis	29	29	0	35.8%	

Out of 52 samples that were fungal suspected infectious keratitis one was PCR negative which means cornea was co-infected with other microbe aside from bacteria. The total positive detection rate was 98.8% as shown in table 3 above.

Table 4: Performance of Direct PCR and Culture for diagnosis of Fungal keratitis

Direct PCR	Culture		Outcome of discrepant analysis, no		Sensitivity %		Specificity %	
	Positive	Negative	Positive	Negative	Culture	PCR	Culture	PCR
Positive	22	29	50	1	22/51(43.1%)	50/51(98.0%)	2/2(100%)	1/1(100%)
Negative	1	1	1	1				
P<0.001								

The performance of culture and direct PCR with corneal scrapings from all patients were analysed. The sensitivity and specificity of direct PCR were 95.6% (22/23) and 96.6% (29/30) respectively (Table 4). After discrepant analysis, a total of 50 true positives were obtained. The sensitivity and specificity of direct PCR was 98.0% and 100% respectively. However, of the 50 true positives, only 22 showed positive results by culture. Therefore, the sensitivity and specificity of culture was 43.3% and 100% respectively.

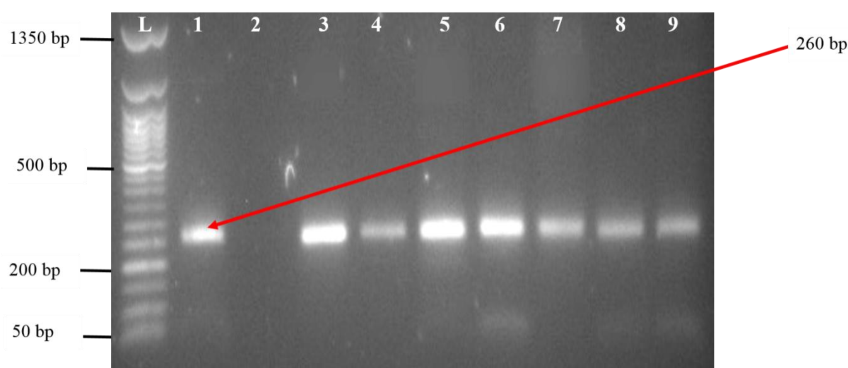

Figure1. DNA amplicons of Fungal Isolates expressed on agarose gel showed presence of band in lane 3, 4, 5, 6, 7, 8, 9, 10 and lane 1 is the marker while no band was found in lane 2.

4. DISCUSSION

In this study, bacterial and fungal strains associated with infectious keratitis were studied among patients of various demographic characteristics. The demographic studies showed high prevalence (59.3%) of infectious keratitis among ages 21 to 49 years and it indicates an active working year for most men who strive to have sustainable socioeconomic status and also provide for the family. Many people were involved in active service such as farming, trading in local materials, retail and petty business that could predisposed them to infectious keratitis from soil or other contaminated materials while trying

to remove sweat, tears or watery mucous from their eyes. Studies done by Saka [9] in Ilorin recorded high participants in the age range 20-80 years (70.3%) as well as Ameen [22] in Egypt that reveals the higher rate in ages between 40 and 50 years which disagrees with our study. The gender distribution in our study reveals higher preponderance of males (72.84%) than female subjects which is in agreement with Ibanga [23] who reported 60.87% of the male patients with suppurative corneal ulcers in Calabar, Nigeria. Joshi [24] and Seal [25] reported similar findings but female preponderances were found in the studies done by Lap-King [26] and Gorski [27] who reported 53.5%, and 65.8% respectively. This variation might be due to geographical locations and their age range differences. All these are attributed to agricultural or domestic workers and labourers, in most cases are residents in rural areas. Occupation is considered as risk factor for infectious keratitis. Higher prevalence rate was found among male farmers compared to artisans and female traders. The high prevalence among male farmers is similar to the report of Bashir et al [3] but Ibanga [23] in Calabar and Bhandari [28] reported more preponderance among industrial labourers. In Nigeria, a large proportion of the population resides in rural areas where farming is a major occupation. Farmers have been observed in studies to be at higher risk of corneal ulcers as they exposed to work-related eye injuries, some of which could be serious. Several studies have demonstrated that corneal injury is an important risk factors for microbial keratitis [23,25,26]. The culture positivity varies widely from place to place and according to the geographic location.
Laboratory testing is very helpful for the correct diagnosis and effective treatment of fungal kerartitis [27], the clinical appearance of which usually mimics that of bacterial keratitis [28]. For fungal keratitis, culture is still considered to be the reference standard for identification of pathogens [28]. Moreover, culture is relatively insensitive and time-consuming. In the present study, the positive detection rate of culture for fungal keratitis (43.1%) was lower than 59.3% culture positive results reported by Ferrer in the study of 20 corneal samples of patients with proven mycotic keratitis [29]. The higher positive results may be due to low corneal scraping samples compared to 81 corneal samples recorded in the present study. Kim et al reported positive detection rates for culture of 56% in their study of fungal keratitis [30] which is higher than the findings in our study. The higher positive detection rate may be due to the fact that each corneal sample was subjected to three tests namely culture, confocal microscopy and PCR.

PCR, as a rapid and sensitive detection technique, has been widely applied for the diagnosis of infectious keratitis [30], with the highest positive detection rates ranging from 80% to 90%. In the present study, a direct PCR assay was applied to the scraping specimen from each patient and the positive detection rate of direct PCR for all scrapings from patients with suspected fungal keratitis. Kuo et al [31] reported culture as the reference standard, the sensitivities of PCR-based techniques ranged from 70% to 100%, whereas the specificities varied from 17% to 94%, according to the results of several related studies. In our study, the sensitivity and specificity of direct PCR was higher compared to other studies [30,31,].

5. CONCLUSION

The direct PCR assay incorporates species-specific primers aid diagnosis of bacterial and fungal agents causing corneal keratitis. Hence, it provides useful information on the pathogens associated with corneal ulcers. We have found that PCR reliably discriminates bacterial and fungal pathogens. We also find that PCR assay provided precise detection of pathogens associated with keratitis compared to culture-based method.

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